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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

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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

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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.

Assessment of purity of preparation and molecular mass of anticoagulant peptide eluted from RP-HPLC column (RP-34, see Mukherjee et al., 2014b) were determined by 12.5% SDS-PAGE (NuPAGENovex® Bis-Tris gels) analysis of reduced and non-reduced proteins as well as MALDI-TOF mass spectrometry (Bruker Ultraflex) analysis of purified sample (~1 μg) (Mukherjee and Mackessy, 2013; Mukherjee et al., 2014b).

2.2. N-Terminal sequencing, peptide mass fingerprinting and secondary structure analyses

Approximately 10 μg of purified protein was blotted onto PVDF membrane followed by Edman degradation in a gas-phase protein sequencer (ABI) to determine the N-terminal sequence. For peptide mass fingerprinting (PMF) analysis using LC-MS/MS (Amazone ion-trap), our previously described procedure was followed (Mukherjee and Mackessy, 2013). The NCBI data base of non-redundant protein sequence (NCBI nr) was used to search the MS/MS spectra. The tryptic peptide sequences of this 7.1 kDa peptide were subjected to a BLAST search in NCBInr against a snake venom protein database (taxid: 8570) using the blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Circular dichroism (CD) measurements of Rusvikunin-II on a JASCO J-815 spectropolarimeter (Tokyo, Japan) were used to determine its secondary structure as described previously (Doley et al., 2004; Mukherjee et al., 2014b). The far UV-CD spectra (190–250 nm) of Rusvikunin-II (0.3 mg/ml in 20 mM potassium-phosphate buffer, pH 7.0) in a quartz cuvette with path length of 0.1 cm were recorded at room temperature (25 °C). The CD spectra was expressed in molar ellipticity [θ] (degrees cm^2/dmol), using 113 as mean residue molecular mass. The CD data were interpreted using CDPRO CLUSTER software (Mukherjee et al., 2014a).

2.3. Anticoagulant activity assay

Citrated goat blood was centrifuged at 5000 rpm for 10 min to prepare the platelet-poor plasma (PPP) and the re-calcification time of PPP in presence of graded concentrations (1.5–15 μg/ml in 20 mM Tris–HCl, pH 7.4) of Rusvikunin, Rusvikunin-II, or the Rusvikunin complex was assayed as described previously (Doley and Mukherjee, 2003; Saikia et al., 2011). A control was run in parallel where PPP was incubated with above buffer only. One unit of anticoagulant activity was defined as an increase in 1 s of clotting time of PPP (treated) compared with clotting time of control PPP (incubated with buffer only) (Doley and Mukherjee, 2003; Doley et al., 2004). In another set of experiments, a fixed amount (10 μg/ml) of Rusvikunin-II or Rusvikunin complex was added to PPP and pre-incubated for 3–10 min before addition of 40 μl of 250 mM $CaCl_2$. The plasma clotting time was recorded and compared with the control PPP (clotting time in presence of 1X PBS).

To determine the thermal stability of anticoagulant activity, a fixed concentration (2 mg/ml in Tris–HCl, pH 7.4) of Rusvikunin, Rusvikunin-II, or Rusvikunin complex was heated from 15 to 60 min at 75 °C at a water bath. After the indicated time period, a measured volume was withdrawn, cooled immediately to room temperature and then plasma clotting activity was determined as above. The anticoagulant activity of unheated (native) Rusvikunin, Rusvikunin-II, or Rusvikunin complex was considered as 100% activity. Data are expressed as percent inhibition of anticoagulant activity compared to activity shown by native (unheated) protein.

2.4. Serine proteases and blood coagulation factors inhibition study

The serine protease inhibitory potency of Rusvikunin was demonstrated in our earlier study (Mukherjee et al., 2014b). However, for a direct comparison under identical experimental conditions, different concentrations of Rusvikunin, Rusvikunin-II, or Rusvikunin complex dissolved in 20 mM Tris–HCl, pH 7.4 were incubated with a fixed concentration of serine protease: plasmin (0.5 μM), tissue plasminogen activator (t-PA) (0.5 μM), trypsin (5 μM), chymotrypsin (5 μM) or blood coagulation factors [factor Xa (0.15 μM), thrombin (0.03 NIH U/ml)], Russelobin (100 nM; a thrombin-like serine protease purified from RVV; see Mukherjee and Mackessy, 2013) was incubated for 30 min at 37 °C in the above buffer, and then protease/coagulation factor activity was assayed using appropriate chromogenic substrates (Mukherjee and Mackessy, 2013; Mukherjee et al., 2014b). The following substrates were used for assays: N-Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide acetate salt (substrate for factor Xa), N-(p-Tosyl)-Gly-Pro-Arg-p-nitroanilide acetate (substrates for thrombin and Russelobin), D-Val-Leu-Lys-p-nitroanilide dihydrochloride (substrate for plasmin), *Nα*-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (substrate for trypsin), and fibrinogen (substrate for thrombin). The activity of t-PA was determined indirectly by converting plasminogen to plasmin by t-PA and the activity of plasmin was then assayed using its chromogenic substrate as above. Activity of the above coagulation factors or serine proteases in the absence of Rusvikunins or the Rusvikunin complex was considered as 100% activity and other values were compared with that (Mukherjee et al., 2014b).

Previously we demonstrated that Rusvikunin dose-dependently inhibited the fibrinogen clotting activity of thrombin (Mukherjee et al., 2014b). In the present study, the thrombin inhibition (fibrinogen clotting activity) potencies of Rusvikunin, Rusvikunin-II and Rusvikunin complex were re-examined under identical conditions by a slight modification of our earlier procedure (Mukherjee et al., 2014b). Briefly, graded concentrations (2.5–20 μg/ml in 20 mM Tris–HCL, pH 7.4) of Rusvikunin or Rusvikunin-II or Rusvikunin complex were incubated with a fixed concentration of thrombin (0.03 NIH U/ml) in a final volume of 260 μl at room temperature for 30 min. Then, 40 μl of 2.5 mg/ml human fibrinogen (final concentration ~7.5 μM) was added and fibrinogen clotting time was recorded.

