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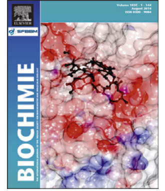

Research paper

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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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.

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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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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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