

Purification and Partial Characterization of a Thrombin-Like Enzyme (AH144) from Venom of Iranian Snake *Agkistrodon Halys*

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ABSTRACT: The snake venom's thrombin-like enzymes comprise a number of serine proteases, which are functionally and structurally related to thrombin. Purification and partial characterization of a thrombin-like enzyme from the venom of the Iranian snake, *Agkistrodon halys*, was the aim of this study. Purification was carried out by a combination of variety of chromatographic methods that included: gel filtration on Sephadex G-50, ion-exchange chromatography on DEAE-Sepharose and HPLC with a C18 column. A trial for the purification of protease resulted in an enzyme with specific activity of 721.2 ($\mu\text{mol}/\text{min}/\text{mg}$), which was purified by 72.1 fold. The purified thrombin-like enzyme designated AH144 was found to have a molecular weight of approximately 30.5 kDa. This thrombin-like enzyme had the highest activity at 37 °C and pH 7.5. Enzyme activity increased as its concentration increased, and the purified enzyme did not have any effect on casein. AH144 demonstrated clotting and proteolytic activities in the presence of the human plasma and the synthetic substrate (BApNA), respectively. Data emphasized the possibility of AH144 for quantitative determination of fibrinogen.

KEY WORDS: Iranian snake venom, *Agkistrodon halys*, Thrombin-like enzyme, Purification, Coagulant activity.

INTRODUCTION

Snake venoms contain a variety of proteolytic enzymes affecting the host coagulation process [1,2]. One of these enzymes responsible for in vitro blood-clotting activity present in several snake venoms is a serine protease which resembles at least in part thrombin, a multifunctional protease that plays a key role in coagulation.

Hence, these enzymes are denominated snake venom Thrombin-Like Enzymes (TLEs), and are widely distributed in the venoms of several genera [3,4]. Thrombin-like Enzymes (TLEs) from snake venoms belong to a class of serine proteases that can cause blood clotting in vitro, a feature exhibited by several snake venoms.

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These enzymes render the blood uncoagulable when acting in vivo apparently by depleting the circulating fibrinogen [5]. They preferentially release either fibrinopeptide A or B, rarely both with equal efficiency, unlike thrombin [1] and cannot activate factor XIII [6]. Therefore, thrombin-like enzymes hydrolyze fibrinogen to produce noncross-linked fibrins which are more susceptible to the lytic action of plasmin than the thrombin-induced cross-linked clots. These clots formed by the action of thrombin-like enzymes are removed from the circulation either by fibrinolysis or via the reticulo-endothelial system [6]. These unique properties enable clinical use of TLEs as defibrinogenating agents; for example, ancrod (Arvin; *Calloselasma rhodostoma*) and batroxobin (Defibrase; *Bothrops atrox moojeni*) [1]. Many snake venoms of the Crotalidae and Viperidae contain proteolytic enzymes which exert some effect on the blood coagulation process [5]. Since fibrinogen-clotting enzyme was first discovered from snake venom in the early years of the last century, a lot of TLEs have been isolated from many different species of snake [6]. At present, more than 30 TLEs primary structures are known that share the active site sequence motif, in which important residues (His57, Asp102, Ser195) are highly conserved [4]. These fibrinogen-clotting enzymes are widely distributed within several pit viper genera (*Agkistrodon*, *Bothrops*, *Lachesis* and *Trimeresurus*), as well as some true vipers (*Bitis* and *Cerastes*) and Colubrid, *Dispholidus typus* [5].

Different methods for TLEs preparation from snake venom, which generally consist of mixtures of more than 20 different pharmacologically active polypeptides, have already been described. These processes are essentially based on chromatographic steps and results the TLEs in varying yield and purity. These processes often use some chromatographic steps (gel chromatography, ion exchange chromatography, affinity chromatography, HPLC or FPLC) to purify and remove foreign proteins and other constituents from the crude enzyme [3-4].

Up to our knowledge no report has been published on purification of this enzyme from Iranian snake *A. halys*. Hence in this study we attempt to purify the enzyme from Iranian snake *A. halys* and study the partial characterization of it.

EXPERIMENTAL SECTION

Materials

Lyophilized crude venom of *A. halys* was prepared by Razi Vaccine and Serum Research Institute of Iran.

