

**PURIFICATION AND PARTIAL CHARACTERIZATION OF A COAGULANT
SERINE PROTEASE FROM THE VENOM OF THE IRANIAN SNAKE *Agkistrodon
halys***

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ABSTRACT: *Agkistrodon halys* is one of several dangerous snake species in Iran. Among the most important signs and symptoms in patients envenomated by this snake is disseminated intravascular coagulation. A thrombin-like enzyme, called AH143, was isolated from *Agkistrodon halys* venom by gel filtration on a Sephadex G-50 column, ion-exchange chromatography on a DEAE-Sepharose and high performance liquid chromatography (HPLC) on a C18 column. In the final stage of purification, 0.82 mg of purified enzyme was obtained from 182.5 mg of venom. The purified enzyme showed a single protein band by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions, and its molecular mass was found to be about 30 kDa. AH143 revealed clotting activity in human plasma, which was not inhibited by EDTA or heparin. This enzyme still demonstrated coagulation activity when exposed to variations in temperature and pH ranging, respectively, from 30 to 40°C and from 7.0 to 8.0. It also displayed proteolytic activities on synthetic substrate. The purified enzyme did not show any effect on casein. We concluded that the venom of the Iranian snake *Agkistrodon halys* contains about 0.45% single procoagulant protein which appears to be a thrombin-like enzyme.

KEY WORDS: Iranian snake, venom, *Agkistrodon halys*, serine protease, chromatography, coagulant activity.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Snake venoms contain numerous enzymes such as glucosidases and proteases, with the latter group including metalloproteases and serine proteases. Several venom serine proteases present important fibrino(geno)lytic, kininogenase and thrombin-like activities (1, 2).

Thrombin-like enzymes (TLE) from snake venoms belong to a class of serine proteases that can result in blood clotting *in vitro*, a feature exhibited by numerous snake toxins. These enzymes render the blood uncoagulable when acting *in vivo*, apparently by depleting the circulating fibrinogen (3, 4). Most of thrombin-like enzymes cleave fibrinogen to release fibrinopeptide A and cannot activate factor XIII (5, 6). Therefore, thrombin-like enzymes hydrolyze fibrinogen to produce non-cross-linked fibrins that are more susceptible to the lytic action of plasmin than thrombin-induced cross-linked clots. These clots formed by TLE action are removed from the circulation either by fibrinolysis or by the reticuloendothelial system (5, 6).

Several venoms from the families Crotalidae and Viperidae contain proteolytic enzymes that exercise some effect on the blood coagulation process (7, 8). Since fibrinogen-clotting enzyme was first discovered in snake venom in the early years of the 20th century, numerous TLE have been isolated from different snake species (5). These enzymes are widely distributed among several pit viper genera (*Agkistrodon*, *Bothrops*, *Crotalus*, *Lachesis* and *Trimeresurus*), as well as in true vipers (*Bitis* and *Cerastes*) and colubrids (*Dispholidus typus*) (9). Some TLE from snake venoms with known complete amino acid sequences are: ancrod (*Calloselasma rhodostoma*, formerly *Agkistrodon rhodostoma*) batroxobin (*Bothrops atrox moojeni*), bilineobin (*A. binlineatus*) and crotalase (*C. adamanteus*) (10-14).

Thrombin-like enzymes are medically promising and act as defibrinogenating agents, such as ancrod and batroxobin, that have been extensively used in victims of thrombosis, myocardial infarction, peripheral vascular disease, acute ischemia and renal transplant rejection (15). Additionally, these enzymes are employed in deep corneal ulcer repair and non-pregnant canine uterus (16, 17). A study on the efficacy of fibrin glue derived from snake venom in colon anastomosis of rats was carried out by Leite *et al.* (14).

To the best of our knowledge, no prior work has been published on purification of this enzyme from *A. halys* venom; hence, in this study, we attempted to it and report its partial characterization.

