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Determination of the fibrinogenolytic activity of *Montivipera raddei* (Radde's mountain viper) venom

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Abstract: Snake venom fibrinogenolytic enzymes have diagnostic and therapeutic value and are important for snakebite pathology. In the present study, the fibrinogenolytic activity of *Montivipera raddei* venom was investigated. Crude venom was incubated with human fibrinogen for different time periods at 37°C. An inhibition study was carried out using different protease inhibitors. The fibrinogenolytic activity was assessed by SDS-PAGE and fibrinogen zymography. An HPLC-based method was used to obtain confirmatory data. *Montivipera raddei* venom predominantly cleaved the Aα chain of fibrinogen in a time-dependent manner. A very slight decrease in band intensity of the Bβ chain was observable after a longer incubation time. Cleavage of fibrinogen was confirmed by HPLC. Zymography revealed that the venom contained 50 and 75 kDa fibrinogenolytic enzymes. Ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline inhibited the overall fibrinogenolytic activity, while phenylmethylsulfonyl fluoride (PMSF) only inhibited the degradation of the Bβ chain. These results indicated that metalloproteinases were major fibrinogenolytic enzymes in the venom. The inhibitor study suggested the presence of serine proteinases that broke down the Bβ chain. With this study, the fibrinogenolytic activity of *M. raddei* venom was shown for the first time. The results will be useful for further isolation and characterization studies.

Keywords: fibrinogen; proteinase; snake venom; SDS-PAGE; zymography

Abbreviations: C-type lectin proteins (CLPs); cysteine-rich secretory proteins (CRISPs); dithiothreitol (DTT); high performance liquid chromatography (HPLC); L-amino acid oxidases (LAAOs); N,N,N′,N′-tetramethylethylenediamine (TEMED); nerve growth factors (NGFs); phospholipase A_2s (PLA_2s); snake venom metalloproteinases (SVMPs); snake venom serine proteinases (SVSPs); sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); thrombin-like snake venom serine proteinases (TL-SVSPs); vascular endothelial growth factors (VEGFs)

INTRODUCTION

Snake venom is composed of a mixture of mainly proteins and peptides with other organic molecules and inorganic ions as minor components. These bioactive proteins affect important physiological processes in the prey [1,2]. Viperid venoms are rich in enzymes and nonenzymatic proteins and peptides, many of which interfere with hemostasis [2,3]. Moreover, these venoms have significant cytotoxic activity [1,4,5]. Viperid venom proteins and peptides can be grouped into several major protein families: enzymes such as snake venom serine proteinases (SVSPs), snake venom Zn^{2+}-metalloproteinases (SVMPs), L-amino acid oxidases (LAAOs), 5'-nucleotidases, hyaluronidases, group II phospholipase A_2s (PLA_2s) and proteins without enzymatic activity, namely the disintegrins, C-type lectin proteins (CLPs), cysteine-rich secretory proteins (CRISPs), nerve growth factors (NGFs), vascular endothelial growth factors (VEGFs), cystatin, natriuretic peptides and Kunitz-type proteinase inhibitors [1,6].

Fibrinogen is a complex fibrous 340 kDa glycoprotein that exists in human plasma at about 1.5-4 g/L concentration in its normal range. It consists of 3 pairs of peptide chains, designated as Aα (66.5 kDa), Bβ (52 kDa) and γ (46.5 kDa). It is essential for hemostasis, wound healing, inflammation, angiogenesis and other important biological processes. Although fibrinogen is a soluble protein, it turns into an insoluble clot when

converted to fibrin by a serine proteinase thrombin. Although this anticoagulation process is a part of a healthy coagulation mechanism, thrombosis also may occur because of the blockage of blood vessels with fibrin clots, which can lead to heart attack, ischemic shock, deep vein thrombosis and other cardiovascular diseases. Various fibrin(ogen)olytics that act directly or by activating plasminogen are widely used in the clinic to treat these conditions [7,8].

