

Identification and partial purification of an anticoagulant factor from the venom of the Iranian snake *Agkistrodon halys*

Ghorbanpur M (1), Zare Mirakabadi A (2), Zokae F (1), Zolfagarrian H (2)

(1) Chemical Engineering Department, Amirkabir University, Tehran, Iran; (2) Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Karaj, Iran.

ABSTRACT: An anticoagulant factor was purified from the venom of the Iranian snake *Agkistrodon halys* by gel filtration on Sephadex G-50 and ion-exchange chromatography on DEAE-Sepharose. In the final stage of purification, the percentage recovery of purified anticoagulant factor was found to be 83%. The purified anticoagulant factor revealed a single protein band in SDS-polyacrylamide electrophoresis under reducing conditions and its molecular weight was about 22 kDa. The purified peptide did not show any effect on casein, BApNA or plasma.

KEY WORDS: snake venom, *Agkistrodon halys*, anticoagulant factor, chromatography.

CONFLICTS OF INTEREST: There is no conflict.

CORRESPONDENCE TO:

ABBAS ZARE MIRAKABADI, Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Karaj, Iran. Phone: +98 261 4502865. Email: abbas.zare8@gmail.com.

INTRODUCTION

Snake venoms are rich sources of pharmacologically active proteins and peptides. They play an important role in incapacitating, immobilizing and digesting prey. Thus toxins have evolved to specifically target various critical points in the physiological systems of prey. Over the years, a number of toxins that affect blood circulation have been isolated and characterized from various snake venoms (1, 2). Within each family of snakes, the venom components seem to be fairly common and similar to one another.

Nerve toxins are generally found in the Hydrophidae and Elapidae venoms whereas hemorrhagic and myonecrotic toxins are generally found in the venoms of the Viperidae and Crotalidae families of snakes (3).

Snake venom toxins affecting hemostasis have been classified by virtue of their overall effect into procoagulant and anticoagulant ones (2-4). Snake venoms have different types of anticoagulant proteins, some of which have enzymatic activity, represented by phospholipase A₂, 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 (1, 3-5). Phospholipases A₂ are esterolytic enzymes that hydrolyze acyl-ester bonds of 1,2-diacyl-3-sn-phosphoglycerides and release fatty acids (3, 6, 7). Snake venoms contain a number of serine and metalloproteinases including fibrino(geno)lytic enzymes (8, 9). Thrombin-like enzymes and fibrinolytic enzymes, which act on fibrinogen, lead to defibrinogenation of blood and a consequent decrease in blood viscosity. Fibrinolytic enzymes have been reported in species of the Crotalidae and Viperidae families including *Agkistrodon contortrix contortrix* and *Agkistrodon acutus* (10-12). L-amino acid oxidases are flavoenzymes that catalyze the stereospecific oxidative deamination of an L-amino acid substrate to a corresponding α -ketoacid by the production of ammonia and hydrogen peroxide (13).

In this study, we report the purification and characterization of an anticoagulation protein from the venom of the Iranian snake species *Agkistrodon halys* that exhibits anticoagulant activity on human blood.

MATERIALS AND METHODS

Material

Fresh crude venom of *A. halys* was obtained directly from a local snake in Iran, lyophilized and preserved at -20°C . DEAE-Sepharose, Sephadex G-50 and C18 columns were purchased from Pharmacia Biotech Company (Sweden). Bovine serum albumin, BApNA (N2-benzoyl-dl-arginine-p-nitroanilide), the kit of standard protein markers and other reagents for enzymatic and biochemical assays were purchased from Sigma (USA). All other chemicals were of analytical reagent grade.

Isolation of the Thrombin-Like Enzyme

Lyophilized crude venom of *A. halys* (200 mg) was dissolved in 8 mL of 50 mM ammonium acetate buffer (pH 7.4) and centrifuged at 5,000 rpm for 15 minutes at 4°C and was filtered by 0.45 microfilter to remove the insoluble materials.

The clear supernatant was applied on a molecular exclusion chromatographic column of Sephadex G-50 (150 x 3 cm), previously equilibrated with the ammonium acetate buffer (pH 7.4) and then eluted with the same buffer. Fractions of 9 mL/tube were collected at a flow rate of 60 mL/hour at 4°C . The obtained fractions were denominated AH1 to AH5, indicating *A. halys* fractions 1 to 5.

