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Biochemical and pharmacological properties of a new thrombin-like serine protease (Russelobin) from the venom of Russell's Viper (*Daboia russelii russelii*) and assessment of its therapeutic potential

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ABSTRACT

Background: Snake venoms are rich sources of bioactive molecules, and several venom-derived proteins have entered clinical trials for use in ischemic disorders; however, late-stage failure of a recent drug candidate due to low *in vivo* efficacy demonstrated the need for new sources of fibrinogenolytic drug candidates.
Methods: A 51.3 kDa thrombin-like serine protease (Russelobin) purified from the venom of Russell's Viper (*Daboia russelii russelii*) was subjected to extensive biochemical characterization, including N-terminal sequencing, substrate specificity, kinetic and inhibitor assays, glycosylation analysis and stability assays. Toxicity and pathology analyses were conducted in NSA mice.
Results: Russelobin has extensive N-terminus identity with a beta-fibrinogenase-like serine proteinase precursor from *Daboia russelii siamensis* venom, a mass of 51.3 kDa and contains extensive N-linked oligosaccharides. Serine protease inhibitors and heparin significantly decreased activity, with much lower inhibition by DTT, antithrombin-III and α_2-macroglobulin. Russelobin preferentially released FPA and slowly released FPB from human fibrinogen, forming a labile fibrin clot readily hydrolyzed by plasmin. The partially deglycosylated enzyme showed significantly lower activity toward fibrinogen and less resistance against neutralization by plasma α_2MG and antithrombin-III. Russelobin was non-cytotoxic, non-lethal and produced no histopathologies in mice, and it demonstrated *in vivo* dose-dependent defibrinogenating activity.
Conclusions: Russelobin is an A/B fibrinogenase with high specificity toward fibrinogen, both *in vitro* and *in vivo*. Extensive glycosylation appears to protect the molecule against endogenous protease inhibitors, prolonging its *in vivo* efficacy.
General significance: Due to its low toxicity, stability and activity as a defibrinogenating agent, Russelobin shows high potential for cardiovascular drug development.

1. Introduction

Russell's Viper (*Daboia russelii russelii*) venom contains an array of proteolytic enzymes which profoundly affect the hemostatic system of snakebite victims [1–3]. Collectively, these multifunctional venom proteases act at different stages of the blood coagulation cascade and contribute significantly to venom-induced toxicity, morbidity and lethality [4–6]. Among these, a specific class of proteases containing serine in the active site and functionally resembling thrombin is known as snake venom thrombin-like enzymes, or SVTLEs [see 7–9 for reviews]. Because of their specific involvement in the cascade reactions, many SVTLEs have been sequenced, with many fewer being subjected to careful evaluation of biological properties. In fact, many sequenced (cDNA-based) SVTLEs have been assigned activities due to apparent homology alone; however, all venom serine proteases share a high degree of sequence similarity, particularly among functionally important residues, and relatively minor surface residue changes can lead to significantly different substrate recognition/binding properties [5,9,10]. Therefore, different structural variants could contain particular and desirable features for developing new fibrinogenolytic drugs with high efficacy. The recent failure of Viprinex, an SVTLE which showed great promise, in late-stage clinical trials [11] further underscores the need to evaluate different sources of potentially therapeutic enzymes.

The SVTLEs are capable of converting fibrinogen to fibrin by preferentially catalyzing hydrolysis of the Arg-Lys bonds of either the Aα or the Bβ-chains of fibrinogen; in some rare instances, they have been reported to cleave both the Aα and Bβ-chains [7–9,12,13]. However, the fibrinogen-clotting activity of SVTLEs is typically significantly lower than that of thrombin [8]. Furthermore, due to the lack of activation of FXIII by SVTLEs, they form non-cross-linked, unstable fibrin monomers which are rapidly degraded by plasmin [8]. As a result, *in vivo*, SVTLEs can deplete the fibrinogen content of the blood and lower its viscosity without affecting other hematological parameters.

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Since fibrinogen is a major risk factor following the onset of cardiovascular diseases, SVTLEs acting as *in vivo* defibrinogenating agents are potential candidates for the development of cardiovascular drugs and for treatment of hyperfibrinogenemia-associated disorders [7–9]. In addition, several SVTLEs currently or potentially find application as anticoagulant agents for the prevention and treatment of a wide range of thrombotic disorders, as a diagnostic reagent for the detection of fibrinogen levels in heparinized blood samples, and in coagulation studies [14].

Most thrombin-like enzymes have been isolated, purified and characterized from venom of snakes belonging to the family Viperidae [7–9], though several have been identified in venoms from the families Elapidae and Dipsadidae [9,15]. However, at present no such enzyme has been purified and characterized from the venom of *D. russelii* (Russell's Viper), a viperid snake responsible for the highest mortality and morbidity in southeast Asia and parts of India [1,16]. The majority of deaths from Russell's Viper bites are attributed to incoagulable blood caused by defibrination, resulting from consumption of the components of the hemostatic system [1,16]. Based on these common coagulopathies, we hypothesized that this venom could contain SVTLEs with high specificity, some of which may have therapeutic potential. Although SVTLEs share many features in common with native thrombin, they differ significantly in many ways. For example, all the thrombin-like enzymes from snake venom possess different biochemical and pharmacological properties from thrombin, and many differ in details of their thrombin-like action [7,8]. Therefore, in addition to drug discovery applications, characterization of a new thrombin-like enzyme from Russell's Viper venom will contribute to our understanding of the mechanistic and structural basis of the thrombin-like actions of SVTLEs, helping to ameliorate the severity of bites from this and other species. In this communication, we report the purification, characterization and potential biomedical application of a new thrombin-like enzyme purified from venom of *D. r. russelii* (of Pakistan origin).

2. Materials and methods

Russell's Viper (*D. r. russelii*) venom (snakes of Pakistan origin) was a gift from Kentucky Reptile Zoo. Pre-cast NuPAGE Novex® Bis-Tris mini Gels, buffers and Mark 12 unstained molecular mass standards were obtained from Invitrogen Inc., USA. Protein concentration standard reagents were purchased from BioRad Inc., USA. All other chemicals used were of analytical grade or better and procured from Sigma-Aldrich, USA.

2.1. Purification of a thrombin-like serine protease from RVV

Lyophilized *D. r. russelii* venom (200 mg) dissolved in 1.5 ml of 25 mM HEPES buffer containing 100 mM NaCl and 5 mM $CaCl_2$ (pH 6.8) was fractionated on a Bio Gel P-100 gel-filtration column (2.8 × 80 cm) previously equilibrated with the same buffer. The flow rate was adjusted to 6 ml/h and fractions of 3.0 ml were collected. Protein was monitored at 280 nm, and peaks were screened for thrombin-like activity using a chromogenic substrate for thrombin (N-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide hydrochloride; see below).

Fractions showing thrombin-like activity were pooled and directly subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on a Vydac Protein C_4 HPLC column (250 mm × 4.6 mm) previously equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The column was washed with 0.1% (v/v) TFA for 6 min, followed by elution of bound proteins with a linear gradient over 56 min from 0 to 100% buffer B (80% acetonitrile (ACN) in H_2O containing 0.1% TFA) at a flow rate of 1 ml/min. The elution of protein was monitored at 280 nm and protein peaks were screened for thrombin-like activity as described below.

2.2. Determination of purity and molecular weight of the protein

The purity and molecular mass of the protein were determined by SDS-PAGE (12.5% NuPAGE Novex® Bis-Tris mini gels) under both reduced and non-reduced conditions. Proteins were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 and destaining with methanol/acetic acid/water (40:10:50). Approximate molecular mass of the purified protein was determined from a plot of log MW of standards *vs.* migration distance.

The molecular mass of the purified protein was also determined by MALDI-TOF mass spectrometry using a Bruker Ultraflex mass spectrometer. One microliter of purified sample (~1 μg) in 0.1% TFA was mixed with 1 μl of α-cyanosinapinic acid matrix (10 mg/ml) dissolved in 50% (v/v) acetonitrile containing 0.1% (v/v) TFA. The mass of the protein was analyzed in linear analysis mode using an acceleration voltage of 25 kV.

2.3. N-terminal sequence and multiple sequence alignment

Approximately 5 μg of TLE from RVV was blotted onto a PVDF membrane and N-terminal sequencing (up to 15 residues) 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 by alignment 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; NCBI). This purified TLE from RVV was named Russelobin.

2.4. Peptide mass fingerprinting and de novo sequencing

For in-gel tryptic digestion of Russelobin, the procedure described by Thiede et al. [17] was followed. The LC/MS/MS of tryptic digested peptides was performed on a Brucker nanoAdvance UHPLC coupled with maXis 4G mass spectrometer using a reverse phase Magic C18AQ 0.1 × 150 mm column. The peptides were eluted from the column with 2–35% ACN gradient at flow rate of 500 nl/min. Spectra are collected over a m/z range of 100–2000 Da. 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 (based on Mascot protein identification) were subjected to a BLAST search in NCBI nr, Swissprot protein sequences (swissprot), and Protein Databank proteins (PDB) 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

Unless otherwise stated, thrombin-like activity was determined by mixing 50 μl of 1.0 mM chromogenic substrate for thrombin (N-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide hydrochloride) with a specific amount of Russelobin and adjusting the total volume to 375 μl with 100 mM HEPES (pH 8.0) containing 100 mM NaCl [18]. The liberation of 4-nitroaniline was determined at 405 nm against a reagent blank. Based on a standard curve, the unit of amidolytic activity is defined as micromoles of 4-nitroaniline released per minute by the enzyme under the assay condition. The following chromogenic substrates were also examined for amidolytic activity assay (Sigma-Aldrich): 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 (substrate for thrombin), and D-Gly-Arg-p-nitroanilide hydrochloride.