Snake venoms, especially viperid venoms are rich sources of fibrin(ogen)olytic enzymes. Thrombin-like snake venom serine proteinases (TL-SVSPs) and SVMPs with fibrinogenolytic activity are among the most abundant enzymes found in viperid venoms and many of them have been purified and characterized from venoms of different species. These enzymes generally cleave the fibrinogen Aα or/and Bβ chains [2,9-12]. During envenomation, these enzymes clearly show various pathological effects by interfering with the hemostatic system processes such as fibrinogenolysis, platelet aggregation and coagulation [1,2,9].

Montivipera raddei (Radde's mountain viper) is one of the venomous viperid snake species distributed in eastern Turkey, Armenia, Azerbaijan, Iran and Iraq, and is adapted to high elevations in rocky mountainous areas [13]. Radde's mountain viper has a total body length of up to 80 cm and mainly feeds on lizards and rodents, small birds and grasshoppers [14]. Research on its venom is limited to only a few functional and toxicity studies [15-18], and two previously published papers that provide insight into the proteomic composition of the venom [5,14]. To the best of our knowledge, no results regarding the fibrinogenolytic activity of *M. raddei* venom have been published to date.

Herein we investigated the fibrinogenolytic activity of *M. raddei* venom for the first time and identified the main protease enzyme group that is responsible for this activity. The results of the study reveal the potential of this understudied venom as a source for new bioactive proteins.

MATERIALS AND METHODS

Ethics statement

Venoms were extracted following the appropriate procedures from live animals under the ethical permission of Ege University Animal Experiments Ethics Committee (Approval No. 2010-43).

Venom samples

The crude lyophilized *M. raddei* venom sample used in the study was pooled from 4 adult female individuals from Aydinkavak village, Kagizman (Kars province of eastern Turkey). The *Macrovipera lebetinus* sample used only in zymography was obtained from 5 adult individuals from Sanliurfa province (southeastern Turkey). The venom was extracted using a paraffin-covered laboratory beaker without exerting pressure on the venom glands [6]. Venom samples were centrifuged at 2000 ×g for 10 min at 4°C to eliminate cell debris. Supernatants were lyophilized using a benchtop freeze-dryer (Millrock Technology, Kingston, NY, USA) and stored at -20°C. Pooled samples were used to reduce the effect of individual venom variation [19].

Reagents

Human plasma fibrinogen, aprotinin, EDTA disodium salt dihydrate, SDS, Bradford's reagent, phosphate-buffered saline (PBS) and iodoacetamide were purchased from Sigma Chemical Co (MO, USA). PMSF, 1,10-phenanthroline, Coomassie Brilliant Blue G-250, dithiothreitol (DTT), ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from Thermo Fisher Scintific (MA, USA), Fluka (Switzerland), Amresco (OH, USA), Fermentas (Lithuania), AppliChem (Germany) and BioFroxx (Germany), respectively. The precision plus unstained protein molecular weight standard was purchased from Bio-Rad Laboratories (USA). All the chemicals used were of analytical or molecular biology grade.

Determination of the protein concentration

Protein quantification was performed according to the Bradford method [20]. Crude venom samples were reconstituted in 50 mM Tris-HCl pH 7.8 buffer. Bovine serum albumin (BSA) was used as a calibration standard at different concentrations (ranging from 0.2 to 1.0 mg/mL). Measurements were carried out on a UV/VIS spectrophotometer (Lambda 25, PerkinElmer, USA) at a wavelength of λ=595 nm. Mean values of 3

technical replicates of the sample and standards were used for calculations.