The fraction (AH2), which showed clotting activity from the gel chromatography step, was pooled and dialyzed overnight at 4°C against distilled water and applied on DEAE-Sepharose CL-6B (2.5 x 20 cm) column, equilibrated with 20 mM Tris buffer, at pH 8.2. In this step, proteins were eluted with a linear gradient of NaCl from 0.0 to 0.5 mM. The flow rate was 17 mL/hour and 5 mL fractions were collected at 4°C . The peaks were monitored at A_{280} .

Blood Collection

Normal pooled plasma was obtained from ten individual healthy donors, without history of bleeding or thrombosis. Blood was centrifuged for 20 minutes at 2,400 g, and the plasma was fresh when used.

Determination of Molecular Weight

12% SDS-PAGE was performed and utilized a low molecular weight standard ranging from 6.5 to 66 kDa (11).

Purity Analysis

Sample aliquots (100 μ L) applied on an HPLC C18 column, were equilibrated with solvent A (H_2O , 0.1% trifluoroacetic acid), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% trifluoroacetic acid) from 0 to 30%, at a flow rate of 0.5 mL/minute for 30 minutes.

Prothrombin Time (PT) Assay

Prothrombin time reagent (200 μ L) and sample aliquots (200 μ L) were pre-incubated for ten minutes at 37°C and mixed for five seconds and then 100 μ L of plasma was added and clotting time was recorded (13). One unit of anticoagulant activity corresponds to an increase of 20 seconds in normal plasma coagulation.

Amidolytic Activity

Proteolytic activity in the samples was assayed using BApNA as substrate. The substrate solution was prepared by dissolving BApNA in 5 mL of dimethyl methyl sulfoxide (DMSO) and adding 95 mL of 0.05 M Tris-HCl buffer (pH 8.2). Proteolytic activity was monitored as BApNA hydrolysis by mixing 50 μ L of sample with 100 μ L of the substrate solution. After ten minutes of incubation at 37°C, the absorbance at 410 nm was measured. One unit of protease activity corresponds to an increase of $A_{410} = 0.001/\text{minute}$ (9).

Coagulant Activity

Plasma (200 μ L) and different amounts of purified factor were pre-incubated briefly at 37°C, then mixed and shaken, and the clotting times were recorded (3, 8, 14).

Proteolytic Activity on Casein

Proteolytic activity was determined by addition of various amounts of the enzyme to a buffer solution of 0.1 M Tris-HCl, pH 9.0, and the final volume was adjusted to 250 μ L, followed by the addition of 750 μ L of 1% (m/v) casein and incubated for 15 minutes at 37°C. The reaction was stopped by adding 1.5 mL of 30% TCA. The resulting proteolysis products in the supernatant solution were evaluated spectrophotometrically at $\lambda = 280$ nm after centrifugation at 5000 rpm for ten minutes. One unit of caseinolytic activity corresponds to an increase of $A_{280} = 0.001/\text{minute}$ (9).

Protein Determination

Protein concentration was measured by the method of Lowry *et al.* (15), using bovine serum albumin (BSA) as standard.

RESULTS

Selection of Snake Species

Crude venom from the Iranian snake species *E. carinatus* and *A. halys* were selected and assayed with PT test. The *E. carinatus* venom coagulated plasma very rapidly, which leads to the conclusion that *E. carinatus* venom contains procoagulant factors. Crude venom of *Agkistrodon halys* was selected next: this venom delayed plasma coagulation. Thus it was concluded that the *A. halys* snake venom contains one or more anticoagulant factors.

Isolation of Anticoagulant Factor

In the initial Sephadex G-50 fractionation of the crude *Agkistrodon halys* venom, five peaks at 280 nm were obtained (AH1 to AH5) as shown in Figure 1. When all the fractions were tested for anticoagulation, it was found that fraction AH2 showed anticoagulation effect. Further purification was carried out by ion exchange chromatography on DEAE-Sepharose resin (Figure 2). In this purification step six fractions were obtained (AH21-AH26) out of which fraction AH21 showed anticoagulant activity. By this purification procedure, about 16.75 mg of purified enzyme was obtained from 182.5 mg of the venom (Table 1).