The venom is lyophilized by Christ Alpha 1-2 lyophilizer made by Martin Christ Company from Germany. The lyophilization carried out at -50 °C for 24 hours. DEAE-Sepharose, Sephadex G-50 and C18 columns were purchased from Pharmacia (Sweden). Bovine serum albumin, BApNA (N2-benzoyl-DL-arginine-p-nitroanilide), kit of standard protein markers and other Reagents for enzymatic and biochemical assays were purchased from Sigma-Aldrich Corporation (USA). Ammonium acetate, Tris, NaCl, Trifluoroacetic acid, Acetonitrile, Sodium citrate, Dimethyl Methyl SulfOxide (DMSO), HCl, Ethylene Di-amine Tetra Acetic Acid (EDTA) were purchased from Merck (Germany).

Isolation of the thrombin-like enzyme

Lyophilized crude venom of *A. halys* (200mg) was dissolved in 8 mL of 50 mM ammonium acetate buffer (pH 7.4) and centrifuged at 5000 rpm for 15 min at 4 °C and was filtered by 0.45 µm filter to remove the insoluble materials.

The clear supernatant was applied on a molecular exclusion chromatographic column of Sephadex G-50 (150 cm × 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/h at 4 °C. The obtained fractions were named AH1 to AH5, Indicating *A. halys* fractions 1 to 5.

The fraction showed clotting activity from the previous gel chromatography step, AH1, was pooled and dialyzed overnight at 4°C against distilled water and applied on DEAE-Sepharose CL-6B (2.5x20cm) 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 M. The flow rate was 17 mL/h and 5 mL fractions were collected at 4°C.

The fraction showed clotting activity from the previous steps was pooled and dialyzed overnight at 4°C and applied on an HPLC C18 column which was equilibrated with solvent A (H₂O, 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/min during 30 min. The peaks were monitored through the A₂₈₀.

Purity and Determination of molecular weight

Purity analysis carried out with HPLC on C18 column as previously described. 12% SDS-PAGE was performed

according to *Laemmili* and using molecular weight standard of low molecular weight ranging from 6.5 KDa to 66 KDa [7].

Characterization of the thrombin-like enzyme Plasma preparation

Normal pooled plasma was made from 10 individual healthy donors, without history of bleeding or thrombosis and mixed with 3.8% Sodium citrate as anticoagulant. Blood was centrifuged for 20 min at 4500 rpm, and the plasma was used freshly.

Prothrombin time (PT) assay

Prothrombin time reagent (200 μ L) and aliquots of sample (200 μ L), pre-incubated for 10 min at 37 °C and mixed. Then 100 μ L of plasma was added and clotting time was recorded [8].

Determination of amidolytic activity

Proteolytic activity in the samples was assayed using method of *Stewart* [9]. 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 7.5). Proteolytic activity was monitored as BApNA hydrolysis by mixing 50 μ L of sample with 100 μ L of the substrate solution. After 10 min incubation at 37 °C, the absorbance at 410 nm was measured. One unit of proteases activity corresponds to an increase of $A_{410} = 0.001/\text{min}$ [3,10].

Coagulant activity

Plasma (200 μ L) and aliquots of sample with different amounts of enzyme (15, 32.5, 48 and 65 $\mu\text{g/mL}$) were pre-incubated briefly at 37 °C, then they were mixed and shaken and the clotting times were recorded [3,6].

Proteolytic activity on Casein

Proteolytic activity was determined using *Sant'Ana* method [3]. Various amounts (15, 32.5, 48 and 65 $\mu\text{g/mL}$) of the enzyme were added to buffer solution 0.1 M Tris-HCl of 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 min at 37 °C. The reaction was stopped by addition of 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 10 min. One unit of caseinolytic activity corresponds to an increase of $A_{280} = 0.001/\text{min}$.

Inhibition of enzyme activity

The effect of Ethylene Di-amine Tetra acetic Acid (EDTA) and heparine were examined by incubation with enzyme in 180 μ L of 20 mM Tris-HCl with optimum pH at 37.0 °C for 5 min. 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 to be 100% activity [3].

Optimal pH and temperature for enzyme activity

The effect of pH on enzyme activity was evaluated by measuring the enzyme activity after incubation at various pHs 4.0 to 9.0 (using 0.1 M Citrate-NaOH buffer for pHs ranging from 7.0 to 10 and using 0.1 M Tris-HCl buffer for pHs ranging from 4.0 to 7.0) at 37 °C for 10 min.