To evaluate the inhibitory effect of Rusvikunin-II or Rusvikunin complex on the prothrombin activating property of factor Xa, graded concentrations of Rusvikunin-II or Rusvikunin complex (125–3000 μg/ml) were incubated with a fixed amount of FXa (20 nM) for 30 min at 37 °C prior to addition of other components of prothrombinase

complex [1.4 μM prothrombin, 100 μM phospholipid vesicles 9:1 phosphatidylcholine (PC): phosphatidylserine (PS) and 3 nM FVa] as described by Mast and Broze (1996). After 30 min of incubation, formation of thrombin was assayed by adding its chromogenic substrate (N-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate salt). From a standard curve of paranitroaniline generated by graded concentrations of thrombin, the amount of thrombin generated from activation of prothrombin was calculated. The prothrombin activation by FXa in the absence of inhibitor was considered as 100% activity.

The degree of neutralization of serine protease inhibition and anticoagulant activity of Rusvikunin-II or Rusvikunin complex by commercial equine monovalent antivenom (MVA) or polyvalent antivenom (PVA), was determined by a previously described method (Mukherjee et al., 2014b). Briefly, individual Rusvikunins or the Rusvikunin complex and MVA/PVA were mixed at different ratios (1:1 to 1:200, w/w) and pre-incubated for 30 min at room temperature. Thereafter, the mixture was assayed for the neutralization of above activities using the corresponding assay system. The activity of individual Rusvikunins or the Rusvikunin complex in the absence of antivenom was considered as 100% activity.

2.5. Spectrofluorometric analysis of protein–protein interactions

The interaction of Rusvikunin-II (0.1 μmol/L) with serine protease (plasmin, trypsin, Factor Xa) was measured spectrofluorometrically (Saikia et al., 2011, 2012). The fluorescence spectra were obtained at an excitation wavelength 280 nm, and the emission spectra were recorded from 290 to 500 nm. Wavelength shifts were measured by taking the midpoint at two-thirds height of the spectra. The maximum fluorescence of free protein (I_0) was also measured (Saikia et al., 2011).

2.6. Biochemical characterization

Total neutral carbohydrate was estimated by the phenol-sulfuric acid method of Dubois et al. (1956) using D-glucose as a standard. A Bio-Rad protein assay kit (BIO-RAD, USA) was used for protein content determination, using γ globulin as a standard. The effect of freeze-thawing on anticoagulant activity of Rusvikunin-II or the Rusvikunin complex was determined by freezing the enzyme at −20 °C followed by thawing at room temperature, and this process was repeated 5 times. The residual anticoagulant activity or serine protease inhibition activity after each cycle of freeze-thawing was determined and compared with the control (Mukherjee and Mackessy, 2013).

2.7. Determination of in vitro pharmacological properties and cytotoxicity

The hemolytic activity of 10.0 μg/ml (in 20 mM K-phosphate buffer containing 150 mM NaCl, pH 7.4) of Rusvikunin-II or Rusvikunin complex on 5% (v/v) washed erythrocytes (obtained from goat blood) was assayed as described (Mukherjee and Mackessy, 2013). The *in vitro* cytotoxicity against mammalian cells Colo-205 (human colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma) and 3T3 (mouse embryo fibroblast) was assayed by adding graded amounts of Rusvikunin-II or Rusvikunin complex (1.0–10.0 μg/ml in 20 mM K-phosphate buffer containing 150 mM NaCl, pH 7.4) to the culture medium containing 1×10^5 cells/ml (Mukherjee and Mackessy, 2013). Cytotoxicity (percent cell death) was assayed by an MTT-based method. Rusvikunin-II or Rusvikunin complex-induced cytotoxicity, if any, was expressed as percent cell death, determined by comparison with values obtained from a standard curve of control cells (Mukherjee, 2007; Mukherjee and Mackessy, 2013).

The Rusvikunin-II or Rusvikunin complex-inflicted nuclear damage in tested cancer cells, if any, was observed by Hoechst 33253 staining. Briefly, after washing both the floating and adherent cells in 1X PBS, cells were fixed in 1% (v/v) formaldehyde (in PBS) for 30 min at room temperature. Thereafter, the cells were washed with 1X PBS, re-suspended in 100 μl respective growth medium and incubated with 5 μl of Hoeschst 33258 (10 mg/ml) for 30 min at 37 °C/5% CO_2 incubator. The cells were washed in 1X PBS and observed under a fluorescence microscope at 400× magnification. The percentage of apoptotic cells were counted from all cells from four random microscopic fields at 400× magnification. The antibacterial activity of Rusvikunin-II or Rusvikunin complex (10.0 μg/ml) was tested against Gram positive *Bacillus subtilis* and Gram negative *Escherichia coli* cells (Mukherjee, 2007). As a positive control, the bacterial cultures were treated with ampicillin and tetracycline to inhibit the growth of Gram positive and Gram negative bacteria, respectively.

2.8. Assessment of in vivo toxicity

For determining the *in vivo* toxicity of Rusvikunin-II or Rusvikunin complex, laboratory inbred, pathogen-free non-Swiss albino mice (NSA) weighing between 18 and 20 g and House Geckos (*Hemidactylus frenatus)* weighing between 1.5 and 3.5 g were used. The experimental protocols using animals were approved by the UNC IACUC (Institutional Animal Care and Use Committee). For toxicity assessment, Rusvikunin-II or Rusvikunin complex (in 0.2 ml of PBS, pH 7.4) was injected *i.p* (1.0–5.0 mg kg^{-1} body weight) into mice. Control animals received only 0.2 ml of PBS, pH 7.4 (placebo). House Geckos received the same dose in a total volume of 75.0 μl. The animals were observed at regular intervals up to 72 h post-injection for death or any physical or behavioral changes (Mukherjee and Mackessy, 2013; Mukherjee et al., 2014b). The procedure to determine *in vivo* blood clotting activity of Rusvikunin-II has been described earlier (Mukherjee et al., 2014b).

