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# Ammodytase, a metalloprotease from *Vipera ammodytes ammodytes* venom, possesses strong fibrinolytic activity

Adrijana Leonardi[a], Jay W. Fox[b], Alenka Trampuš-Bakija[c], Igor Križaj[a,*]

[a] *Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, 1000 Ljubljana, Slovenia*
[b] *Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, USA*
[c] *Medical Centre Ljubljana, Division of Pediatrics, 1525 Ljubljana, Slovenia*

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**Abstract**

Ammodytase, a high molecular mass metalloproteinase with fibrinogenolytic and fibrinolytic activities, was purified from long-nosed viper (*Vipera ammodytes ammodytes*) venom by gel filtration, affinity and ion-exchange chromatographies. The enzyme is a single-chain glycoprotein with apparent molecular mass of 70 kDa and isoelectric point of 6.6. Ammodytase shows very weak hemorrhagic activity, and only at doses higher than 20 μg. Consistent with this, it partially degrades some components of the extracellular matrix *in vitro*. It cleaves the Aα-chain of fibrinogen preferentially at peptide bonds $Glu^{441}$–$Leu^{442}$ and $Glu^{539}$–$Phe^{540}$. Its preference for bulky and hydrophobic amino acids at the P1′ position in substrates is demonstrated by its hydrolysis of only the $Gln^{4}$–$His^{5}$ and $Tyr^{16}$–$Leu^{17}$ bonds in the B-chain of insulin. Ammodytase is able to dissolve fibrin clots. It neither activates nor degrades plasminogen and prothrombin, and has no effect on collagen- or ADP-induced platelet aggregation *in vitro*. LC/MS and MS/MS analyses of its tryptic fragments demonstrated that ammodytase is a P-III class snake venom metalloproteinase composed of metalloproteinase, disintegrin-like and cysteine-rich domains. Its similarity to hemorrhagins from *V. a. ammodytes* venom, accompanied by very low toxicity, makes ammodytase a promising candidate as an antigen to prepare antisera against these most dangerous components of the viper's venom. Moreover, its ability to degrade fibrin clots suggests its clinical use as an antithrombotic agent.


*Keywords:* Snake venom; *Vipera ammodytes ammodytes*; Metalloproteinase; Fibrinogenase; Fibrolase; Antihemorrhagic; Antithrombotic

## 1. Introduction

Snake venoms (SVs) contain many biologically active components that affect hemostasis. These may interact with platelets, clot or degrade fibrinogen, activate plasminogen, prothrombin and different clotting factors (White, 2005). Fibrin(ogen)olytic enzymes have been isolated from venoms of Viperidae, Elapidae and Crotalidae snakes (Markland, 1998; Swenson and Markland, 2005). The majority of SV fibrin(ogen)olytic enzymes are zinc metalloproteinases, and are grouped, together with the trans-membrane A Disintegrin-like and Metalloproteinase-containing proteins (ADAMs), among reprolysins, a subfamily of the M12 family of metalloproteinases (Rawlings and Barett, 1995; Jia

*Corresponding author. Tel.: +386 1 477 36 26; fax: +386 1 477 39 84.
*E-mail address:* igor.krizaj@ijs.si (I. Križaj).

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et al., 1996). Some SV fibrin(ogen)olytic enzymes are hemorrhagic, others are not. According to their proteolytic preference towards the Aα- or Bβ-chain of fibrin(ogen), they are classified as α- and β- fibrin(ogen)ases. In contrast to serine proteinases, which induce fibrinolysis through activation of plasminogen, SV fibrinolytic enzymes act directly on fibrin and are not inhibited by serpin proteinase inhibitors in the blood. The absence of effective inhibitors in the blood has raised interest in the potential therapeutic use of SV non-hemorrhagic fibrinolytic metalloproteinases to dissolve occlusive thrombi. Alfimeprase, a recombinant fibrinolytic enzyme derived from SV metalloproteinase (SVMP) fibrolase from *Agkistrodon contortrix contortrix* venom, has recently been introduced into clinical trials (Toombs, 2001a; Ouriel et al., 2005; Moll et al., 2006).

*Vipera ammodytes ammodytes* is the most venomous European snake. The most pronounced consequences of envenomation by this snake are necrosis and hemorrhage and, to a lesser extent, neurotoxic effects (Lukšić et al., 2006). By analyzing the components of *V. a. ammodytes* venom that affect hemostasis, we have already described two potently hemorrhagic metalloproteinases, VaH1 and VaH2 (Leonardi et al., 2001). Here we report another venom component acting on hemostasis with characteristics that make it a very promising candidate for clinical applications. This metalloproteinase, ammodytase, is essentially non-toxic and expresses potent fibrinolytic activity.

## 2. Materials and methods

Crude *V. a. ammodytes* venom was obtained from the Institute of Immunology, Zagreb, Croatia. All other chemicals were of analytical or sequence grade.

### 2.1. Purification

The initial stages of purification of ammodytase were essentially as described in Leonardi et al. (2001). Crude venom was first fractionated on a Sephacryl S-200 column, followed by affinity chromatography on Concanavalin A-Sepharose and anion-exchange chromatography on DEAE-Sephacel.

#### 2.1.1. FPLC on Mono-S

Unbound proteins from DEAE-Sephacel chromatography were applied on a Mono-S HR 5/5 FPLC column (Amersham Biosciences, Bucks, UK) equilibrated with 50 mM MES, 2 mM $CaCl_2$, pH 6.5. Proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M in the equilibration buffer over 30 min at a flow rate of 1 ml/min. Fibrinogenolytic and hemorrhagic activities of pooled fractions were tested by methods described in Sections 2.3. and 2.5., respectively. In tests, 1 μM PMSF was present to inhibit activities associated with serine proteases.

#### 2.1.2. FPLC on Superdex 75

Ammodytase in fraction D of the Mono-S FPLC separation step was separated from remaining impurities on a Superdex 75 column (Amersham Biosciences, Bucks, UK), equilibrated with 20 mM imidazole, 0.3 M NaCl, 2 mM $CaCl_2$, pH 7.0. The flow rate was 0.5 ml/min. Throughout the purification, the proteins were traced by monitoring absorbance at 280 nm. Fibrinogenolytic and hemorrhagic activities of pooled fractions were tested as described in Section 2.1.1.

### 2.2. SDS-polyacrylamide gel electrophoresis and isoelectric focusing

The isolated protein was analyzed by SDS-PAGE on 12% (w/v) polyacrylamide gel under reducing conditions according to Laemmli (1970). Molecular mass standards used were from Fermentas (Lithuania). IEF was performed using a PhastSystem (Amersham Pharmacia Biotech, Sweden), following the manufacturer's instructions. PhastGel isoelectric focusing (IEF) 4–6.5 and 3–9 slabs ($0.35 \times 43 \times 50$ mm) and pI standards (3.5–9.3) were used. Proteins were visualized by staining with Coomassie Brilliant Blue R250.

### 2.3. Fibrinogenolytic assay

The method of Ouyang and Teng (1976) was used to assess the fibrinogenolytic activity of ammodytase. First, 62.5 μl of a solution of human fibrinogen (16 mg/ml) in 50 mM Tris/HCl, 2 mM $CaCl_2$, pH 8.5, and 37.5 μl of the ammodytase (3.5 μg) were mixed and incubated at 37 °C. Then, 5 μl aliquots were taken after 5, 15, 30, 60 and 120 min. Fibrinogen fragments were separated by SDS-PAGE on 12% (w/v) polyacrylamide gel under reducing conditions, electro-transferred to a polyvinylidenedifluoride (PVDF) membrane according to the manufacturer's instructions (Millipore,