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Pharmacological properties and pathophysiological significance of a Kunitz-type protease inhibitor (Rusvikunin-II) and its protein complex (Rusvikunin complex) purified from *Daboia russelii russelii* venom

Ashis K. Mukherjee [a, b, *], Stephen P. Mackessy [b]

[a] *Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784 028, Assam, India*
[b] *School of Biological Sciences, University of Northern Colorado, Greeley, CO 80639-0017, USA*

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ABSTRACT

A 7.1 kDa basic peptide (Rusvikunin-II) was purified from a previously described protein complex (Rusvikunin complex, consists of Rusvikunin and Rusvikunin-II) of *Daboia russelii russelii* venom. The N-terminal sequence of Rusvikunin-II was found to be blocked, but peptide mass fingerprinting analysis indicated its identity as Kunitz-type basic protease inhibitor 2, previously reported from Russell's Viper venom. A tryptic peptide sequence of Rusvikunin-II containing the N-terminal sequence HDRPTFCNLFPESGR demonstrated significant sequence homology to venom basic protease inhibitors, Kunitz-type protease inhibitors and trypsin inhibitors. The secondary structure of Rusvikunin-II was dominated by β-sheets (60.4%), followed by random coil (38.2%), whereas α-helix (1.4%) contributes the least to its secondary structure. Both Rusvikunin-II and the Rusvikunin complex demonstrated dose-dependent anticoagulant activity; however, the anticoagulant potency of latter was found to be higher. Both inhibited the amidolytic activity of trypsin > plasmin >> FXa, fibrinogen clotting activity of thrombin, and, to a lesser extent, the prothrombin activation property of FXa; however, the inhibitory effect of the Rusvikunin complex was more pronounced. Neither Rusvikunin-II nor Rusvikunin complex inhibited the amidolytic activity of chymotrypsin and thrombin. Rusvikunin-II at 10 μg/ml was not cytotoxic to Colo-205, MCF-7 or 3T3 cancer cells; conversely, Rusvikunin complex showed ~30% reduction of MCF-7 cells under identical experimental conditions. Rusvikunin-II (5.0 mg/kg body weight, i.p. injection) was not lethal to mice or House Geckos; nevertheless, it showed *in vivo* anticoagulant action in mice. However, the Rusvikunin complex (at 5.0 mg/kg) was toxic to NSA mice, but not to House Geckos, suggesting it has prey-specific toxicity. Rusvikunin complex-treated mice exhibited dyspnea and hind-limb paresis prior to death. The present study indicates that the Kunitz-type protein complex Rusvikunin from Russell's Viper venom significantly contributes to venom toxicity, and an important biological role in venoms appears to be facilitation of prey subjugation.

1. Introduction

Russell's Viper (RV; *Daboia russelii russelii*) bites are responsible for a heavy toll on human life in the southeast

* Corresponding author. Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784 028, Assam, India. Tel.: +91 7896003886; fax: +91 3712 267005/267006.
E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

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Asian countries including India (Warrell, 1989; Mukherjee et al., 2000). Unfortunately, minimal attention has been focused on the treatment and prevention of snakebite in these Asian countries, and snakebite is currently considered one of the neglected tropical diseases in this region (Gutiérrez et al., 2013; Bhaumik, 2013). In the Indian subcontinent, RV is considered as a category I medically important snake, and the RV envenomed patient warrants immediate medical attention.

Snakes use their venom primarily to subdue large, quick-moving prey such as rodents, allowing prey to be dispatched chemically and remotely before being swallowed. This facilitates prey capture by large-bodied snakes such as RV, which thrive on abundant rodent prey near rice field. Secondarily, the venom is also used by the snake in self-defense, and this is why rice farmers are major victims of RV bites. Further, geographical variation in venom composition results in significant differences in clinical manifestations following RV envenomation in different parts of Southeast Asia (Warrell, 1989; Mukherjee et al., 2000; Prasad et al., 1999). However, irrespective of the geographic location, the most common symptom of RV envenomation is interference in blood coagulation of victims, and several other notable complications, such as renal failure, are also commonly observed (Warrell, 1989; Mukherjee et al., 2000; Prasad et al., 1999).