3. Results and discussion

3.1. Purification of a low molecular mass anticoagulant peptide from RVV

Gel-filtration (GF) pooled fractions 131–135 (see Supplementary Fig. S1A) showing anticoagulant activity

were eluted from MonoS 5/50 GL cation exchange column (with a NaCl concentration gradient) as a single, sharp symmetrical peak as a protein complex, named Rusvikunin complex; the high salt concentration required for elution indicated the basic nature of this protein complex (see Supplementary Fig. S1B). Two Kunitz-type protease inhibitors isolated from the venom of *Oxyuranus scutellatus* and *Oxyuranus microlepidotus* showed plasma kallikrein inhibitory activity and were also reported to be present as protein complexes (Earl et al., 2012) suggesting that these Kunitz-type protease inhibitor complexes serve a basic function common to venoms of different snake families. RP-HPLC fractionation of the Rusvikunin complex resulted in its separation into two protein peaks, a larger peak (retention time 34.2 min, RP-34) and a smaller peak with a retention time of 44.1 min (see Supplementary Fig. S1C). The large peak (RP-34) showed anticoagulant activity and displayed a sharp, single protein band of approximately 7.4 kDa on SDS-PAGE under reducing conditions (Fig 1A). However, the non-reduced protein showed a broad band in the range of 18–28 kDa, suggesting self-aggregation of protein in a native state (Fig 1A).

By MALDI-TOF-MS analysis, the molecular mass of RP-34 was determined as 7108.364 Da, also confirming the purity of preparation (Fig. 1B). This 7.1 kDa purified peptide prolonged the Ca^{2+}-clotting time of PPP, suggesting it is anticoagulant in nature (see below). The yield of this peptide, named Rusvikunin-II, was 7.8% of total venom protein. The proportion of Rusvikunin-II in RVV is significantly higher as compared with NA-CI (0.08%), a Kunitz-type protease inhibitor from *Naja atra* venom (Zhou et al., 2004), and Rusvikunin (0.01%) from *D. r. russelii* venom (Mukherjee et al., 2014b). However, due to a lack of data on percent composition of other snake venom Kunitz-type protease inhibitors, the yield of Rusvikunin-II could not be compared with other Kunitz-type protease inhibitors.

The molecular mass of snake venom Kunitz-type protease inhibitors are reported in the range of 6.4–7.5 kDa (see Table 1), and in general, consist of 55–67 amino acids with six cysteine residues (Zhou et al., 2004; Lu et al., 2008; Earl et al., 2012; Qiu et al., 2013; Guo et al., 2013; Mourão and Schwartz, 2013; Mukherjee et al., 2014b; Change and Tsai, 2014). Estimating the mean residual molecular mass per amino acid as 113 Da, Rusvikunin-II likely consists of 62–63 amino acid residues, consistent with Kunitz-type protease inhibitors.

3.2. Identification and secondary structure of Rusvikunin-II

The N-terminal sequence of Rusvikunin-II was found to be blocked and therefore could not be sequenced through Edman degradation. This finding is in accordance to a Kuntiz-type trypsin inhibitor CBPTI-1 purified from *D. r. siamensis* venom, which also had a modified N-terminus (Guo et al., 2013). However, analysis of tryptic digest peptides (42% sequence coverage) of Rusvikunin-II showed it had significant identity with protease inhibitor 2, a 60 amino acid long peptide purified from *D. r. siamensis* venom (NCBI accession P00900). The molecular function of this peptide (by sequence homology only) was attributed to serine-type endopeptidase inhibitory activity, although neither its biological activity nor functions were characterized experimentally. By PMF analysis, one tryptic peptide sequence of Rusvikunin-II was found to contain the N-terminal sequence HDRPTFCNLFPESGR (1–15 residues) (Table 2). Although no putative conserved domain was detected, BLAST analysis of this N-terminal sequence (Table 2) as well as another tryptic sequence (RIYYNLESNK) of Rusvikunin-II showed significant sequence homolog to venom basic protease inhibitors, Kunitz-type protease inhibitors and trypsin inhibitors isolated from *D. r. russelii* and *D. r. siamensis* venoms (data not shown). In the first 15 amino acid residues of N-terminal sequence, the only variation observed was at position 10, where A was replaced by F in Rusvikunin-II (Table 2). It should be noted that minor substitutions in the primary structure of Kunitz-type protease inhibitors from snake venoms may result in significant differences in biological activities of these molecules (Guo et al., 2013).

The far-UV spectrum of Rusvikunin-II is shown in Fig. 2. Similar to the secondary structure of Rusvikunin (Mukherjee et al., 2014b), the absence of negative peaks at 209 nm and 229 nm is indicative of a lack of significant amounts of α-helical structure in Rusvikunin-II. The analysis of CD data by CDPRO CLUSTER software suggested that the secondary structure of Rusvikunin-II is composed of 60.4% β-sheet, 38.2% random coil, 1.4% α-helix and 0% turn. Therefore, except for some minor variation, the secondary structure of Rusvikunin-II clearly resembles that of Rusvikunin (58.4% β-sheet, 37.2% random coil, 3.4% α-helix and 0% turn; Mukherjee et al., 2014b). These data strongly support the identification of Rusvikunin-II as a member of the Kunitz-type protease inhibitors present in Russell's Viper venom.

3.3. Comparison of inhibition of serine proteases by Rusvikunin-II and Rusvikunin complex

Rusvikunin, Rusvikunin-II and the Rusvikunin complex showed inhibition of amidolytic activity of serine proteases in the following order of preference: trypsin (Fig. 3A) >> plasmin (Fig. 3B) >>> FXa (Fig. 3C); however, Rusvikunin did not show inhibition of amidolytic activity of FXa (Mukherjee et al., 2014b). The Rusvikunin complex under identical experimental conditions produced greater inhibition of serine proteases ($p < 0.01$) as compared with the inhibition produced by Rusvikunin or Rusvikunin-II (Fig. 3A–C). Both Rusvikunin-II and the Rusvikunin complex failed to inhibit the amidolytic activity of chymotrypsin or thrombin. Furthermore, the inability of Rusvikunin-II or Rusvikunin complex to inhibit the amidolytic or protease activity of Russelobin supports our earlier observation that snake venom Kunitz-type protease inhibitors have probably undergone positive selection favoring the inhibition of protease(s) of prey to induce pathogenicity while avoiding targeting of the snake's own venom components (Mukherjee et al., 2014b).