Enzyme activity was assayed at different temperatures ranging from 20 °C to 70 °C using BApNA as a substrate. The assay was conducted at pH 7.5 using 50 mM Tris-HCl buffer containing 0.02 M CaCl_2 for 5 min in a temperature controlled water bath. Thereafter, the heat treated samples were rapidly cooled in an iced bath, and residual activity was assayed using BApNA as a substrate at pH 7.5 and 25 °C for 5 min, as previously described [10].

Protein determination

Protein concentration was measured by the method of *Lowry et al* using BSA as a standard [11].

RESULTS AND DISCUSSION

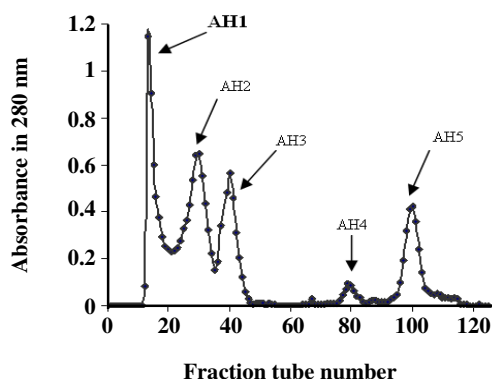
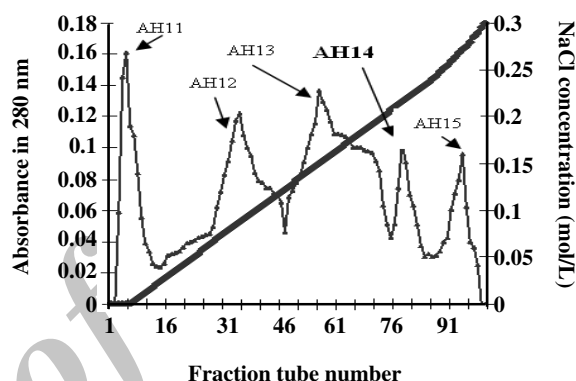
Isolation of the thrombin-like enzyme

The coagulant enzymes which can convert fibrinogen to fibrin gel are widely distributed in crotalidae snake venoms [3]. The selected venom for this study was from the same family group. In the initial Sephadex G-50 fractionation of the crude venom five peaks having absorbance at 280 nm were obtained (AH1-AH5) as shown in (Fig. 1). When all the fractions were tested

Table1: Purification of a thrombin-like enzyme (AH144) from *A. halys* venom.

Step	Protein (mg)	Specific activity* ($\mu\text{mol}/\text{min}/\text{mg}$)	Total amidase activity ($\mu\text{mol}/\text{min}$)	fold	Yield (%)
Venom	182.5	10	1801.9	1	100
AH1	48.12	32.39	1558.6	3.24	91
AH14	1.8	622.2	1120	62.22	65
AH144	0.7	721.2	146	72.1	36

* Activity was determined using BApNA as the substrate.

**Fig. 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 buffer ammonium acetate 50mM and PH 7.5.****Fig. 2: DEAE-Sepharose chromatography of AH1 obtained from Sephadex G-50. The AH1 pooled fractions were applied to DEAE-Sepharose column (2.5 x 20 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.2). Series 1: Absorbance in 280 nm, Series 2: NaCl Concentration (mol/l)**

for coagulation, it was found that fraction AH1 showing pro-coagulation test positive. The yield of pro-coagulant enzyme fraction (AH1) was calculated and found to be 91% (Table 1). In this report, yield was defined as the amount of protein in the outlet of chromatography to the total amount of initial protein.

Further purification was carried out by ion exchange chromatography on DEAE-Sepharose resin. In this step of purification 5 fractions were obtained (AH11-AH15) of which fraction AH14 showed pro-coagulant activity (Fig. 2). The total yield of the fraction AH14 was 65%. The fraction AH14 that showed coagulant activities were pooled, dialyzed and applied to a reverse-phase HPLC C18 column. Four peaks (AH141-AH144) were obtained (Fig. 3); AH144 had coagulant activity. The PT-test reached its maximum at the elution time of 9 and 12 min. The purification procedure of AH144 is summarized

in Table 1. By this purification procedure, about 0.7 mg of purified enzyme was obtained from 182.5 mg of the venom.

