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Original Article

Isolation, characterization, and expression of the *Montivipera raddei* (Caucasus viper) venom metalloprotease-like protein

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Abstract

Background and purpose: Snake venom contains numerous proteolytic enzymes, including metalloproteases. This study aimed to isolate, characterize the metalloproteinase from *Montivipera raddei* venom and insert its cDNA into a host cell genome for expressing the protein's potential use as a coagulation factor.

Experimental approach: Initially, *M. raddei* venom was analyzed using SDS-PAGE and ion-exchange chromatography. All purified fractions were assessed using a prothrombin time (PT) assay. Immunoblot analysis and MALDI-TOF/TOF MS spectrometry confirmed the metalloproteinase active domain in the obtained fraction. All of the confirmations were studied using the I-TASSER server. To produce the recombinant enzyme, metalloproteinase cDNA was isolated from the total RNA of the Caucasus viper venom gland tissue and cloned into pEX-A2-H plasmid, containing homologous sequences with CHO-S cell genome. Also, it was confirmed by western blotting and PT test.

Findings/Results: Two fractions from *M. raddei* venom had PT values of 6 and 21 s, respectively. We confirmed the presence of a 25-KDa procoagulant metalloprotease (Met). The results indicated successful expression of the Met protein in the recombinant CHO-S cells. The Met protein was structurally similar to the target in the PDB ID. 2e3x, regulatory subunit of the blood coagulation factor X- and IX-activating enzyme. The Met protein also contained a similar binding domain with 60 amino acids, comparable to those in *E. carinatus*. and *E. pyramidum leakeyi* metalloproteinases.

Conclusion and implications: The Met protein may be a potent candidate recombinant coagulant drug. Remarkably, the thrombin time for this protein was 3 s.

Keywords: Cloning; Chromatography; Metalloproteinase; Venom proteins.

INTRODUCTION

Biologically active natural products have played a prominent role in human health and survival, serving as natural remedies throughout history. Plants and animals have long been the primary source of active pharmaceutical agents in traditional medicine systems, which are used to alleviate and treat a wide range of human diseases (1). Over the last century, numerous natural-based drugs have been developed to treat cancer and infectious, cardiovascular, and neurological diseases (2).

Venoms are highly complex mixtures of biologically active substances produced and stored in cells or organs of venomous animals for defensive and/or offensive purposes. Venoms have fascinated scientists for over a century and are now heavily utilized in pharmaceutical and biomedical sciences due to their valuable substances (3).

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Medically important venomous snakes come from 3 families: Atractaspidae, Elapidae, and Viperidae. The Viperidae family is widespread, with 3 subfamilies including Azemiopinae, Crotalinae, and Viperinae (4). *Montivipera raddei*, commonly known as the zigzag mountain viper or the Caucasus viper, is found in the northwestern mountainous regions of Iran, especially in the highlands of Zanjan Province. According to Siigur (4) and Gutierrez's (5) studies, *Montivipera raddei* venom contains a highly complex mixture of valuable proteins and peptides with potential pharmaceutical applications. This type of snake hunts its prey by inducing thromboembolic stroke and cardiovascular collapse from pulmonary embolism, indicating the presence of a potent coagulant factor in its venom (5). However, no study has specifically analyzed the coagulant agent of *M. raddei* venom. The present study focused on the prothrombin-like activity of *M. raddei* venom and aimed to identify the strongest coagulation factor.

MATERIALS AND METHODS

Preparation of the Viper Venom

The preparation of the viper venom followed the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals. The venom of the Caucasus viper was prepared by the Laboratory of Live Animals, College of Agriculture and Natural Resources, Tehran University. The venom was transferred to the Cellular and Molecular Biology Research Center laboratory and immediately stored at -80 °C. All animal procedures were conducted in accordance with the ethical principles of the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences (Code of Ethics: IR.SBMU.RETECH.REC. 1398.162).