The strength of inhibition of amidolytic activity of trypsin, plasmin and FXa by Rusvikunin-II, measured in terms of IC_{50} value, was determined as 398 ng/ml, 10.1 μg/ml and 1001 μg/ml, respectively, whereas, the IC_{50} value for the inhibition of amidolytic activity of trypsin, plasmin and

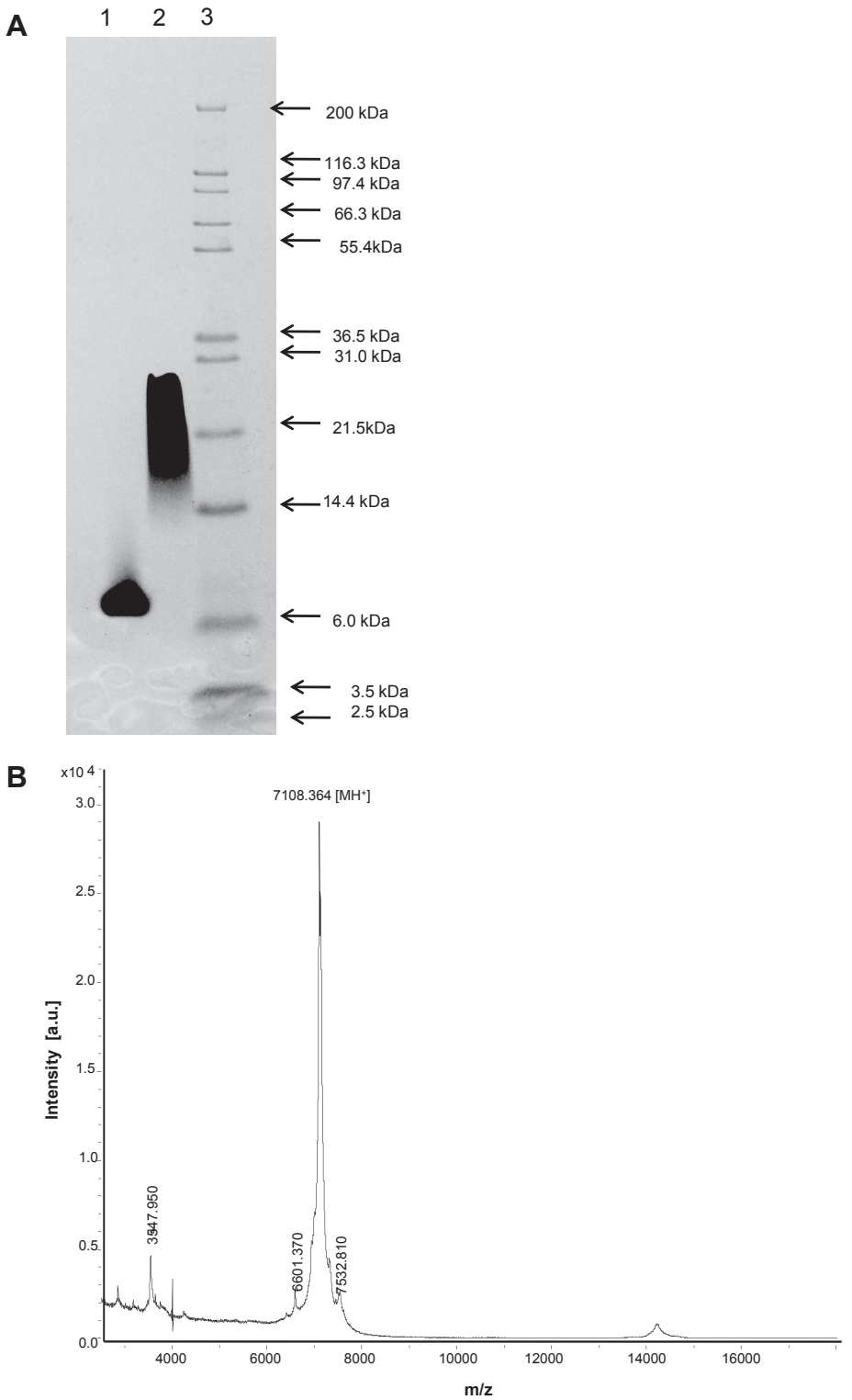

Fig. 1. A. Assessment of purity and determination of molecular mass of Rusvikunin-II by 12.5% SDS-PAGE analysis. Lanes 1 and 2, reduced and non-reduced Rusvikunin-II (6.0 μg); lane 3, protein mass standards: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), BSA (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), insulin B chain (3.5 kDa), insulin A chain (2.5 kDa). B. Determination of mass of Rusvikunin-II (1.0 μg) by MALDI-TOF-MS analysis using α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml) dissolved in 50% (v/v) acetonitrile in water and containing 0.1% (v/v) TFA.

Table 1
A comparison of the molecular masses of typical Kunitz-type serine protease inhibitors isolated from snake venoms.

Description	Species	Molecular mass	Reference
Rusvikunin-II	*Daboia russelii russelii*	7108.364 Da	Present study
Rusvikunin	*Daboia russelii russelii*	6936.89 Da	Mukherjee et al., 2014b
CBPTI-1	*D. russelii siamensis*	7555 Da	Guo et al., 2013
CBPTI-2	-do-	6928 Da	-do-
CBPTI-3	-do-	6997 Da	-do-
Bungaruskunin	*Bungarus fasciatus*	6752.8 Da	Lu et al. 2008
Kunitz-type trypsin inhibitor	*Eristicophis macmahonii*	6.5 kDa	Siddiqi et al., 1991
NA-CI	*Naja atra*	6403.8 Da	Zhou et al., 2004
Kunitz-type plasma kallikrein inhibitor	*Oxyuranus scutellatus*	7.0 kDa	Earl et al., 2012

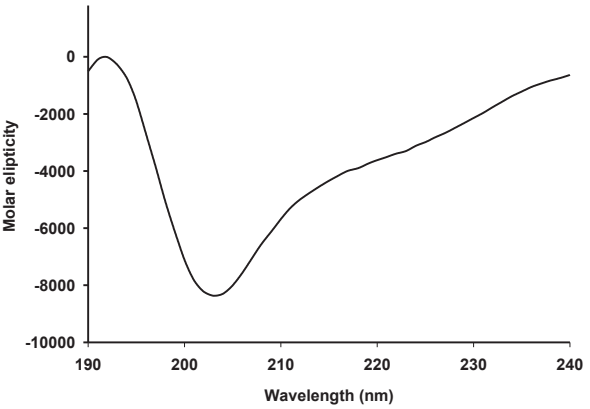

Fig. 2. Determination of the secondary structure of Rusvikunin-II. The CD measurements were performed as described in Materials and Methods and the data was interpreted using CDPRO CLUSTER software. The CD signal is expressed as mean residue ellipticity [θ] (degrees cm^2/dmol), using 113 Da as the mean residue molecular mass.