2.6. Assay of fibrinogen clotting activity

The fibrinogen clotting time was determined using a BBL-Fibrinosystem (Biomedical Technology Services, USA). Briefly, 0.1 ml 50 mM Tris–HCl, pH 7.4 containing 100 mM NaCl was mixed with 0.1 ml of 2.6 mg/ml of human fibrinogen (fraction-I) in buffer, and then 0.1 ml of test enzyme (human thrombin 1 NIH U/ml, or Russelobin) was added. All solutions were prewarmed, and the clotting time (in seconds) was measured at 37 °C. For controls, 0.1 ml of enzyme was replaced with buffer.

2.7. Determination of fibrinogen degradation pattern

2.7.1. SDS-PAGE analysis

Samples of 0.1 ml of human fibrinogen (2.6 mg/ml in 50 mM Tris–HCl, pH 7.4 containing 100 mM NaCl) were incubated with different amounts of Russelobin, and the final volume was adjusted to 0.2 ml with 50 mM Tris–HCl, pH 7.4 containing 100 mM NaCl. After incubation for 8 h at 37 °C the reaction was stopped by adding 0.2 ml of 4x SDS-PAGE LDS dye (Invitrogen) containing 15 mM DTT, and the tubes were incubated at 75 °C for 20 min. A control was run in parallel where instead of enzyme, 0.1 ml of buffer was incubated with fibrinogen. The peptides were separated by 12.5% NuPage SDS-PAGE at 170 V, and protein bands were visualized by staining with Coomassie Brilliant Blue R-250 and destaining with methanol/acetic acid/water (40:10:50).

2.7.2. HPLC and mass spectrometric analyses

For the assay of fibrinopeptides A and B released from fibrinogen, 0.5 ml of human fibrinogen solution (3 mg/ml in 0.1 M potassium phosphate buffer containing 100 mM NaCl, pH 7.4) was incubated with 0.1 ml of either human thrombin (1 NIH Unit/ml) or various doses of Russelobin (10–240 nM) for 8 h at room temperature (~23 °C). The fibrin clot was then removed with a glass rod and the mixture was centrifuged at 12,000 rpm for 10 min (Microfuge 18 Centrifuge, Beckman Coulter, USA). The resulting supernatant was filtered through a 0.2 μm membrane filter and concentrated by vacuum drying. The released fibrinopeptides A and B were separated on a Waters HPLC using a Jupiter reverse-phase C_{18} column (250 × 4.6 mm) previously equilibrated with 0.1% (v/v) TFA. After washing the column with 6.0 ml of 0.1% TFA (solvent A), the bound proteins were eluted with solvent B (80% ACN with 0.1% v/v, TFA). From a standard curve of human fibrinopeptides A and B (Sigma-Aldrich, USA) eluted from the RP-HPLC column under identical conditions, the amount of thrombin or Russelobin-induced release of fibrinopeptides A and B from human fibrinogen was calculated. A control was also run in parallel where fibrinogen solution alone was incubated with 0.1 ml of PBS, pH 7.4. The mass of peptide peaks eluted from RP-HPLC analysis was determined with a MALDI-TOF-MS (Bruker Ultraflex, GmbH) as above. Briefly, the RP-HPLC fractions were dried under vacuum, dissolved in 1.0 μl of 50% ACN (v/v) containing 0.1% TFA and then directly spotted on a MALDI target (1.0 μl matrix: 10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA).

2.8. Protease assay and substrate specificity

The proteolytic activity of enzyme against casein, bovine serum albumin, bovine serum gamma globulin, human plasma fibrinogen (fraction I) and fibrin was determined by a modification of the method described by Mukherjee et al. [19]. One unit (U) of protease activity is defined as 1.0 μg of tyrosine equivalent liberated per min per ml of enzyme. From a standard curve of tyrosine (at 280 nm) the protease activity was calculated. The peptide bond specificity of Russelobin against the oxidized B-chain of bovine insulin was tested following the procedure described by Weldon and Mackessy [6].

2.9. Esterase activity upon TAME and BAEE

Esterolytic activity was assayed by a spectrophotometric method using N_α-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) and N_α-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates. TAME esterase activity (in 50 mM Tris–HCl, 100 mM KCl, pH 8.1) was determined as described by Costa et al. [20]. One unit of TAME-esterase activity is defined as an increase in absorbance of 0.01 at 244 nm during the first 10 min of the reaction at 37 °C. For the BAEE esterase activity assay, the procedure described by Rutkowski [21] was followed. This assay was carried out in 100 mM Tris–HCl, pH 8.0 at 37 °C for 10 min. One unit of BAEE-esterase activity is defined as an increase of 0.01 absorbance unit at 254 nm during the first 5 min of the reaction at 37 °C. To examine the effect of Ca^{2+} on enzymatic activity, the assay was carried out in presence or absence of 10 mM $CaCl_2$. For every experiment, a control was run in parallel where instead of enzyme, an equivalent volume of buffer was used. Activity was expressed as units of TAME or BAEE /mg protein.

2.10. Other biochemical characterization

Protein content was assayed using the Bio-Rad protein assay kit (BIO-RAD, USA) following the instructions of the manufacturer and using bovine serum gamma globulin as a standard. Optimum conditions for Russelobin activity were determined by incubating 2 nmol of enzyme at different pH (7–11) and temperature ranges (30–65 °C) followed by measuring the amidolytic activity with 0.13 mM BzPheValArg-pNA (final concentration). Kinetics parameters were calculated by incubating 2 nmol of enzyme with different concentrations (0.01 to 0.13 mM) of BzPheValArg-pNA, and the K_m and V_{max} values were calculated from a Lineweaver-Burke plot.

Stability of aqueous solution of Russelobin (2 mg/ml) at 4 °C was determined by withdrawing 1.0 μg of enzyme at an interval of 7 days for 28 days and then assaying amidolytic activity as above (BzPheValArg-pNA). Enzyme activity at 0 day was considered as 100% activity, and residual amidolytic activity at various time intervals was compared with that. The effect of freeze-thawing on enzyme activity was determined by freezing the enzyme at −20 °C followed by thawing at room temperature, and this process was repeated 5 times. The residual activity after each cycle of freeze-thawing was determined and compared with the control (enzyme activity before freeze-thawing).

2.11. Determination of carbohydrate content and role of glycosylation on enzyme function

The total neutral sugar content of Russelobin was determined following the phenol-sulfuric acid colorimetric method [22]. To determine the extent of N-linked or O-linked oligosaccharides, as well as sialic acid content, the TLE (5 μg) was treated with PNGase, O-glycosidase and neuraminidase, respectively following the instructions of the manufacturer (New England Biolabs Inc, Ipswich, MA). After denaturation of enzyme, the reaction was incubated for 4 h at 37 °C and the reaction products were visualized by 12.5% NuPage SDS-PAGE under reducing conditions.

For partial deglycosylation without denaturing the enzyme (native deglycosylation), the denaturation step was eliminated. Russelobin (3 μg) was treated with 2000 units of PNGase for 24 and 36 h at 37 °C and the reaction products were visualized by 12.5% SDS-PAGE under reducing conditions. Differences in biochemical properties (fibrinogenolytic and amidolytic activities, optimum pH and temperature, thermostability, inhibition by protease inhibitors) between the native (glycosylated) and partially deglycosylated enzymes were then compared.

2.12. Effect of enzyme inhibitors

Inhibition of enzyme activity toward BzPheValArg-pNA or a native substrate (fibrinogen) was assayed as previously described after pre-incubation of 50 nmol of enzyme in 0.1 M HEPES buffer, containing 100 mM NaCl, pH 8.0 for 30 min at 37 °C containing one of the following inhibitors (final concentration): benzamidine-HCl (0.5–5 mM), aprotinin (100 μM), dithiothreitol (5–10 mM), diNa-EDTA (5–10 mM), heparin (100 IU/ml), soybean trypsin inhibitor (100–150 μg), α_2-macroglobulin (100 μg), Antithrombin-III (100 μg), TPCK (100 μM), TLCK (100 μM), iodoacetamide (5 mM), and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (5 mM). The remaining enzyme activity after treatment with inhibitors was expressed as percent activity remaining relative to the control preparation (without inhibitors). The IC_{50} values (the concentration of inhibitor reducing enzyme activity to 50% of its original activity) were calculated by incubating various concentrations of inhibitor with Russelobin and the dose-response data were analyzed by linear regression.

2.13. FPA- and FPB-mediated inhibition of fibrinogenolytic activity

In order to examine product inhibition of Russelobin, 250 nM enzyme was incubated with graded amounts (1.0 to 5.0 nmol) of either individual fibrinopeptide A or fibrinopeptide B or with both FPA and FPB for 30 min at room temperature. This was followed by measuring the fibrinogenolytic activity as above against a control (enzyme without FPs A and/or B).

