Characterization of a Kunitz-type protease inhibitor peptide (Rusvikunin) purified from *Daboia russelii russelii* venom

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

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

ABSTRACT

The snake venom may be considered as a potent source of untapped therapeutic proteins and peptides. The peptide mass fingerprinting and N-terminal sequence alignment of a 6.9 kDa peptide named Rusvikunin from *Daboia russelii russelii* venom show the presence of putative conserved domains of the KU superfamily. Further, BLAST analysis of two of the *de novo* peptide sequences of Rusvikunin demonstrates significant sequence homology with serine proteases reported in the NCBI database. Rusvikunin possesses conserved cysteine residues and Arg15 at the P1 position. It inhibits amidolytic activity of trypsin ($IC_{50} = 50$ nmol/l), plasmin ($IC_{50} = 1.1$ μmol/l), and fibrinogen clotting as well as plasma clotting activity of thrombin ($IC_{50} = 1.3$ μmol/l); however, it does not inhibit the amidolytic activity of chymotrypsin, thrombin, factor Xa, and tissue plasminogen activator. Rusvikunin is a glycoprotein, demonstrates dose-dependent BAEE-esterase activity. It does not show lethality in mice or *in vitro* cytotoxicity against mammalian cells but shows *in vivo* anticoagulant activity 6 h after i.p. injection in the mouse model. The commercial polyvalent and monovalent antivenom failed to inhibit the functional properties of Rusvikunin. The possible biomedical applications of Rusvikunin in the treatment and/or prevention of cardiovascular disorders such as thrombosis and trypsin-induced inflammation are suggested.

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1. Introduction

Death or morbidity due to snakebite is a significant and recurrent occupational hazard in many parts of the world. Numerous venomous species occur in Asia, but Russell's Vipers (family Viperidae) are broadly distributed in at least 10 south-east Asian countries, including the Indian subcontinent [1]. Envenomation by Russell's Vipers (*Daboia russelii russelii*) accounts for much of the snakebite-caused mortality and it is therefore considered as a category I medically important snake in India [2].

The mechanisms of toxicity and structure–function properties of several different major components of Russell's Viper venom (RVV), such as proteinases, phospholipase A_2, myotoxins and L-amino acid oxidase, have been explored [2–6]. However, studies from our laboratory have provided convincing evidence of the presence of several low molecular weight toxins (<8 kDa) in venom

of *D. r. russelii* (unpublished observation). Functional characterization of such novel components of venom will not only advance our understanding of the molecular mechanism(s) of pathogenicity of snakebite, but also has the potential to contribute to the discovery of therapeutically important proteins from RVV as lead drug molecules.

Kunitz-type serine protease inhibitors are low molecular mass snake venom peptides which consist of 50–60 amino acid residues and are homologous with the conserved Kunitz motif present in bovine pancreatic trypsin inhibitor, or BPTI [7–11]. The majority of these have been shown to possess classical disulfide-rich alpha/beta fold structures with a conserved active site (P1 site) responsible for binding and subsequent inhibition of specific serine proteases [8–10]. Despite structural similarities, Kunitz-type serine protease inhibitors are reported to exhibit a wide variety of biological functions, such as inhibition of one or more serine proteases, blocking of ion-channels, interference with blood coagulation, inflammation and fibrinolysis [8–10,12]. Nonetheless, many other biological functions of this class of biomolecules, including their pathophysiological significance in snakebite, remain to be explored. Studies have also provided convincing evidence of the therapeutic potential of Kunitz-type/BPTI peptides from snake

* Corresponding author at: Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784 028, Assam, India. Tel.: +91 7896003886; fax: +91 3712267005/267006.

E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

venom, and therefore exploration of further areas for biomedical applications such as cardiovascular drug development is a promising and challenging task [13].

The goal of the present study is the functional characterization of a 6.9 kDa basic protein (named Rusvikunin) purified from the venom of Russell's Viper (*D. r. russelii*) of Pakistan origin. Further, possible biomedical applications of Rusvikunin in the treatment and/or prevention of cardiovascular disorders such as thrombosis and trypsin-induced inflammation are suggested. To the best of our knowledge, this report provides the first evidence of antithrombin activity of Kunitz-type protease inhibitor isolated from snake venom.

2. Materials and methods

Pre-cast NuPAGE Novex® Bis-Tris Mini Gels, buffers and Mark 12 unstained molecular mass standards were obtained from Invitrogen Inc, USA. Russell's Viper (*D. r. russelii*) venom of Pakistan origin was a gift from the Kentucky Reptile Zoo. Protein concentration standard reagents were purchased from BioRad Inc, USA. All other chemicals used were of analytical grade and procured from Sigma-Aldrich, USA. ATCC®MTT Cell Proliferation Assay kit was procured from American Type Culture Collection, Manassas, VA. The diagnostic biochemistry kits were procured from Fisher Scientific. Equine monovalent antivenom (lyophilized) against crude Russell's Viper venom was a gift from Vins Bioproducts Limited, India.

2.1. Purification of a low molecular mass anticoagulant peptide from Russell's viper venom

The Bio Gel P-100 gel filtration of lyophilized *D. russelii russelii* venom (500 mg dry weight) was done as described by us [5]. The low molecular mass gel-filtration fractions (tubes 131–135) were pooled, desalted and concentrated by centrifugation using Nanosep 3 K Omega membrane filters. These proteins were then subjected to separation on a MonoS 5/50 GL cation exchange column using an AKTA Purifier Fast Protein Liquid Chromatography System (GE Healthcare). The column was first washed with equilibration buffer A (20 mM MES, pH 6.0) for 10 min to remove any unbound protein, and the bound proteins were eluted with a gradient of buffer B (20 mM MES containing 1.0 M NaCl, pH 6.0) for 60 min. The elution of proteins was monitored at 280 nm and 0.75 ml fractions were collected.

The MonoS 5/50 GL peak was further fractionated on a Jupiter C_{18} reverse-phase high pressure liquid chromatography column (250 mm × 4.6 mm) previously equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA), using a Waters HPLC system operating under Empower software. After washing the column with 0.1% TFA for 10 min, the bound proteins were eluted with a linear gradient over 75 min from 0 to 40% (v/v) acetonitrile (ACN) containing 0.1% (v/v) TFA (flow rate of 1 ml/min). The elution of protein was monitored at 280 nm and the protein peaks were screened for anticoagulant activity.