FXa by Rusvikunin complex under identical experimental conditions was determined as 200 ng/ml, 6.2 μg/ml, and 602 μg/ml, respectively. The extent of protease inhibition did not vary by changing the pH from 7.0 to 10, and the potency of inhibition could not be altered by increasing the pre-incubation time of Rusvikunin-II or Rusvikunin complex with serine proteases (trypsin/plasmin/FXa) from 5 to 30 min (data not shown). This indicates that similar to Rusvikunin (Mukherjee et al., 2014b), binding of Rusvikunin-II or Rusvikunin complex with serine proteases is a very rapid event and is largely independent of the pH of the medium. Conversely, Change and Tsai (2014) reported slow inhibition of plasmin by a Kunitz-type peptide inhibitor isolated from RVV.

Like other Kunitz-type serine protease inhibitors isolated from snake venoms, Rusvikunin-II has attained its specific inhibitory activity by binding of its main protease contact site (P1) to the active site (S1 binding pocket) of trypsin/plasmin/FXa in a substrate-like conformation (Yang et al., 1998; Guo et al., 2013). A minor substitution in the P1 site of Kunitz-type protease inhibitors may result in a significant change in their specificity towards serine proteases (Yang et al., 1998). Kunitz-type protease inhibitors possessing basic residues Arg or Lys at the P1 position specifically inhibit trypsin; when the P1 site of the inhibitor is occupied by a large hydrophobic residue such as Met, Phe, Leu, Trp or Tyr, it exhibits anti-chymotrypsin activity (Guo et al., 2013; Qiu et al., 2013; Wan et al., 2013). Therefore, possession of antitrypsin activity and lack of chymotrypsin inhibition by Rusvikunin-II may be anticipated by the presence of Arg15 at the P1 position (Table 2). However, exception to this rule is displayed by Chinese Russell's Viper (*D. r. siamensis*) venom Kunitz-type protease inhibitor (CBPTI-2), a close homolog to Rusvikunin-II that inhibited both trypsin and chymotrypsin (Guo et al., 2013). The Kunitz/BPTI homologs from snake venoms are encoded by multigene families that resulted in their diversification by positive Darwinian selection (Zupunski et al., 2003). Regardless of possessing an almost identical three dimensional structures or close sequence homology, even minor substitutions in amino acid residues in the primary structure of Kunitz/BPTIs may show differences in protease inhibition (Zupunski et al., 2003; Mourão and Schwartz, 2013).

Excitation of Rusvikunin-II at 280 nm shows a maximum fluorescence emission at 345.5 nm (Supplementary Fig. S2). However, interaction of Rusvikunin-II with trypsin did not result in an increase in fluorescence intensity or a shift in fluorescence maxima

Table 2
Multiple sequence alignment of N-terminal sequence of Rusvikunin-II with other known protease inhibitors from snake venoms.

Accession/Reference	Description	Species	N-terminal sequence	
			1	15
This work	Rusvikunin-II	*Daboia russelii russelii*	HDRPTFCNLFPESGR	
Mukherjee et al., 2014b	Rusvikunin	*D. russelii russelii*	HDRPTFCNLAPESGR	
Guo et al. (2013)	CBPTI-2	*D. russelii siamensis*	HDRPTFCNLAPESGR	
P00990.1	Venom basic protease inhibitor II	*D. russelii siamensis*	HDRPTFCNLAPESGR	
Q2ES50.1	Kunitz protease inhibitor 1	*D. russelii russelii*	HDRPTFCNLAPESGR	
A8Y7P4.1	Trypsin inhibitor B4	*D. russelii siamensis*	HDRPTFCNLAPESGR	
AFE83617.1	Kunitz-type protease inhibitor	*D. russelii russelii*	HDRPTFCNLAPESGR	
AFB74192.1	Protease inhibitor	*D. russelii russelii*	HDRPTFCNLAPESGR	
A8Y7P2.1	Trypsin inhibitor B2	*D. russelii siamensis*	HDRPTFCNLAPESGR	
A8Y7P3.1	Trypsin inhibitor B3	*D. russelii siamensis*	HDRPTFCNLAPESGR	

