Biochimie xxx (2014) 1–10

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

A new peptide (Ruviprase) purified from the venom of *Daboia russelii russelii* shows potent anticoagulant activity via non-enzymatic inhibition of thrombin and factor Xa

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ARTICLE INFO

Article history:
Received 2 May 2014
Accepted 7 July 2014
Available online xxx

Keywords:
Anticoagulant peptide
Biosensor analysis
Daboia russelii russelii
Factor Xa inhibitor
Protein–protein interaction
Thrombin inhibitor

ABSTRACT

Compounds showing dual inhibition of thrombin and factor Xa (FXa) are the subject of great interest owing to their broader specificity for effective anticoagulation therapy against cardiovascular disorders. This is the first report on the functional characterization and assessment of therapeutic potential of a 4423.6 Da inhibitory peptide (Ruviprase) purified from *Daboia russelii russelii* venom. The secondary structure of Ruviprase is composed of α-helices (61.9%) and random coils (38.1%). The partial N-terminal sequence (E^1-V^2-X^3-W^4-W^5-W^6-A^7-Q^8-L^9-S^{10}) of Ruviprase demonstrated significant similarity (80.0%) with an internal sequence of apoptosis-stimulating protein reported from the venom of *Ophiophagus hannah* and *Python bivittatus*; albeit Ruviprase did not show sequence similarity with existing thrombin/FXa inhibitors, suggesting its uniqueness. Ruviprase demonstrated a potent *in vitro* anticoagulant property and inhibited both thrombin and FXa following slow binding kinetics. Ruviprase inhibited thrombin by binding to its active site via an uncompetitive mechanism with a *Ki* value and dissociation constant (K_D) of 0.42 μM and 0.46 μM, respectively. Conversely, Ruviprase demonstrated mixed inhibition (Ki = 0.16 μM) of FXa towards its physiological substrate prothrombin. Furthermore, the biological properties of Ruviprase could not be neutralized by commercial polyvalent or monovalent antivenom. Ruviprase at a dose of 2.0 mg/kg was non-toxic and showed potent *in vivo* anticoagulant activity after 6 h of intraperitoneal treatment in mice. Because of the potent anticoagulant property as well as non-toxic nature of Ruviprase, the possible application of the peptide as an antithrombotic agent for combating thrombosis-associated ailments appears promising.

1. Introduction

In response to a vascular injury, the complex phenomenon of blood clotting requires the activation of zymogens of the coagulation cascade [1]. Thrombin and activated factor X (FXa) are the key components of blood coagulation [1,2]. FXa is the major component of the prothrombinase complex, which also comprises factor Va, negatively charged phospholipids, and calcium ions [2]. The prothrombinase complex eventually converts prothrombin to thrombin in the liver, which catalyzes the final stage of the blood coagulation cascade by cleaving fibrinogen to fibrin [1]. Besides, thrombin also activates procoagulant factors V, VIII, and XIII, in addition to contributing to the generation of a platelet plug [3].

Abbreviations: ALP, alkaline phosphatase; AT-III, antithrombin-III; BLASTP, basic local alignment search tool; CD, circular dichroism; CK-MB, creatine kinase; FXa, factor Xa; MAV, Russell's viper monovalent antivenom; OD, optical density; PBS, phosphate buffered saline; PLA_2, phospholipase A_2; PAV, polyvalent antivenom; pNA, p-nitroanilide; PPP, platelet poor plasma; RVV, Russell's viper venom; RU, response unit; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; TFA, trifloro acetic acid.

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http://dx.doi.org/10.1016/j.biochi.2014.07.006
0300-9084/

Endogenous inhibitors of FXa and thrombin such as antithrombin-III (AT-III) maintain a balance of these enzymes in the blood circulation, thus preventing the formation of unwanted clots [1]. Any defect in the delicate balance between the coagulation factors, such as thrombin and FXa, and their endogenous inhibitors can lead to serious hemostatic disorders such as the formation of superfluous thrombi in the blood vessels [4]. This can result in severe complications such as myocardial infarction and other cardiovascular disorders [4]. FXa and thrombin are, therefore, important pharmaceutical targets for the treatment and prevention of thrombotic associated ailments [1,4]. Antithrombotic agents such as thrombin inhibitors are still under development for the treatment and prophylaxis of atrial fibrillation to avoid thromboembolism [5]. However, the research focus has recently shifted toward discovering dual inhibitors of thrombin and FXa that have a broader action mechanism. To date, most of these dual inhibitors have been chemically synthesized for better clinical efficacy and are still undergoing clinical trials [6]. Characterization of naturally occurring anticoagulant compounds possessing both thrombin and FXa inhibiting properties has been gaining focus for developing superior peptide-based therapeutic molecules to ameliorate cardiovascular disorders; nevertheless, discovering such potent molecules is still in its infancy worldwide.

Snake venoms are reportedly rich in serine protease inhibitors, many of which are functionally characterized as FXa or thrombin inhibitors [7–12]. Russell's viper (*Daboia russelii russelii*) is one of the most dreaded species of snake found throughout Southeast Asia including India [13,14]. Like the venom of most venomous snake species, Russell's viper venom (RVV) is also a complex mixture of many toxic proteins and peptides that meddle in various pathophysiological processes, particularly hemostasis [11,15–20]. Additionally, the presence of several low molecular weight toxins (<8 kDa) in venom of *D. r. russelii* is also evident [12,21]. From Russell's viper venom a few serine protease inhibitors showing excellent thrombin inhibitory activity have been characterized that belong to the phospholipase A_2 (PLA_2) superfamily, or the Kunitz-type protease inhibitors [11,12,22]. However, there has been no report identifying FXa inhibitors from snake venom. Therefore, the goal of the present study is the pharmacological characterization and elucidation of the mechanism of anticoagulant action of a newly isolated, low molecular mass (4.4 kDa) peptide purified from *D. r. russelii* venom. Furthermore, we will also explore the physiological significance and therapeutic potential of the isolated peptide. To the best of our knowledge, this is the first report on the functional characterization of a snake venom peptide demonstrating dual inhibition of thrombin and FXa via non-enzymatic mechanisms.

2. Material and methods

2.1. Chemicals and venom

D. r. russelii venom was obtained from Calcutta Snake Park, Kolkata. Sephadex G-50 (fine grade) and CM Cellulose were obtained from Pharmacia Fine Chemicals, Sweden. All fine chemicals and chromogenic substrates were purchased from Sigma Chemical Co., USA. Coagulation proteins were purchased from Calbiochem, Germany. Polyvalent antivenom (PAV), effective against *D. russelii*, *Naja naja*, *Echis carinatus* and *Bungarus caeruleus* venoms) was purchased from the Serum Institute of India Limited, Pune, India (Batch No. A2512012). Russell's viper monovalent antivenom (MAV) was obtained from VINS Bioproduct Limited, India (Batch No. 30AS11001). All kits used for serum analysis were purchased from Crest Biosystem, Goa. All other reagents were of analytical grade.

2.2. Purification of an anticoagulant protein

The procedure for the fractionation of crude *D. r. russelii* venom (25.0 mg) on a CM-cellulose (20 × 60 mm^2) column has been described elsewhere [21]. The fractions eluted with 300 mM potassium phosphate buffer, pH 8.0, showing significant anticoagulant activity were pooled, desalted and lyophilized. The active fraction was dissolved in a minimum volume of equilibration buffer and applied to a Sephadex G-50 gel filtration column (1 × 60 cm^2) previously equilibrated with 20 mM potassium phosphate buffer, pH 7.0. Elution was carried out with the same buffer at room temperature (~25 °C). The flow rate was adjusted to 24.0 ml/h and 1.0 ml fractions were collected.