2.2. Determination of purity and molecular weight of anticoagulant protein

The molecular weight and purity of preparations were determined by 12.5% SDS-PAGE (NuPAGE Novex® Bis-Tris Mini Gels) under both non-reduced and reduced conditions as described previously [5]. The purity as well as molecular weight of the purified protein was also determined by MALDI-TOF mass spectrometry (Bruker Ultraflex).

2.3. N-terminal sequencing

The N-terminal sequencing (15 residues) of PVDF membrane-blotted protein (5 μg) was performed by Edman degradation in a gas-phase protein sequencer (PPSQ-10) connected to an on-line PTH analyzer and a CR-7A data processor. Protein homology searches were performed using the online BLASTP (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Multiple alignments of homologous sequences from snake venom were performed using COBALT (Constraint-based Multiple Alignment Tool; NCBI).

2.4. Peptide mass fingerprinting and de novo sequencing

The in-gel tryptic digestion of alkylated and reduced protein was done following the procedure described by Thiede et al. [14]. For PMF and *de novo* sequencing, LC/MS/MS of tryptic digested peptides was performed on a Brucker nano Advance UHPLC coupled with maXis 4G mass spectrometer using a reversed phase Magic C18 AQ 0.1 × 150 mm column [5]. The MS/MS spectra were searched against the NCBI data base of non-redundant protein sequence (NCBI nr) using the Mascot database search engine (version 2.3). The *de novo* sequences of the peptides obtained from Mascot protein identification were subjected to a BLAST search in NCBI nr against a snake venom protein database (snakes, taxid: 8570) using the blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Assay of amidolytic activity

The following chromogenic substrates (final concentration 0.2 mM) were examined for amidolytic activity assay following a previously described procedure [5,15]: N-Benzoyl-Pro-Phe-Arg-*p*-nitroanilide hydrochloride (substrate for plasma kallikrein), *N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (substrate for trypsin), D-Val-Leu-Lys-p-nitroanilide dihydrochloride (substrate for plasmin), N-Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide acetate salt (substrate for factor Xa), N-(p-Tosyl)-Gly-Pro-Arg-p-nitroanilide acetate and N-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide hydrochloride (substrates for thrombin), and N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (substrate for chymotrypsin).

2.6. Assay of anticoagulant activity

Platelet-poor plasma (PPP) from citrated goat blood was prepared and plasma re-calcification times by graded amounts of purified peptide were measured by our previously described procedure [16,17]. A control was run in parallel where buffer was added and plasma clotting time was determined under identical experimental conditions. One unit of anticoagulant activity has arbitrarily been defined as crude venom/purified peptide that induces a 1-second increase in the clotting of the PPP compared with the clotting time of normal plasma [16,17]. In another set of experiments, a fixed amount of Rusvikunin was added to PPP and the mixture was pre-incubated for 3–10 min before the addition of $CaCl_2$. The plasma clotting time was recorded and compared with the control (clotting time without addition of Rusvikunin).

2.7. Determination of serine protease inhibition activity

Inhibitory activity of Rusvikunin against a panel of serine proteases (trypsin, chymotrypsin, thrombin, plasmin, factor Xa, t-PA and Russelobin, a thrombin-like serine protease isolated from RVV [5] was also determined. A fixed concentration of serine protease

(5 μmol/l trypsin or Russelobin; 0.5 μmol/l plasmin, thrombin or t-PA; 0.15 μmol/l FXa) was pre-incubated with graded concentrations of Rusvikunin at 37 °C for 30 min, followed by assay of residual enzyme activity (except t-PA) with the respective chromogenic substrate (0.2 mM) as described by Mukherjee and Mackessy, 2013 [5]. The following chromogenic substrates were used for amidolytic activity assay: N-α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (substrate for trypsin), N-(p-Tosyl)-Gly-Pro-Arg-p-nitroanilide acetate (substrate for thrombin), N-Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide acetate salt (substrate for factor Xa) and, D-Val-Leu-Lys-p-nitroanilide dihydrochloride (substrate for plasmin). The inhibitory activity against t-PA was assessed by an indirect method; plasminogen was converted by t-PA to plasmin and the activity of latter protease was assayed using D-Val-Leu-Lys-p-nitroanilide dihydrochloride as a substrate. The percent inhibition (PI) of serine protease activity was calculated by considering the activity of enzyme in absence of inhibitor as 100%.

The FXa inhibition by Rusvikunin, if any, was also assessed by incubating 0.5 μM Rusvikunin with 0.26 μM FXa (two times its physiological concentration) at room temperature for 30 min. A control was set up where the factor Xa was incubated with buffer instead of Rusvikunin under identical experimental conditions. Thereafter, prothrombin (14 μM) was added and incubated for another 60 min at 37 °C. Formation of thrombin from prothrombin by FXa alone or in presence of Rusvikunin was assayed by analyzing the prothrombin activation products by 12.5% SDS-PAGE. The activity of FXa alone was considered as 100% activity and other value was compared with that.

The time-dependent inhibition was also assayed by pre-incubating Rusvikunin with serine protease (trypsin/plasmin/thrombin) from 5–30 min and then assaying the enzyme activity against the preferred chromogenic substrate for trypsin/plasmin and against fibrinogen for thrombin. The effect of pH on trypsin and plasmin inhibition was determined by incubating the inhibitor with trypsin or plasmin at different pH (7.5–9.0) and then assaying the activity of enzyme against the suitable chromogenic substrate.

2.8. Assay of inhibition of plasmin and thrombin towards their physiological substrates

To assay the inhibition of activity of plasmin toward its physiological substrate fibrin, 10 μl of thrombin (1 NIH U/ml) was added to 90 μl of human fibrinogen solution (2.5 mg/ml) and the fibrin clot was allowed to form at room temperature. To this clot, 5 μg of human plasmin, pre-incubated with different doses of Rusvikunin (1.0–6.0 μmol/l), was added and the final volume was made up to 50 μl with 50 mM Tris-HCl, pH 7.4. The mixture was incubated at 37 °C for 45 min and then 50 μl of 10% (v/v) ice-cold TCA was added to stop the reaction. The mixture was centrifuged at 10,000 rpm for 10 min and release of tyrosine in the supernatant was determined at 280 nm. The fibrinolytic activity of plasmin alone (without inhibitor) was considered as 100% activity and inhibitory effect of Rusvikunin on plasmin (antifibrinolytic activity) was expressed as percent reduction in its fibrinolytic activity in the presence of inhibitor [18].