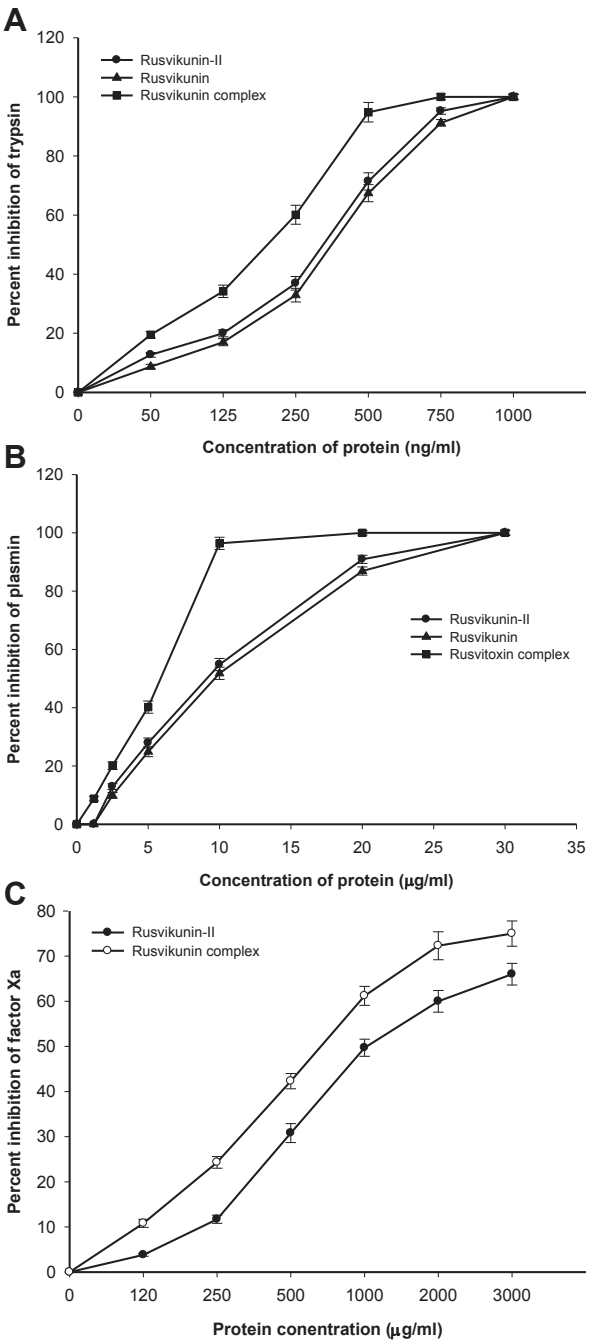

Fig. 3. Dose-dependent inhibition of amidolytic activity of (A) trypsin (5 μM), (B) plasmin (0.5 μM), and (C) FXa (0.15 μM) by Rusvikunin, Rusvikunin-II or Rusvikunin complex dissolved in 20 mM Tris–HCl, pH 7.4. Incubation of serine protease with Rusvikunins or Rusvikunin complex was carried out for 30 min at 37 °C before the assay of amidolytic activity in the assay systems as described in the text. The values are means ± SD of triplicate determinations.

(Supplementary Fig. S2). The same result was obtained with plasmin and factor Xa (data not shown). This result may be due to non-modification of the microenvironment of the Trp residue in Rusvikunin-II by these protease molecules (Saikia et al., 2011; Mukherjee et al., 2014b). Therefore, the spectrofluorometric results did not rule out an interaction between Rusvikunin-II or Rusvikunin complex with the above serine proteases.

3.4. Biochemical properties and anticoagulant activity

The neutral carbohydrate content of Rusvikunin-II was found to be 15.5 μg/mg protein. A direct comparison of the dose-dependent anticoagulant activity among Rusvikunin, Rusvikunin-II and Rusvikunin complex demonstrated that the Rusvikunin complex showed slightly higher ($p < 0.01$) anticoagulant activity (Fig.4A). Kinetics of the heat-inactivation study demonstrated that anticoagulant activity of Rusvikunin, Rusvikunin-II and the Rusvikunin complex decreased with increasing heating time; nevertheless, the anticoagulant activity of Rusvikunin complex was found to be less susceptible to heat denaturation compared with Rusvikunin-II (Fig 4B). However, both Rusvikunin-II as well as the Rusvikunin complex showed significant stability against 5 cycles of freeze-thawing (data not shown).

Many biological functions of snake venom components are initiated and executed by biochemical interactions between molecular components in the form of a protein complex (Mukherjee, 2010; Doley and Kini, 2009). The formation of protein complexes in snake venom often eliminates non-specific binding, in addition to enhancing binding to the pharmacological target molecule(s) (Doley and Kini, 2009). Therefore, the natural interaction between Rusvikunin and Rusvikunin-II to form a protein complex in RVV apparently augmented many of their biological functions (anticoagulant action and inducing lethality in target prey) and stability, playing a significant role in pathogenesis following RV bites.

Both Rusvikunin-II and the Rusvikunin complex dose-dependently inhibited the prothrombin activating potency (formation of thrombin) of FXa; however, their potency was significantly different ($p < 0.05$), with greater inhibition produced by Rusvikunin complex (Fig. 5A). Since Rusvikunin alone is unable to inhibit FXa (Mukherjee et al., 2014b), it is suggested that the interaction between Rusvikunin and Rusvikunin-II, forming a stable protein complex, is responsible for the observed increase in biological potency. Supporting this prediction is the observation that tissue factor pathway inhibitor (TFPI), a Kunitz-type multivalent protease inhibitor, inhibits FXa via its second Kunitz-type domain (Wun et al., 1988). Because the complete primary structure of Rusvikunin-II is not yet known, sequence homology could not be compared with the second Kunitz-type domain of TFPI. Nevertheless, the primary structure of venom basic protease inhibitor-II (Takahashi et al., 1974), which shows significant identity to Rusvikunin-II (by PMF), has ~52% sequence identity with the Kunitz domain 2 of TFPI (Wun et al., 1988). However, the FXa inhibitory potency of Rusvikunin-II was significantly less than a previously reported Kunitz-type FXa inhibitor from tick saliva (Batista et al., 2010). Determination of the complete primary structure of Rusvikunin-II, which is part of our ongoing analysis of RVV, may shed light on its mechanism of FXa inhibition.