The gel-filtration fraction (GF-IV) containing low molecular mass proteins and showing anticoagulant activity was further purified on a reversed phase C_{18}-μ-Nova pack column by using the HPLC system (Waters, Milenium-2000). Briefly, about 50.0 μg of GF-IV was dissolved in 20.0 μl of solvent A (0.1% v/v trifloro acetic acid (TFA) in 5% v/v acetonitrile) and subjected to the RP-HPLC column previously equilibrated with solvent A. Bound proteins were eluted using a gradient of 5–95% solvent B (0.1% v/v TFA in 95% v/v acetonitrile in H_2O) from 5.0 to 37.0 min at a flow rate of 1.0 ml/min. Protein elution was monitored at 280 nm, and protein peaks were screened for anticoagulant activity on platelet poor plasma (PPP). The active fraction showing anticoagulant activity was named "Ruviprase".

The purity of preparation as well as molecular mass of Ruviprase was determined by MALDI-ToF-ToF mass spectrometric analysis (MALDI ToF/ToF Analyzer, 4800 Plus MDS SCIEX, Applied Biosystems) using 5.0 μg protein, as described previously [11].

2.3. N-Terminal sequencing and secondary structure determination

About 10.0 μg of the isolated protein was subjected to Edman degradation using a fully automated Perkin–Elmer Applied Biosystems 492 pulsed-liquid phase protein sequencer (Procise) with an on-line 785A PTH-amino acid analyzer. Protein homology searches were performed by using the BLASTP (Basic Local Alignment Search Tool) program. The secondary structure of Ruviprase was determined by measuring the circular dichroism (CD) spectrum (Jasco J715 Spectropolarimeter, Europe), as described previously [21]. CDPRO CLUSTER software was used to determine the secondary structure of Ruviprase [21].

2.4. Anticoagulant assay

For determination of the anticoagulant property of Ruviprase, plasma recalcification activity and prothrombin time test was performed with graded amounts of Ruviprase, as described previously [14,18]. Graded amounts of commercially available anticoagulants such as heparin and warfarin were also taken for comparison of the recalcification activity of PPP. One unit of anticoagulant activity is defined as an increase of 1 s clotting of PPP compared to the clotting time of normal PPP [14,18].

2.5. Serine protease inhibition

Ruviprase (2.4 μM) was incubated with either thrombin (0.16 μM)/FXa (0.21 μM)/trypsin (0.24 μM)/plasmin (0.12 μM) for 30 min at 37 °C. To the mixture, the appropriate chromogenic substrate (T1637 for thrombin/F3301 for FXa/B3133 for trypsin/V0882 for plasmin) was added, and an amidolytic assay was done, as described previously [12]. The activity of the above serine proteases towards their respective chromogenic substrates in the

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absence of Ruviprase was considered as 100% and other values were compared to this.

2.6. Inhibition of amidolytic activity of thrombin

Thrombin (3.0 μl, 10 NIH/ml) was incubated with different concentrations of Ruviprase (0–2.4 μM) for 30 min at 37 °C. Then, 0.2 mM chromogenic substrate for thrombin (T1637) was added (0.2 mM) and substrate hydrolysis was measured at 405 nm for 20 min at 30 s intervals [23,24]. The residual activity of thrombin was calculated by considering the absorbance after 20 min of substrate hydrolysis in the absence of inhibitor as 100%. The dose response curve for thrombin inhibition was fitted using GraphPad Prism 5.0 to calculate the Hill co-efficient and IC_{50} value.

2.7. Determination of inhibitory constant (Ki) for thrombin inhibition

Thrombin (3.0 μl, 10 NIH Unit/ml) was pre-incubated with two different concentrations of Ruviprase (0.6 μM and 1.2 μM) at 37 °C for 30 min. Then, various concentrations (0.1–0.8 mM) of chromogenic substrate for thrombin (T1637, Sigma) were added to the mixture, and the amidolytic assay was done as described above. For the kinetics analysis, the reaction rate (V) was plotted against substrate concentration (S) at each inhibitor concentration, and the data was fitted to a hyperbolic Michaelis–Menten model using GraphPad Prism 5.0 software.

The inhibitory constant (*Ki*) was determined using the uncompetitive model for enzyme inhibition and the same software. The inhibitory constant was determined by using the model shown below,

$$V_{App} = \frac{V_{max}}{1 + \frac{I}{\alpha Ki}} \quad (1)$$

$$Km_{App} = \frac{Km}{1 + \frac{I}{\alpha Ki}} \quad (2)$$

$$V = \frac{V_{App} \times S}{Km_{App} + S} \quad (3)$$

where

αI is the inhibition constant, expressed in the same units as I,
V_{maxApp} and V_{max} denote the maximum velocity in the presence and absence of the inhibitor, and
Km_{App} and Km denote the Michaelis constants in the presence and absence of inhibitor, respectively.

2.8. Effect of Ruviprase on fibrinogen clotting activity of thrombin

The effect of Ruviprase on the fibrinogen clotting activity of thrombin was evaluated following the procedures described by Ciprandi et al. [23].Thrombin (3.0 μl, 10 NIH unit/ml) was incubated with different concentrations of Ruviprase (0–4.8 μM) for 30 min at 37 °C. To this mixture, 100 μl of fibrinogen solution (2.5 mg/ml) was added, and the volume of the final reaction was adjusted to 100 μl with 1× phosphate buffered saline (PBS), pH 7.4. Fibrin clot formation was inspected by reading the absorbance at 605 nm at 30 s interval for 60 min. The residual activity of thrombin was calculated by considering the absorbance value after 60 min of fibrinogen clotting in the absence of Ruviprase (100% activity). Dose-response curves for the inhibition of thrombin catalyzed fibrinogen clotting were fitted using GraphPad Prism 5.0 to calculate the Hill co-efficient and IC_{50} value.

2.9. Platelet aggregation assays

The effect of Ruviprase on thrombin-induced platelet aggregation was evaluated using goat platelet rich plasma following the protocol described by Horn et al. [25]. Briefly, citrated platelet rich plasma was centrifuged at 650×*g* for 10.0 min and the pellet was washed twice in a tyrode buffer (5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1% glucose and 0.25% bovine serum albumin). The pellet was re-suspended in the same buffer, and the volume was adjusted to give an optical density (OD) of 0.15 at 650 nm. Thrombin (0.2 μg, final concentration, 0.2 unit/ml) was pre-incubated for 30 min at 37 °C with or without Ruviprase (0.6 μM and 1.2 μM) in a final volume of 50 μl with tyrode buffer. To the mixture, 100 μl of platelet solution was added, and the decrease in absorbance was monitored for 10 min at 650 nm.

2.10. Thrombin inhibition by AT-III in the presence or absence of heparin

The effect of Ruviprase on thrombin inhibition produced by AT-III in the presence or absence of heparin was performed as per the protocol described by Arocas et al. [26]. Briefly, thrombin equivalent to 0.16 μM (3 μl, 10 NIH/ml) was pre-incubated with different concentrations of Ruviprase (0.6 μM) for 30 min at 37 °C in a final reaction mixture of 100 μl adjusted with 1× PBS, pH 7.4. Heparin free AT-III (100 nM) or 25 nM AT-III in the presence of 0.5 U/ml heparin was added to the samples and the residual activity of thrombin was measured by T1637 hydrolysis after 10 min at 405 nm.