To study the inhibitory effect of Rusvikunin on fibrinogen clotting activity of thrombin, 250 μl of human fibrinogen solution (2.5 mg/ml) was incubated with 10 μl of thrombin (1 NIH U/ml) pre-incubated with different doses of Rusvikunin (0–9.0 μmol/l) and the final volume was adjusted to 300 μl. The time of clot formation (in sec) was recorded with a coagulometer and percent inhibition of Rusvikunin-mediated clotting ability of thrombin was calculated as above. Further, release of fibrinopeptides A and B by thrombin either in the presence or absence of graded concentrations of Rusvikunin was also determined by RP-HPLC analysis [5]. From a standard curve of fibrinopeptides A and B,

the amount of these peptides released by thrombin either in the presence or absence of Rusvikunin was determined [5].

In another set of experiments, 5.0 nM of thrombin was incubated with graded concentrations of Rusvikunin at room temperature for 10 min. Then 300 μl of PPP was added to this mixture and plasma clotting time was measured and compared with control (thrombin without Rusvikunin). To assure the reproducibility, all assays were carried out in triplicate.

2.9. Determination of protein–protein interaction by fluorescence spectroscopy

Measurement of interaction of purified Rusvikunin with serine proteases (trypsin, plasmin, and thrombin) and fibrinogen was studied using a Perkin Elmer (LS55) fluorescence spectrometer with an excitation wavelength of 280 nm, emission wavelength was monitored from 290 to 500 nm, with excitation and emission slits of 5 nm. All measurements were carried out in triplicate after thermal equilibrium in a thermo stated cell holder maintained at 30 °C ($\pm$0.05 °C). Wavelength shifts were measured by taking the midpoint at two-thirds maximum height of the spectrum. The maximum fluorescence of free protein (I_0) was also measured [3].

2.10. Effect of antivenom on protease inhibitory activity of Rusvikunin

In order to determine the degree of neutralization of trypsin/plasmin/thrombin inhibitory activity of Rusvikunin by a commercial equine monovalent antivenom (against crude RVV), Rusvikunin and antivenom were mixed in different ratios (1:1 to 1:100, w/w). The mixture was pre-incubated for 30 min at room temperature and thereafter, the protein was assayed for neutralization of above stated activities in the corresponding assay system. The activity in absence of antivenom was considered as 100% activity and other values were compared with this.

2.11. Assessment of in vitro pharmacological properties

Dose-dependent hemolytic activity (0.5–1.5 μmol/l) of purified Rusvikunin against 5% (v/v) washed erythrocytes from goat were assayed as described earlier [5]. Briefly, to 5% (v/v) erythrocyte suspension (0.5 ml) different amounts of Rusvikunin (0.5–5.0 μM) in a final volume of 0.5 ml were added and the mixture was incubated for 60 min at 37 °C. Tubes were then centrifuged at 10,000 rpm for 5 min, and the absorbance of the supernatant was measured at 540 nm. Dose-dependent 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 (0.5–1.5 μmol/l) to culture medium containing 1×10^5 cells/ml [5]. Cytotoxicity (percent cell death) was assayed by an MTT-based method following the instructions of the manufacturer. Enzyme-induced cytotoxicity, if any, was expressed as percent cell death as determined by comparison with values obtained from a standard curve of control cells [5]. An anticancer drug cytosine-β-D-arabinose furanosidase hydrochloride (Sigma) at a concentration of 1 mg/ml and respective growth media were used as positive and negative controls, respectively.

Rusvikunin induced nuclear damage in Colo-205 and MCF-7 cells, if any, was measured by Hoechst 33253 staining. Briefly, both floating and trypsinized adherent cells were collected and washed in PBS. The cells were then fixed in 1% formaldehyde (in PBS) for 30 min at room temperature. After washing the cells with PBS, they were re-suspended in 100 μl respective growth medium and incubated with 5 μl of Hoechst 33258 (10 mg/ml) for 30 min at 37 °C/5% CO_2 in a humidified incubator. Thereafter, cells were washed in 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 (0.1–5.0 μM) was assayed against 1.0 ml mid-logarithmic culture (O.D.$_{630}$ ~0.3) of Gram negative *Escherichia coli* and Gram positive *Bacillus subtilis* bacteria [19]. As a positive control, the bacterial cultures were treated with ampicillin and tetracycline to inhibit the growth of *E. coli* and *B. subtilis*, respectively.

2.12. Assessment of in vivo toxicity towards mice and lizards

Laboratory inbred, pathogen-free non-Swiss albino mice (strain NSA) weighing between 18 and 20 g and House Geckos (*Hemidactylus frenatus*) weighing between 1.5 and 3.5 g were used for *in vivo* toxicity experiments. All experimental protocols using animals were approved by the UNC IACUC (protocol 9401). For toxicity assessment, purified Rusvikunin (in 0.2 ml of PBS, pH 7.4) was injected i.p (1.0 mg/kg to 4.0 mg/kg body weights) into mice. The 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 μl. The animals were observed at regular intervals up to 72 h post-injection for death or any physical or behavioral changes.

For determining the *in vivo* blood clotting activity of Rusvikunin, groups of three NSA strain albino mice weighing 28–30 g were i.p. injected with Rusvikunin at two doses (2.0 and 4.0 mg/kg) in a total volume of 0.2 ml PBS, pH 7.2. The control group of mice received the injection of the same volume of PBS only. The blood clotting time from treated or control group of mice were determined 8 h after i.p injection.

2.13. Statistical analysis

Statistical analysis of the data was done by Student's *t* test using the software Sigma Plot 11.0 for Windows (version 7.0). The value of $p \leq 0.05$ was considered as significant.

3. Results

3.1. Purification of a low molecular mass anticoagulant peptide

Gel filtration fractions 131–135, which showed strong anticoagulant activity (Fig. 1A), were pooled, desalted and then applied to a Mono S 5/50 GL cation exchange FPLC column. Rusvikunin eluted from the column with 0.3 M NaCl as a sharp symmetrical peak (a protein complex) (Fig. 1B). This protein complex was termed Rusvikunin complex. Interestingly, FPLC cation exchange under several different chromatographic conditions could not separate the components of Rusvikunin complex. The RP-HPLC fractionation of Rusvikunin complex resulted in its separation into two protein peaks-one large peak (retention time 34.2 min, RP-34) and a small peak with a retention time of 44.1 min (RP-44; Fig. 1C). The HPLC peak RP-44 prolonged the clotting time of platelet poor plasma (see below) and displayed a single band on SDS-PAGE in both reduced and non-reduced conditions and its molecular mass was determined as ~7 kDa (Fig. 1D). The molecular mass of this purified protein by MALDI-TOF-MS was found to be 6936.89 Da (Fig. 1E). This protein, named Rusvikunin, represents 0.01% of the total protein of RVV.