MATERIALS AND METHODS

Material

Fresh crude venom of *A. halys* was obtained directly from local Iranian snakes, lyophilized and stored at -20°C . DEAE-Sepharose, Sephadex G-50 and C18 columns were purchased from Pharmacia (Sweden). Bovine serum albumin BApNA (N2-benzoyl-DL-arginine-p-nitroanilide), standard protein marker kits and other reagents for enzymatic and biochemical assays were purchased from Sigma. All chemicals were of analytical reagent grade.

Isolation of 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 . Subsequently, the supernatant was filtered on a 0.45 microfilter to remove insoluble materials.

Clear supernatant was submitted to molecular exclusion on a Sephadex G-50 chromatographic column (150 x 3 cm), previously equilibrated with 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 . Obtained fractions were denominated consecutively AH1 to AH5 to indicate *A. halys* fractions 1 to 5.

Fractions revealed clotting activity during the gel chromatography step. AH1 was pooled and dialyzed overnight at 4°C against distilled water and applied on DEAE-Sepharose column (2.5 x 20 cm), 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. Flow rate was 17 mL/hour and 5-mL fractions were collected at 4°C .

The fraction that had shown clotting activity in the previous step was pooled and dialyzed overnight at 4°C and applied on an 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 30%, at a flow rate of 0.5 mL/minute during 30 minutes. The peaks were monitored at 280 nm.

Blood Collection

Normal pooled plasma comprised samples from ten healthy donors, without history of bleeding or thrombosis. Blood was centrifuged for 20 minutes at 2,000 rpm, and the plasma was freshly used for test.

Determination of Molecular Mass

SDS-PAGE (12%) was performed according to Laemmli (18), utilizing a standard of low molecular masses ranging from 6.5 to 66 kDa.

Purity Analysis

Analysis of purity was carried out by HPLC on a C18 column as previously described.

Prothrombin Time (PT) Assay

Prothrombin time reagent (200 μ L) and sample aliquots (200 μ L) were preincubated for ten minutes at 37°C and mixed. Then, 100 μ L of plasma was added and clotting time was recorded (19).

Amidolytic Activity

Sample proteolytic activity was assayed using the method of Laemmli (18). 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 through the expression of 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 was measured at 410 nm. One unit of protease activity corresponds to an increase of $A_{410} = 0.001/\text{min}$ (18).

Coagulant Activity

Plasma (200 μ L) and sample aliquots with different amounts of enzyme (2.5, 5.0, 10.0, 20.0 or 50.0 μ g) were briefly preincubated at 37°C. After that, they were mixed and shaken and then clotting times were recorded (15, 20, 21).

Proteolytic Activity on Casein

Proteolytic activity was determined by the Sant'Ana method (22). Various amounts (10, 20, 30, 40, 50 or 60 mg) of the enzyme were added to the buffer solution 0.1M Tris-HCl, pH 9.0, and the final volume was adjusted to 250 μ L, followed by 750 μ L of 1% (m/v) casein and incubated for 15 minutes at 37°C. The reaction was stopped by addition of 1.5 mL of 30% TCA. Resulting proteolysis products in the supernatant solution were evaluated spectrophotometrically after centrifugation at 1,600 g for 10

minutes at 280 nm. One unit of caseinolytic activity corresponds to an increase of $A_{280} = 0.001/\text{min}$ (2).

Inhibition of Enzyme Activity

The effect of ethylenediaminetetraacetic acid (EDTA) and heparin were examined by incubation with enzymes in 180 μL of 20 mM Tris-HCl, with optimum pH at 37°C for five minutes. After mixing each of the inhibitors and 10 mL of purified enzyme, the remaining activity was determined by measuring the hydrolysis of synthetic BApNA. The remaining coagulating activity was calculated as percent activity in relation to the control preparation incubated without inhibitors, which was considered 100% (18).

Effect of pH and Temperature

The effect of pH on enzyme activity was evaluated by measuring residual enzyme activity after incubation at different pH, ranging from 4.0 to 6.0 with 0.1 M citrate-NaOH buffer while between 7.0 and 10 with 0.1 M Tris-HCl buffer, at 37°C for ten minutes.