2.14. Susceptibility of fibrin formed by Russelobin and human thrombin to plasmin degradation

To determine the plasmin-mediated degradation of fibrin formed by Russelobin or human thrombin, 100 μl of human fibrinogen (2.6 mg/ml in PBS, pH 7.4) in a total volume of 150 μl was incubated with 0.01 NIH U of human thrombin or 250 nmol of Russelobin and the fibrin clot was allowed to form at room temperature. To these clots, 10 μl of 150 U/ml of human plasmin was added and incubated for 45 min at 37 °C. The reaction was terminated by addition of 50 μl of 10% (w/v) ice-cold TCA and the release of acid-soluble oligopeptides was determined by using the BioRad protein assay as per manufacturer instructions. A control was run in parallel where the plasmin was added to reaction mixture after 45 min of incubation at 37 °C and just before the addition of TCA. One unit of fibrinolytic activity is defined as an increase of 0.01 absorbance/min at 595 nm compared to controls.

2.15. Pharmacological properties

For *in vitro* cytotoxicity assays, Colo-205 (human colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma) and 3T3 (mouse embryo fibroblast) cells were cultured in RPMI-1640, Eagle's MEM, and Dulbecco's Modified Eagle's Medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum. Various dilutions of Russelobin (1.0 to 10.0 μg/ml) were added to cells aliquoted in a 96-well plate at a concentration of 1×10^5 cells/ml. The final volume was adjusted to 100 μl/well and the cells were allowed to grow for 48 h at 37 °C in a humidified 5% CO_2 incubator. Cytotoxicity (percent cell death) was assayed by an MTT-based method following the instructions of the manufacturer (ATCC ®MTT Cell Proliferation Assay kit, American Type Culture Collection, Manassas, VA). The absorbance of cultures after addition of detergent was measured at 570 nm. For controls, cell cultures were treated with respective growth medium (negative control), and medium without cells was used for blanks. Cytotoxicity was expressed as percent cell death determined by comparison with values obtained from a standard curve of control cells.

The hemolytic activity of Russelobin was assayed as described earlier [2]. Briefly, blood was collected in 3.8% trisodium citrate (9:1) from anesthetized mice by cardiac puncture, washed twice in isotonic saline, and a 5% (v/v) erythrocyte suspension was prepared in PBS, pH 7.4. To 0.5 ml of erythrocyte suspension, graded amounts (1.0 to 10.0 μg/ml) of Russelobin were added and the final volume was adjusted to 1.0 ml with assay buffer. The mixture was incubated for 60 min at 37 °C; tubes were placed in ice to stop the reaction. After centrifugation at 10,000 rpm for 5 min, the absorbance of the supernatant was measured at 540 nm. One hundred percent hemolysis was achieved by adding 1% Triton X-100 instead of Russelobin to erythrocyte suspensions. A control was run in parallel where enzyme was replaced with 0.1 ml of PBS, pH 7.4.

In vitro plasma clotting activity was assayed using platelet poor mouse plasma by following our previously described procedure [2].

2.16. In vivo toxicity toward mice and lizards

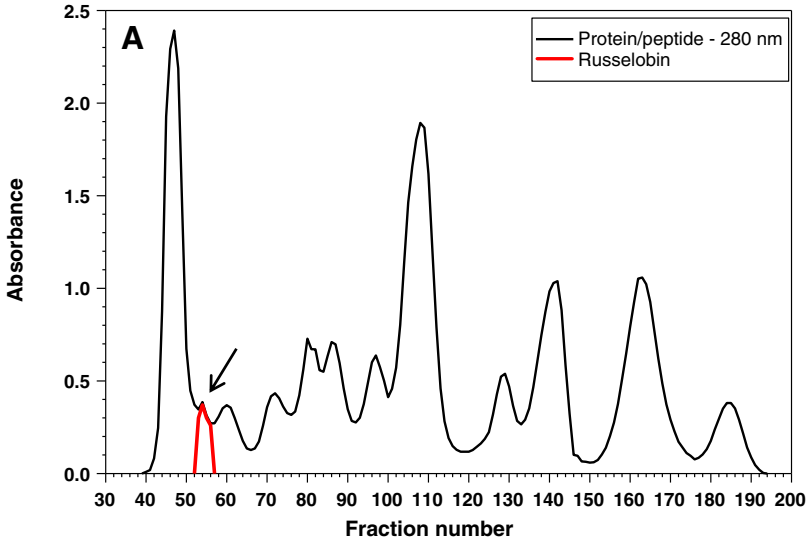
Pathogen-free, laboratory inbred non-Swiss albino mice (strain NSA) weighing between 18 and 20 g and House Geckos (*Hemidactylus frenatus*) weighing between 1.5 and 3.5 g were used for *in vivo* toxicity experiments. All experimental protocols using animals were approved by the UNC IACUC (protocol 9401). For toxicity assessments, Russelobin dissolved in 0.2 ml of PBS, pH 7.4 was injected intraperitoneally (1.0 mg kg^{-1} to 5.0 mg kg^{-1} body weight) into mice. The control animals received only 0.2 ml of PBS, pH 7.4. House Geckos received the same dose in a total volume of 75.0 μl. The animals were observed at regular intervals up to 72 h post-injection for any physical or behavioral changes.

In order to examine the morphological alterations and/or intravascular coagulation produced by purified Russelobin, mice were sacrificed by cervical dislocation 5 h after the injection of Russelobin (5 mg/kg) or normal saline (control) and the heart, liver, lung and kidney were dissected out. The tissues were washed with PBS, pH 7.2 to remove the adherent blood and then placed in 10% buffered formaldehyde. The fixed tissues were dehydrated in graded alcohol series, embedded in paraffin and processed routinely for light microscopic observation after hematoxylin-eosin staining (Colorado Histo-Prep, Ft. Collins, CO).

2.17. Defibrinogenating activity

The *in vivo* defibrinogenating activity was determined by i.p. injection of Russelobin at two doses (0.5 or 1 mg/kg, dissolved in 0.2 ml PBS, pH 7.4) to groups of three NSA strain albino mice weighing 28–30 g. The control group of mice (placebo) received the injection of same volume of PBS, pH 7.2. After 5 h, the animals were anesthetized by an overdose of Na-pentobarbital and bled by cardiac puncture. Blood was collected into a glass test tube and time to clot formation (24 °C) was recorded. For quantitative determination of plasma fibrinogen concentration, in a separate set of experiments, blood was collected in 3.8% trisodium citrate (9:1) and plasma was separated by centrifuging the blood at 2500 rpm for 10 min. The fibrinogen content of plasma obtained from control and Russelobin-treated mice was determined by using a Ca-thrombin reagent as described by Burmester et al. [23].

Fig. 1. A. Fractionation of crude *D. russelii* venom on size-exclusion BioGel P-100 column (2.8 × 80 cm). The arrow indicates elution of proteins showing thrombin-like activity (peak 2). B. Fractionation of gel-filtration peak 2 on a C_4 RP-HPLC column. Russelobin eluted at 34.2 min. C. Determination of purity and molecular mass of Russelobin by SDS-PAGE under reduced (lanes 2–4) and non-reduced (lanes 5–7) conditions. Lane 1, protein molecular markers; lanes 2 and 5, crude RVV (20 μg); lanes 3 and 5, gel-filtration fraction (8 μg); lanes 4 and 7, Russelobin (2.5 μg).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Protein/peptide - 280 nm
Russelobin
Absorbance
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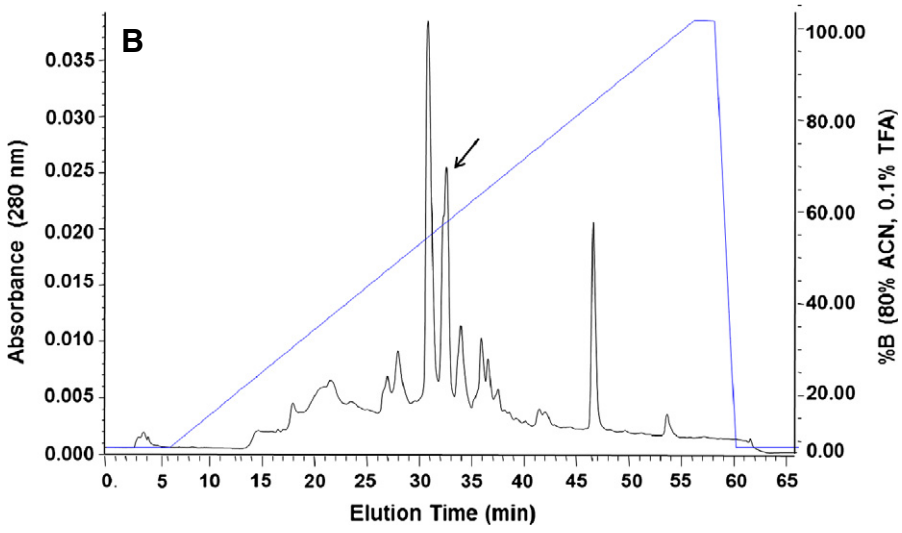
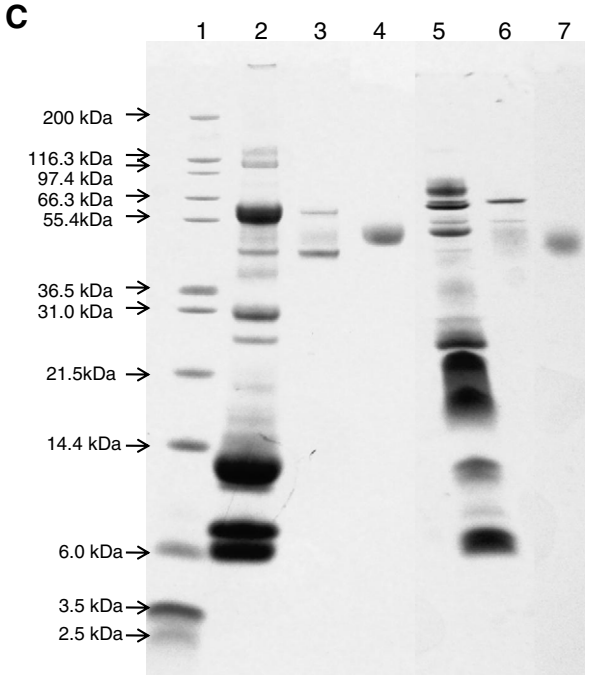