Table 1. Purification of an anticoagulant from *A. halys* venom

Step	Protein (mg)	Specific activity* (unit/mg)	Total amidase activity (unit)	Purification fold	Yield (%)
Venom	182.5	0.9	164.25	1	100
AH2	57.3	2.69	154.3	3	94
AH21	16.75	7.64	128.1	8.5	83

*Activity was determined using plasma as the substrate.

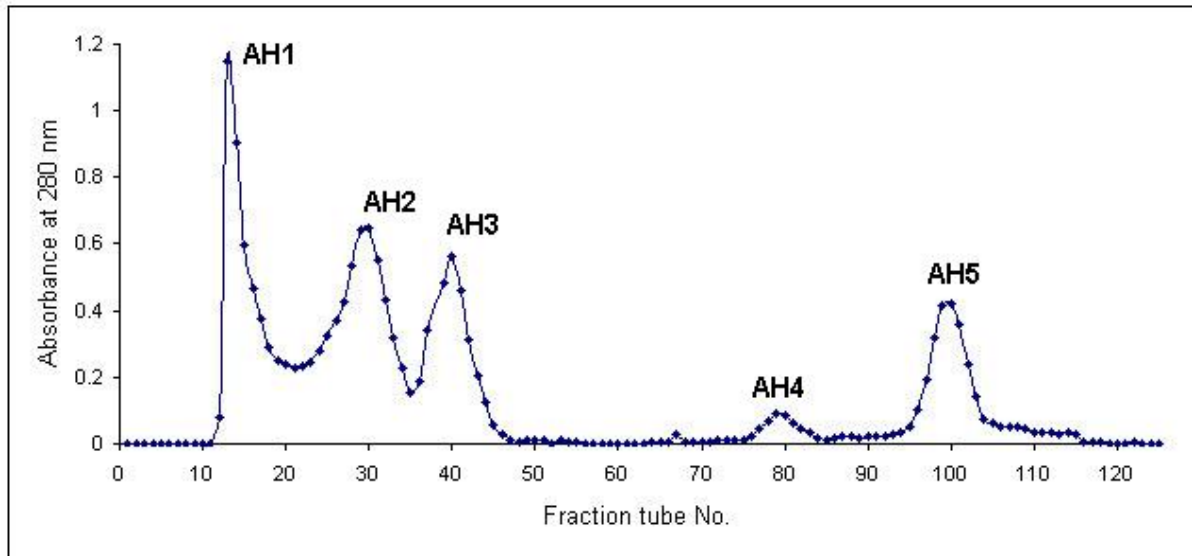


Figure 1. Sephadex G-50 chromatography of Iranian *Agkistrodon halys*. Crude venom (182.5 mg) was applied to Sephadex G-50 column (3 x 150 cm) using ammonium acetate buffer with molarity 50 mM and pH 7.5. Flow rate was 60 mL/hour and 9 mL fractions were collected from each tube.