Additionally, enzyme activity was assayed at diverse temperatures ranging from 20 to 70°C using BApNA as a substrate. The assay was conducted at pH 7.5 with 0.05 M Tris-HCl buffer containing 0.02 M CaCl_2 for five minutes in a temperature-controlled water bath. Thereafter, heat-treated samples were rapidly cooled in an ice bath and residual activity was measured using BApNA as substrate at pH 7.5 and 25°C for five minutes, as previously described (18).

Protein Determination

Protein concentration was measured according to Lowry *et al.* (23) using BSA as a standard.

RESULTS

Isolation of the Thrombin-Like Enzyme

In the Sephadex G-50 fractionation of the crude venom, five peaks with absorbance at 280 nm were obtained (AH1 to AH5) as shown in Figure 1. All fractions were tested for coagulation, it was found that AH1 fraction was positive for procoagulation. PT assay revealed that AH1 fraction had a 10 $\mu\text{mol}/\text{min}/\text{mg}$ activity. The yield of procoagulant enzyme fraction (AH1) was calculated and found to be 36% (Table 1).

Further purification was carried out by ion exchange chromatography on DEAE-Sephacrose. In this step, five fractions were obtained (AH11 to AH15), out of which AH14 fraction revealed procoagulant activity (Figure 2). The total yield of this fraction was 56% that accounted for 0.98% of total venom. AH14 fraction was pooled, dialyzed and applied to a C18 reversed-phase HPLC column. Four peaks (AH141 to AH144) were obtained (Figure 3) and AH143 presented coagulant proteolytic activity. The PT-test reached its elution peaks at 9 and 12 minutes (Figure 3). The purification procedure of AH143 is summarized in Table1. Through this technique, about 0.82 mg of purified enzyme was obtained from 182.5 mg of venom.

Table 1. Purification of a thrombin-like enzyme (AH143) from *A. halys* venom

Step	Protein (mg)	Specific activity* ($\mu\text{mol}/\text{min}/\text{mg}$)	Total amidase activity ($\mu\text{mol}/\text{min}$)	Yield (%)
Venom	182.5	10	1801.9	100
AH1	48.12	32.39	1558.6	91
AH14	1.8	622.2	1120	65
AH143	0.82	469.7	328.8	36

* Activity was determined using BApNA as substrate.