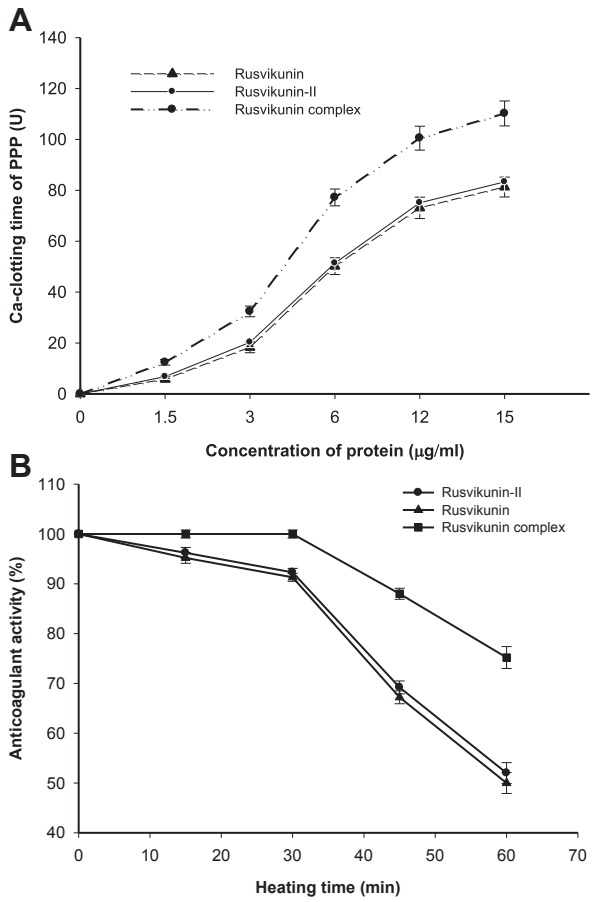

Fig. 4. (A) A comparison of the dose-dependent anticoagulant activity of Rusvikunin, Rusvikunin-II or Rusvikunin complex under identical experimental conditions. Rusvikunin, Rusvikunin-II or Rusvikunin complex were pre-incubated for 5 min with 300 μl of goat platelet poor plasma (PPP) at 37 °C and then 40 μl of 250 mM $CaCl_2$ was added to initiate the fibrin clot formation. Values are mean ± SD of triplicate determinations. (B) Effect of heating at 75 °C for the indicted time period on anticoagulant activity of Rusvikunins and Rusvikunin complex (2 mg/ml in 20 mM Tris–HCl, pH 7.4). The anticoagulant activity was determined as above. Values are mean ± SD of triplicate determinations.

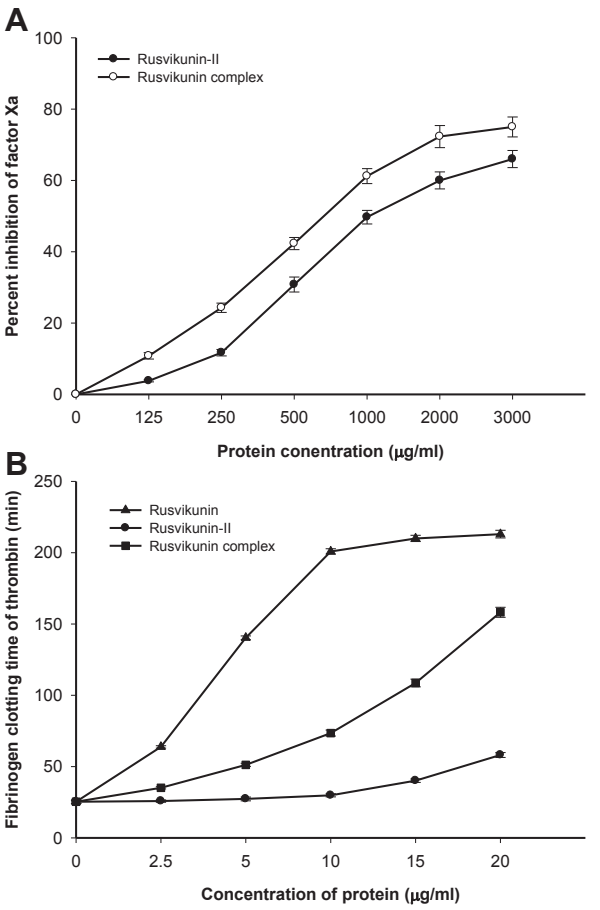

Fig. 5. Comparison of the inhibition potency between Rusvikunin, Rusvikunin-II and Rusvikunin complex (125–3000 μg/ml) under identical experimental conditions. (A) Inhibition of prothrombin activation by FXa (20 nM). (B) Inhibition of fibrinogen clotting activity of thrombin (0.03 NIH U/ml). The experimental details are described in Materials and Methods. Values represent mean ± SD of triplicate determinations.

Rusvikunin displayed significantly higher ($p < 0.01$) dose-dependent inhibition of fibrinogen clotting activity of thrombin as compared with the Rusvikunin complex or Rusvikunin-II (Fig.5B). The catalytic site of thrombin displays amidolytic activity against small chromogenic substrates such as S-2238 (Bode, 2006), and failure to inhibit the amidolytic activity of thrombin by Rusvikunin, Rusvikunin-II or Rusvikunin complex suggests none of them binds to the catalytic site of thrombin. Thrombin also possesses two positively charged regions named anion binding exosites (ABE) I and II; ABE-I is important for the binding of thrombin to fibrinogen. Inhibition of fibrinogen clotting activity of thrombin, but no inhibition of amidolytic activity, by Rusvikunin-II or the Rusvikunin complex indicates that binding to an exosite, rather than the active site, is the mechanism of inhibition. This activity is similar to Rusvikunin (Mukherjee et al., 2014b), which also binds to ABE-I of thrombin. Cardiovascular disorders such as thrombosis are the leading cause of the death throughout the world and anticoagulant drugs, particularly FXa and thrombin inhibitors, are the preferred therapeutic agents for the treatment of thrombosis (Batista et al., 2010). The Rusvikunins thus represent compelling lead compounds for new anticoagulant drug development.

3.5. *Neutralization of serine protease inhibition and anticoagulant activity by commercial antivenom*

Intravenous administration of antivenom is the only therapy of choice for the hospital management of snakebite. Due to geographic and species-level variation in snake venom composition (Mackessy, 2010), as well as low immunogenicity of low molecular weight components of venom, antivenom may not be able to neutralize all the toxic components of a venom (Mukherjee and Maity, 1998). At an antigen:antivenom (protein:protein) ratio of 1:100, trypsin inhibitory activity or anticoagulant activity of Rusvikunin-II or Rusvikunin complex was not neutralized

Table 3
Neutralization of trypsin inhibition and anticoagulant activity of Rusvikunin II and Rusvikunin complex by commercial polyvalent and monovalent antivenoms. Values are mean ± S.D. of triplicate determinations.