2.11. Effect of Ruviprase on the prothrombin activation property of FXa and determination of inhibitory constant (Ki) for FXa inhibition

To understand the effect of the interaction of Ruviprase with FXa, we studied the effect of Ruviprase on the physiological function (*i.e.*, prothrombin activation) of FXa. Briefly, FXa (0.02 μM) was pre-incubated with two different concentrations of Ruviprase (0.6 μM and 1.2 μM) at 37 °C for 30 min. Various concentrations (1.0 μM–5.0 μM) of prothrombin were then added to the mixture in a final reaction volume of 100 μl adjusted with 1× PBS, pH 7.4 and incubated for an additional 60 min at 37 °C. After the stipulated time interval, 2.0 μl of chromogenic substrate for thrombin (10 mM, T1637, Sigma) was added to the mixture, and the release of p-nitroanilide (pNA) was monitored at 405 nM after 10 min incubation at 37 °C). Dose-response curves for inhibition of FXa mediated prothrombin activation by Ruviprase were fitted using GraphPad Prism 5.0 to calculate the Hill co-efficient and IC_{50} value. For kinetic analysis, the reaction rate (V) was expressed as nanomoles of thrombin generated/min/μM FXa and plotted against the prothrombin concentration (S) at each inhibitor concentration, and the data was fitted to a hyperbolic Michaelis–Menten model using GraphPad Prism 5.0 software. The inhibitory constant (*Ki*) was determined using the mixed-model for enzyme inhibition using the same software. The inhibitory constant was determined by the software using the model given below,

$$V_{App} = \frac{V_{max}}{1 + I/(\alpha \times Ki)} \quad (4)$$

$$Km_{App} = \frac{Km(1 + I/Ki)}{1 + I/(\alpha \times Ki)} \quad (5)$$

$$V = \frac{V_{App} \times S}{Km_{App} + S} \quad (6)$$

where

I denotes the inhibitor concentration,
V_{App} and V_{max} denote the maximum velocity in the presence and absence of the inhibitor, and
Km_{App} and Km denote the Michaelis constants in the presence and absence of inhibitor, respectively.
$\alpha = 3.92$ (software predicted value)

2.12. Effect of pre-incubation on the inhibitory activities of Ruviprase

To determine whether Ruviprase binds thrombin/FXa with slow or fast kinetics, Ruviprase was pre-incubated with thrombin/FXa for different time intervals (0–30 min) at room temperature. Thereafter, the amidolytic activity of thrombin and prothrombin activation property of FXa was analyzed in the corresponding assay systems, as described above.

2.13. Spectrofluorometric analysis of binding of Ruviprase with thrombin/FXa

Interaction of Ruviprase with thrombin/FXa was measured by intrinsic fluorescence analysis using a fluorescence spectrometer (LS 55, Perkin Elmer, Palo Alto, CA) following the protocol described by Mukherjee et al. [12]. Briefly, thrombin/FXa was mixed with Ruviprase in a 1:1 molar ratio either in the absence or presence of Ca^{2+} (5 mM), and the change in the fluorescence intensity at an excitation of 280 nm was measured. All the binding experiments were done in triplicate to ensure the reproducibility.

2.14. Surface plasmon resonance (SPR) analysis of Ruviprase binding with thrombin

Binding affinity was measured using Biacore X100 (GE Healthcare, Freiburg, Germany). Briefly, thrombin (0.4 mg/ml in 10 mM sodium acetate buffer, pH 5.0) was immobilized on a CM5 sensor chip using the manufacturer's protocol (~5000 RU). Binding was measured in multi-cycle mode using different concentrations of Ruviprase (0 μM–3 μM) dissolved in 20 mM potassium phosphate buffer with Tween 20 (0.25% v/v), pH 7.4 (25 °C; flow rate of 10 μl/min). Between each cycle, the chip was regenerated using glycine, pH 2.5. The dissociation constant (K_D) was calculated by plotting the Response Unit (RU) at steady state (R_{eq}) versus the concentration of Ruviprase and fitting the data to a 1:1 binding model using Sigmaplot 11.0 [27].

2.15. Other biochemical characterizations

The total carbohydrate was estimated by the phenol-sulfuric acid method using glucose as a standard [28]. The protein content was estimated by the method of Lowry et al. [29]. PLA_2 and protease activity were determined as described previously [14].

2.16. Effect of antivenom on the anticoagulant and inhibitory activities of Ruviprase

In order to determine the degree of neutralization of the anticoagulant and inhibitory activities of Ruviprase towards thrombin/FXa by PAV and MAV, Ruviprase was mixed with each of these antivenoms in different ratios (1:1 to1:100, w/w). The mixture was pre-incubated for 30 min at room temperature, and thereafter, the PPP recalcification activity, Ruviprase mediated inhibition of the fibrinogen clotting activity of thrombin and prothrombin activation property of FXa were measured in their corresponding assay system. The activity in absence of PAV/MAV was considered as 100% activity and other values were compared with this.

2.17. Acute in vivo toxicity assessment on BALB/c mice

Pathogen free, laboratory inbred BALB/c mice (25–35 g) were obtained from the Central Facility of Animal House, Defense Research Laboratory, Tezpur, Assam. The animals were maintained at 33–36 °C with relative humidity ≥75%. Acute *in vivo* toxicity of Ruviprase was evaluated in BALB/c mice as per the protocol of the OECD/OCED guidelines 425 (2001) and as approved by the Institutional Animal Ethical Committees of Tezpur University and the Defense Research Laboratory, Tezpur, Assam (Approval no: DORD-Pro/TUAEC/10-56/14/Res-09). Briefly, groups of BALB/c mice ($n = 6$) were injected intraperitoneally with 2 mg/kg dose of Ruviprase dissolved in 0.25 ml of 1× PBS (pH 7.4). Groups of mice ($n = 6$) injected with 1× PBS, pH 7.4, served as a control (placebo). The animals were observed at regular intervals for up to 48 h for any behavioral change, viz. body weight, food and water intake, grip strength, and rectal temperature. Whole blood clotting time was measured by the capillary glass method [30] recorded after 6 h of treatment of the animal. After 48 h of treatment, the animals were sacrificed and blood was collected immediately by cardiac puncture. Hematological parameters were analyzed by Hematology Auto Analyzer MS4-S (Melet Schloesing Laboratories, Osny, France). The plasma obtained from the control as well as from the treated groups of mice blood was analyzed for different biochemical parameters, viz. total protein, glucose, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), creatine kinase (CK-MB), cholesterol, triglyceride, and urea and uric acid by using commercial diagnostic kits and following the manufacturers' instructions. All biochemical parameters were analyzed on an autoanalyzer (Biochemical Systems International SRL Model 3000 evolution, Florence, Italy). For histological analysis of liver, kidney and heart morphology, the tissues were fixed in 10% formalin, processed routinely and stained with hematoxylin and eosin for light microscopic observation at 40× magnification.

2.18. Statistical analysis

Data are expressed either as means or as means ± standard deviation of three experiments or unless otherwise indicated. The statistical analysis of the data was done using the student's *t*-test. The value of $p \leq 0.05$ was considered as significant. Statistical analysis and data fitting was performed using SigmaPlot 11.0 software.