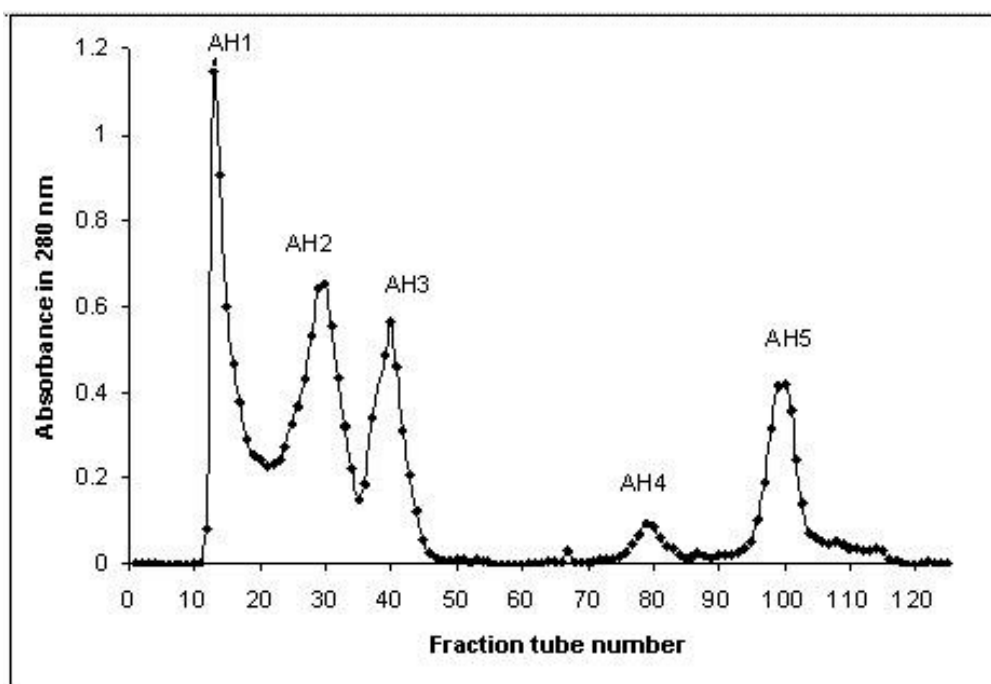


Figure 1. Crude venom of *Agkistrodon halys* (182.5 mg) was applied to Sephadex G-50 column (3 x 150 cm) using buffer ammonium acetate 50 mM and pH 7.5. Flow rate was 60 mL/hour and 9-mL fractions per tube were collected.

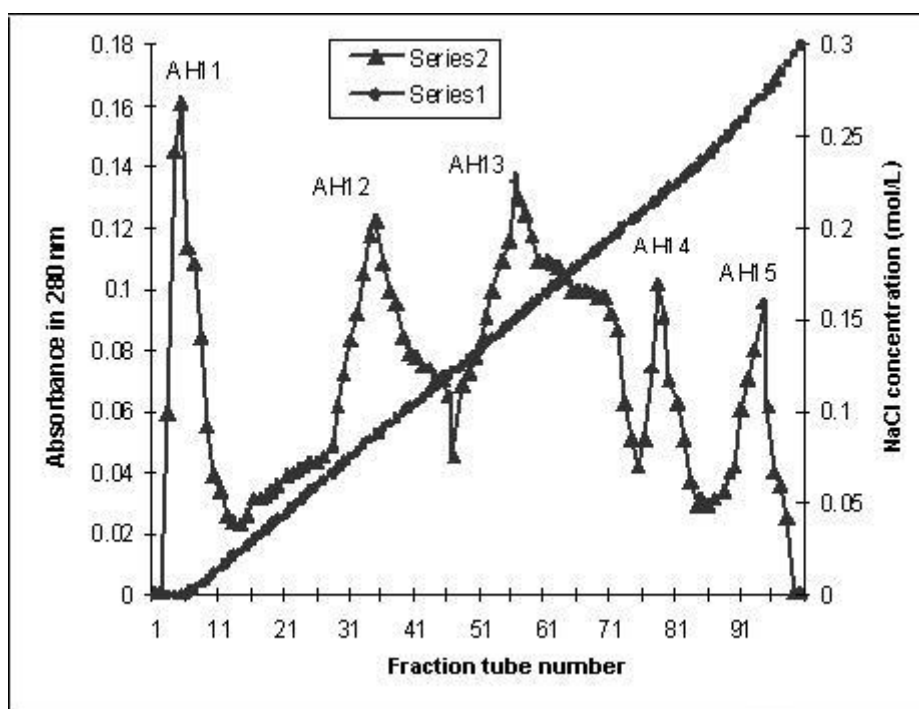


Figure 2. DEAE-Sepharose chromatography of AH1 fraction obtained from Sephadex G-50. The pooled fractions from Figure 1 were applied to DEAE-Sepharose column (2.5 x 20 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.2). Proteins were eluted with a linear concentration gradient of NaCl from 0 to 0.5 M and 4.5-mL fractions per tube were collected.