Isolation and identification of the coagulant proteins

Ion exchange chromatography

Due to the negative charge and acidic isoelectric point (pI) value of metalloproteinases, a positively charged resin was applied in ion-exchange chromatography for the purification of the coagulant proteins. First, 50 mg of crude venom from *M. raddei* was diluted in 15 mL of distilled water and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered with a 0.45-µm Millipore filter to remove all insoluble materials and then loaded on a diethylaminoethyl cellulose (DEAE-C) column. The column was equilibrated with 20 mM Tris-HCl buffer (pH = 8) and eluted with a gradient of NaCl concentrations from 0.1 to 0.6 M at a flow rate of 20 mL/h calculated manually. The absorbance of collected fractions was recorded at 280 nm.

Coagulant activity

Two peaks of concentration were observed in the previous step at 2.3 and 1.86 mg/mL. Prothrombin time (PT) was applied to evaluate coagulation time in extrinsic pathways. In this test, 100 µL of PT reagent and 100 µL of fractions, including F1 and F2 (Fig. 1) obtained from the ion exchange column, were mixed and pre-incubated at 37 °C for 5 min. Then, 100 µL of fresh citrate plasma was added, and clotting time was recorded.

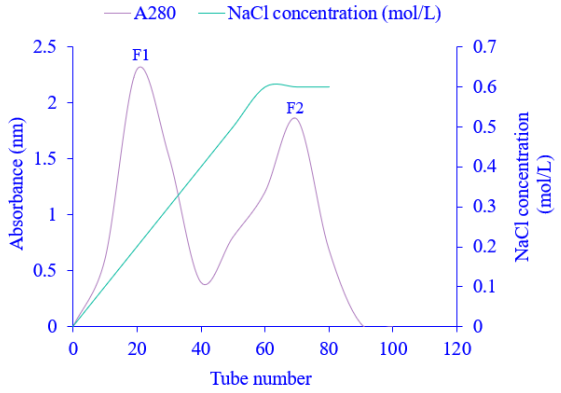

Fig. 1. Diethylaminoethyl (DEAE)-cellulose chromatography curve. The profile of eluted *M. raddei* venom proteins (purple curve) in a linear gradient of NaCl concentration (green curve).

Production of anti-commercial ecarin-like specific serum

To detect the metalloproteinase in the purified coagulant fractions, a polyclonal antibody was prepared. A 4-week-old BALB/c mouse (Pasteur Institute, Tehran, Iran) was injected intraperitoneally with a 1:1 (v/v) mixture of 200 µg/mL commercial ecarin metalloproteinase (Merck, Germany) and Freund's complete adjuvant (Sigma, Germany) under sterilized conditions. The second and third injections were administered 12 and 14 days after the first injection with the mixture of antigen and Freund's incomplete adjuvant. The animal serum was then analyzed with ELISA.

Evaluation of the coagulant fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

The total concentration of proteins in each sample was determined using the Bradford method (Roche Molecular Biochemical, USA). Fraction precipitation was performed by adding cold acetone (Roche Molecular Biochemical, USA). Protein components in each fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Approximately 30 µg/µL of each sample was loaded onto a 12% (w/v) SDS-PAGE gel and subjected to electrophoresis for 3 h at 100 V and 70 mA. Coomassie brilliant blue R-250 was used to visualize proteins on the gel (6).

The presence of the metalloproteinase in each fraction was determined by Western blotting. Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane. The membrane was blocked with Tris-HCl buffered saline (TBS) containing 3% skimmed milk powder for 45 min, then washed by TBS with 0.1% Tween 20. The membrane was incubated with the mouse serum as the source of anti-metalloproteinase antibodies (1:1000 in TBS) and after washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (ab6789, Abcam, UK) (1:10000 in TBS) was used. Protein bands were detected with 3,3′-diaminobenzidine and H_2O_2 reaction.

Matrix-assisted laser desorption/ionization-time of flight/time-of-flight mass spectroscopy analysis

To analyze matrix-assisted laser desorption/ionization-time of flight/time-of-flight (MALDI TOF/TOF) mass spectroscopy (MS) the protein fragments from the extracted sample were subjected to enzymatic digestion. The resulting peptides were separated using a combination of ion exchange and reverse-phase chromatography in the liquid chromatography system. Automated fraction collection and robotic spotting of a MALDI plate resulted in a properly annotated sample plate for presentation to the mass spectrometer. The Mascot Server 3.1 (Matrix Science, UK) provided fully automated sample acquisition protocols. At first, MS data was acquired. Precursor ions were automatically selected by several gating criteria, including molecular weight and abundance. A subsequent MS/MS analysis of precursor ions formed the data table for the ProteinPilot software to systematically identify proteins based on these precursors (Proteomics International Pty Ltd Company, Western Australia).