3. Results

3.1. Purification, identification, and secondary structure of Ruviprase

Fractionation of RVV anticoagulant proteins eluted with 300 mM potassium phosphate buffer (pH 8.0) from a CM-cellulose column on a Sephadex G-50 gel filtration column resulted in separation of four protein peaks (GF-I, GF-II, GF-III, and GF-IV) (Supplementary Fig. S1). The fractions of both GF-III and GF-IV contained low molecular mass proteins and demonstrated anticoagulant property. However, GF-IV was selected for further studies as it demonstrated a better anticoagulant property as compared to GF-III. The peak GF-IV showing potent anticoagulant activity was subjected to fractionation through RP-HPLC, which resulted in a single peak exhibiting anticoagulant activity. The active fraction was termed Ruviprase and contributed to 0.1% of the total RVV proteins (Fig. 1(A), Supplementary Table S1). The purity and molecular mass of Ruviprase was determined by MALDI-MS as 4423.6 Da (Fig. 1(B)). The partial N-terminal amino

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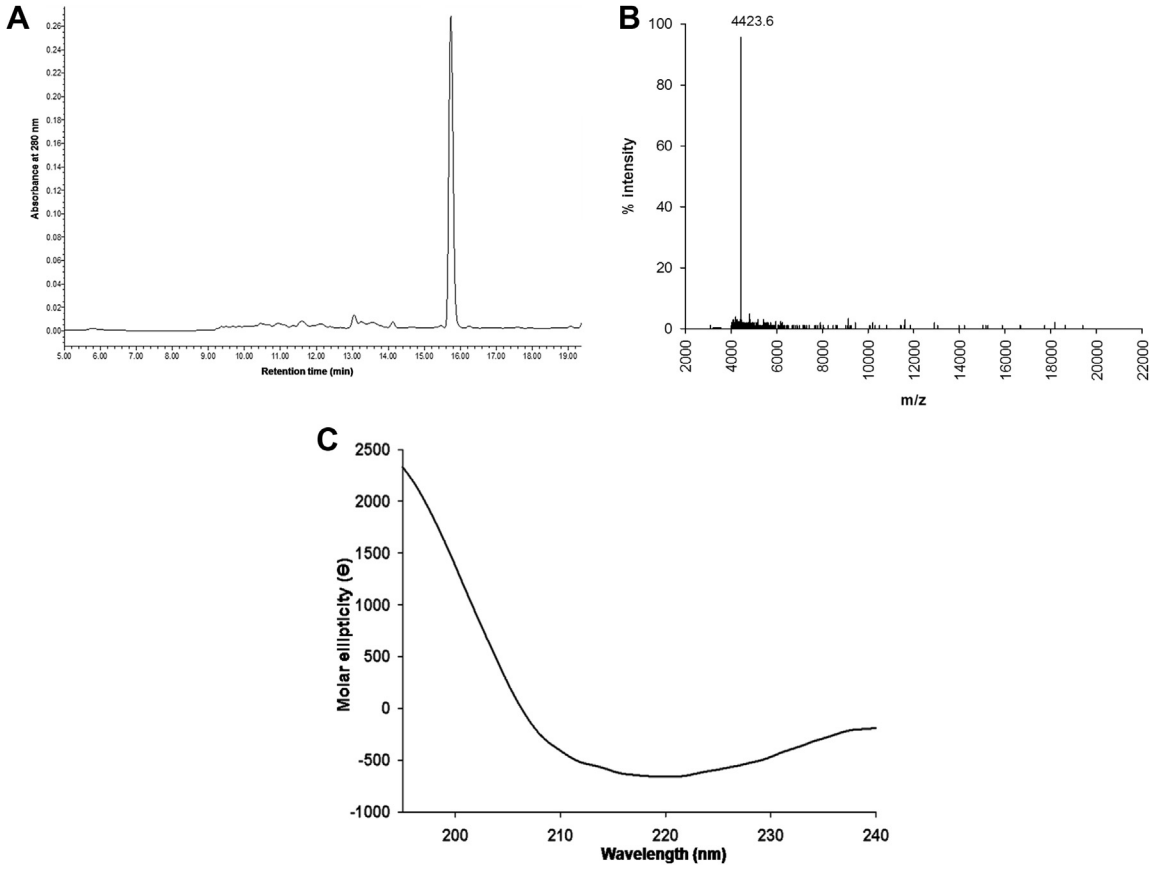

Fig. 1. Purification and biochemical characterizations of Ruviprase. (A) RP–HPLC analysis of Ruviprase. RP-HPLC analysis of the active fraction (GF-IV) on C_{18} column at a 0–100% gradient of B (95% acetonitrile) in the presence of A (5% acetonitrile). (B) MALDI-ToF-ToF mass spectra of Ruviprase. (C) Cirular dichroism (CD) spectra of Ruviprase. Native Ruviprase (0.3 mg/ml) was dissolved in 20 mM potassium phosphate buffer pH 7.0 and the far UV-CD spectra was recorded at room temperature (~25 °C)between 195 and 250 nm against the appropriate buffer (blank).

acid sequence of Ruviprase (E^1-V^2-X^3-W^4-W^5-W^6-A^7-Q^8-L^9-S^{10}) showed maximum sequence homology (80.0%) with the internal sequence of the apoptosis-stimulating factor of p53 protein 2 (Accession no: ETE62279.1) from venom of *Ophiophagus hannah* and apoptosis-stimulating factor of p53 protein 2 isoform X2 and X1 (Accession no: XP_007426348.1 and XP_007426347.1, respectively) from venom of *Python bivittatus*. A variation in the partial *N*-terminal sequence of Ruviprase was observed only at two positions; at position 2, I was replaced by E in Ruviprase. Also, the amino acid residue at position 3 (E in similar peptides) could not be identified in Ruviprase and hence denoted as X. Nevertheless, it is to be noted that the anticoagulant property of these proteins has never been investigated. Analysis of the CD spectrum of Ruviprase revealed that it was primarily composed of α-helices (61.9%) with the characteristic minimum at 222 nm (Fig. 1(C)). Random coils (38.1%) contributed to the rest of this protein structure (Fig. 1(C)).

3.2. Anticoagulant activity and inhibition of serine proteases

Ruviprase prolonged the plasma recalcification time in a dose-dependent manner (Fig. 2(A)). Moreover, Ruviprase demonstrated analogous *in vitro* anticoagulant potency as compared to that of commercial anticoagulants viz. heparin and warfarin (Fig. 2(A)). Ruviprase also prolonged the prothrombin time in a dose-dependent manner (Fig. 2(B)). Ruviprase was found to be devoid of carbohydrate content, and it lacked PLA_2 or protease activity (data not shown).

Ruviprase failed to inhibit the amidolytic activity of plasmin, trypsin, and FXa (data not shown), but it significantly inhibited the amidolytic activity of thrombin in a dose-dependent manner (Fig. 3(A)). The IC_{50} value and Hill co-efficient of thrombin inhibition (amidolytic activity) were determined as 0.23 μM and 1.08, respectively. The Michaelis–Menten plot (Fig. 3(B)) indicated a decrease in both the *Km* as well as V_{max} values of thrombin for its substrate (T1637) in the presence of Ruviprase. The kinetics data indicate that Ruviprase inhibits thrombin in an uncompetitive manner. The *Ki* value for the inhibition of thrombin by Ruviprase was determined as 0.42 μM. Additionally, Ruviprase did not enhance thrombin inhibition produced by Heparin-AT-III complex (Supplementary Table S2).

The fibrinogen clotting activity of thrombin was also inhibited by Ruviprase in a dose-dependent manner (Fig. 3(C)). The IC_{50} value and Hill co-efficient for the inhibition of fibrinogen clotting activity of thrombin by Ruviprase were determined at 1.52 μM and 5.21, respectively. Ruviprase also inhibited thrombin-induced platelet aggregation at the tested doses (Fig. 3(D)).