B
Absorbance (280 nm)
%B (80% ACN, 0.1% TFA)
Elution Time (min)


C
1 2 3 4 5 6 7
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66.3 kDa
55.4kDa
36.5 kDa
31.0 kDa
21.5kDa
14.4 kDa
6.0 kDa
3.5 kDa
2.5 kDa

2.18. Statistical analysis

Results were presented as means ± standard deviation (S.D.). Statistical analysis of the data was done using Student's t test in SigmaPlot 2011 for Windows (version 7.0). The value of $p \leq 0.05$ was considered as significant.

3. Results

3.1. Purification of a thrombin-like enzyme from RVV

Crude RVV was fractionated via size exclusion chromatography into 12 peaks (Fig. 1A); tubes 51–54 showed thrombin-like enzyme (TLE) activity. The pooled fractions yielded 8 peaks on RP-HPLC (Fig. 1B). The fraction with a retention time of 34.2 min showed thrombin-like activity and was found to be homogenous by 12.5% SDS-PAGE. It displayed a single band under both reducing and non-reducing conditions and the molecular weight of purified protein was 51.3 kDa as determined by SDS-PAGE (Fig. 1C). The enzyme eluted as a single, sharp symmetrical peak on RP-HPLC using a C_4 column, demonstrating the purity of preparation (data not shown). By MALDI-TOF mass spectroscopy, the molecular mass of the purified protein was determined as 38,728.3 Da (Supplementary Fig. S1). The enzyme was named Russelobin (Russell's Viper thrombin-like enzyme), and a summary of the purification is displayed in Table 1.

3.2. N-terminal amino acid sequence and multiple sequence alignment

The N-terminal amino acid sequence of Russelobin shared significant sequence homology (87–100%) with N-terminal sequences of some previously described thrombin-like serine proteases from snake venom (Table 2). The N-terminal sequences of snake venom thrombin-like enzymes are generally highly conserved; variation from Russelobin sequence were observed only at position 2 (V replaced with I or P) and at position 14 (S replaced with F).

3.3. Peptide mass fingerprinting and de novo sequences of Russelobin

LC/MS/MS analysis of tryptic digested peptides of Russelobin identified 8 unique peptides with greater than 95% probability (Table 3). When these *de novo* peptide sequences were subjected to BLAST search in NCBI database against a snake venom protein database, they showed significant similarity (score 249.4, rank 1, 23.4% sequence coverage) with a serine β-fibrinogenase-like protein precursor from *Daboia russelii siamensis* (GenBank accession ADP88560.1). The *de novo* sequences of the peptides aligned with this fibrinogenase are shown in Supplementary Fig. S2.

3.4. Amidolytic activity

Russelobin showed highest activity toward N-Bz-Phe-Val-Arg-pNA, followed by Nα-Tosyl-Gly-Pro-Arg-pNA. However, in the case of human thrombin, this substrate specificity was reversed (Fig. 2). Russelobin also showed weak amidolytic activity against the chromogenic substrates for plasmin, trypsin and factor Xa (Fig. 2). Nevertheless, Russelobin demonstrated significantly higher specific activity ($p<0.001$) in hydrolyzing all the tested chromogenic substrates compared to human thrombin. The kinetic parameters Km, Vmax and kcat of Russelobin with N-Bz-Phe-Val-Arg-pNA at 37 °C were determined as 66.7 ± 1.3 μM, 100 ± 6.0 μmol pNA/min/mg protein, and 858 ± 4.9 s^{-1}, respectively.

Table 1
Summary of purification of thrombin-like enzyme from *Daboia r. russelii* venom. Data represent a typical experiment. Unit is defined as micromoles of pNA liberated/min.

Purification steps	Total protein (mg)	Total activity (units)	Protein yield (%)	Specific activity (units/mg)	Purification (fold)
Crude RVV	173.7	45.4	100	0.26	1.0
GF-fraction	7.968	25.6	4.59	3.2	12.3
RP-HPLC	0.926	21.7	0.53	23.4	89.6

3.5. Biochemical characterization

The biochemical properties and enzymatic activities of Russelobin are shown in Table 4. The enzyme showed optimum activity at 45 °C and at pH 9.0. Russelobin demonstrated remarkable storage stability, as it retained 100% activity even after storage for more than one month (at 4 °C as well as −20 °C). Furthermore, five cycles of freezing at −20 °C and subsequent thawing at room temperature did not significantly affect the enzyme activity.

3.6. Carbohydrate content determination

The total neutral carbohydrate content of Russelobin was determined as 67.7 μg/mg of protein, which represents 6.7% of total protein mass (Table 4). When treated with PNGase under denaturing conditions for the removal of N-linked sugars, Russelobin showed a protein band of 22.2 kDa on SDS-PAGE (Supplementary Fig. S3). Therefore, it may be inferred that the N-glycosylated oligosaccharides constituted 56.7% of total mass of Russelobin (Table 4). Under non-denaturing conditions, complete deglycosyation by PNGase could not be achieved, and the partially deglycosylated enzyme showed a protein band of 36.5 kDa (Supplementary Fig. S3). Treatment of Russelobin with neuraminidase or O-glycosidase did not result in a change in the SDS-PAGE migration pattern (Supplementary Fig. S3).

3.7. Protease and esterolytic activity

The enzyme did not show proteolytic activity against bovine serum albumin and bovine serum gamma globulin but demonstrated caseinolytic and human fibrinogenolytic activity (Table 4). With an increase in fibrinogen concentration from 2.0 to 8.0 μM, fibrinogenolytic activity of Russelobin increased linearly, and at a fibrinogen concentration of 12.0 μM a steady state was reached. Interestingly, a further increase in fibrinogen concentration led to an inhibition of fibrinogenolytic activity of Russelobin (Fig. 3). Therefore, Russelobin demonstrated classic Michelis-Menton behavior at a low substrate concentration. The Km and Vmax values of fibrinogenolytic activity (2.0 to 12.0 μM fibrinogen concentration) were determined as 7.6 μM and 20.5 μg L-tyrosine equivalent/min/mg protein, respectively. Russelobin did not show TAME-esterase activity but it hydrolyzed BAEE with a specific activity of 1200 ± 21.1 (mean ± S.D.) unit/mg protein. BAEE-esterase activity was inhibited by 50% in the presence of 10 mM Ca^{2+} (Table 4), but Na^{+} did not influence esterase activity (data not shown).

3.8. Fibrinogen clotting activity and release of FPA and FPB

At 800 nM, Russelobin induced clotting of human fibrinogen within 31.2 ± 5.1 s (mean ± S.D., n = 3), whereas 7 μM (0.33 NIH unit/ml) of human thrombin under the identical conditions was required to clot fibrinogen within 28.0 ± 1.6 s (mean ± S.D., n = 3). However, as opposed to human thrombin, which produced a rigid clot with time, the fibrinogen clot induced by Russelobin was viscous and soft in nature, easily destroyed by applying physical force such as mild vortexing.

SDS-PAGE analysis showed that at 50 nM Russelobin, only the Aα-chain of fibrinogen was degraded and the Bβ and γ-chains of fibrinogen remained intact (Fig. 4). With an increase in Russelobin concentration to 200 nM, the Bβ-chain was partially degraded, but the γ-chain of fibrinogen remained undegraded.

Table 2
Multiple sequence alignment of N-terminal sequence of RVVTLE with other known serine proteases from snake venom. NR: not reported.