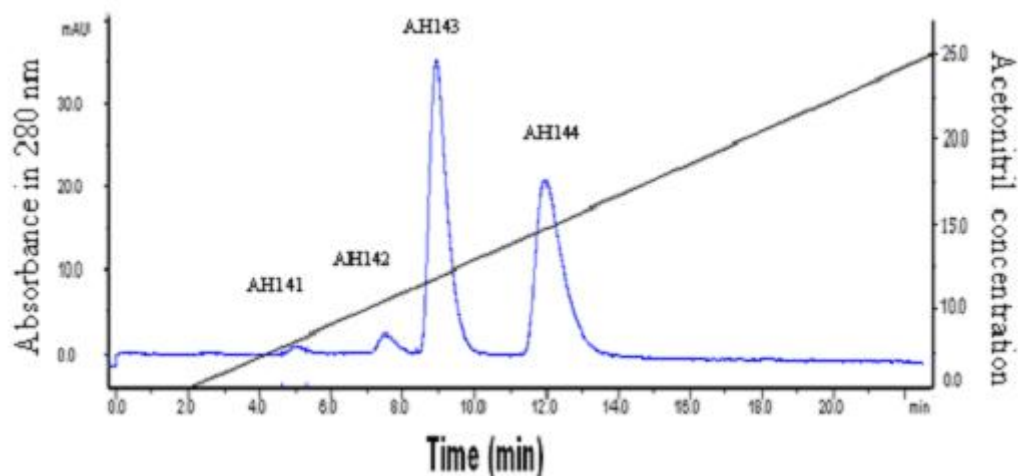


Figure 3. HPLC chromatography of AH14 fraction obtained from DEAE-Sepharose chromatography. AH14 was applied on a HPLC C18 column equilibrated with solvent A (H₂O, 0.1% TFA) and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% TFA) from 0 to 30%, at a flow rate of 0.5 mL/minute during 30 minutes.

Purity and Determination of Molecular Mass

The homogeneity of purified enzymes was confirmed by SDS-PAGE and HPLC as shown in Figures 4 and 5, respectively. Isolated AH143 revealed higher purity, analyzed by C18 reversed-phase HPLC (Figure 4). The chromatographic profile of

AH143 at 280 nm did not show the contaminating peptide. Furthermore, this enzyme showed a single band by SDS-PAGE (Figure 6) whereas its molecular mass was estimated to be about 30 kDa under reducing conditions.

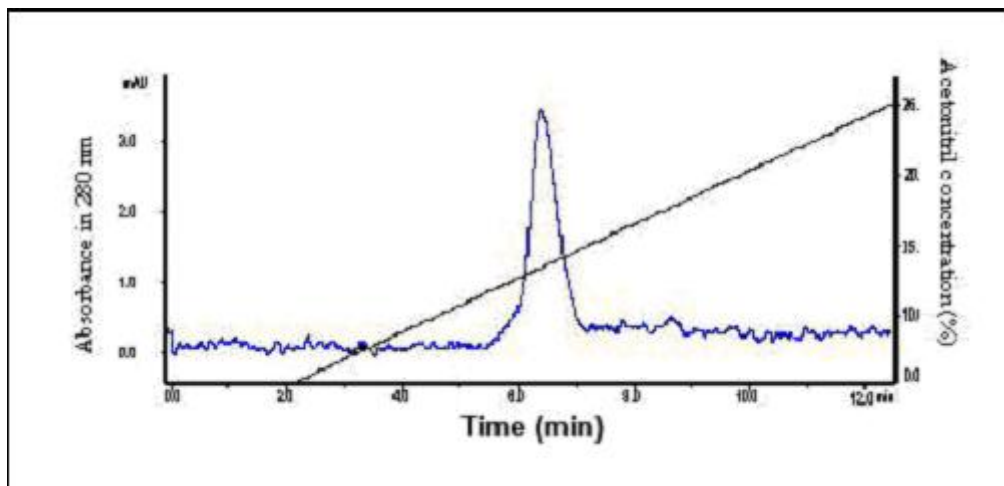


Figure 4. Purity assay of AH143 by HPLC.

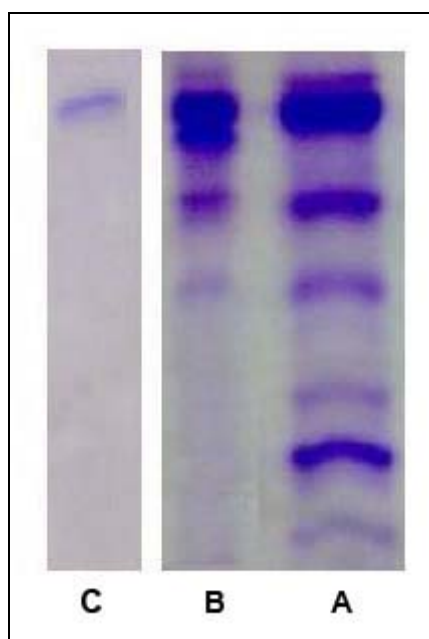


Figure 5. AH1 (A), AH14 (B) and AH143 (C) were analyzed by 12.5% SDS-PAGE in the presence of 1% 2-mercaptoethanol.