Exploration of the metalloproteinase coding DNA in the Caucasus viper's venom glands

Total RNA extraction and complementary DNA synthesis

Total RNA from the Caucasus viper's venom gland tissue was extracted using a Total RNA purification kit (Jena Bioscience, Germany) by grinding in a microtube according to the manufacturer's instructions. The RNA sample was treated by DNase I (1 IU/1 µg RNA) (Fermentas, Lithuania). Complementary DNA (cDNA) was synthesized using 1 µg of total RNA, oligo dT primer (2 pmol, Fermentas, Lithuania), and M-MuLV Reverse Transcriptase (100 U, Fermentas, Lithuania). The tubes were incubated at 42 and 70 °C for 60 and 5 min, respectively. The concentration of cDNA was measured using a nano-drop spectrophotometer.

Amplification of metalloproteinase gene with PCR

The metalloproteinase gene of *Montivipera raddei* glands was amplified by polymerase chain reaction (PCR) with the addition of a 90 bp nucleotide fragment similar to the pEX-A2-H insertion site (homologous sequences) to enter into the expression vector. Specific primers for the PCR-amplified metalloproteinase gene were designed based on the ecarin sequence of *Echis carinatus* (NCBI GenBank accession number: D32212). The PCR reaction was performed in a total volume of 20 µL, including 1.25-unit *Pfu* (*Pyrococcus furiosus*) DNA polymerase (Fermentas, Germany), 0.30 µM each of forward (5′GCCCAGTTCCGCCCATTCTC CGCCCCATGGCTGACTAATTTTTTTTATTT ATGCAGAGGCCGAGGCCGCCTCGGCCTC TGAGCTAATGATCCAGGTGCTGCTGGTG ACCA3′) and reverse (5′AGATGC AAGTTGCA CTTCAATTGCAGCAGCGG CGCTGTCCAT GTGCTGGCGTTCGAATTTAGCAGCGGTTT CTTTCATCAGCACGCTGCCCTTGGCGGGG GCGGGCCACT3′) oligonucleotide primers, 25 ng cDNA, and nuclease-free water. The PCR cycle was set at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 40 s, 60 ºC for 40 s, and 72 ºC for 1 min. Finally, the PCR-amplified fragment was analyzed by electrophoresis in 2% (w/v) agarose gel.

Cloning the metalloproteinase isolated gene into pEX-A2-H plasmid

The PCR product was excised from the gel and purified using a PCR clean-up kit (Qiagen, USA), according to the manufacturer's instructions. The isolated DNA fragment was cloned into pEX-A2-H using the homologous recombination process after transformation into *Escherichia coli* (*E. coli*) Dam$^-$ (Fig. 2).

Briefly, about 1 µL of DNA fragments was mixed with 25 µL of electrocompetent *E. coli* *Dam*$^-$ cells (containing pEX-A2-H plasmid with entry location) (7). Electroporation was performed with a Gene Pulser electroporator (Eppendorf, Germany) at 2500 V. The cells were suspended in 1 mL of super optimal broth (SOB) medium and incubated in a rotary shaker with 120 rpm for 1 h at 37 °C. Next, bacterial cells were spread onto SOB agar plates containing 20 mM of $MgSO_4$ and 25 µg/mL of ampicillin and incubated at 37 °C overnight.

In silico confirmation of the isolated metalloproteinase sequence

The cloned gene was sequenced using specific primers of the original ecarin (Pishgaman, Iran) in both directions, following the dideoxynucleotide chain termination method. The obtained sequence was translated *in silico* using a translation tool (http://www.expasy.ch/ tools/dna.html). The deduced protein sequence was then analyzed using the basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify similar proteins. The physicochemical properties of the resulting amino acid sequence were investigated using ProtParam (8). Subsequently, the protein sequence was run through the I-TASSER server for protein structure prediction and structure-based function annotation (9).

Additionally, metalloproteinase protein sequences from *Echis carinatus* (Q90495) and *Echis pyramidum leakeyi* (E9JGJ0) were selected and aligned with obtained metalloprotease (Met) protein sequence using COBALT, a multiple-sequence alignment tool (10). This analysis characterized the similar binding region.