Accession/ reference	Description	Species	N-terminal sequence 1 15	Identity (%)	Molecular mass
This work	Thrombin-like serine protease	*Daboia russelii russelii*	VVGGDECNINEHRSL	–	51.3 kDa
ADP88560.1	Serine beta-fibrinogenase-like protein precursor	*Daboia russelii siamensis*	VVGGDECNINEHRSL	100	NR
P86531.1	Vipera russelli proteinase RVV-V homolog 2	*Daboia russelii pulchella* (now *D. r. russelii*)	VVGGDECNINEHRSL	100	NR
P80899.1	Protein C activator/Thrombin-like snake venom serine protease	*Gloydius halys*	VVGGDECNINEHRSL	100	NR
P81883.1	Fibrinogen-clotting enzyme TL-BJ isoform 2	*Bothrops jararaca*	VVGGDECNINEHRSL	100	31 kDa
P33588.1	Protein C activator/Thrombin-like enzyme	*Agkistrodon bilineatus*	VVGGDECNINEHRSL	100	35 kDa
Q7LZF5.1	Thrombin-like enzyme catroxobin-1	*Crotalus atrox*	VVGGDECNINEHRSL	100	25 kDa
Q9PRW2.1	Alpha-fibrinogenase A3	*Crotalus atrox*	V I GGDECNINEHRSL	93	35 kDa
P81882.1	Fibrinogen-clotting enzyme TL-BJ isoform 1	*Bothrops jararaca*	V I GGDECNINEHRSL	93	30 kDa
Q9PRW4.1	Serine protease alpha-fibrinogenase A1	*Crotalus atrox*	V I GGDECNINEHRSL	93	33 kDa
P0DJF7.1	Thrombin-like enzyme purpurase	*Cryptelytrops purpureomaculatus*	VVGGDECNINDHRSL	93	35 kDa
P0C578.1	Thrombin-like enzyme okinaxobin-2	*Ovophis okinavensis*	VVGGDECNINEHRFL	93	37.5 kDa
Q9PRW3.1	Alpha-fibrinogenase A2	*Crotalus atrox*	VPGGDECNINEHRSL	93	34 kDa
Q7SZE2.1	Bradykinin-releasing enzyme KR-E-1/; AltName: Thrombin-like enzyme defibrase	*Agkistrodon caliginosus* (=*Gloydius ussuriensis*)	VIGGDECNINEHRSL	93	NR
P82981.1	Thrombin-like enzyme contortrixobin	*Agkistrodon contortrix contortrix*	VVGGDECNINEHRFL	93	26 kDa
P0C590.1	Thrombin-like enzyme calobin-2	*Agkistrodon caliginosus*	VIGGDECNINEHRFL	87	41 kDa
P20005.1	Thrombin-like enzyme okinaxobin-1	*Trimeresurus okinavensis*	VIGGDECNINEHRFL	87	37.5 kDa

Fibrinopeptide release from fibrinogen after incubation with Russelobin is depicted in Fig. 5. The release of fibrinopeptides A and B was confirmed by MALDI-TOF-MS analysis of the RP-HPLC peaks of fibrinogen degradation products. The peptide peaks eluting at 16.73 min and 17.33 min showed masses of 1537.229 [M + H] and 1553.159 [M + H], respectively, corresponding to the masses of FPA and FPB. Russelobin at 40 nM did not release detectable quantities of either fibrinopeptide A or B; however, after increase in the enzyme concentration to 80 nM, preferential release of FPA was observed. The release of FPB was observed at a much slower rate, approaching the maximum value only after 8 h of incubation at room temperature (data not shown). Human thrombin, on the other hand, at a concentration of 5.0×10^{-5} NIH U/ml, released almost equal amount of FPA (1.2 nmol) and FPB (1.1 nmol) after 8 h at room temperature (data not shown).

3.9. Effect of FPA and FPB on fibrinogenolytic activity of Russelobin

Russelobin-mediated degradation of fibrinogen in the presence of FPA or FPB, or with both FPA and FPB, did not result in inhibition of fibrinogenolytic activity as compared to controls lacking fibrinopeptides (data not shown). This result suggests free FPA or FPB do not influence the fibrinogenolytic activity of Russelobin.

3.10. Plasmin mediated degradation of fibrin formed by Russelobin and human thrombin

Under identical experimental conditions, plasmin was 3.3 times more efficient in degrading fibrin formed from fibrinogen by the action of Russelobin than the fibrin clot formed by human thrombin (data not shown), demonstrating the more labile nature of the Russelobin-catalyzed clot.

3.11. Effect of inhibitors

Serine protease inhibitors such as benzamidine and AEBSF significantly ($p<0.001$) inhibited the amidolytic activity of Russelobin (Table 5). The IC_{50} values of benzamidine and AEBSF for inhibition of amidolytic activity were determined as 4.0 mM and 3.7 mM, respectively. Heparin at a concentration of 100 U/ml inhibited approximately 45% amidolytic activity of the enzyme. Antithrombin-III (inhibits thrombin and factor Xa), α_2-macroglobulin (a broad spectrum protease inhibitor of blood) and DTT (disulfide bond reducing agent) also showed minor but statistically significant ($p<0.05$) inhibitory effects on the amidolytic activity of Russelobin (Table 5). However, TLCK (inhibitor of trypsin-like serine proteases), TPCK (inhibitor of chymotrypsin-like serine proteases), soybean trypsin inhibitor-I (inhibitor of trypsin), aprotinin (serine protease inhibitor), iodoacetamide (inhibitor of cysteine protease) and EDTA (inhibitor of metalloproteases) did not inhibit amidolytic activity of Russelobin (Table 5).

The coagulant activity of Russelobin was also inhibited to a significant extent by benzamidine and AEBSF (Table 5). This inhibitory effect of benzamidine and AEBSF on fibrinogenolytic activity was also confirmed by SDS-PAGE analysis of fibrinogen degradation; in the presence of either inhibitor, the Aα and Bβ-chains of fibrinogen were not degraded (Fig. 4).

3.12. Role of glycosylation on stability and enzyme activity

No significant differences in biochemical properties of native and deglycosylated Russelobin, including temperature and pH optima, thermal stability, effect of freeze-thawing on enzyme activity, and hydrolysis of the chromogenic substrate Bz-Phe-Val-Arg-pNA, were detected. However, the partially deglycosylated enzyme hydrolyzed human fibrinogen at a significantly lower rate ($p<0.001$) than the native enzyme (Fig. 6A). Interestingly, among the inhibitors tested,

Table 3
The de *novo* peptide sequences generated for Russelobin by LC/MS/MS and matched to ADP88560.1 by MASCOT search.

Calculated mass	z	Scores	Sequence	Modifications	ADP88560.1 sequence #
1339.6844	3	72.9	R.NNAEIRLPEER.F	None	83–93
1610.7471	3	63.1	E.LVVGGDECNINEHR.C	Carbamidomethyl:8	24–37
1633.8213	3	34.5	R.NNAEIRLPEERFF.C	None	83–95
1160.6302	2	44.7	R.AAYKGLPAQSR.T	None	174–184
1497.6630	2	118.5	E.VVGGDECNINEHR.C	Carbamidomethyl:7	25–37
2344.2209	3	44.3	K.TSTYIAPLSLPSSPPRVGSVCR.I	Carbamidomethyl:21	120–141
1685.8988	2	88.4	K.TSTYIAPLSLPSSPPR.V	None	120–135
1217.6517	3	27.6	R.AAYKGLPAQSR.T	Carbamidomethyl:4	174–184

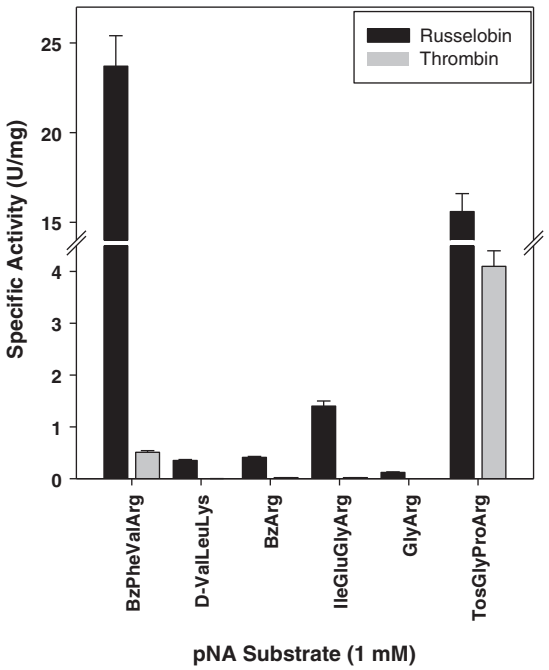

Fig. 2. Comparison of amidolytic activity of Russelobin and human thrombin toward various chromogenic substrates. The values are means ± S.D of six experiments.

α_2-macroglobulin and antithrombin-III showed a significantly higher inhibition ($p<0.05$) of amidolytic (Fig. 6B) and fibrinogenolytic activity by partially deglycosylated Russelobin as compared to the native enzyme (data not shown).

3.13. Specificity on oxidized B-chain of insulin

Insulin B-chain cleavage by Russelobin and comparison with several representative snake venom proteases is depicted in Table 6. Russelobin hydrolyzed the insulin B-chain slowly, and about 40% of insulin B-chain remained intact after 24 of incubation (Supplementary Fig. S4). Russelobin preferentially cleaved oxidized insulin B-chain at positions Leu_{15}-Tyr_{16} and Tyr_{16}-Leu_{17} resulting in the formation of peptides Phe_1-Ala_{14}, Phe_1-Leu_{15}, Phe_1-Tyr_{16}, Leu_{15}-Ala_{30}, Tyr_{16}-Ala_{30} and Leu_{17}-Ala_{13}. At a much lower rate (approximately 10–20-fold lower), Russelobin also catalyzed cleavage of peptide Phe_1-Ala_{14} at positions Phe_1-Val_2, His_5-Leu_6 and His_{10}-Leu_{11}, generating the peptides Val_2-Ala_{14}, Val_2-His_{10},Leu_6-Ala_{14} (Table 6).

