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Thrombolytic protein from cobra venom with anti-adhesive properties

Chandrasekhar Chanda, Angshuman Sarkar, Dibakar Chakrabarty*

Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, K.K. Birla Goa Campus, Zuarinagar, Goa 403726, India

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ABSTRACT

A metalloproteinase anticoagulant toxin of molecular weight 66 kDa has been purified from the venom of Indian monocled cobra (*Naja kaouthia*). This toxin named as NKV 66 cleaved fibrinogen in a dose and time dependent manner. The digestion process was specific to Aα chain and cleaved fibrinogen to peptide fragments. NKV 66 completely liquefied the fibrin clots developed *in vitro* in 18 h. Plasma recalcification time and thrombin time were significantly prolonged following treatment of plasma with NKV 66. NKV 66 significantly inhibited ADP and collagen induced platelet aggregation in a dose dependent manner. It showed disintegrin like activity on A549 cells cultured *in vitro*. About 40% inhibition of adherence of A549 cells to matrix was observed following NKV 66 treatment also NKV 66 treated A549 cells were drastically inhibited from passing through the matrix in cell invasion assays *in vitro*, suggesting anti-adhesive properties of NKV 66.

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1. Introduction

Neuromuscular and circulatory systems are the two major physiological communication systems targeted by many toxins to spread the toxicity in widely in a short time. Over the years, a number of toxins affecting blood circulation have been isolated and characterized from various snake venoms [1,2]. Some of them affect platelet aggregation as well as blood coagulation cascade. Whereas, some can affect the platelet systems only. Venom proteins affecting blood coagulation can functionally be classified as pro-coagulant or anticoagulant toxins on the basis of their ability to shorten or prolong the blood-clotting process.

Metalloproteinases constitute a family of enzymes which are classified by the nature of most prominent functional group in their active site. These are generally proteolytic enzymes involving a metal ion in their catalytic mechanism. Most metalloproteases are zinc-dependent, but some also use cobalt. Snake venom metalloproteinases (SVMP) comprise a subfamily of zinc-dependent enzymes of varying molecular mass, which are responsible for the hemorrhagic and proteolytic effects [3–5]. Phylogenetically, snake venom metalloproteinases are closely related to mammalian ADAM (a disintegrin and metalloproteinase) family of proteins. Structural analysis of these snake venom metalloproteinases classifies them into four groups (P-I–P-IV) [6].

Many of the snake venom metalloproteinases were found to possess fibrin(ogen)olytic property by digesting C terminal end of fibrinogen and making it unavailable for polymerization [7,8]. These fibrinogenolytic proteins are classified into α-fibrinogenases and β-fibongenases depending on the digestion of Aα or Bβ chain of fibrinogen [9–11]. Some snake venom metalloproteinases with disintegrin domain were found specifically inhibiting integrin functions [12,13]. Disintegrins comprise a family of non-enzymatic, low molecular weight, cystein-rich peptides usually derived from the proteolytic processing of snake venom metalloproteinases (SVMPs). Inhibition of platelet aggregation is mostly due to the non-proteolytic domain *i.e.*, the disintegrin domain of the SVMPs [14–16]. Very few reports of metalloproteinase anticoagulant toxins are currently available from the Elapid snake venoms. Natrahagin from *Naja atra* [17] and proteinase F1 from *Naja nigricollis* [18] were reported preferentially cleaving the Aα chain and slowly the Bβ chain. Ohagin, another metalloproteinase from *Ophiophagus hannah* venom [19] specifically cleaved the Aα chain of fibrinogen. In this article, we present purification, biochemical

Abbreviations: SVMP, snake venom metalloproteinase; RP-HPLC, Reversed phase high performance liquid chromatography; EDTA, Ethylene di-amine tetra acetate; ADP, Adenosine di-Phosphate; CM-Sephadex, Carboxymethyl- Sephadex; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, Phenyl methane sulfonyl fluoride.

* Corresponding author.
E-mail addresses: diba27@yahoo.com, dibakarchakrabarty@goa.bits-pilani.ac.in (D. Chakrabarty).

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characterization and anticoagulant studies of a novel metalloproteinase anticoagulant protein (66 kDa) purified from the venom of Indian monocled cobra.

2. Materials and methods

Dry pooled *Naja kaouthia* venom was purchased from Calcutta snake park, Kolkata, India. CM-sephadex C-50, ADP, bovine fibrinogen and bovine thrombin were purchased from Sigma–Aldrich, India. Collagen was purchased from Chronolog Corporation, USA. Protein molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. A549 cells were obtained from National Centre for Cell Science, Pune, India.

2.1. Purification of NKV 66

Dry cobra venom (50 mg) was reconstituted in 20 mM phosophate buffer, pH 7.4, overnight at 4 °C. The solution was then centrifuged at 5000 rpm for 5 min at 4 °C to discard cell debris and other particulate matters. The clear supernatant was then fractionated using CM-sephadex C-50 cation exchange column (1.5 cm × 10.5 cm) with a flow rate of 0.5 ml/min. The adsorbed fractions were eluted with a linear gradient of NaCl (0–0.5 M) in 3 column volumes of running buffer. Fraction size was restricted to 1 ml.