3.2. N-terminal sequence, peptide mass fingerprinting and multiple sequence alignment

The homology search in the NCBI database (taxid: snake) of the first 15 N-terminal amino acid residues of purified Rusvikunin (HDRPTFCNLAPESGR) demonstrated 100% sequence identity with the N-terminal sequences of some previously reported Kunitz-type protease inhibitors/trypsin inhibitors from Russell's Viper venom (Table 1). The N-terminal sequences of Russell's Viper venom protease inhibitors are all highly conserved.

When the tryptic peptide sequences of purified Rusvikunin (RP-44) were subjected to a BLAST search in the NCBI database, they showed significant similarity with venom basic protease inhibitor 2 (BPI-2) reported from *D. r. siamensis* venom (*Supplementary Fig.S1*). The peptide mass fingerprinting analysis of Rusvikunin also indicates that it is a C1-type protease inhibitor. Based on PMF analysis and N-terminal sequence alignment, purified Rusvikunin (RP-44) showed significant sequence similarity with venom basic protease inhibitors, Kunitz-type protease inhibitors and trypsin inhibitors from Viperidae venoms (Table 2). Like other trypsin inhibitors, the highly conserved cysteine residues and arginine (R15) at the P1 site were observed. Furthermore, the KU superfamily of putative conserved domains, which are common features of BPTI/Kunitz family of serine protease inhibitors, were detected in Rusvikunin by BLAST analysis. This reinforces that RP-44 is structurally similar to other Kunitz-type serine protease inhibitors from snake venom. Therefore, based on structural similarity, RP-44 peptide was named Rusvikunin (Russell's viper Kunitz-type protease inhibitor).

3.3. Biochemical characterization

Rusvikunin did not show amidolytic activity against any chromogenic substrates tested except a very marginal activity against the substrate for thrombin (N-Bz-Phe-Val-Arg-pNA. HCl), with a specific activity of 7.4 Units/mg proteins (data not shown). However, unlike thrombin, Rusvikunin did not show fibrinogen clotting activity. The secondary structure of Rusvikunin as determined by CD analysis shows it is made up of 58.4% β-sheet, 37.2% random coil, 3.4% α-helix and 0% turn (Fig. 2). Rusvikunin retained 100% serine protease (trypsin/plasmin/thrombin) inhibitory activity even after 5 cycles of freeze-thawing.

3.4. Inhibition of serine proteases

The inhibitory activity of Rusvikunin against a panel of serine proteases demonstrated that it showed significant inhibition of trypsin towards its chromogenic substrate BApNA with an IC$_{50}$ value of 50 nmol/l (Fig. 3A), followed by plasmin (IC$_{50}$ = 1.1 μmol/l) (Fig. 3B). However, no detectable inhibition of thrombin, chymotrypsin, t-PA, factor Xa or Russelobin (thrombin-like serine protease from RVV) towards their chromogenic substrates was observed under identical experimental conditions. Interestingly, Rusvikunin dose-dependently inhibited the fibrinogen clotting and plasma clotting activity of thrombin (Fig. 3C) with an IC$_{50}$ value of ~1.3 μmol/l.

As demonstrated by both SDS-PAGE and RP-HPLC analyses, Rusvikunin did not degrade trypsin, plasmin or t-PA, and a high molecular mass complex of Rusvikunin with trypsin or plasmin was not detected (data not shown).Rusvikunin at a concentration of 5.0 μM did not inhibit the prothrombin activation by factors Xa/Va because equal amount of thrombin was formed by FXa/Va complex in presence or absence of prothrombin (data not shown).

Rusvikunin-mediated inhibition of the above serine proteases was not strongly pH dependent, because equal inhibition was observed in the pH range of 7.5–9.5. Furthermore, the inhibitory effect of Rusvikunin was unaltered when pre-incubated with trypsin, plasmin or thrombin for different times (5–30 min) before chromogenic substrate addition, suggesting that binding of Rusvikunin with these serine proteases was a rapid event. Increasing the chromogenic substrate concentration in the reaction mixture did not overcome Rusvikunin-mediated inhibition, indicating Rusvikunin did not compete with substrate. Kinetic analysis
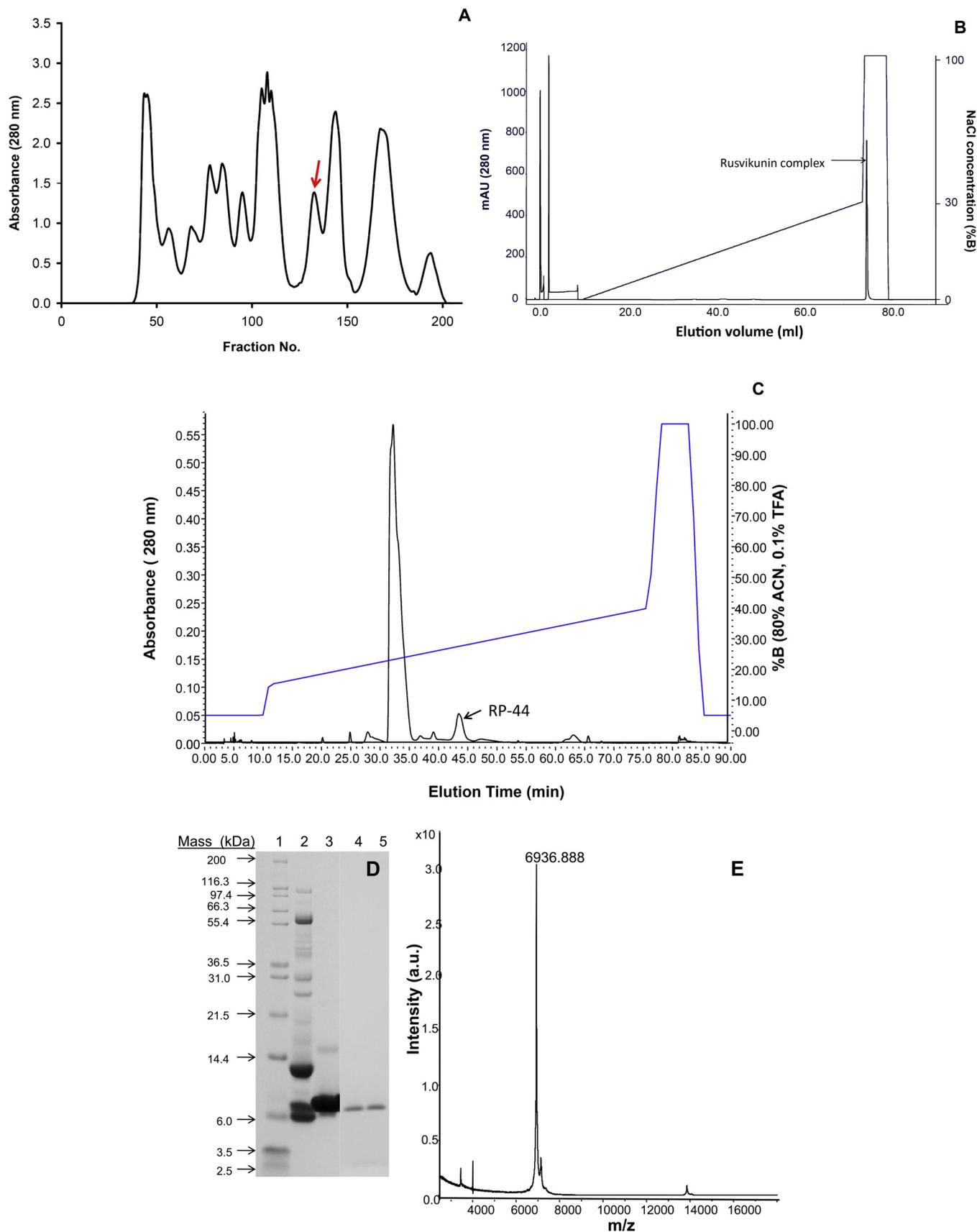
Fig. 1. (A) Fractionation of crude *D. russelii russelii* venom on size-exclusion BioGel P-100 column (2.8 × 80 cm). The arrow indicates elution of proteins showing fibrinogenolytic activity. (B) FPLC cation exchange of BioGel peak in (A) using a Tricorn MonoS 5/50 column. (C) Fractionation of FPLC-cation exchange eluted protein complex (Rusvikunin complex) on a C18 RP-HPLC column. Rusvikunin was eluted at 44.2 min (RP-44). (D) Determination of purity and molecular mass of Rusvikunin by SDS-PAGE; Lane 1, protein molecular markers; lane, 2 reduced crude RVV (20 μg); lane 3, reduced gel-filtration fraction (8 μg); lanes 4 and 7, reduced and non-reduced Rusvikunin (3.0 μg), respectively. (E) MALDI-TOF-MS of Rusvikunin (∼2 μg).