Antigen (Rusvikunin): antivenom ratio (protein:protein)	Percent inhibition of activity			
	Rusvikunin-II		Rusvikunin complex	
	Trypsin inhibition	Anticoagulant	Trypsin inhibition	Anticoagulant
Polyvalent antivenom				
1:10	0	0	0	0
1:50	0	0	0	0
1:100	0	0	3.1 ± 0.4	5.2 ± 0.8
1:200	14.1 ± 2.1	15.8 ± 1.9	21 ± 2.2	26 ± 2.4
Monovalent antivenom				
1:10	0	0	0	0
1:50	0	0	0	0
1:100	0	0	4.3 ± 0.4	8.1 ± 0.4
1:200	16.1 ± 1.1	17.9 ± 1.3	27 ± 2.1	33 ± 1.9

($P < 0.05$) by commercial monovalent or polyvalent antivenom (Table 3). This result corroborates well with our earlier finding (Mukherjee et al., 2014b). The lack of neutralization of pharmacological properties of Rusvikunin-I and the protein complex by commercial antivenoms further supports the need for a well-designed immunization protocol with differential amounts of specific venom components in order to improve the efficacy, quality and safety of commercial antivenom and provide better management of snakebite patients (Mukherjee and Maity, 1998; Mukherjee et al., 2014b).

3.6. *Cytotoxicity and* in vivo *toxicity*

At 10 μg/ml, Rusvikunin-II did not show cytotoxicity against any of the cancer cells tested following 72 h of incubation; in sharp contrast, the Rusvikunin complex showed ~30% inhibition of MCF-7 cells under identical experimental conditions. Neither Rusvikunin-II nor Rusvikunin complex was hemolytic in *in vitro* conditions. Furthermore, Rusvikunin-II or Rusvikunin complex (at 10 μg/ml) did not inhibit the growth of *E. coli* or *B. subtilis*, demonstrating a lack of antibacterial activity; these results are in accordance with the pharmacological properties demonstrated by Rusvikunin, another member of the Rusvikunin complex (Mukherjee et al., 2014b). Rusvikunin-II at 4.0 mg/kg significantly prolonged the *in vivo* clotting time of blood of treated mice (23 ± 3 min; mean ± SD, $n = 3$, $p < 0.05$), compared with clotting time of control mice (2.6 ± 1.2 min; mean ± SD, $n = 3$).

Similar to Rusvikunin (Mukherjee et al., 2014b), the *i.p* injection of Rusvikunin-II at 5.0 mg/kg body weight was not lethal to NSA mice or House Geckos, nor did they induce any behavioral changes/adverse effects in treated animals. However, administration of Rusvikunin complex at the same dose (2.5–2.8 mg/ml in mice) resulted in death of two of three mice within 4 h after injection, although all the three House Geckos survived without apparent symptoms at this dose. Rusvikunin complex-treated mice at the above dose showed the symptoms of increased respiration rate, dyspnea, difficulty in movement and hind-limb paresis before death. However, the third mouse gradually recovered 6 h after the injection. Effects on experimental animals of RVV from eastern India induced hind limb paresis (Prasad et al. 1999); conversely, neurotoxic symptoms have never been observed in RV envenomed patients from this region (Mukherjee et al., 2000). Therefore, it is probable that Rusvikunin complex inflicts discrete effects in both humans and rodents. Alternatively, it is reasonable to assume that the estimated concentration of Rusvikunin complex in adult human blood (~5.2 μg/ml) even after a lethal bite is insufficient to show the apparent neurotoxic symptom.

The significance of Kunitz-type protease inhibitors and their physiological complex in the pathophysiology of snakebite has remained unclear. We suggest that these non-toxic, Kunitz-type inhibitors (Rusvikunin and Rusvikunin-II) interact synergistically, and the increased toxicity provides a definitive advantage for the Russell's Viper by inducing lethal toxicity in its prey (or human) after a bite (Doley and Kini, 2009; Mukherjee, 2010). Rusvikunin complex can produce lethal toxicity in mice at a dose of 5 mg/kg, and so a primary function of this protein complex in RVV is likely targeted at rapidly incapacitating small mammal prey. Furthermore, lack of biological activity against House Geckos suggests prey-specific toxicity of the Rusvikunin complex, similar to specific effects observed following envenomation from other vipers (Gibbs and Mackessy, 2009) and from some rear-fanged snakes (Mackessy et al., 2006; Pawlak et al., 2009). Moreover, the concentration of Rusvikunin complex in an adult human victim could approach ~5 μg/ml blood and at this concentration, Rusvikunin complex has strong *in vitro* anticoagulant effects, facilitating toxic effects of other venom components such as serine proteases, metalloproteases and phospholipases A_2. In view of the fact that the most common clinical manifestation of RV envenomation is incoagulable blood, it appears probable that the Rusvikunin complex contributes to the overall toxicity of RV bites.

4. Conclusion

We report the purification and characterization of a 7.1 kDa Kunitz-type protease inhibitor (named Rusvikunin-II) possessing anticoagulant activity from RVV. This peptide forms a complex with another Kunitz-type protease

inhibitor (Rusvikunin) from the same venom, termed the Rusvikunin complex. Rusvikunin-II showed highest specificity in inhibition of amidolytic activity of trypsin, followed by plasmin and then FXa, by non-covalently binding with these serine proteases; nevertheless, inhibition produced by Rusvikunin complex was more pronounced. Further, it appears that the anticoagulant action of Rusvikunin-II or the Rusvikunin complex in humans is primarily attributed to inhibition of the fibrinogen clotting activity of thrombin, though in target prey of RV such as in rodents, additional inhibition of FXa may result in further increasing the anticoagulant activity of the Rusvikunin complex. Our study suggests that a primary function of the Rusvikunin complex in RV venom is to immobilize and/or kill rapid-moving prey.

Ethical statement

In the present paper entitled-"Pharmacological properties and pathophysiological significance of a Kunitz-type protease inhibitor (Rusvikunin-II) and its protein complex purified from *Daboia russelii russelii* venom" All experimental protocols using animals were approved by Institutional Animal Ethical Committee of UNC, USA.

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Conflict of interest

We declare that there is no conflict of interest in this study entitled-" Pharmacological properties and pathophysiological significance of a Kunitz-type protease inhibitor (Rusvikunin-II) and its protein complex isolated from *Daboia russelii russelii* venom".

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.06.016.

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