Table 4
Biochemical properties and activities of Russelobin. Values are mean ± S.D of triplicate determinations.

Biochemical properties	Values
Optimum activity	pH 9.0, 45 °C
Activity after 5 cycles of freeze-thawing (control 100%)	97.6 ± 0.5%
Carbohydrate content (%)	
Neutral	6.7
N-linked	56.7
Protease and esterase activity (substrate conc.)	Specific activity (U/mg protein)
Casein (10 mg/ml)[a]	82 ± 10.6
Bovine serum albumin (10 mg/ml)[a]	0
Bovine serum γ-globulin (10 mg/ml)[a]	0
Human fibrinogen (10 mg/ml)[a]	20.2 ± 1.9
TAME (1 mM)[b]	0
BAEE (1 mM)[c]	1200 ± 21.1
BAEE (1 mM) containing 10 mM Ca^{2+}	600 ± 13.2

[a] Unit = μg of L-tyrosine equivalent liberated/min after 60 min incubation at 37 °C.
[b] Unit = change in 0.01 absorbance unit/min at 244 nm at 37 °C.
[c] Unit = change in 0.01 absorbance unit/min at 254 nm at 37 °C.

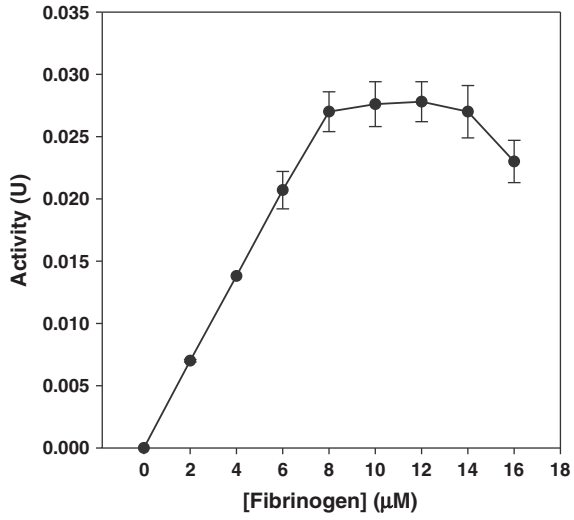

Fig. 3. Effect of substrate (fibrinogen) concentration on protease activity of Russelobin. The reaction mixture was incubated at 37 °C for 60 min and the release of oligopeptides was measured at 595 nm. Values are means ± S.D. of triplicate determinations.

3.14. Cytotoxicity, hemolytic activity and in vivo toxicity of Russelobin

At a dose of 10 μg/ml, Russelobin did not show cytotoxicity toward tested mammalian cells after 48 h of incubation; however, at the same dose, it resulted in minor hemolysis (3.0 ± 0.2%) of mouse erythrocytes.

Injection of Russelobin (IP) at a dose of 5 mg/kg body weight did not cause mortality in mice or house geckos, and no behavioral changes were noticed in animals up to 3 days post-injection. Light microscopic examination of tissues from Russelobin-treated mice did not show morphological alterations, evidence of intravascular coagulation or extravasation of erythrocytes (Supplementary Fig. S5).

3.15. Defibrinogenating activity

Russelobin demonstrated dose-dependent *in vivo* defibrinogenation in mice. Blood obtained from treated mice showed prolonged *in vitro* coagulation time as compared to blood from control mice (Fig. 7). This result corroborated well with the dose-dependent decrease in fibrinogen content of plasma of treated mice as compared to control mice.

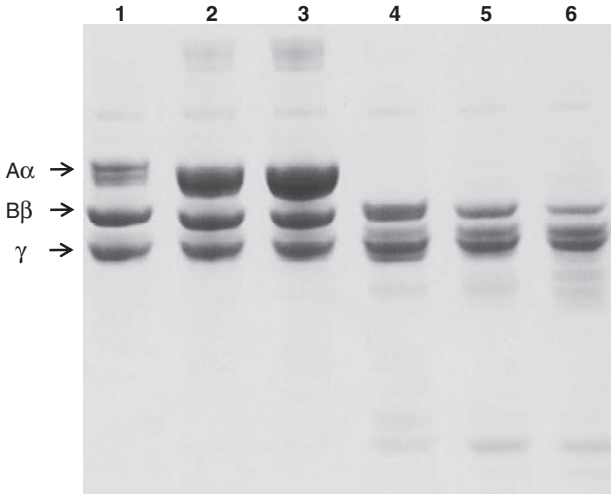

Fig. 4. Dose-dependent fibrinogen (2.6 mg/ml) degradation by Russelobin and inhibition by benzamidine-HCl and AEBSF. Lane 1, fibrinogen control; lane 2, 2 mM benzamidine-HCl with 200 nM Russelobin; lane 3, 2 mM AEBSF with 200 nM Russelobin; lane 4, 50 nM Russelobin; lane 5, 100 nM Russelobin; lane 6, 200 nM Russelobin.

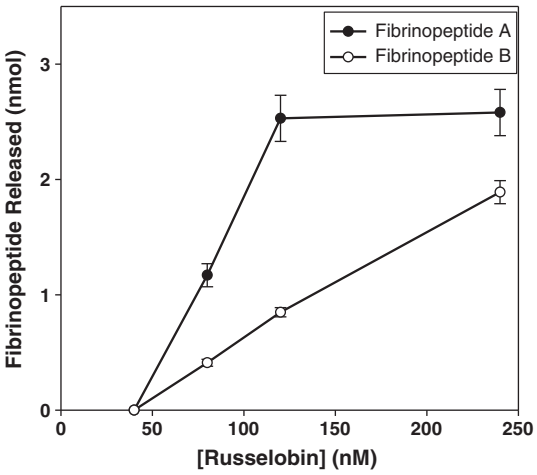

Fig. 5. RP-HPLC determination of dose-dependent FPA and FPB release from fibrinogen by Russelobin. The experimental procedure is described in the text. Values are means ± S.D. of triplicate determinations.

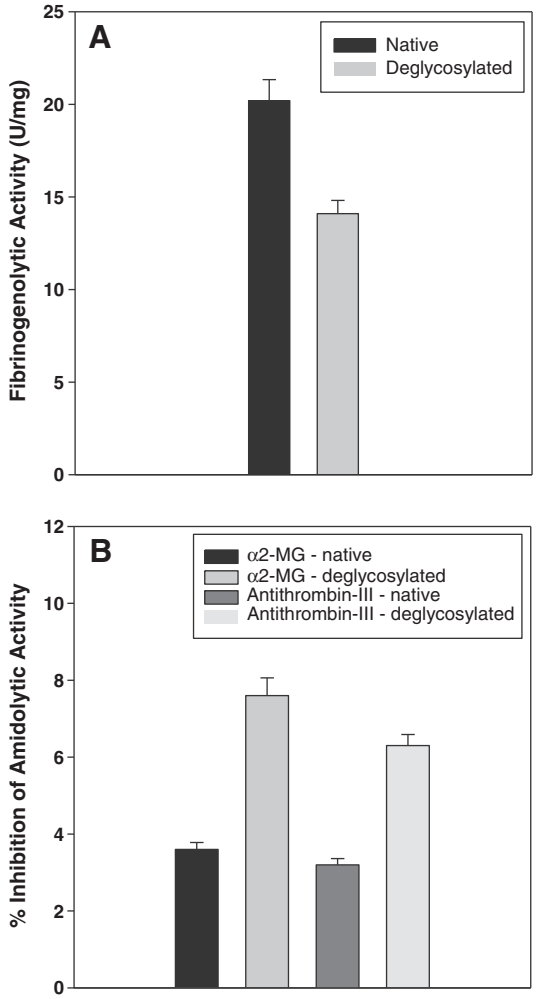

Fig. 6. Effects of deglycosylation of Russelobin on activity toward natural and model substrates. A. Fibrinogenolytic activity. B. Percent inhibition of amidolytic activity by endogenous protease inhibitors (α_2 macroglobulin and antithrombin-III). All treatments showed small but statistically significant differences between native and deglycosylated Russelobin (A, $p<0.001$; B, $p<0.05$). All values are means ± S.D. of three independent experiments.