Determination of the fibrinogenolytic activity by SDS-PAGE

The fibrinogen digestion kinetics of *M. raddei* venom was evaluated according to the method described previously [21] using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with some modifications. Five µL of human plasma fibrinogen solution (2.5 mg/mL in 50 mM Tris-HCl pH 7.8) was incubated with 5 µL of *M. raddei* venom (125 µg/mL crude venom in 50 mM Tris-HCl pH 7.8) for different time intervals (10, 30, 60 and 120 min) at 37°C. The reaction was stopped by adding 4 µL of SDS-PAGE loading buffer containing 0.313 M Tris HCl pH 6.8, 10% SDS (w/v), 0.05% bromophenol blue (w/v), 50% glycerol (v/v) and 1 µL of 2 M DTT, followed by heating at 95°C for 5 min. After reduction and denaturation, the samples were loaded onto an SDS-PAGE gel consisting of 4% stacking and 12% resolving gels and run at 120 V. After the electrophoretic procedure, the gel was stained with 0.12% (w/v) Coomassie Brilliant Blue G-250, scanned and analyzed for fibrinogen chain hydrolysis. Densitogram graphics of gel lanes were created using GelAnalyzer 19.1 software [22].

SDS-PAGE-based inhibition study

The inhibitory effect of different protease inhibitors against the venom's fibrinogenolytic activity was also investigated using SDS-PAGE. Two metalloproteinase inhibitors, EDTA and 1,10-phenanthroline, and 2 serine proteinase inhibitors, PMSF and aprotinin, were used and preincubated with venom for the inhibition study. Five µL of 100 mM EDTA, 10 mM aprotinin, 100 mM 1,10-phenanthroline and 100 mM PMSF were added to 45 µL of 250 µg/mL *M. raddei* venom in separate microtubes and incubated for 30 min at room temperature. Only buffer was added to one tube. The incubation was continued for 60 min at 37°C after the addition of fibrinogen (5 mg/mL), bringing the total volume to 100 µL. This procedure allowed the inhibitors to inhibit the proteinases of interest. The reaction was stopped by adding loading dye and reducing agent and SDS-PAGE was carried out as described above.

Fibrinogen zymography

The fibrinogenolytic activity of *M. raddei* venom was also evaluated using the SDS-PAGE-based fibrinogen zymography method. Human plasma fibrinogen was added and solubilized in the 12% resolving gel solution at a final concentration of 1 mg/mL and letting the solution polymerize together with fibrinogen. 12.5 µl of *M. raddei* and *M. lebetinus* venoms (both at 2 mg crude venom per mL concentration, in 50 mM Tris-HCl, pH 7.8) were mixed with 4 µL of SDS-PAGE loading buffer without reducing agent (DTT). Samples were loaded onto the gel and electrophoresis was performed. The gel was incubated in washing buffer containing 2.5% Triton X-100, 50 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$, 1 µM $ZnCl_2$ and 100 mM NaCl for 2×30 min at room temperature, followed by incubation in an incubation buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 100 mM NaCl) overnight at 37°C. After washing with distilled water for 10 min gels were stained with Coomassie Brilliant Blue G-250 for 60 min. After washing with distilled water, the gel was visualized.

High performance liquid chromatography (HPLC) analysis

An HPLC-based reversed-phase (RP) method was used to confirm the results with an additional technique. The aim was to obtain supporting data for our results. Chromatographic separation was performed on a C18 column (Agilent Poroshell 120 EC-C18, 2.7 µm particle size, 3.0 × 50 mm) using an Agilent 1220 Infinity HPLC system equipped with a diode array detector (DAD). Fibrinogen at a final concentration of 0.5 mg/mL was prepared in PBS solution, mixed with *M. raddei* venom (20:1 fibrinogen:venom ratio) and then incubated for 1 h at 37°C. DTT (at 0.1 M final concentration) was added as a reducing agent, followed by the addition of urea (4.2 M final concentration) and incubation for 2 h at 55°C. Iodoacetamide was added as an alkylating agent at 30 mM final concentration and incubated for 30 min at room temperature in the dark. Buffer was added instead of venom to the negative control tube and DTT and iodoacetamide were not added to the intact fibrinogen sample. Reverse-phase separation was achieved using a gradient method with mobile phases A (deionized water with 0.1% trifluoroacetic acid) and

B (acetonitrile with 0.1% trifluoroacetic acid) at a flow rate of 0.5 mL/min. The gradient was applied as follows: 0-1 min 95% A, 1-8 min 95% to 80% A, 8-10 min 80% to 20% A, 10-12 min 20% to 95% A. Five µL of the samples were injected into the column and the chromatograms were recorded at 280 nm.