However the total yield of the enzyme accounted for 36 % only. This may be due to the partial loss of the biological activity of the enzyme in purification procedure. On the other hand, in HPLC chromatography we were able to isolate a protein with same type of activity but with different molecular weight or polarity [3,5,6].

Characterization of the thrombin-like enzyme

The purity of AH144 was confirmed by SDS-PAGE and HPLC As shown in Fig. 4 and Fig. 5, respectively. Isolated AH144 showed high purity as analyzed by C18 reverse phase HPLC. The chromatographic profile of AH144 sample at 280 nm does not show the

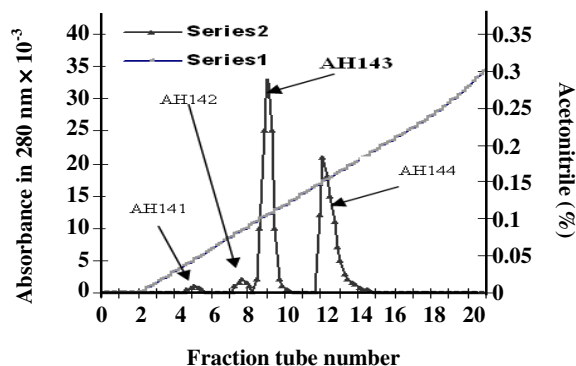


Fig. 3: HPLC chromatography of AH14 obtained from DEAE-Sepharose chromatography. AH14 was applied on an HPLC C18 column which was equilibrated with solvent A (H_2O , 0.1% Trifluoroacetic acid), and eluted with a concentration gradient of solvent B (Acetonitrile, 0.1% Trifluoroacetic acid) from 0 to 30%, at a flow rate of 0.5 mL/min during 30 min

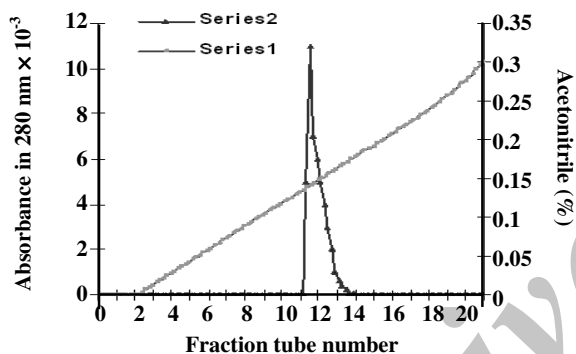


Fig. 4: Purity assay of AH144 by HPLC. Series 1: Absorbance in 280 nm, Series 2: NaCl Concentration (mol/l).

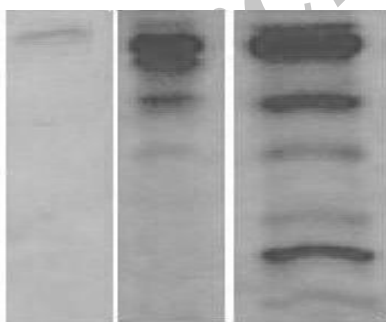


Fig. 5: SDS-PAGE of AH1 (Sephadex G-50 chromatography) (A), AH14 (DEAE-Sepharose chromatography) (B) and AH144 (HPLC chromatography) (C) was analyzed on 12.5% SDS-polyacrylamide gel in the presence of 1% 2-mercaptoethanol.

contaminating peptide. This enzyme showed a single band in SDS-PAGE too. The molecular weight of this enzyme was estimated to be about 30.5 kDa under reduced condition. The molecular weight of this thrombin-like AH144 is similar to other enzymes of the same class already studied such as: BjuusuSP-I (37 kDa) [3], leucurobin (35 kDa) [5] and calobin II (41 kDa) [6].

AH144 showed no proteolytic activity upon casein and show clotting activity upon human plasma (Fig. 6). Amidolytic activity assay showed that AH144 had serine protease activity on BApNA. This enzyme, at concentration of 15 $\mu\text{g/mL}$ of protein coagulated plasma at 20 sec and at lower concentrations less than 15 $\mu\text{g/mL}$ did not show any coagulant activity. The coagulation time of plasma decreased with increased concentration of enzyme. The amidolytic activity of AH144 was not affected by a metal chelator (EDTA) and heparin.