4. Discussion

Snake venoms consist of a myriad of biologically active proteins, and several, including thrombin-like SVSPs, have been developed either as potential drugs for the treatment of cardiovascular disorders or as diagnostic reagents [see 8,9 for recent reviews]. One of the most notable examples of this class of proteinase is Ancrod (Viprinex), a serine proteinase originally isolated from the venom of *Calloselasma* (formerly *Agkistrodon*) *rhodostoma* [9]. Although Ancrod showed great potential for the treatment of acute ischemic stroke during the initial stages of drug development, in 2008, Ancrod failed in phase 3 clinical trials, mainly due to a lack of efficacy [9]. Therefore, there is an urgent need to discover safe and effective drugs to treat cardiovascular and hyperfibrinogenemia-associated disorders, as these diseases will continue to increase in the near future. In the present study, we purified and characterized a new thrombin-like enzyme from Russell's Viper venom with the intent of uncovering novel and potentially more effective fibrinogenolytic therapeutics. Snake venoms and other natural products have great potential to be used as drugs and in drug development [24–26], and while many SVTLEs have been sequenced, relatively few have been subjected to careful biochemical and biological characterization [9]. The observed diversity of pharmacological actions of these structurally conservative venom components highlights the absolute necessity of thorough characterization, and small surface residue changes can give rise to very significant changes in activity [10], most of which are not apparent from structural studies alone.

Table 5
Effect of inhibitors on amidolytic activity of RVVTLE. Values are mean ± S.D of triplicate determinations.

Inhibitor*	Enzyme activity ($U \times 10^{-3}$)
Control (no inhibitor)	47.4 ± 0.1
Benzamidine-HCl (5 mM)	21.2 ± 3.5[b]
AEBSF (5 mM)	18.9 ± 0.4 [b]
Heparin (100 U/ml)	26.3 ± 4.9 [b]
EDTA (10 mM)	47.5 ± 2.2
Soybean trypsin inhibitor-I (1:150)	46.8 ± 1.1
α2-macroglobulin (1:25)	45.7 ± 0.2 [a]
Antithrombin-III (1:25)	45.9 ± 0.5 [a]
Aprotinin (100 μM)	48.5 ± 0.8
TPCK (100 μM)	46.5 ± 2.9
TLCK (100 μM)	46.5 ± 1.4
DTT (10 mM)	42.5 ± 0.3 [a]
IAA (5 mM)	47.5 ± 2.2

*Amount inhibitor is given as concentration or mass ratio (enzyme:inhibitor); significant difference with respect to control: [a]$p<0.05$, [b]$p<0.001$.

4.1. Russelobin is a new thrombin-like enzyme from RVV

The mass of Russelobin is greater than the typical masses of thrombin-like enzymes purified from snake venom. With few exceptions, such as Bilineobin (57 kDa) from *Agkistrodon bilineatus* venom [27], most TLEs have masses of 30–35 kDa, and the presence of high mass thrombin-like enzymes in snake venom has not been reported. The N-terminal amino acid sequence of Russelobin clearly demonstrates that it is a thrombin-like serine protease from Russell's Viper venom, and this identification was confirmed by peptide mass fingerprinting (PMF) matching. Although no putative conserved domains could be detected, Russelobin shared considerable sequence homology with a serine beta-fibrinogenase-like protein precursor (28 kDa) from *D. r. siamensis* venom [3]. However, Russelobin (from *D. r. russelii* venom) is a much larger serine proteinase compared to beta-fibrinogenase, leading us to conclude that Russelobin is a new and previously uncharacterized thrombin-like serine protease from Russell's Viper venom.

Table 6
A comparison of the cleavage sites of snake venom proteinases on the oxidized B-chain of bovine insulin. Grey arrows for Russelobin represent low frequency secondary sites of hydrolysis of primary cleavage products.

Protease	Protease type	1	5	10	15	20	25	30	Reference
		F-V-N-Q-H-L-C-G-S-H-L-V-E-A-L-Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A							
Russelobin	Serine protease	↑	↑	↑	↑↑↑		↑		Present study
VLCTLP	Serine protease				↑		↑		[35]
MPB1	Serine protease			↑	↑↑		↑		[36]
MSP1	Serine protease						↑	↑	[36]
MSP2	Metalloprotease						↑	↑	[36]
Alsophinase	Metalloprotease				↑↑				[6]
Protease from *Ophiophagus hannah*	Metalloprotease		↑↑	↑	↑↑				[37]

4.2. Inhibition studies indicate Russelobin is a serine protease and differs from many other SVTLEs

Inhibition studies with different protease inhibitors are used consistently to identify the nature of the active site in a protease molecule. In addition, they may also highlight important structural differences between two functionally related protease molecules. The classical serine protease inhibitors such as AEBSF and benzamidine significantly inhibit the protease activity of Russelobin by irreversibly binding with the serine residue present in the active site of the enzyme and by competitive inhibition of binding of an arginine reside (P1 site specificity for SVSPs) [8]. Many of the SVTLEs, as well as thrombin, are inhibited by serine protease inhibitors [5]. Furthermore, the inability of EDTA (a metalloprotease inhibitor) or iodoacetamide (cysteine protease inhibitor) to inhibit proteolytic activity of Russelobin confirmed its identity as a serine protease; however, some SVTLEs were recently shown to be inhibited by both PMSF (serine protease inhibitor) and by EDTA [28], though this appears to be an exceptional case. The minor inhibitory effect of DTT on Russelobin protease activity is likely due to partial reduction of disulfide bonds, but the enzyme is remarkably resistant to significant reduction under the conditions utilized (10 mM DTT).

Effects of inhibitors highlighted significant differences amongst the SVTLEs at and around the active site of these enzymes [8]. For example, Russelobin, like thrombocytin [29], was significantly inhibited by thrombin inhibitors such as heparin (and weakly inhibited by antithrombin-III). However, batroxobin [12], cerastocytin [30], cerastotin [31] and contortrixobin [32] were insensitive to these inhibitors. Two different regions (anion binding exosites, ABE-I and ABE-II) are the key sites on thrombin molecules for binding and subsequent catalysis of its natural substrates, and antithrombin-III and heparin bind to these sites, preventing the catalytic activity of thrombin [7]; SVTLEs lacking these sites are not inhibited by antithrombin-III and/or heparin. Therefore, it may be concluded that the anion binding site(s) is conserved in Russelobin. Another example of the difference in the active site structure of Russelobin, as compared to many other SVTLEs such as cerastocytin and cerastotin [30,31], is the inability of TPCK and TLCK to inhibit Russelobin, whereas both of the *Cerastes*-derived toxins are inhibited by these active site-directed ketones.

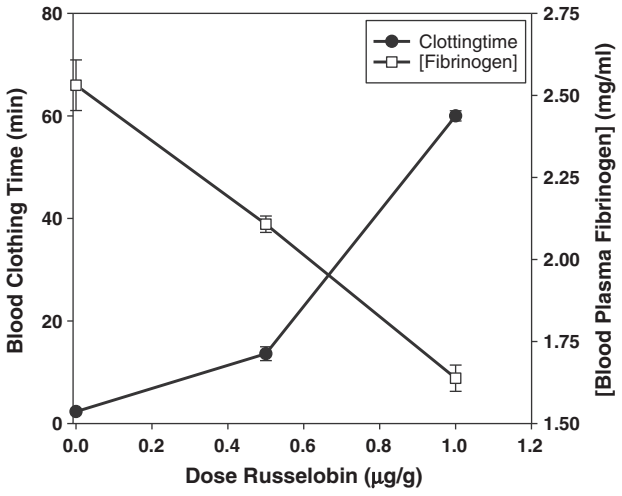

Fig. 7. Dose-dependent *in vivo* defibrinogenating activity of Russelobin 5 h after i.p. injection in mice; blood clotting time and plasma fibrinogen concentrations show an inverse relationship. All values are means ± S.D. of triplicate determinations.

4.3. Russelobin shows a high degree of selectivity toward fibrinogen

Although SVTLEs share a significant sequence similarity with thrombin, they often show different substrate specificities and manifest different biological activities [5,9]. This difference in specificity/activity corroborates well with our present observations showing reverse substrate specificity between Russelobin and human thrombin with respect to hydrolysis of chromogenic substrates BzPheValArg-pNA and N-(p-Tosyl)-GlyProArg-pNA. Similarly, many of the SVTLES (and thrombin) are reported to hydrolyze BApNA (a chromogenic substrate for trypsin) and TAME [33]; Russelobin did not hydrolyze either substrate. The presence of subsites S1 (Asp199) and S2 (Gly 228) in SVTLEs was reported to play an important role in binding of basic substrates such as TAME and BApNA [33]. Therefore, determinants of substrate recognition and the molecular basis of protease specificity may be quite subtle, involving the contribution of several factors rather than just a few critical sites in the protease molecule, producing the differences in substrate specificity observed among SVTLEs [5].

Russelobin demonstrated a high degree of selectivity toward fibrinogen without detectable proteolysis of other common plasma proteins, suggesting that fibrinogen is the primary physiological substrate for Russelobin. The reason(s) for inhibition of fibrinogenolytic activity of Russelobin at a higher substrate concentrations (>12 μM) and its physiological significance remain uncertain. This observation is in agreement with the reports showing that fibrinogen clotting capacity of thrombin-like enzymes such as alborase from *Cryptelytrops albolabris* and purpurase from *Cryptelytrops purpureomaculatus* were also dependent on fibrinogen concentration [13,34]. At a substrate (fibrinogen) concentration of greater than 5 mg/ml (~7.5 μM), the fibrinogen clotting time by these enzymes was substantially prolonged. However, fibrinogen degradation product-mediated inhibition of fibrinogenolytic activity of Russelobin (other than by fibrinopeptides A and B) at higher substrate concentrations cannot be ruled out at present.