Although Ruviprase failed to inhibit the amidolytic activity of FXa towards its chromogenic substrate F3301, it inhibited the physiological function of FXa (prothrombin activation) in the absence of the prothrombinase complex (Fig. 4). The Michaelis–Menten plot showing inhibition of FXa (prothrombin activation) by Ruviprase indicated a mixed type of inhibition (Fig. 4). A decrease in the V_{max} value with a subsequent increase in the *Km* value of FXa towards prothrombin in the presence of Ruviprase was observed. The *Ki* value for the inhibition of FXa (towards

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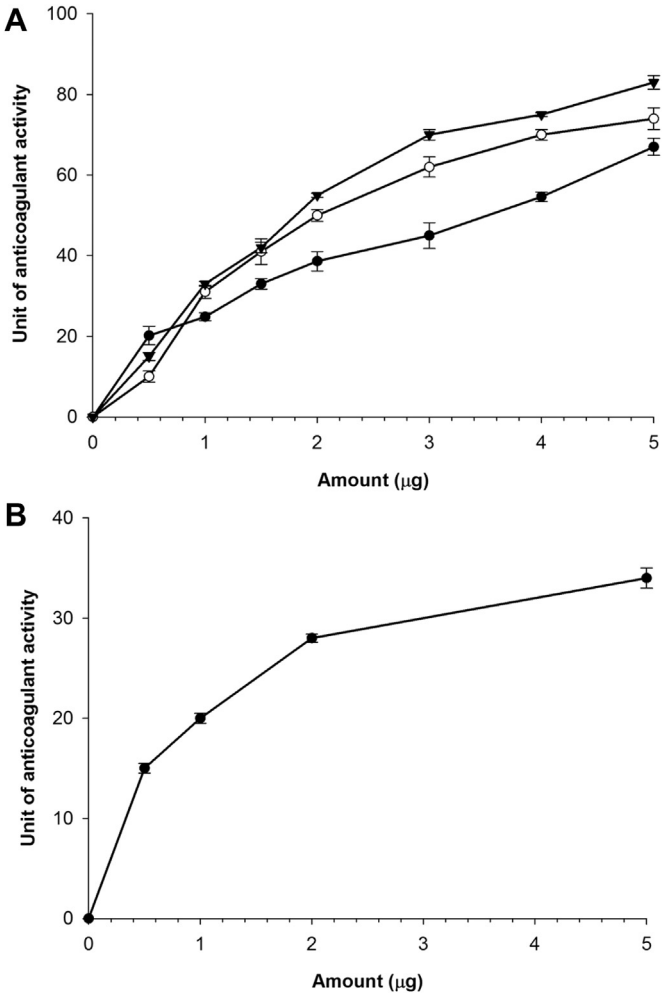

Fig. 2. (A) Comparison of dose dependent plasma recalcification activity of Ruviprase (▼) with warfarin (○) and Heparin (●). (B) Dose dependent prothrombin time of Ruviprase. One unit of anticoagulant activity is defined as increase in 1 s clotting of plasma compared to clotting time of normal plasma.

prothrombin activation) by Ruviprase was determined as 0.16 μM. The IC_{50} value and Hill co-efficient for the inhibition of prothrombin activation by FXa were determined as 0.46 μM and 2.08, respectively.

Furthermore, it was observed that with increased pre-incubation of Ruviprase with thrombin or FXa, the inhibitory activities of Ruviprase were also enhanced concomitantly; however, saturation was observed after 30 min of pre-incubation of Ruviprase with thrombin/FXa (Fig. 5).

3.3. Interaction study of Ruviprase with thrombin/FXa

Excitation of Ruviprase/thrombin/FXa at 280 nm resulted in an emission maximum at 345 nm (Fig. 6(A)). The interaction between Ruviprase and thrombin resulted in an increase in the emission fluorescent signal even in the absence of Ca^{2+} (Fig. 6(A)). A similar phenomenon was also observed in the interaction between Ruviprase and FXa (Fig. 6(B)). Nevertheless, the presence of millimolar concentrations of Ca^{2+} increases the binding strength of Ruviprase with thrombin or FXa (Fig. 6(A–B)).

The binding of Ruviprase to thrombin was confirmed by SPR analysis. A multi-cycle binding study was performed, and the dissociation constant was determined using steady state data (Fig. 6(C)). The dissociation constant (K_D) of Ruviprase for thrombin was found to be 0.46 μM.

3.4. Neutralization of Ruviprase by commercial antivenom

Both polyvalent antivenom (PAV) and monovalent antivenom (MAV), at a dose of 1: 100 (Ruviprase: PAV/MAV) ratio, failed to neutralize the inhibitory effects of Ruviprase on the fibrinogen clotting activity of thrombin or the prothrombin activation property of FXa, as well as the anticoagulant property of Ruviprase (data not shown).

3.5. Acute in vivo toxicity assessment on BALB/c mice

At a dose of 2 mg/kg body weight, Ruviprase was non-lethal to mice and the treated animals did not show any noticeable behavioral changes. However, the *in vitro* whole blood clotting time of the treated group of mice (255.0 ± 8.0 s) was found to be significantly higher ($p < 0.05$) as compared to the whole blood clotting time of control group of mice (180.0 ± 9.0 s) under identical experimental conditions. Biochemical analysis of serum from Ruviprase-treated mice (48 h post treatment) showed that apart from alkaline phosphatase (ALP) and cholesterol, no significant changes were observed in the serum level of any of the other tested parameters as compared to these levels in the control group of mice (Supplementary Table S3). However, the elevated levels of both ALP and cholesterol were found to be within the normal range. Besides, none of the hematological parameters of mice was found to be significantly affected by Ruviprase treatment (Supplementary Table S4). Histopathological study of liver, kidney, and heart tissues from Ruviprase-treated mice did not show any morphological alterations in these tissues as compared to tissues from the control group of mice (data not shown).

4. Discussion

A number of potential inhibitors of thrombin and FXa have been reported from snake venom and from the salivary gland secretions of hematophagous insects [7,10–12,23–25]. Venom of snakes belonging to the Viperidae family also possesses specific serine protease inhibitors [7,11,12] which belong to the C-type lectin protein family (snaclecs), the PLA_2 superfamily, or the Kunitz-type protease inhibitors. However, to date, there has been no report on the characterization of a dual inhibitor of thrombin and FXa from snake venom.

Most of the serine protease inhibitors from snake venom have a molecular mass in the range of 6–29 kDa [7,10–12] although low molecular mass thrombin and FXa inhibitors (<5 kDa) have also been isolated from hematophagous insects such as tick [22,31,32]. To the best of our knowledge, Ruviprase, with a molecular mass of 4423.6 Da, is the smallest inhibitor of thrombin as well as FXa reported to date from snake venom. To ascertain whether Ruviprase is accidently produced during the extraction or drying of venom, we purified Ruviprase from several different batches of RVV that were from Eastern Indian origin. It is noteworthy that the results were identical in each case, suggesting that Ruviprase is produced and processed in the venom gland of Russell's Viper.