Table 1

Multiple sequence alignment of N-terminal sequence of RP-44 with other known protease inhibitors from snake venom. *Sequence of mature peptide.

Accession/Reference	Description	Species	N-terminal sequence	Residues
This work	Rusvikunin	*Daboia russelii russelii*	HDRPTFCNLAPESGR	1–15
P00990.1	Venom basic protease inhibitor II	*D. russelii siamensis*	HDRPTFCNLAPESGR	1–15
Q2ES50.1	Kunitz protease inhibitor 1	*D. russelii russelli*	HDRPTFCNLAPESGR	1–15
AFE83617.1	Kunitz-type protease inhibitor	*D. russelii russelii*	HDRPTFCNLAPESGR	1–15*
AFB74192.1	Protease inhibitor	*D. russelii russelii*	HDRPTFCNLAPESGR	1–15*
A8Y7P4.1	Trypsin inhibitor B4	*Daboia russelii siamensis*	HDRPTFCNLAPESGR	1–15*
A8Y7P3.1	Trypsin inhibitor B3	*Daboia russelii siamensis*	HDRPTFCNLAPESGR	1–15*
A8Y7P2.1	Trypsin inhibitor B2	*Daboia russelii siamensis*	HDRPTFCNLAPESGR	1–15*
A8Y7N8.1	Trypsin inhibitor C5	*Daboia russelii siamensis*	HDRPTFCNLAPESGR	1–15*
Guo et al. (2013)	CBPTI-2	*D. russelii siamensis*	HDRPTFCNLAPESGR	1–15

Table 2

Partial sequence of Rusvikunin aligned with homologous sequences from snake venom using CLUSTALO1.0.

Inhibitors	Sequence	
Protease inhibitor C5	MSSGGLLLLLALLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNPDSNKCE-VF	60
Protease inhibitor C1	MSSGGLLLLLGLLTLWAELTPISGQDRPKFCNLAPESGRCRGHLRRIYYNPDSNKCE-VF	60
Kunitz-type prot.inhi.	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCK-VF	60
Protease inhibitor B3	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCE-VF	60
Protease inhibitor B2	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCN-VF	60
Protease inhibitor 1	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCK-VF	60
Protease inhibitor	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRAHLRRIYYNLESNKCE-VF	60
Protease inhibitor B4	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCE-VF	60
Rusvikunin	----------------------HDRPTFCNLAPESGR------RIYYNPDSNKCEYVF	36
	:***.********** ***** :****: **	
Protease inhibitor C5	FYGGCGGNDNNFETRKKCRQTCGAPRKGRPT	90
Protease inhibitor C1	FYGGCGGNDNNFETRKKCRQTCGAPRKGRPT	90
Kunitz-type prot.Inhi.	FYGGCGGNDNNFETRDECRQTCG----GK--	84
Protease inhibitor B3	FYGGCGGNDNNFSTRDECRHTCV----GK--	84
Protease inhibitor B2	FYGGCGGNDNNFETRDECRQTCG----GK--	84
Protease inhibitor 1	FYGGCGGNANNFETRDECRQTCG----GK--	84
Protease inhibitor	FYGGCGGNDNNFSTWDECRHTCV----GK--	84
Protease inhibitor B4	FYGGCGGNDNNFSTWDECRHTCV----GK--	84
Rusvikunin	FYGGCGGNDNNFETR---QTCGAPR-----	67
	******* ***.* :**	

*Indicates identical residues in all sequences; (:) = highly conserved; (.) = moderately conserved.

The GenBank accession number of aligned sequences were: Rusvikunin (present study), Protease inhibitor C5 (gi|239977257), Protease inhibitor C1 (gi|239977246), Kunitz-type protease inhibitor (gi|380842421), Protease inhibitor B3 (gi|239977251), Protease inhibitor B2 (gi|239977248), Protease inhibitor 1 (gi|123913156), Protease inhibitor (gi|377657518), Protease inhibitor B4 (gi|239977254).