4.4. Oxidized insulin β-chain cleavage by Russelobin is different from cleavage patterns of other venom proteases

Due to its known amino acid sequence, diversity of peptide bonds and short chain length, the oxidized B-chain of insulin has been commonly used to determine the specificity of venom protease molecules [6,35,36]; however, we are aware of no published reports of attempts to determine cleavage sites by SVTLEs using this substrate. The low rate of hydrolysis of insulin B-chain by Russelobin corroborates well with the finding of Reichel et al. [36], showing that venom metalloproteases hydrolyze insulin B-chain much more rapidly than non-thrombin-like

serine proteases. Russelobin catalyzed slow preferential hydrolysis at Val15 and Leu16 and it shows several additional minor cleavage sites at residues which are mostly not shown by other venom proteases. Therefore, Russelobin appears distinct from most of the venom proteases with respect to cleavage of insulin B-chain [6,35–37]. While a number of proteases (such as trypsin, plasmin, papain, tissue cathepsins) have been shown to be capable of hydrolyzing substrates at the carboxyl end of arginine, they also show hydrolysis of lysine, glycine, or tyrosine-containing substrates; thrombin appears to be unique in having its action limited to arginine substrates.

4.5. Physiological significance of glycosylation on Russelobin

A survey of the literature shows that most SVTLEs are glycoproteins containing several Asn-N-linked or Ser-O-linked glycosylation sites in the primary structure; however, their carbohydrate content may vary tremendously, from 5 to 62% of the total protein mass [8,9]. Russelobin, like the majority of other SVTLEs, contains N-linked glycans and does not possess O-linked carbohydrates or sialic acid residues. Nevertheless, a few glycosylated SVTLEs, such as TLBan from *Bothrops andianus* [28] and BjussuSP-I from *Bothrops jararacussu* [33], were shown to contain both N-linked carbohydrates and sialic acid moieties in their structures. It is noteworthy that complete removal of N-linked carbohydrates from Russelobin under native condition (without denaturation of the enzyme) was not possible, suggesting that all of the carbohydrate moieties of Russelobin are not readily accessible to PNGase hydrolysis. The Asn-linked carbohydrate moieties from BJ-48, a TLE from *Bothrops jararacussu* venom, were also found to be partially resistance against PNGase-F hydrolysis under non-denaturing condition [38].

The physiological significance or the role of carbohydrate moieties on functioning of SVTLEs is still unclear, and many contradictory and/or fragmentary data have been presented [8]. Partially deglycosylated Russelobin, like elegaxobin II [39] and BjussuSP-I [33], demonstrated a marked reduction of fibrinogenolytic activity without affecting its activity toward small molecule such as N-Bz-Phe-Val-Arg-pNA. The carbohydrate moieties of SVTLEs therefore appear to play a significant role in physiological substrate recognition and/or enhancement of catalytic activity of the enzyme [33,39]. In a sharp contrast to these observations, Leme et al. [40] have reported an increase in fibrinogenolytic activity of partially deglycosylated *Bothrops* protease A as compared to the native enzyme. It has also been reported that glycosylation stabilizes the tertiary structure of some of the SVTLEs rather than influencing the catalytic activity of enzyme [41]. In the case of Russelobin, no differences in thermostability between native and deglycosylated enzyme were observed.

Another significant effect of glycosylation of Russelobin was the increased resistance of the native (glycosylated) enzyme to physiologically relevant inhibitors such as α_2-MG and antithrombin-III. Glycosylation may therefore provide greater stability and resistance to host (victim) defense mechanisms involving endogenous serine protease inhibitors [9]. It has been demonstrated that removal of sialic acid from Batroxobin from *B. atrox* enhanced its degree of neutralization by α_2-MG present in human serum [12,40].

4.6. Russelobin forms a tenuous clot by preferentially releasing FPA and slowly releasing FPB from fibrinogen

Fibrinogen-dependent clotting capacity of thrombin or SVTLEs is due to the ability to cleave the Arg-Lys bonds on α and β-chains of fibrinogen, converting it to fibrin polymer and resulting in release of fibrinopeptide(s) A, B, or both A and B from fibrinogen [8]. With the exceptions of a few SVTLEs such as albolabrase [13], bilineobin [27] and brevinase [42], most SVTLEs preferentially catalyze cleavage of either Aα [33] or Bβ [27,32] fibrinogen chains, but not both. Russelobin preferentially hydrolyzed Aα chain of fibrinogen and slowly released Bβ chain and therefore it may be classified as an AB fibrinogenase, belonging to the less common class of A/B thrombin-like serine proteases from snake venoms.

A comparison of plasmin-mediated degradation of the fibrin clot formed by thrombin and Russelobin showed that plasmin is more efficient in degrading the fibrin clot formed by Russelobin, which may be attributed to non-crossed linked, labile (soft) clot formed by Russelobin. In sharp contrast, thrombin forms a covalently crossed-linking stable fibrin polymer which is a stable thrombus, and therefore the native fibrin clot formed by thrombin differs from that formed by SVTLEs [8,9]. This indicates that *in vivo*, Russelobin likely acts as a potent defibrinogenating agent, because the fibrin clot would easily be dissolved by the plasmin. Uncontrolled bleeding seen after human envenomation by this and other species with high TLE content venoms is consistent with defibrinogenation [1,16]. Our data with mice also supports this conclusion.

4.7. The in vivo defibrinogenating activity and non-toxic nature of Russelobin support its therapeutic potential

Studies have shown that elevated levels of fibrinogen in plasma (hyperfibrinogenemia) are associated with increased risk of cardiovascular disorder and thrombosis [43]. Moreover, elevated level of fibrinogen synthesized by some cancers also promotes the growth of lung and prostate cancer cells through interaction with fibroblast growth factor 2 [44]. Abnormally high levels of fibrinogen therefore increase risk of morbidity due to various disease states, emphasizing the need for defibrinogenating drugs. During the last decade, many SVTLEs have been purified and biochemically characterized; however, little attempt has been made to investigate the potential toxicity and pharmacological properties of most of these enzymes. Several SVTLEs have been found to induce adverse side effects such as platelet aggregation *in vitro* [28], myotoxicity, liver necrosis and lymphocytic interstitial pneumonitis [45], and neurotoxicological symptoms [46] in experimental animals. It is therefore noteworthy that Russelobin, under the *in vitro* and *in vivo* test conditions reported above, showed no adverse reactions in inbred mice.

Severe systemic hemorrhage is one of the serious manifestations of human envenomation by Russell's Viper, and mortality is also a significant concern [1,47]. On average, a large adult Russell's Viper may produce approximately 250 mg dried venom in a single extraction (pers. comm., Mr. D. Mitra, Calcutta Snake Park, India). In the highly unlikely scenario that *all* of this venom was delivered in a single bite to a human victim and because Russelobin represents ~0.5% of the total venom protein, approximately 1.25 mg of Russelobin could be delivered in a single bite. If the blood volume of an average adult human is approximately 5 l, this injected dose of Russelobin is equivalent to 4.8 nM in the blood (assuming full delivery to the blood). However, Russelobin was found to be non-lethal at a dose of 5 mg/kg in mice (assuming a total blood volume of 1.5 mL: approx. 1.6 μM), suggesting that this protease does not contribute significantly to RVV-induced bleeding or lethality. The lack of histologically detectable changes to several tissues, lethal toxicity or cytotoxicity on mammalian cells reinforces our conclusion that Russelobin is non-toxic at doses tested, supporting its suitability for clinical applications.

Somewhat enigmatically, *in vitro* Russelobin, like other SVTLEs, induced clotting of platelet-poor mouse plasma by virtue of its effects on fibrinogen; however, *in vivo* it showed defibrinogenating activity. As discussed above, the fibrin monomers formed by Russelobin are not cross-linked into stable thrombi due to absence of factor XIIIa, instead leading to microclot formation. This tenuous thrombus is therefore quickly dissolved by plasmin, resulting in hypofibrinogenemia [8]. In snakebite victims, this process leads to a consumptive coagulopathy by degradation of plasma fibrinogen. In a controlled clinical setting, Russelobin could result in defibrinogenation which would benefit the pathologies associated with certain cancers and cardiovascular disorders.

5. Conclusion

Biological and biochemical characterization, N-terminal sequencing and PMF unequivocally indicate that Russelobin is a non-toxic, thrombin-like serine protease from Russell's Viper venom. Glycosylation of Russelobin appears to provide some physiological advantages against neutralization by endogenous protease inhibitors and may lead to unimpeded interaction with its physiological substrate resulting in higher fibrinogenolytic activity. The substrate specificity and insulin B-chain cleavage pattern, as well as inhibition of enzymatic activity by various protease inhibitors, show that Russelobin possesses properties which are distinct from many of the SVTLEs reported to date. The *in vitro* fibrinogen clotting and *in vivo* defibrinogenating activities of Russelobin are attributed to preferential release of FPA and slower release of FPB from fibrinogen, an uncommon property of most SVTLEs. In summary, therapeutic application of Russelobin for the treatment of hyperfibrinogenemia-associated disorders is indicated by *in vivo* and *in vitro* characteristics of the enzyme.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.02.007.

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