The mention characters of isolated enzyme included molecular weight [3,5,6], plasma Clotting activity [3,5,6,12], amidolytic activity [3,5,6] and activity in presence of heparin [3,5] characterize AH144 as a thrombin-like enzyme isolated from Iranian *Agkistrodon halys* venom. Since AH144 is not inhibited by heparin, the enzyme can be used in quantitative determination of fibrinogen, especially in the plasma of patients under heparin treatment as well as in the blood laboratory for routine assays of coagulation factors as a research tool and as a research tool.

Optimal temperature for AH144 catalysis was evaluated as its amidolytic activity on BApNA. AH144 activity after incubation at different pH ranging from 7-8 was high (Fig. 7A), and its activity was stable at temperature within the range of 20 $^{\circ}\text{C}$ to 42 $^{\circ}\text{C}$ (Fig. 7B). The maximum Activity of AH144 was at pH 7.5. The optimum temperature for its activity was 37 $^{\circ}\text{C}$. At temperatures above 50 $^{\circ}\text{C}$, the enzyme lost about 64% of its activity and it was completely inactivated at 60 $^{\circ}\text{C}$.

CONCLUSIONS

This article reports a procedure for the isolation of a highly purified thrombin-like enzyme from Iranian *Agkistrodon halys* venom, which was named AH144. This venom contain about 0.38% of a pro-coagulant protein which it seems to be thrombin-like enzyme. The molecular weight of this enzyme was estimated to be about 30.5 kDa. Optimum temperature and pH for its activity was 37 $^{\circ}\text{C}$ and 7-8.

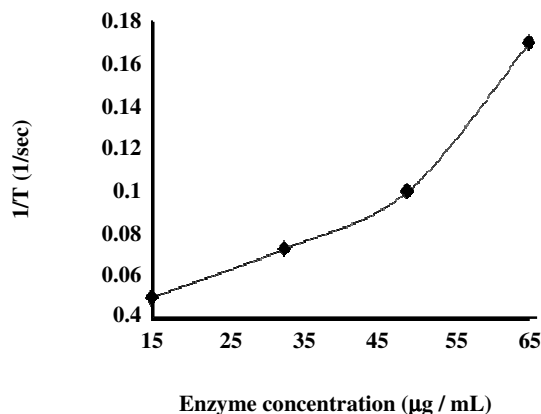


Fig. 6: Clotting activity of AH144 upon human plasma.

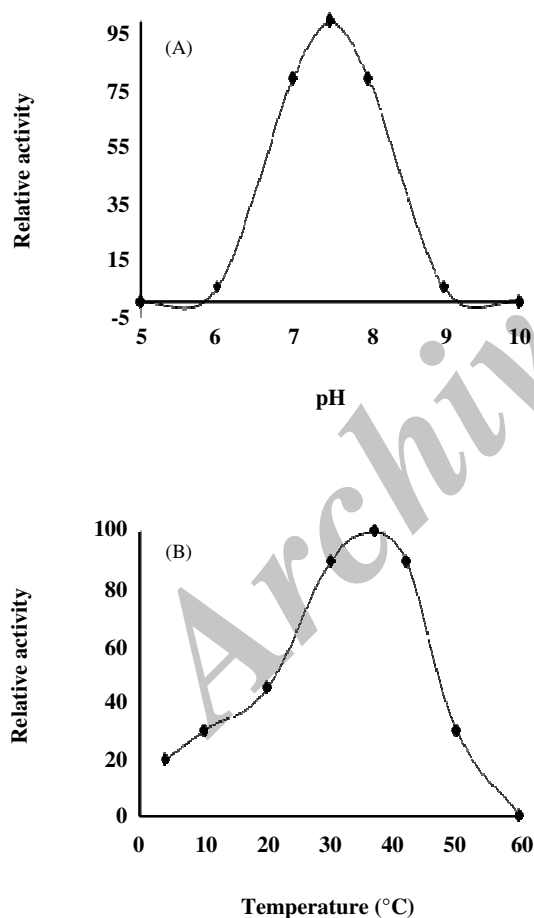


Fig. 7: Activity dependence of AH144 on pH (A) and temperature (B).

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