(plotting $1/v$ vs. $1/s$ in presence or absence of Rusvikunin) showed that its inhibition of the catalytic activity of trypsin/plasmin was non-competitive in nature, because in the presence of inhibitor, apparent K_m values remained the same; however, the V_{max} values were altered (data not shown).
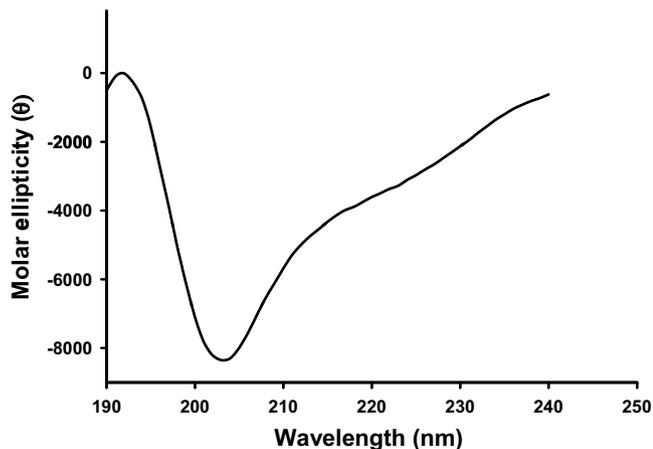

Fig. 2. Far UV spectra of Rusvikunin by CD spectroscopy to determine its secondary structure. The absence of predominant α-helical structure is indicated by absence of negative peaks at 209 and 222 nm.

3.5. Spectrofluorometric analysis of interaction of Rusvikunin with trypsin, thrombin and plasmin

The interaction between Rusvikunin and trypsin/fibrinogen at a 1:1 molar ratio was studied by a spectrofluorometric method. Excitation of trypsin or thrombin at 280 nm shows an emission maximum at 362 nm and 366 nm, respectively. However, no change in the fluorescence intensity of trypsin or thrombin post-incubation with Rusvikunin was detected (*Supplementary Figs. S2A and S2B*). The identical result was obtained upon incubation with plasmin.

3.6. The in vitro and in vivo pharmacological properties of Rusvikunin and neutralization by commercial antivenom

Under *in vitro* conditions, Rusvikunin dose-dependently prolonged the Ca-clotting time of citrated goat plasma (Fig. 4A) and demonstrated antiplasmin activity by significantly inhibiting the plasmin-mediated degradation of fibrin (Fig. 4B). At a concentration of 1.5 μmol/l, Rusvikunin did not show cytotoxicity against Colo-205, MCF-7 or 3T3 cells after 72 h of incubation and it was devoid of hemolytic activity against washed mammalian erythrocytes. At a dose of 1:100 Rusvikunin:antivenom (protein:protein) ratio, the serine protease inhibitory activity or anticoagulant activity of Rusvikunin was not inhibited ($P < 0.05$) by commercial polyvalent or monovalent antivenom (data not shown).
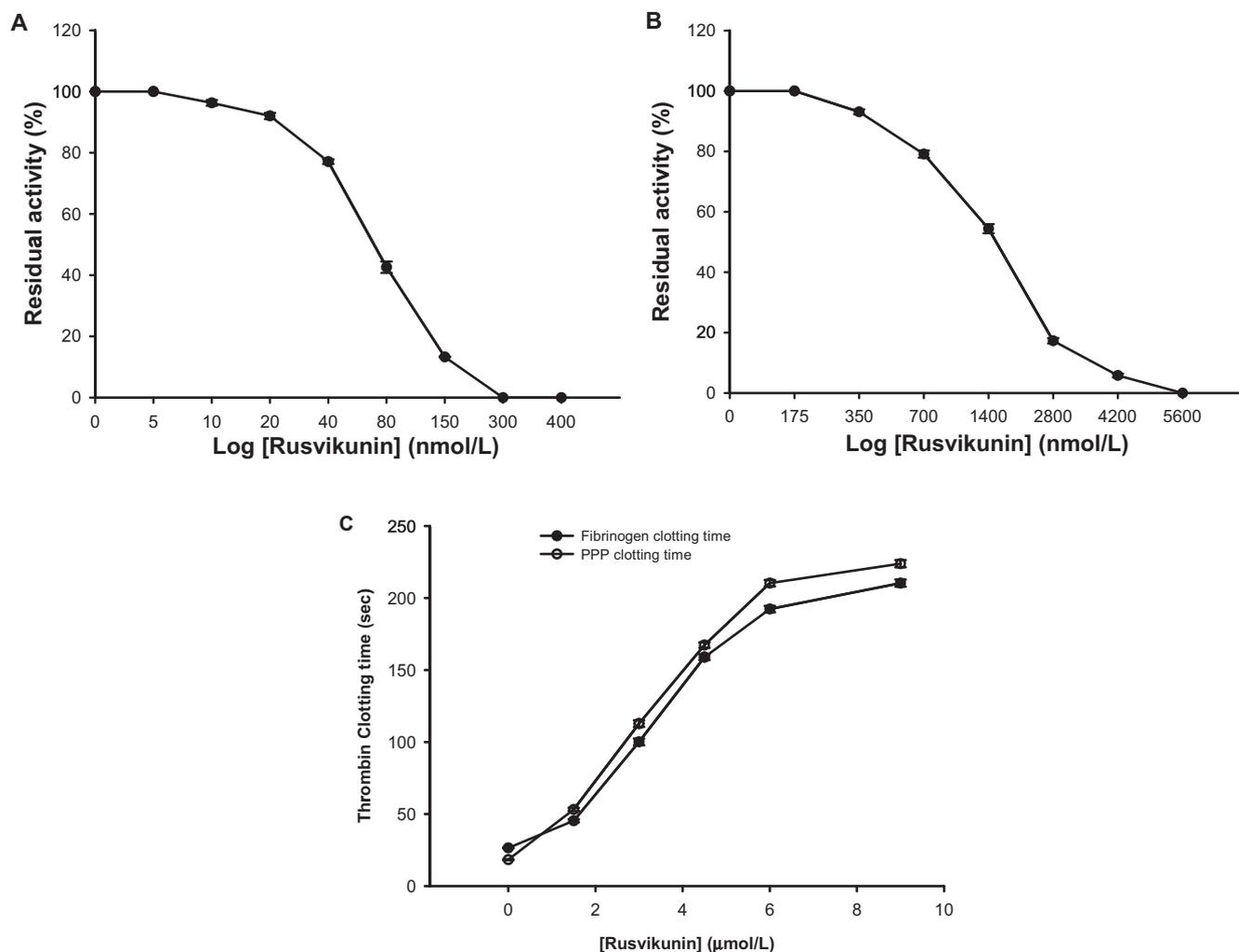
Fig. 3. Dose-dependent inhibitory activity of Rusvikunin against (A) amidolytic activity of trypsin (5 μM), (B) amidolytic activity of plasmin (0.5 μM), and (C) fibrinogen clotting and PPP clotting activity of thrombin (0.5 μM). Values are mean ±S.D. of triplicate determinations.