Partial N-terminal sequence analysis of Ruviprase suggests that, unlike most of the thrombin or FXa inhibitors isolated from snake venom, Ruviprase does not belong to the C-type lectin protein family (snaclecs) or Kunitz-type protease inhibitors isolated from venom. Instead, the partial N-terminal sequence of Ruviprase displayed significant homology with the internal sequence of an apoptosis-inducing peptides (10 kDa) identified by analysis of the venom-gland transcriptome of the *O. hannah* and *P. bivittatus*,

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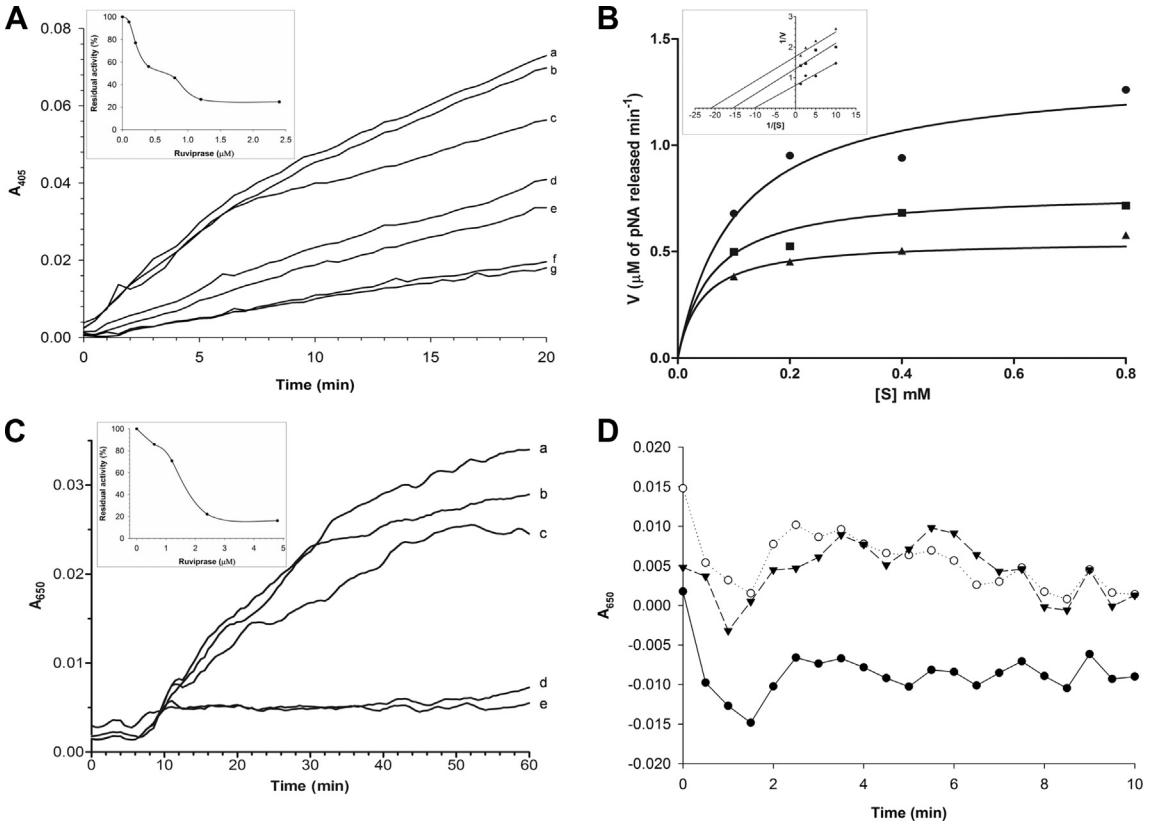

Fig. 3. (A) Inhibition of the amidolytic activity of thrombin by Ruviprase. Thrombin (0.03 NIH/ml) was preincubated with (a) 0 μM; (b) 0.1 μM; (c) 0.2 μM; (d) 0.4 μM; (e) 0.8 μM; (f) 1.2 μM; or (g) 2.4 μM Ruviprase for 30 min at 37 °C. Chromogenic substrate for thrombin (T1637, 0.2 mM) was added and hydrolysis of the substrate was monitored for 30 min at 405 nm at 30 s intervals. Inset: Dose response curve of inhibition of the amidolytic activity of thrombin by Ruviprase. Residual activity of thrombin was calculated by considering the absorbance after 20 min of substrate hydrolysis in the absence of inhibitor as 100%. (B) Michaelis–Menten plots and Lineweaver–Burk plots (inset) showing inhibition of thrombin activity towards T1637 by (●) 0 μM; (■) 0.6 μM; or (▲) 1.2 μM Ruviprase. The plots are the means of 3 independent measurements. (C) Inhibition of thrombin induced fibrinogen clotting by Ruviprase. Thrombin (3 μl, 10 NIH/ml) was preincubated with (a) 0 μM; (b) 0.6 μM; (c) 1.2 μM; (d) 2.4 μM; or (e) 4.8 μM for 30 min at 37 °C prior to addition of fibrinogen (50 μg) in a final reaction mixture of 100 μl. Fibrin-clot formation was measured by monitoring the change in absorbance at 650 nm. Inset: dose–response curve of inhibition of thrombin induced fibrinogen clotting by Ruviprase. Residual activity of thrombin was calculated by considering the absorbance after 60 min of fibrinogen clotting in the absence of inhibitor as 100%. (D) Effect of Ruviprase on thrombin induced platelet aggregation. Thrombin (0.2 μg, 0.2 NIH unit/ml) was incubated with (●) 0 μM; (▼) 0.6 μM or (○) 1.2 μM Ruviprase, for 10 min at 37 °C. Washed platelet suspension was added to the mixture and decrease in absorbance was monitored at 650 nm for 10 min.

whose effect on the coagulation system is unknown. Secondary structure analysis showed that Ruviprase is primarily composed of α-helices and random coils, unlike some of the thrombin inhibitors isolated from tick such as Vareigin, which is a typical random coil protein [31], and snake venom Kunitz-type protease inhibitors such as Rusvikunin, which is primarily made of β-sheets and random coils [12]. The secondary structure of Ruviprase showed partial similarity to snake venom thrombin inhibitors such as Bothrojaracin, a snaclec, which typically consists of α- helices and β- sheets [33]. These data indicate that Ruviprase is a novel anticoagulant peptide isolated from RVV or any snake venom.

The catalytic activity of thrombin is regulated by the recognition domains – exosite-I and exosite-II, in addition to the active site [34,35]. Exosite-I of thrombin is the fibrinogen-binding site, whereas exosite-II is responsible for heparin binding [34,35]. Both sites are distant from the catalytic pocket but are involved in the specific binding of thrombin to several macromolecular substrates, inhibitors and modulators [34,35]. The binding of Ruviprase with thrombin or FXa was evident by fluorescence spectroscopy and SPR analyses. The binding was enhanced in the presence of Ca^{2+}, suggesting that divalent ions might contribute to increased protein–-protein interaction. However, Ruviprase demonstrated potent inhibitory effects on thrombin and FXa, even in the absence of divalent ions. Ruviprase binding resulted in the inhibition of the catalytic activity of thrombin towards its small chromogenic substrate, suggesting its interaction with the active site. The catalytic inhibition of thrombin by Ruviprase followed an uncompetitive model of inhibition similar to that shown by rAaTI, a thrombin inhibitor isolated from the saliva of *Aedes aegypti* [36]. The mode of Ruviprase mediated thrombin inhibition was different from that of other potent snake venom thrombin inhibitors such as TI-Nh [10] and Rusvikunin [12], which follow a mixed model or non-competitive model of thrombin inhibition, respectively.