RESULTS

Fibrinogenolytic activity: SDS-PAGE results

The aim of the present study was to investigate the fibrinogenolytic activity of *M. raddei* venom by SDS-PAGE, fibrinogen zymography and HPLC. The total protein concentrations of *M. raddei* and *M. lebetinus* samples were calculated as 540.4 and 491.7 µg/mL, respectively. Three chains of fibrinogen (Aα, Bβ and γ chains) were clearly observed as separate bands after reducing SDS-PAGE (Fig. 1A). The polypeptide chains were assigned to SDS-PAGE bands based on their molecular weights [8]. Fibrinogen samples were incubated with *M. raddei* venom for different time intervals and compared with negative controls containing fibrinogen alone. Negative controls were prepared separately for each time point to determine if the incubation time affected fibrinogen chains. SDS-PAGE bands of 3 different chains were not affected during the incubation time in the study (up to 2 h); all the Aα, Bβ and γ chains were clearly observable with similar intensities (Fig. 1A). Venom alone was loaded in one of the wells with the same protein amount as used for fibrinogen incubation samples to find out if venom proteins in the incubation samples influenced the results. No intense bands that were enough to interfere with the results were observed (Fig. 1A, lane 10).

Based on the results, *M. raddei* venom cleaved the fibrinogen Aα chain starting from 10 min after incubation with an almost complete degradation after 30 min (Figs. 1A and 2). A band at about 43 kDa and additionally some other lighter bands (at about 23, 20, 16 kDa) were observed below the band of the γ chain, which were not observable in fibrinogen-only wells (Fig. 1A). *M. raddei* venom did not hydrolyze the fibrinogen Bβ and γ chains significantly under our experimental conditions, as it did the Aα chain. However, a very slight decrease in the band intensity