Several uncharacterized peptides, in the molecular weight range of 4–8 kDa, have been observed in the venom of *D. r. russelii*, and recently, two were purified and characterized in our laboratories (Mukherjee et al., 2014a, b). Functional characterization of such novel components of Russell's Viper venom (RVV) may reveal their biological function and help advance our understanding of their molecular mechanism(s) of toxicity, in target prey as well as in RV-envenomed victims. The low molecular mass peptides (<10 kDa) in snake venoms are frequently represented by cytotoxins, three-finger toxins, cardiotoxins, neurotoxins, and Kunitz-type protease inhibitors (Shelke et al., 2002; Gomes et al., 2007; Mackessy, 2010; Guo et al., 2013; Mukherjee et al., 2014b; Change and Tsai, 2014). Snake venom protease inhibitors are homologous to the conserved Kunitz motif present in bovine pancreatic trypsin inhibitor which possesses classical disulfide-rich α/β-fold structures with a conserved active site (P1 site); this structural feature accounts for the molecule's capacity to inhibit one or more specific serine proteases such as trypsin, chymotrypsin, elastase, thrombin and activated factor X (Earl et al., 2012; Qiu et al., 2013; Guo et al., 2013; Mourão and Schwartz, 2013; Mukherjee et al., 2014b). Despite significant structural similarities, Kunitz-type serine protease inhibitors exhibit a wide variety of biological functions, such as blocking of ion-channels and interference with blood coagulation, inflammation and fibrinolysis (Earl et al., 2012; Qiu et al., 2013; Guo et al., 2013; Mukherjee et al., 2014b). Nonetheless, many other biological functions in this class of biomolecules, as well as their pathophysiological significance in snakebite, remain to be explored. Furthermore, a single venom may contain several Kunitz-type serine protease inhibitors (Guo et al., 2013) which exist in venom as non-covalent protein complexes (Earl et al., 2012; Mukherjee et al., 2014b). Several individual Kunitz-type serine protease inhibitors isolated from the same venom have been biochemically characterized; however, their biological functions and roles in snakebite have never been explored.

Our recent study has shown that Rusvikunin, a 6.9 kDa Kunitz-type serine protease inhibitor isolated from venom of *D. r. russelii* of Pakistan origin, forms a basic complex (Rusvikunin complex) with another low molecular mass peptide (7.1 kDa) from the same venom (Mukherjee et al., 2014b). In the present study, we report the purification and *in vitro* and *in vivo* pharmacological characterization of this 7.1 kD peptide (named Rusvikunin-II) as well as that of the Rusvikunin complex. We show that the Rusvikunin protein complex has greater thermostability, and its pharmacological properties are more pronounced, as compared with individual components of the complex, and its natural biological role likely involves subduing agile mammalian prey. To the best of our knowledge, this is the first report demonstrating the biological role of a Kunitz-type protease inhibitor complex from snake venom.

2. Methods

Venom of *Daboia r. russelii* was a gift from Kentucky Reptile Zoo, USA. Protein concentration standard reagents were purchased from BioRad Inc., USA. Trypsin (from bovine pancreas), plasmin (from human plasma), thrombin (human plasma), and activated factor X (bovine plasma) were obtained from Sigma–Aldrich, USA. Pre-cast NuPAGE Novex® Bis-Tris gels, buffers and Mark 12 unstained molecular mass standards were obtained from Life Technologies (Invitrogen Inc.), USA. All other chemicals used were of analytical grade and procured from Sigma–Aldrich, USA. Lyophilized monovalent antivenom produced against crude Russell's Viper venom was a gift from Vins Bioproducts Limited, India. Polyvalent antivenom (against *Naja naja, D. r. russelii, Bungarus caeruleus, Echis carinatus)* was procured from Bharat Serum and Vaccines Limited, Ambernath, India.

2.1. Isolation and purification of a low molecular mass anticoagulant peptide

Four hundred fifty mg (protein) of crude *D. russelii russelii* venom was fractionated on a BioGel P-100 gel-filtration column as described previously (Mukherjee and Mackessy, 2013). The low molecular mass gel-filtration fractions (tubes 131–135) showing appreciable anticoagulant activity (see below) were pooled, desalted and then subjected to separation on a MonoS 5/50 GL cation exchange column coupled with ÄKTA Purifier Fast Protein Liquid Chromatography System (Wipro GE Healthcare) by following our previously described procedure (Mukherjee et al., 2014b). The MonoS 5/50 GL protein peak showing anticoagulant activity was re-fractionated on a Jupiter C_{18} reversed-phase high performance liquid chromatography column (250 mm × 4.6 mm) pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) (Mukherjee et al., 2014b). The elution of protein was monitored at 280 nm and the protein peaks were screened for anticoagulant activity.