It has been well established that fibrinogen binding by thrombin is mediated through exosite-I, which is followed by the enzymatic cleavage of fibrinogen leading to its clotting [34]. Further, thrombin-induced aggregation of platelets is mediated by enzymatic cleavage of protease-activated receptors (PAR-1 and PAR-4 present on the surface of platelets) by thrombin [3]. As such, the enzymatic activity of thrombin is necessary for fibrinogen clotting and platelet aggregation [3]. Ruviprase inhibited the amidolytic activity, fibrinogen clotting, and platelet aggregation property of thrombin to an equal extent; therefore, it may be assumed that binding of Ruviprase to the catalytic site of thrombin results in inhibition of its enzymatic activity with a subsequent inhibition of its fibrinogen clotting or platelet aggregation property. Therefore, Ruviprase does not hamper substrate binding (fibrinogen) to exosite-I of thrombin. The heparin-AT-III complex strongly inhibits the catalytic properties of thrombin by binding to the heparin-binding site (exosite-II) of thrombin [34,35]. Pre-incubation of

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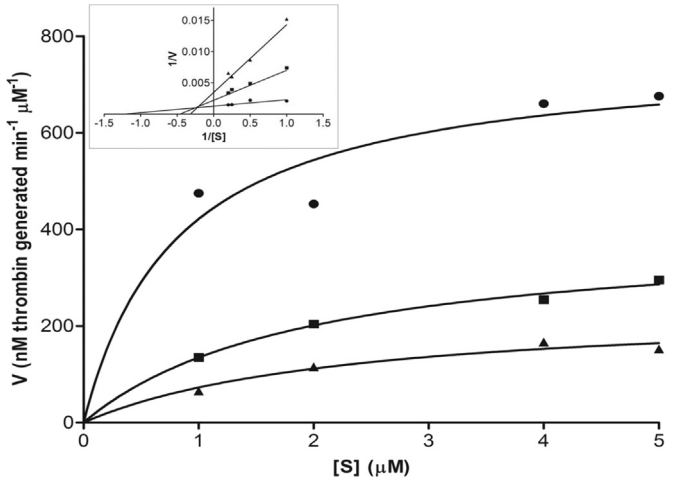

Fig. 4. Kinetic analysis of FXa inhibition by Ruviprase. Michaelis–Menten and Lineweaver–Burk plots (inset) showing inhibition of FXa mediated prothrombin activation by (●) 0 µM; (■) 0.6 µM; or (▲) 1.2 µM Ruviprase. The plots are the means of 3 independent measurements.

Ruviprase/thrombin prior to addition of heparin/AT-III lead to a partial decrease in the thrombin inhibitory property of the latter, suggesting that Ruviprase, in addition to binding to the active site, also binds to exoiste-II (the heparin binding site) of thrombin. Therefore, the mechanism of thrombin inhibition by Ruviprase is distinctly different from known snake venom thrombin inhibitors such as Bothrojaracin [7], TI-Nh [10], RVAPLA$_2$ [11] and Rusvikunin [12] as well as tick thrombin inhibitors such as Savignin [37] and Boophilin [38]. However, so far the data is not sufficient to conclude that Ruviprase binds to exosite-II. Further structural analysis is essential to pinpoint the thrombin binding region of Ruviprase.

Biosensor analysis suggested that the affinity of Ruviprase towards thrombin is higher than that of thrombin inhibitors isolated from hematophagus insects [39]. However, it showed lower affinity ($Kd = 0.46$ µM) in comparison to C-type lectins isolated from snake venom [7]. Ruviprase demonstrated a higher *Ki* value for thrombin inhibition compared with some other snake venom thrombin inhibitors such as Bothrojaracin (0.15 nM) [7], TI-Nh (73.0 nM) [10] and RVAPLA$_2$ (0.38 µM) [11]. Nevertheless, unlike these inhibitors, Ruviprase also demonstrated inhibition of the PTH activating property of FXa; therefore, it turns out to be a superior anticoagulant agent compared to the snake venom thrombin inhibitors described to date.

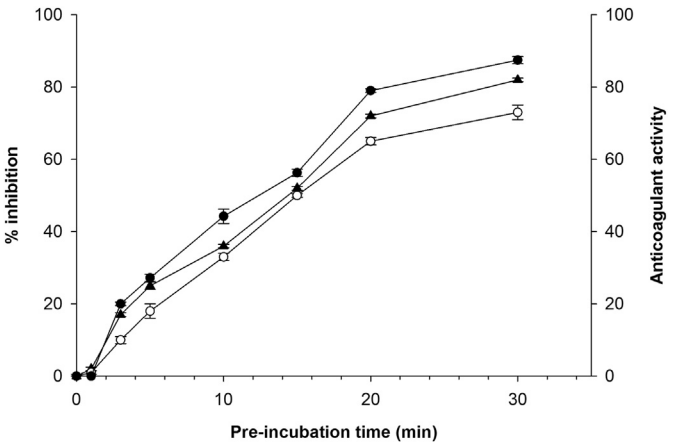

Fig. 5. Effect of pre-incubation on the anticoagulant activity (●) and inhibitory effects of Ruviprase on thrombin's amidolytic activity (○) and prothrombin activation by FXa (▼).

The FXa inhibitors are basically classified under two groups-direct and indirect FXa inhibitors [34]. Direct FXa inhibitors are those which inhibit FXa directly without the requirement of any intermediate, whereas indirect FXa inhibitors demonstrate an AT-III mediated FXa inhibition [40]. To date there has been no report on the characterization of FXa inhibitor from snake venom. Ruviprase demonstrated direct FXa inhibition via a mixed model of inhibition, indicating that Ruviprase can inhibit free FXa or prothrombin (substrate) bound FXa. Besides, since Hill's co-efficient for binding of Ruviprase to thrombin (1.08) or FXa (5.21) was more than 1.0, a positive cooperative binding between the above coagulation factors and the inhibitor (Ruviprase) can be predicted. Additionally, binding of Ruviprase to thrombin or FXa follows slow binding kinetics because the binding strength of Ruviprase to thrombin/FXa increases with increased pre-incubation time. Taken together, the anticoagulant nature of Ruviprase is substantially attributed to its non-enzymatic mechanism of thrombin and FXa inhibition, and it furnishes an example of a component of snake venom exerting its biological activity through protein (venom)-protein (predator/victim) interaction [10–12].

Due to the low immunogenicity of low molecular mass snake venom proteins and peptides, neutralization of such venom components by commercial antivenoms is always a challenge [41]. The inability of both commercial polyvalent and monovalent antivenom to neutralize the physiological properties of Ruviprase clearly suggests the necessity of a better antivenom therapy for the efficient management of snakebite patients.

Ruviprase, at the tested dose of 2 mg/kg (intraperitoneal), did not induce mortality in mice even after 48 h of injection, suggesting that it is non-lethal. Ruviprase represents 0.1% (w/w) of total RVV protein and on average an adult Russell's viper may inject 225–250 mg of venom (the total amount in a bite) to its victim [42]. Therefore, after a full Russell's viper bite, 180–200 µg of Ruviprase gets injected, which corresponds to a nanomolar concentration (7.4 nM–8.2 nM) of Ruviprase in the blood of an adult victim. Therefore, it is unlikely that Ruviprase exerts anticoagulant activity in an adult victim; however, its pathobiological effect in mice (concentration in blood ~1.6 µM) and rats (concentration in blood ~2.5 µM) cannot be ruled out. There are reports of several weak or non-toxic proteins/peptides of venom that exert a synergistic effect with other components of venom to enhance the lethality of venom [43,44]. Therefore, association of Ruviprase with other component(s) of the same venom to form a protein complex might contribute to the overall lethality of RVV envenomation (Mukherjee, A.K., Thakur, R, unpublished observation).