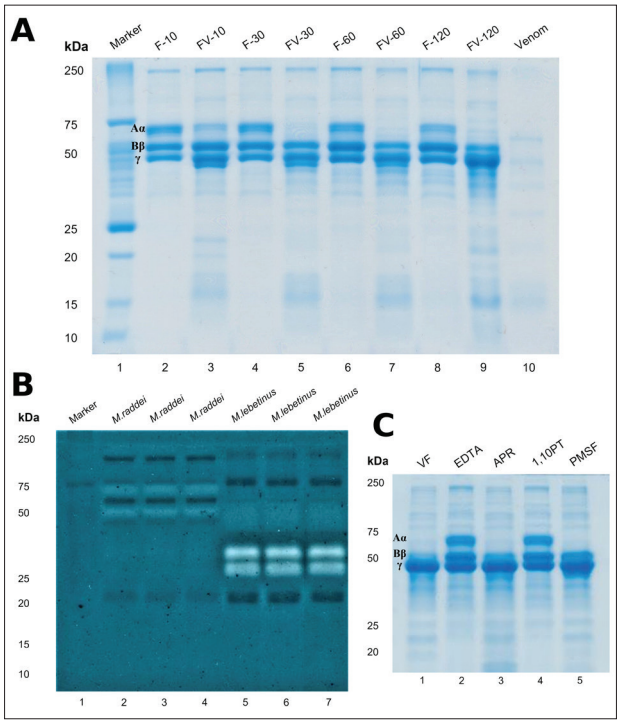

Fig. 1. A – Reducing SDS-PAGE gel image showing the fibrinogenolytic activity of *M. raddei* venom at different time intervals. Lane 1: molecular weight marker; lanes 2, 4, 6, 8: control fibrinogen incubated without venom for 10 (F-10), 30 (F-30), 60 (F-60) and 120 (F-120) min, respectively; lanes 3, 5, 7, 9: fibrinogen incubated with *M. raddei* venom for 10 (FV-10), 30 (FV-30), 60 (FV-60) and 120 (FV-120) min, respectively; lane 10: *M. raddei* venom alone loaded with the same amount as in other wells with fibrinogen and venom. **B –** Fibrinogen zymogram gel image of *M. raddei* and *M. lebetinus* venom samples. **C –** SDS-PAGE gel image showing the inhibition of the venom fibrinogenolytic activity after pre-incubating with different protease inhibitors. Lane 1 (VF) includes fibrinogen sample incubated with venom. Lanes 2 (EDTA), 3 (APR), 4 (1,10PT) and 5 (PMSF) – fibrinogen and venom preincubated with EDTA, aprotinin, 1,10-phenanthroline and PMSF, respectively.

of the Bβ chain was observable after 60 and 120 min of incubation with venom (Figs. 1A and 2).

Fibrinogenolytic activity: zymography results

The fibrinogenolytic activity of *M. raddei* venom was further confirmed by fibrinogen zymography and compared with the venom of *M. lebetinus* as a positive control since it contains different types of fibrinogenases [23]. The experiment was carried out in triplicate and all samples were loaded onto the same gel. Lighter bands on the dark background

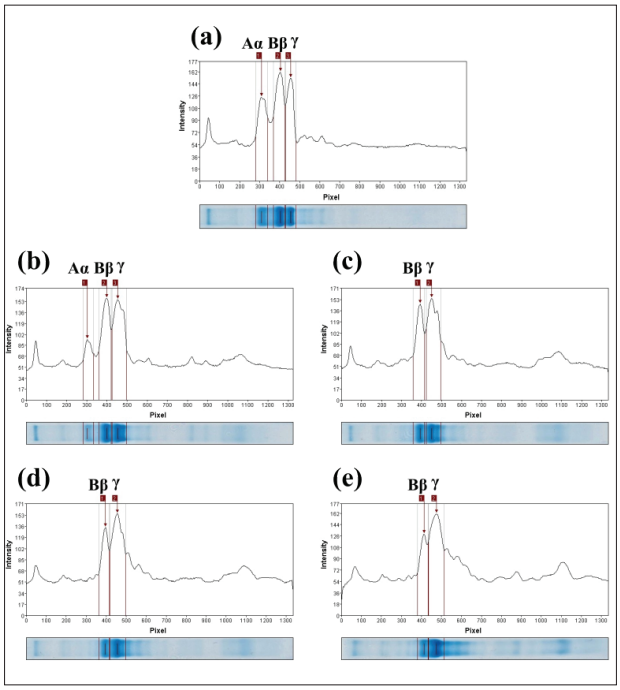

Fig. 2. SDS-PAGE bands belonging to the fibrinogen chains incubated without **(A)** and with *M. raddei* venom for 10 **(B)**, 30 **(C)**, 60 **(D)** and 120 **(E)** min. Note the disappearance of the Aα chain after 30 min of incubation and a slight decrease in the peak of the Bβ chain after 60 and 120 min.