Caseinolytic, Clotting and Amidolytic Activity Assays

AH143 showed no proteolytic activity on casein while coagulant activity assay demonstrated that it presented serine protease activity on BAPNA. AH143 also showed clotting activity on human plasma (Figure 6) and at a concentration of 15 $\mu\text{g}/\text{mL}$ it was able to coagulate plasma in 20 seconds. However, at lower

concentrations (less than 15 $\mu\text{g/mL}$), this enzyme did not show any coagulant activity. The time of plasma coagulation decreased with increased concentrations of AH143.

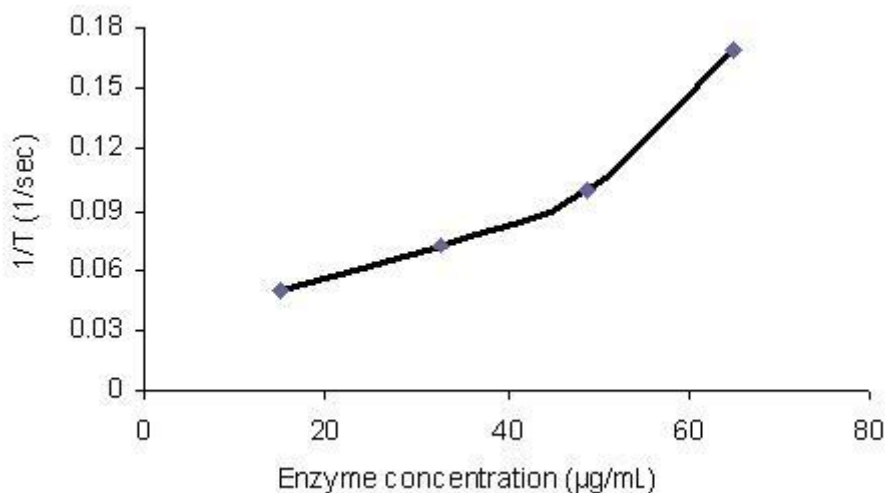


Figure 6. Clotting activity of AH143 on human plasma.

Inhibition of Enzyme Activity

The amidolytic activity of AH143 was not affected by metal chelator (EDTA) or heparin.

Effect of pH and Temperature on Activity

The optimum temperature for AH143 catalysis was evaluated in terms of amidolytic activity on BApNA. AH143 activity after incubation at different pH, ranging from 7 to 8, was high (Figure 7A) and was stable at temperatures ranging from 20 to 40°C (Figure 7B). AH143 maximum activity was found to be at 7.5. At pH 6.0, AH143 activity was about 50% of the maximum; at pH 9.0, it was about 65%; while almost 95% of the activity was observed at pH 8.0.

AH143 displayed progressively higher hydrolysis rates from 20 up to 40°C. The optimum temperature for its activity was 37°C. The enzyme lost almost 60% of its maximum activity at temperatures above 50°C, being completely inactivated at 60°C.