Injection of Rusvikunin at a dose of 4 mg/kg body weight did not cause mortality in mice or house geckos, and no behavioral changes were observed in animals up to 72 h post-injection. The treated animals did not show features of neurotoxicity, and light microscopic examination of cardiac, hepatic and renal tissues from Rusvikunin-treated mice did not show morphological alterations, evidence of intravascular coagulation or extravasation of erythrocytes (micrographs not shown). Six hour after *i.p* administration, Rusvikunin dose-dependently prolonged the *in vitro* coagulation time of blood of treated mice as compared to control mice (Fig. 5).

4. Discussion

Animal venoms contain a variety of potent biological activities with great potential for use as drugs or in drug development [6,13,20], and reptile venoms have been a particularly successful source for novel drug discovery [21,22]. Snake venom trypsin inhibitors have been explored for their clinical applications in the treatment of trypsin-mediated inflammatory reactions in cardiovascular and nervous system disorders, as well as in pancreatitis [23,24]. In 1972, Takahashi et al. [7] described a protease inhibitor isolated from Russell's viper venom showing specific inhibition of trypsin. Rusvikunin, with a molecular mass of 6.9 kDa, is typical of size of Kunitz-type/BPTI peptides from snake venom, which consist of 57–65 amino acids residues and contain six cysteines [8–11]. Based on a mean residual molecular mass of 113 Da per amino acid, Rusvikunin likely consists of approximately 60–61 amino acid residues, and it shows significant sequence identity with Kunitz-type protease inhibitors from snake venom. An analysis of peptide sequences of snake venom Kunitz-type/BPTIs suggests their evolution by gene duplication followed by subsequent diversification, resulting in a wide variety of biological functions by different isoforms of this class of venom peptides [10,25].

Kunitz-type serine protease inhibitors were reported to show inhibitory activity against trypsin or chymotrypsin, and in some rare instances, both [9–11,26,27]. Moreover, several of these inhibitors were also shown to inhibit protein C, plasmin and plasma kallikrein [8,27]. Rusvikunin was found to inhibit trypsin specifically, and to a significantly lesser extent, it showed anti-plasmin and anti-thrombin (fibrinogen clotting activity) effects; this is the first report showing an antithrombin effect of Kunitz/BPTI homologs from RVV. Specific inhibitory activity of Kunitz-type protease inhibitors results from binding of the main protease contact site (P1) to the active site (S1 binding pocket) of serine proteases in a substrate-like conformation. However, unlike the scissile bond of the appropriate protein substrates, the peptide bond of inhibitors undergoes extremely slow hydrolysis [8,28]. A minor substitution in the P1 site of the inhibitor may lead to a change in its specificity towards proteases [28]. Kunitz-type protease inhibitors
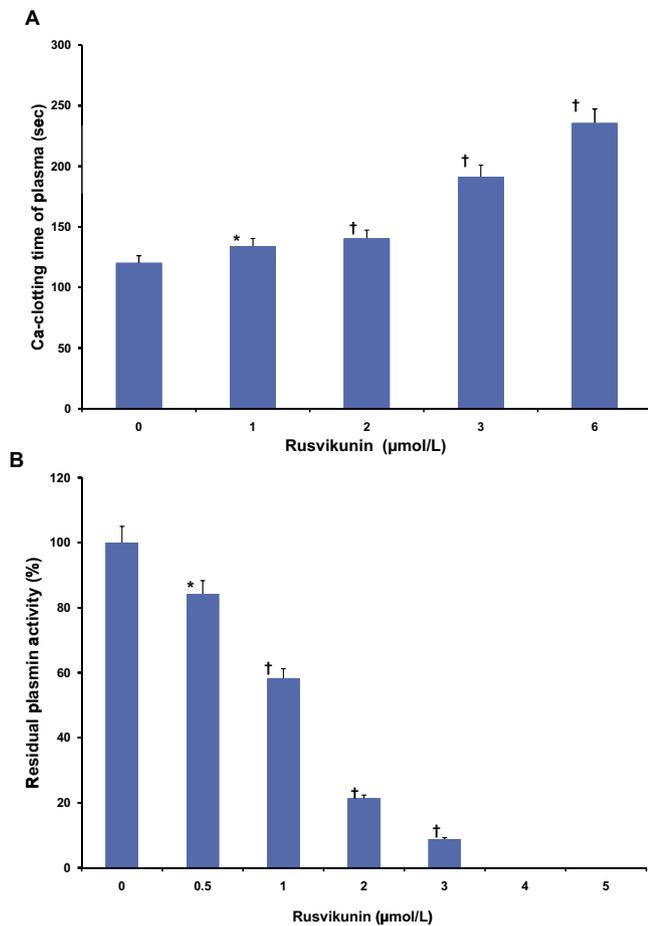
Fig. 4. (A) Dose-dependent in vitro anticoagulant activity of Rusvikunin. (B) Dose-dependent inhibition of fibrin degrading activity of plasmin by Rusvikunin. Values are mean ±S.D. of triplicate determinations. Significance of difference with respect to control ($^*p < 0.05$, $^†p < 0.001$).
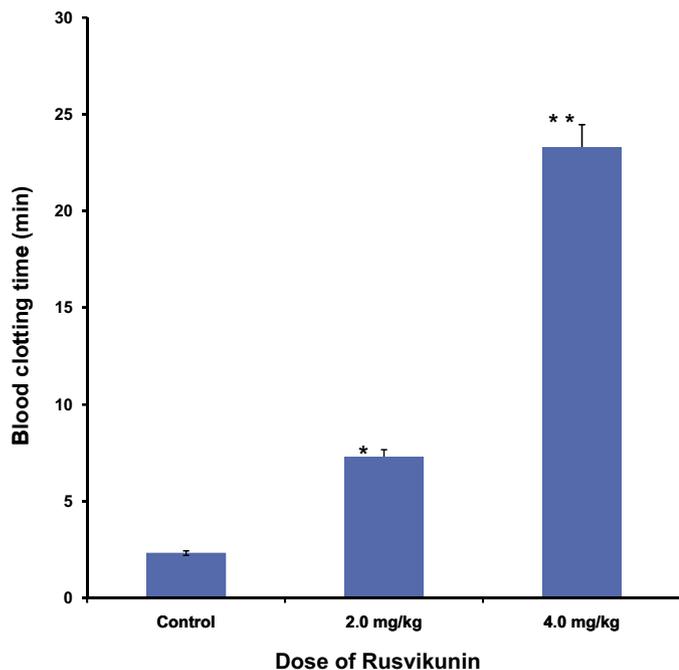