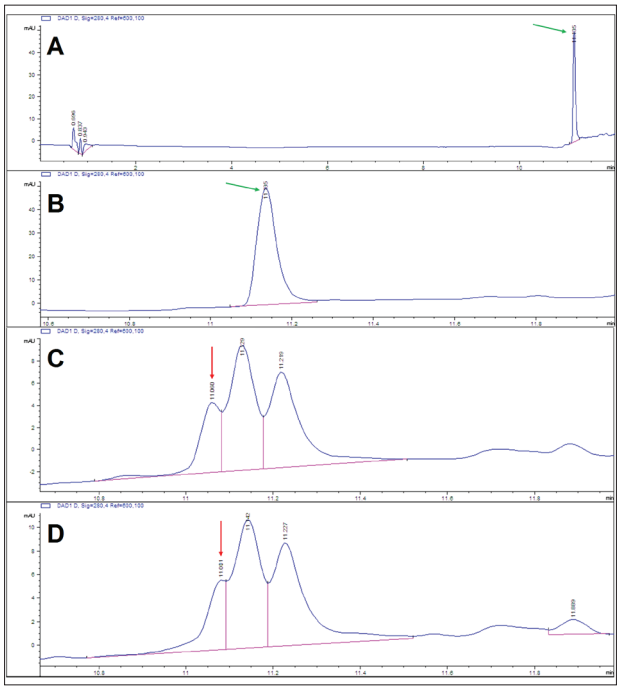

Fig. 3. HPLC chromatograms of intact fibrinogen (**A** and **B**), reduced fibrinogen only (**C**) and fibrinogen treated with venom and subsequently reduced (**D**). Reduced fibrinogen samples with or without venom were obtained by adding DTT and iodoacetamide after incubation, which were not added to the intact fibrinogen samples.

indicated fibrinogenolytic activity. Based on the results, the activity of the fibrinogenolytic enzymes in *M. raddei* venom was lower when compared to *M. lebetinus*. However, the fibrinogenolytic activity of *M. raddei* venom was clearly confirmed, indicating the presence of at least 2 of the main enzymes responsible for this activity with about 50 and 70 kDa (Fig. 1B). Additionally, a faint lighter zone was observed at about 35 kDa, which is difficult to visualize on a zymogram gel. The molecular weights of the main fibrinogenolytic enzymes present in *M. lebetinus* venom were in the range of about 25-40 kDa.

Inhibition of the fibrinogenolytic activity

The results of the inhibition study as revealed by SDS-PAGE are presented in Fig. 1C. The experiment was carried out with an incubation time of 1 h. Venom samples were preincubated with different protease inhibitors. In the inhibition study, only venom (without inhibitors) degraded the fibrinogen Aα chain completely and the Bβ chain slightly, confirming the previous result. When the gel image was examined, it was clearly observed that the Aα chain of the fibrinogen was completely degraded in the wells containing the venom preincubated with aprotinin and PMSF, whereas it remained unhydrolyzed after preincubation with 1,10-phenanthroline and EDTA. Moreover, the Bβ chain was slightly hydrolyzed in the presence of aprotinin while it was not degraded after incubation of the venom with 1,10-phenanthroline, EDTA and PMSF (Fig. 1C).

Confirmation of the fibrinogenolytic activity by HPLC

An RP-HPLC-based method with a C18 column was used to assess and confirm the fibrinogenolytic activity. The retention time of the intact pure human fibrinogen was determined as 11.1 min (Fig. 3A, B). Three peaks were observed at the same retention time between 11.0-11.2 min in the chromatogram of reduced fibrinogen (Fig. 3C), incubation with venom decreased the intensity of the peak at 11 min (Fig. 3D).

DISCUSSION

Until now, snake venom fibrinogenolytic enzymes have been purified and characterized from the venoms of different snake species with a special focus on TL-SVSPs [3,12,24,25]. Fibrinogenolytic TL-SVSPs generally cleave the fibrinogen Bβ chain preferentially and have lower activity against the Aα chain, while fibrinogenolytic SVMPs mainly degrade the Aα chain. However, some SVSPs and SVMPs may primarily degrade the Aα and Bβ chains, respectively [3,10,12]. A few enzymes also break down the γ chain of fibrinogen, generally after prolonged incubation [3]. These enzymes are important for the pathology of snakebites since they interfere with various aspects of the blood coagulation mechanism [1,2,9]. Fibrinogenolytic SVSPs and SVMPs (with special attention to TL-SVSPs) have been investigated as potential therapeutic agents in the treatment of various diseases such as myocardial infarction, ischemic stroke and thrombotic disorders [11,26,27]. Moreover, TL-SVSPs have been used in hematology laboratories since the early 90s for the diagnosis of various clotting disorders. Batroxobin is one of the well-known examples of this class used in diagnostics [28].