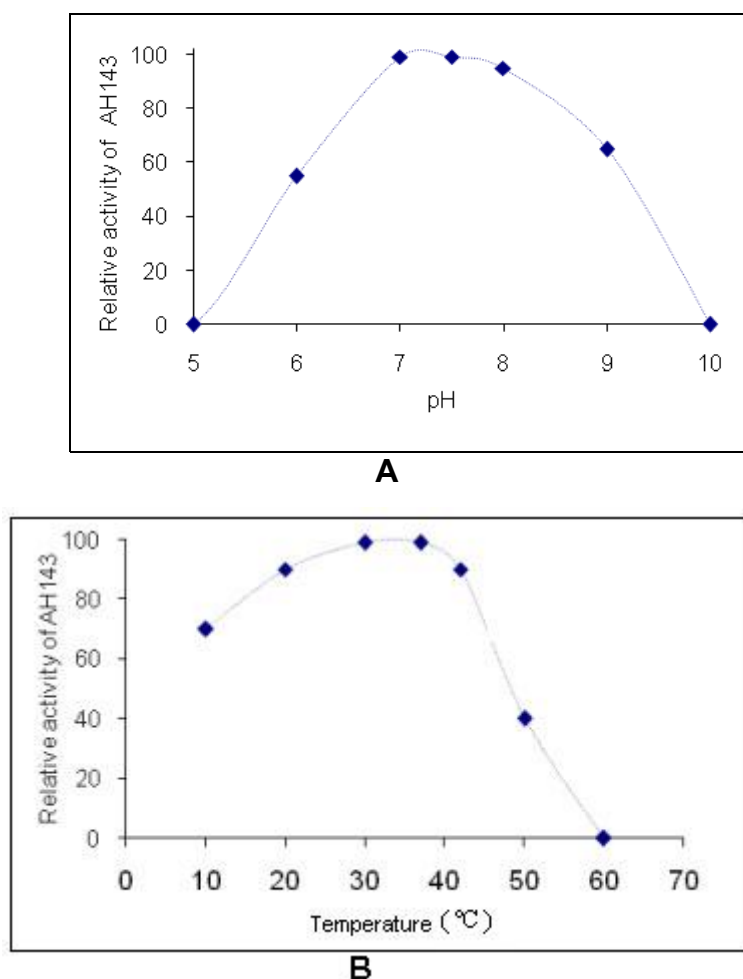


Figure 7. AH143 activity depends on pH (A) and temperature (B).

DISCUSSION

Coagulant enzymes that can convert fibrinogen into fibrin gel are widely distributed in Crotalidae snake venoms (8, 22, 24). The selected venom for this study was from the same family group.

This article reports an efficient and relatively simple procedure for the isolation of a highly purified thrombin-like serine protease from the venom of Iranian *Agkistrodon halys*, called AH143. This enzyme was isolated and purified by a combination of gel filtration on Sephadex G-50 (Figure 1), ion-exchange chromatography on DEAE-Sepharose (Figure 2) and HPLC on C18 column. However, the total yield of the enzyme accounted only for 36% (Table 1). This may be due to the partial loss of the biological activity of the enzyme. On the other hand, HPLC was able to isolate a protein with same type of activity, but with different molecular mass (results

unpublished) which could be the reason for reduced specific activity in the purification final step.

In conventional methods, the thrombin-like protein represent around 1% of the venom (2), but through the method herein described, it accounted for approximately 0.83% of the venom protein, out of which AH143 was found to be 0.45% (Table 1). The amount of TLE in snake venoms from diverse species or in the same species from various places can be different (6, 7). Analyses of AH143 activity in various conditions of temperature and pH were carried out in accordance with Nikai *et al.* (13). The temperature under which the purification was performed was based on the observation that the enzyme solution was stable at 4°C (4, 7). Several reports indicate that the optimum pH for TLE activity is in the range of 7.0 to 8.5, being practically inactive at 5 (4).

The molecular mass of AH143 is similar to other thrombin-like enzymes already studied (6, 13). In addition, molecular mass and enzymatic activities – including plasma clotting, amidolytic activity and not inhibition by heparin – characterize AH143 as a thrombin-like enzyme isolated from *Agkistrodon halys* venom (Figures 5, 6 and 7) (4, 15, 20, 21, 25, 26).

Since AH143 is not inhibited by heparin, it can be used in quantitative determination of fibrinogen as well as in plasma of patients under heparin treatment and in the laboratory for routine assays of coagulation factors (4).

In conclusion, the venom of Iranian snake (*Agkistrodon halys*) contain about 0.45% of single procoagulant protein with molecular mass of about 30 kDa which it seems to be a thrombin-like enzyme.

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