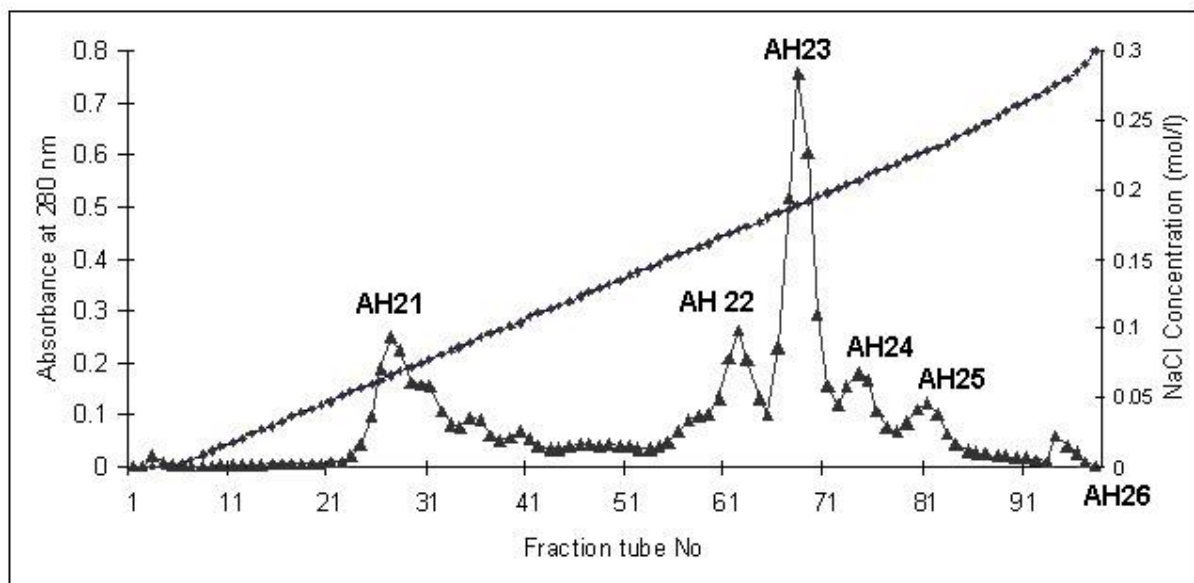


Figure 2. DEAE-Sepharose chromatography of AH2 obtained from Sephadex G-50. The pooled AH2 anticoagulant fraction was applied to DEAE-Sepharose column (2.5 x 20 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.2). Proteins were eluted with a linear concentration gradient of NaCl from 0.0 to 0.5 M, and 4.5 mL fractions were collected from each tube. Series 1: NaCl concentration (mol/L) and series 2: absorbance at 280 nm.

Purity and Determination of Molecular Weight

The homogeneity of purified enzyme was confirmed by SDS-PAGE and HPLC as shown in Figures 3 and 4, respectively. Isolated anticoagulant factor (AH21) showed high purity as analyzed by C18 reverse phase HPLC. This factor also showed a single band in SDS-PAGE. The molecular weight of this anticoagulant factor was estimated to be about 22 kDa under reduced conditions.

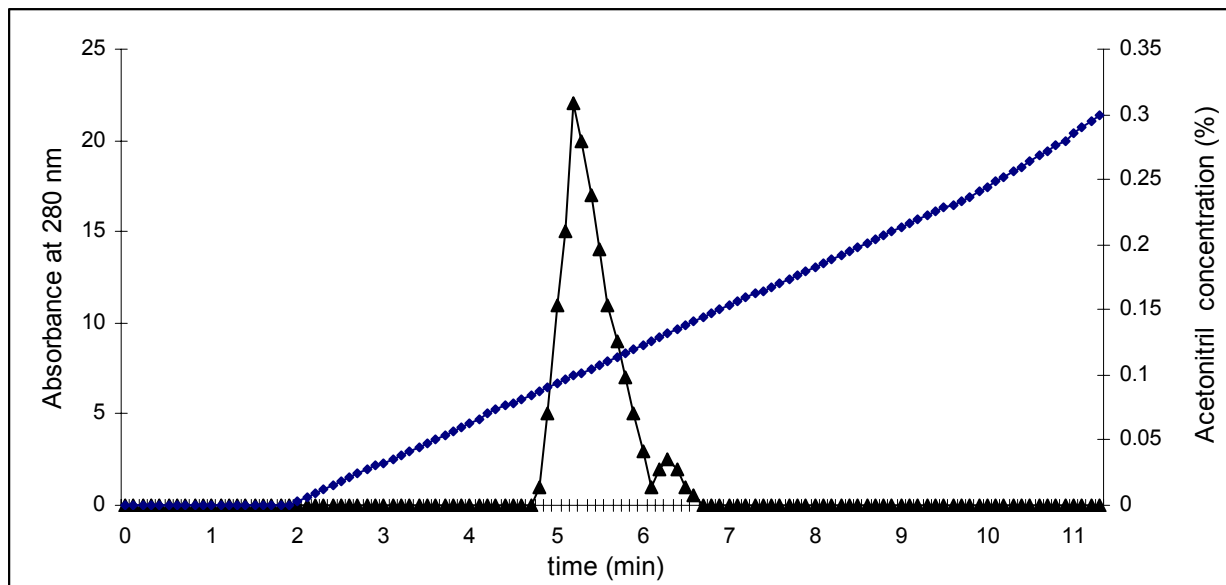


Figure 3. Purity assay of anticoagulant factor by HPLC. Series 1: acetonitril concentration (%) and series 2: absorbance at 280 nm.