Based on the results of the study, it can be concluded that *M. raddei* venom contains fibrinogenolytic enzymes that degrade mainly the Aα chain of fibrinogen. Moreover, the results indicated that these enzymes could also degrade the Bβ chain, but the data were not as prominent as those obtained for the Aα chain. Therefore, it can be concluded that *M. raddei* venom shows its fibrinogenolytic activity primarily by cleaving the Aα chain starting from 10 min incubation and secondarily by cleaving the Bβ chain with lower activity. Additional bands observed after venom treatment (at about 43, 23, 20, 16 kDa below the γ chain band) are likely due to the cleavage of the Aα chain, and this result suggests the presence of multiple cleavage sites. Similar effects on fibrinogen were also reported for other viperid venoms and as well as the venoms of other groups [12,29].

Our follow-up study showed that the α- and β-fibrinogenolytic activities of *M. raddei* venom were inhibited by EDTA and 1,10-phenanthroline, which are metalloproteinase inhibitors [30], whereas the serine proteinase inhibitors aprotinin and PMSF [31] did not exhibit an inhibitory effect against the degradation of the Aα chain. According to these results, it was concluded that the main enzyme class responsible for the fibrinogenolytic activity of *M. raddei* venom were the metalloproteinases that degrade primarily Aα and secondarily Bβ chains. Moreover, the results of the PMSF treatment, which seems to inhibit the slight degradation of Bβ chain, also point to the possible presence of serine proteinases that cleaved this chain.

EDTA and 1,10-phenanthroline are metal ion chelators and they inhibit most SVMPs by interacting with the zinc ion at the active site [12,30]. The serine proteinase inhibitor PMSF, which irreversibly binds to serine residues at the catalytic site, was shown to reduce the activity of many SVSPs [25,31]. But aprotinin generally has no inhibitory effects on SVSPs [25,31]. The inhibition of β-fibrinogenase activity by aprotinin may be due to the different binding efficiencies and interaction sites of these molecules. Our findings corroborate previous data in the literature regarding the inhibition of SVMPs and SVSPs.

HPLC was used to confirm our results in addition to SDS-PAGE. The peak of the intact fibrinogen was divided into 3 peaks after reduction, indicating the separation of 3 fibrinogen chains. Although the resolution of the separation was not ideal in the reversed-phase method using the C18 column, we observed a decrease in one of these peaks as a supportive result. Taking into consideration the SDS-PAGE images, the lower peak may contain the Aα chain. For a more reliable interpretation based solely on HPLC, the resolution should be increased by using different methods (e.g. ion exchange, gel filtration), and separated peaks should be analyzed by SDS-PAGE and/or mass spectrometry for the identification of fibrinogen chains. HPLC-based methods can then also be used for assessing the fibrinogenolytic activities of snake venoms.

The fibrinogenolytic activity of *Montivipera xanthina*, a closely related species to *M. raddei*, was assessed by tricine SDS-PAGE in a previous study, with the results indicating that *M. xanthina* venom contained fibrinogenolytic enzymes that mainly cleaved the Aα chain of fibrinogen [29]. Although the SDS-PAGE results were not as clear as those obtained for the Aα chain, the authors stated that *M. xanthina* venom also contained β-fibrinogenase. Although they asserted that the activity was not inhibited by EDTA,

there is a prominent difference in the band intensities of the Aα chain after incubation with only venom for 10 and 120 min, whereas such a difference was not clearly observable in samples containing EDTA. This result raises the possibility of at least partial inhibition of the fibrinogenolytic activity in the venom by EDTA. It was also reported that this activity was partially inhibited in the presence of a protease inhibitor cocktail containing serine, cysteine and metalloprotein inhibitors [29]. Comparing the results of the present study with this paper, it can be concluded that both *M. raddei* and *M. xanthina* venoms predominantly contain α-fibrinogenases and possibly β-fibrinogenolytic enzymes with much lower activities. The major fibrinogenolytic enzyme group of *M. raddei* venom was clearly shown to be a metalloproteinase in the present study, exhibiting strong inhibition of activity by EDTA and 1,10-phenanthroline, whereas this inhibition by *M. xanthina* venom was not significant, albeit maintaining a possible partial inhibition [29]. The venom of *M. xanthina* did not cause observable degradation of the Aα chain after 10 min of incubation [29] while our results showed that *M. raddei* venom started to degrade the Aα chain at the same incubation time, suggesting that the fibrinogenolytic activity of *M. raddei* venom is slightly stronger than that of *M. xanthina*.