Thrombosis-associated disorders such as cardiovascular diseases are a leading cause of mortality in both developing and developed countries [45,46]. Unfortunately, many natural anticoagulants used as antithrombotic drugs are associated with adverse side effects. Heparin and warfarin are two of the potent commercial anticoagulants used for the treatment of thrombosis-associated ailments. Heparin does not possess anticoagulant activity by itself; however, it shows anticoagulant effect by binding with AT-III present in plasma leading to thrombin/FXa inhibition [39]. Warfarin, on the other hand, inhibits the synthesis of biologically active forms of the calcium-dependent clotting factors II, VII, IX and X, as well as the regulatory factors—protein C, protein S, and protein Z [46]. Both of these compounds demonstrate severe adverse effects mostly accompanied with bleeding complications. As such, snake venom derived potent anticoagulants have been considered as an alternative for the development better antithrombotic agents [7,10–12]. Additionally, low toxicity anticoagulants are in great demand for therapeutic use in the prevention and treatment of

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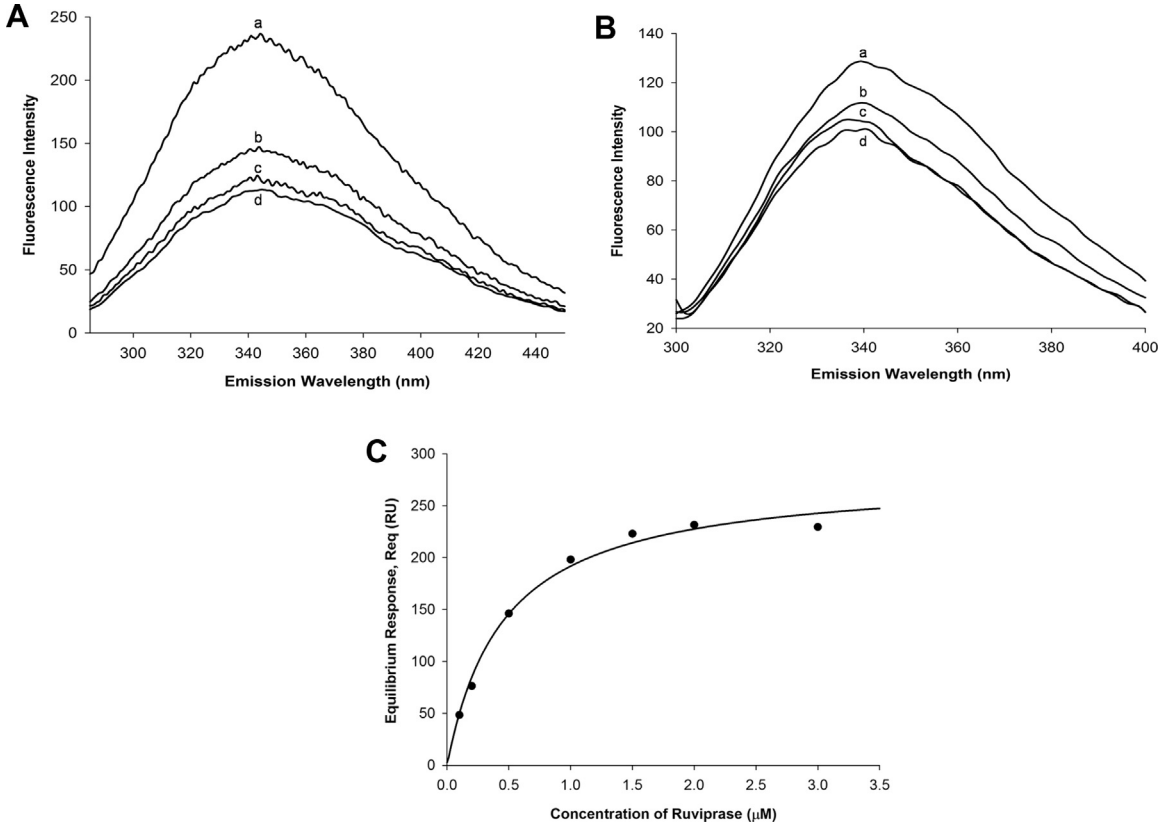

Fig. 6. (A) Fluorescence spectra showing interaction of Ruviprase with thrombin. (a) thrombin (0.6 μM) in presence of Ruviprase (0.6 μM) and 5 mM Ca^{2+}, (b) thrombin (0.6 μM) and Ruviprase (0.6 μM) in absence of 5 mM Ca^{2+}, (c) Thrombin (0.6 μM) and, (d) Ruviprase (0.6 μM). (B) Fluorescence spectra showing interaction of Ruviprase with FXa. (a) FXa (0.2 μM) in presence of Ruviprase (0.6 μM) and 5 mM Ca^{2+}, (b) FXA (0.2 μM) and Ruviprase (0.6 μM) in absence of 5 mM Ca^{2+}, (c) FXa (0.2 μM) and, (d) Ruviprase (0.6 μM). (C) Equilibrium binding assay of Ruviprase with thrombin by SPR. Sensorgram depicting interaction of Ruviprase with thrombin. Thrombin was immobilized at high density (~5000 RU) via amine coupling using a Biacore CM5 sensor chip. Inset: One site saturation binding curve of Ruviprase for thrombin. The concentrations of Ruviprase were: 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 and 3.0 uM; the data is fitted to the equation, $R_{eq} = RU_{max} \times C/(K_D + C)$. The $K_D = 458.7$ nM $R^2 = 0.9890$.

occlusive thrombosis. Ruviprase at the therapeutic dose of plasmin (2 mg/kg) [47] does not show toxicity or adverse pharmacological effects but demonstrates strong anticoagulant activity. This suggests the suitability of Ruviprase for development of peptide-based therapeutic drugs for treatment of cardiovascular ailments [7,10–12].

5. Conclusion

RVV is regarded as a gold mine of potential therapeutic molecules which affect the process of hemostasis. In this communication, we report for the first time the isolation and functional characterization of a non-lethal, low molecular mass dual inhibitor of thrombin and FXa from RVV or any snake venom. Ruviprase demonstrated slow binding direct inhibition of both thrombin and FXa via different non-enzymatic mechanisms. The potent *in vitro* and *in vivo* anticoagulant effects of Ruviprase suggest the pharmacological significance of Ruviprase. This new basic knowledge about Ruviprase permits us to foresee the development of it as a therapeutic agent to prevent unwanted blood clot (thrombus) formation. Future studies on the structural aspects of Ruviprase designed to enable us to understand its binding mechanisms as well as its therapeutic potential in terms of its inhibitory effects on thrombin and FXa are warranted. Moreover, since Ruviprase displayed sequence similarity with apoptosis-inducing peptides isolated from snake venom, the pro-apoptotic property and anticancer potential of Ruviprase likewise requires further investigation.

Author contribution

AKM designed experiments, analyzed data and edited the manuscript; RT designed kinetics study of inhibiton, performed laboratory analysis, analyzed the data and prepared the manuscript; AK and BB performed and analyzed SPR data; DS helped in purification of the peptide; DP performed N-terminal sequence of the peptide; PC provided help in animal experimentation.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgments

Ms. R. Thakur, Ms. D. Saikia and Mr. A. Kumar were recipients of DST-INSPIRE Senior Research Fellowship, CSIR Senior Research Fellowship and IIT Guwahati Institutional fellowships, respectively. This work was supported partially by Research Grants to AKM and DP from the DBT-twinning project (Grant no. BT/38/NE/TBP/2010), Department of Biotechnology (DBT), New Delhi and instrument support under the DST-FIST grant to the Department of MBBT, TU. The BiaCore facility at IIT Guwahati is supported by research grant from Department of Biotechnology, India (project no. BT/01/NE/PS/08). The authors thank ContentConcepts, Chennai, India for providing copy-editing and proof-reading services for the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.07.006.

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Please cite this article in press as: R. Thakur, et al., A new peptide (Ruviprase) purified from the venom of *Daboia russelii russelii* shows potent anticoagulant activity via non-enzymatic inhibition of thrombin and factor Xa, Biochimie (2014), http://dx.doi.org/10.1016/j.biochi.2014.07.006