Fractions with anticoagulant properties from the purification I were pooled and subjected to further fractionation (Purification II) on a CM-sephadex C-50 column (1.5 cm × 10.5 cm). Fractions were eluted with a shallow linear gradient of NaCl (0.2–0.5 M). Fraction size was restricted to 1 ml with a flow rate of 0.5 ml/min. An anticoagulant protein, named Lahirin was reported in JBSc in 2011 [20] by this group. But later it was found that the Lahirin is not in its purest form. It was contaminated with a very small quantity of a metalloproteinase anticoagulant protein and the anticoagulant properties shown by Lahirin were partly due to the simultaneous effects of two proteins. Purification to homogeneity of the metalloprotease from semi-purified fraction obtained from purification II was achieved using reverse phase-HPLC. One milligram of semi-purified fraction was dissolved in 200 μl of water containing 0.1% tri-fluoroacetic acid (TFA; solution A). The solution was applied to a Shimadzu C18 reverse phase column. Elution was performed at a flow rate of 1 ml/min. A linear gradient of 0–80% acetonitrile was maintained with solution B (acetonitrile containing 0.1% TFA). Elution of proteins was monitored at A_{220} nm. Eluted fractions in major peaks were immediately concentrated by removing acetonitrile using Genevac centrifugal vacuum concentrator.

Active semi-purified fractions from purification II were pooled and concentrated against sucrose. 100 μl of sample along with 10 μl of blue dextran and 10 μl of bromophenol blue was applied to a sephadex G-75 column (50 cm × 1 cm). Fractionation was performed using three bed volumes of 20 mM K-Phos (pH 7.4) with 0.1 M NaCl. Absorbance values of the fractions were measured at $A_{280\ nm}$. Molecular mass of NKV 66 was determined by MALDI-TOF/TOF mass spectrometric analysis.

2.2. Assay of fibrinogenolytic activity

Bovine fibrinogen fraction I (30 μg) was incubated with different doses of purified fractions at 37 °C for 2 h. The incubated mixture was then subjected to SDS-PAGE on 12% polyacrylamide gel. Protein bands were viewed by staining the gel with 0.2% Coomassie brilliant blue R250. Fibrinogenolytic activity of toxins was monitored by comparing the band patterns and extent of fibrinogen digestion.

2.3. Assay of fibrinolytic activity

Fibrin plate method of Astrup and Mullertz [21] was followed with slight modifications. Fibrinogen type I (3.3 mg) and ammonium sulfate (70 mM) were dissolved in 0.2 ml of 20 mM potassium phosphate buffer, pH 7.4. Five microliters of thrombin was added to the above solution and transferred immediately to a 0.5 ml microfuge tube. The solution was allowed to clot by incubating at 25 °C for 2 h. Test samples were applied on top of the clot and incubated at 37 °C for 18 h. Russell's viper whole venom and 0.85% saline were used as positive and negative controls, respectively.

2.4. Platelet aggregation assay

Platelet aggregation assays were performed in whole human blood as follows. Nine parts of whole human blood was mixed with 1 part of 3.8% (w/v) sodium citrate. A Chrono-Log whole blood aggregometer was used to monitor platelet aggregation. Five hundred microliters of 0.85% saline was incubated at 37 °C for 5 min and mixed with equal volumes of citrated whole blood for each assay. Blood samples were then treated with different concentrations of NKV 66 for 2 min. Agonists of platelet aggregation in appropriate concentrations were added immediately to the above treated blood sample and impedance patterns were monitored. Blood samples treated with platelet aggregation agonist alone were taken as positive control. Blood samples without any treatment (*i.e.*, agonists or NKV 66) were taken as negative controls.

2.5. Inhibition studies

Inhibition studies of NKV 66 were performed on fibrin clots developed *in vitro* by pre-treating NKV 66 with 2 mM EDTA, 1 mM PMSF, and heating to 100 °C for 1 min in a boiling water bath.

2.6. Anticoagulant activity

Coagulation tests were performed *in vitro* on human platelet poor plasma (PPP) which was obtained following method described by [22]. Briefly nine volumes of human blood was mixed with one volume of ACD and centrifuged at 1100 RPM for 10 min at room temperature. The supernatant obtained was platelet rich plasma (PRP). PRP was then centrifuged at 4000 RPM for 15 min at room temperature and the supernatant obtained was PPP.

2.7. Recalcification time

Recalcification time of human PPP was determined following the method of Langdell et al. (1953) [23] with slight modifications. Briefly, 250 μl of 50 mM Tris–HCl buffer (pH 7.4) and 250 μl of PPP was mixed in a glass container. Three doses of NKV 66 (10 μg, 20 μg, 50 μg) were incubated with PPP for 3 min at room temperature. Clotting was initiated by the addition of 100 μl of 100 mM $CaCl_2$.

2.8. Thrombin time

Thrombin time was determined following the method described by Jim (1957) [24] with slight modifications. 250 μl of 50 mM Tris–HCl buffer (pH 7.4) and 250 μl of PPP was mixed in a glass container. Three doses of NKV 66 (10 μg, 20 μg, 50 μg) were incubated with PPP for 3 min at room temperature. Clotting was initiated by the addition of three NIH units of standard thrombin reagent.

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