Viperid venoms contain several major protein families with or without enzymatic activity. These venoms are rich in proteinases (SVMPs and SVSPs) that act on the blood coagulation mechanism [1,6,14,32]. Venom characterizations in viperids at the protein family level were achieved by mainly using mass spectrometry-based proteomics (so-called venomics) approaches [33]. In two previous studies, it was revealed that *M. raddei* venom contained SVMP, SVSP, PLA_2, LAAO, CLP, CRISP, VEGF, NGF, disintegrin, bradykinin-potentiating/C-type natriuretic peptides and Kunitz-type serine proteinase inhibitor [5,14]. In addition to the SDS-PAGE results in our study, it was also found by fibrinogen zymography that the main fibrinogenolytic enzymes in *M. raddei* venom had molecular weights of about 70 and 50 kDa. Based on our current knowledge of the protein composition of viperid venoms, the molecular weights of P-II and P-III SVMPs range between 30-60 and 60-100 kDa, respectively [3,34]. Moreover, venomic characterization of *M. raddei* from the same region used in the present study identified a 60 kDa metalloproteinase band by mass spectrometry-based partial sequencing [5], and P-III SVMPs were identified from 46, 67 and 56 kDa bands of *M. raddei* venom from Armenia [14]. Thus, metalloproteinases were detected as one of the major protein families of *M. raddei* venom. All our findings corroborate well with literature data and indicate that the main fibrinogenolytic enzyme group in *M. raddei* venom is a metalloproteinase from the P-II/P-III class. Moreover, serine proteinases were identified from SDS-PAGE bands of about 35, 37 and 43 kDa [5,14]. The faint zone at about 35 kDa observed in our zymography results confirmed our SDS-PAGE-based inhibition experiment, suggesting the presence of fibrinogenolytic serine proteinases acting on the Bβ chain with lower activity.

CONCLUSIONS

This is the first study describing the presence of fibrinogenolytic zinc-metalloproteinases and serine proteinases in *Montivipera raddei* venom by *in vitro* experiments. Although general proteomic characterization of *M. raddei* venom disclosed the presence of these enzyme classes previously, their fibrinogenolytic activity was not demonstrated. Using SDS-PAGE, fibrinogen zymography and HPLC, *M. raddei* venom was shown to contain fibrinogenolytic metalloproteinases that cleave the Aα and Bβ chains and serine proteinases that break down the Bβ chain. The degradation kinetics of β-fibrinogenases should be confirmed by further studies with longer incubation times. The results obtained in the present work will guide future isolation/purification and characterization studies and provide the groundwork for the identification of new thrombin-like enzymes and fibrinogenolytic metalloproteinases as well as the evaluation of their biotechnological potential for diagnostic and therapeutic use. Moreover, the results will be useful for the assessment of the pathology of *M. raddei* snakebite. Further studies aiming to purify and characterize the active enzymes and identify the degradation products and cleavage sites will provide better insight into the biochemical properties of *M. raddei* fibrinogenases.

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Author contributions: Conceptualization, supervision and funding acquisition: NI; experimental (methodology): FSA; writing, original draft preparation: NI, and FSA; writing–review and editing: NI, and FSA. All authors have read and agreed to the published version of the manuscript. This paper originates from the M.Sc. thesis of the first author, supervised by the second author.

Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: All data underlying the reported findings have been demonstrated in the article.

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