Fig. 5. Dose-dependent *in vitro* blood clotting time of control and Rusvikunin-treated mice. Values are mean ±S.D. of triplicate determinations. Significance of difference with respect to control ($^*p < 0.01$, $^{**}p < 0.001$).

possessing a basic residue Arg or Lys at the P1 position show trypsin inhibitory activity, whereas an inhibitor with a P1 site occupied by a large hydrophobic residue (such as Met, Phe, Leu, Trp or Tyr) exhibits anti-chymotrypsin activity [9–11,27]. Therefore, potent antitrypsin activity and the lack of chymotrypsin inhibition by Rusvikunin suggest that Arg15 is at the P1 position. It is to be noted that in contrast to Rusvikunin, CBPF-2 (GenBank: AM411362) which is closely homologous with Rusvikunin [10], inhibited both trypsin and chymotrypsin. According to Zupunski et al. [29], the Kunitz/BPTI homologs from snake venoms are encoded by multigene families that resulted in diversification by positive Darwinian selection. This reinforces the observation that despite having a very close structural similarity, even a minor difference in the primary structure of Kunitz/BPTIs may lead to altered specificities towards proteases.

In addition to possessing a catalytic site with amidolytic activity against small chromogenic substrates, thrombin has two positively charged regions named anion binding exosites (ABE) I and II [30]. The ABE-I is important for the binding of thrombin to fibrinogen [30]. Inhibition of the fibrinogen clotting activity of thrombin by Rusvikunin suggests that like the leech anticoagulant hirudin [30], Rusvikunin binds to ABE-I. Nevertheless, this binding does not inhibit the catalytic site of thrombin which was evident from the same amidolytic activity against chromogenic substrate for thrombin displayed by unbound and Rusvikunin bound thrombin. Interestingly, Rusvikunin did not inhibit Russelobin, a thrombin-like serine protease isolated from the same venom [5]. This suggests that snake venom Kunitz-type protease inhibitors have probably undergone positive selection favoring the inhibition of protease(s) of prey, inducing pathogenicity in prey while avoiding targeting of its own venom components.

The inhibitor studies have provided convincing evidence that like many other Kunitz/BPTI reported from snake venom, Rusvikunin inhibits serine proteases non-enzymatically by binding to the active site or an allosteric site of these serine proteases in a non-competitive manner [8–10]. Our spectrofluorometric results suggest that although Rusvikunin may bind with trypsin/plasmin/thrombin or fibrinogen, this binding induces a negligible change in fluorescence signal of either fibrinogen or trypsin molecules. Plasmin, which also shows fibrinogenolytic activity, also induces a negligible change in the fluorescence emission of native fibrinogen (Mukherjee, A. K. unpublished observations). However, a change in fluorescence intensity depends on modulation of the microenvironment of tryptophan residue(s) of interacting protein molecules [3], and in the absence of such modulation, change in the fluorescence intensity of two interacting proteins may not be detected.

Cardiovascular disorders such as thrombosis are a leading cause of death worldwide, and anticoagulants can be used *in vivo* to mitigate or prevent thrombotic disorder, myocardial infarction and stroke. Unfortunately, many natural anticoagulants used to prevent blood clotting show adverse side effects, such as bleeding complications. Rusvikunin, on the other hand, was non-lethal and did not show adverse pharmacological effects in experimental animals. Further, administration of Rusvikunin did not alter biochemical parameters tested in mice, and Rusvikunin was also devoid of *in vitro* cytotoxicity against mammalian cells. Low toxicity anticoagulants are in great demand for therapeutic use in the prevention and treatment of occlusive thrombosis [31], supporting the suitability of Rusvikunin for drug design applications as a peptide-based cardiovascular drug [6,13].

The *in vivo* as well as *in vitro* anticoagulant activity of Rusvikunin is supported by the ability of micro molar concentrations of Rusvikunin to inhibit the fibrinogen clotting activity of thrombin by a non-catalytic mechanism. However, antiplasmin activity is a key regulator of fibrinolysis and its complete deficiency causes a

severe bleeding disorder [32], so Rusvikunin, with low antiplasmin activity, may potentiate this observed anticoagulant effect.

Intravenous administration of polyvalent or monovalent antivenom is the only accepted therapy after Russell's viper or any other snakebite; however, in India and in Pakistan polyvalent antivenom is most widely used. Due to geographical and species variations in venom composition, 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 [33,34]. The inability of neutralization of Rusvikunin by commercial poly-valent or monovalent antivenom suggests the requirement of a well-designed immunization protocol with different components of venom for further improving the quality, efficacy and safety of antivenom for the efficient management of snakebite patients [35].

The physiological significance of the presence of Kunitz-type protease inhibitors in snake venom is still obscure, and the non-toxic nature and minor proportion of Rusvikunin in whole RVV indicates that it does not directly induce toxicity in prey. However, the pathophysiological effect of Rusvikunin after a Russell's Viper envenomation in target prey (mouse/rat) or in humans may be sig-nificant, because micromolar concentrations of Rusvikunin prolong the clotting time of plasma and also show antiplasmin activity; anticoagulation is the major clinical manifestation following RV envenomation in humans [2,4]. Rusvikunin likely exerts a syner-gistic effect with other venom components to enhance the lethality of venom [36]. Exploration of the physiological role of this intrigu-ing class of venom components is the goal of studies currently in progress.

5. Conclusion

This is the first report describing the functional character-ization of a Kunitz-type BPTI (Rusvikunin) from venom of *D. russelii russelii* of Pakistan origin. In addition to displaying trypsin, plasmin and thrombin inhibitory activity via a non-enzymatic mechanism, Rusvikunin demonstrated *in vivo* potent anticoag-ulation effect by targeting thrombin of treated mice. Clinical application of Rusvikunin as a peptide-based drug to ameliorate cardiovascular disorders, such as ocular thrombosis, trypsin-mediated inflammatory reactions and anti-bleeding disorders due to antiplasmin deficiencies, is indicated by its biochemical prop-erties and non-toxic nature. Exploration of the pathophysiological roles of Rusvikunin in RV envenomation is the goal of studies cur-rently in progress.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac. 2014.02.058.

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