Figure 4. SDS-PAGE of crude venom of (A) *Agkistrodon halys*, (B) AH2 and (C) AH21, analyzed on 12.5% SDS-polyacrylamide gel in the presence of 1% 2-mercaptoethanol.

Caseinolytic Clotting Assay and Amidolytic Activity

AH21 did not exert any proteolytic activity on casein, coagulant activity on BAPNA or clotting activity upon human plasma.

DISCUSSION

Venoms of Colubridae snakes are a rich source of novel compounds that may have applications in medicine and biochemistry (16). This article reports a relatively simple procedure for the isolation of an anticoagulant factor from Iranian *Agkistrodon halys* venom, denominated AH22, which was isolated and purified by a combination of gel filtration on Sephadex G-50 (Figure 1) and ion-exchange chromatography on DEAE-Sepharose (Figure 2). By this procedure the total enzyme purification yield was about 83% (Table 1), thus enabling the conclusion that the protocol utilized was highly efficient. Fractionation and purification of snake venom constituents have been carried out through several chromatography methods (6-8, 16). An acidic phospholipase A₂ was purified from *Agkistrodon halys pallas* venom by a two-step procedure comprised of gel filtration chromatography on Sephadex G-100 and ion exchange chromatography on DEAE Sephadex A-50 (17). Another phospholipase A₂ from *B. leucurus* venom was purified by a three-step procedure involving gel filtration on Sephacryl S-200, ion exchange chromatography on Q-Sepharose and reverse phase HPLC on Vydac C4 column (6).

By using the method described herein, about 16.75 mg of anticoagulant factor was obtained from 182.5 mg of the venom of *Agkistrodon halys* (equal to 9.2% of total venom protein). Therefore; it appears that the venom of the Iranian snake *Agkistrodon halys* causes blood coagulation and, in this manner, may cause some hemostatic disorders in the victim. The amount of this anticoagulant is quite high and comprises four percent of the entire venom (6).

Snake venom toxins that prolong blood coagulation are proteins or glycoproteins with molecular masses ranging from 6 to 350 kDa (1). The molecular weight of the factor under denaturing conditions was estimated to be 22 kDa. Thus, this anticoagulant factor should belong to the low-molecular-weight group of these factors; some anticoagulant factors, along with their molecular weights, reported in the literature are: l-amino acid oxidase from *Agkistrodon blomhoffii ussurensis* weighing 108.8 kDa (18); anticoagulant protein, halyxin, from *Agkistrodon halys brevicaudus* venom at

29 kDa (19); and metalloproteinase from *Philodryas patagoniensis* presenting 53 kDa (5).

Venoms from snake species belonging to the genus *Agkistrodon* (*A. contortrix contortrix*, *A. c. mokasen*, *A. c. pictigaster*, *A. piscivorus*, *A. p. leucostoma*, *A. halys halys*, *A. blomhoffi ussuriensis* and *A. bilineatus*) contain protein C activators. They are glycoproteins with a molecular mass ranging from 36 to 40 kDa. On account of its molecular mass (22 kDa), we concluded that the anticoagulant obtained in the present work does not belong to this low-molecular-weight group.

As described in the Introduction section, snake venom toxins that prolong blood coagulation are proteins or glycoproteins that inhibit blood coagulation by different mechanisms. Some of these anticoagulant proteins such as phospholipase A₂, metalloproteinases (α -fibrinogenase) and serine proteases exhibit enzymatic activities, whereas others including C-type lectin-related proteins and three-finger toxins do not exhibit any enzymatic activity (1, 5). We also isolated two serine proteases, namely AH143 and AH144, from *Agkistrodon halys* venom (under publishing).

This purified anticoagulant factor did not show any effect upon casein, BAPNA or human plasma, and thus appears to be another type of anticoagulant protein rather than protease. We also carried out a simple test on this factor by using red blood cells in which no effect was observed (not reported), probably indicating that this factor is not a phospholipase A₂.

CONCLUSION

An anticoagulant factor was purified from the venom of *Agkistrodon halys*. In the final stage of purification, 83% recovery was obtained. The methods applied in the purification were suitable and allowed a high degree of toxin recovery. The anticoagulant factor isolated in the present work was not characterized as protease, since no proteolytic effect was observed on casein, BAPNA or human plasma.

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