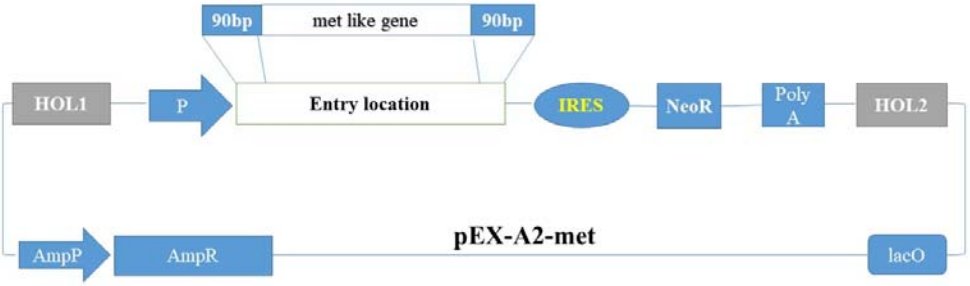

Fig. 2. Demonstration of the recombination between homologous sequences and the modified met gene. AmpR, ampicillin resistance gene; lacO, lacO promoter; Hol, homologous sequences; P, promoter; IRES, EMCV internal ribosome entry site; NeoR, neomycin resistance encoding sequence; PolyA, poly A signal sequence; Met, metalloprotease.

Transfection of the Chinese hamster ovary-S cell line, expression, and purification of the recombinant Met protein

The Chinese hamster ovary (CHO)-S (CHO-S) cell line, a gift from Prof. Kazemi, was used as a platform to express the metalloproteinase gene. Initially, the CHO-S cells were adapted to growth in a serum-free-CHOCLON medium (INOCLON, Iran) through daily passaging. The pEX-A2-H vector, which included 28s rDNA homologous sequences of the CHO-S genome, was used to transfer the Caucasus viper metalloproteinase gene (met) into the CHO-S cell genome.

The pEX-A2-met expression vector (10 µg) was transfected into CHO-S cells by electroporation at 500 v and 5 µs using a Multiporator (Eppendorf, Germany). The electroporation reaction involved 1 × 10^6 cells and 10 µg plasmid. Transfected cells were then seeded in a 24-well plate containing CHOCLON medium at 37 °C and 5% Co2. After 24 h, 300 µg/mL of G418 antibiotic (Sigma, USA) was added. The medium was changed after 24 h, and the cultured cells were collected after 3 days to purify the Met protein. The expression system was intracellular. For protein extraction, the medium was removed after centrifuging at 8000 rpm for 15 min. The cell pellet was lysed with a lysis buffer and sonicated for 20 pulses at 80% amplitude. The supernatant was separated by centrifuging at 10000 rpm for 10 min. For protein purification, an S-protein agarose column (Novagen, USA) was used. The purified protein was assessed with SDS-PAGE and Western blot using anti-ecarin mouse serum as the primary polyclonal antibody (1:1000 dilution) and HRP-conjugated anti-mouse antibody (1:10000 dilution) (Abcam, UK).

Coagulation activity of the recombinant expressed metalloproteinase

The concentration of the Met recombinant protein was first measured using a nano-drop spectrometer. Coagulation activity was then determined with a PT kit as described earlier.

RESULTS

Ion-exchange chromatography and coagulant activity assay of the isolated fractions

The optical absorbance of the fractions obtained from DEAE-C chromatography was analyzed at 280 nm. Two fractions, including F1 and F2 with higher protein concentrations (Fig. 1), were eluted using 0.2 and 0.6 mol/L of NaCl, respectively. The PT values for the fractions F1 and F2 were 6 and 21 s, respectively.

Western blot analysis of 25 kDa isolated protein with ecarin-specific serum polyclonal antibody

The SDS-PAGE pattern of purified protein fractions from the weak anion exchanger (DEAE) was shown in Fig. 3A. Western blot analysis using an anti-ecarin polyclonal antibody confirmed the presence of the procoagulant metalloprotease among the isolated proteins. The molecular weight of the procoagulant metalloprotease protein was approximately 25 kDa (Fig. 3B).

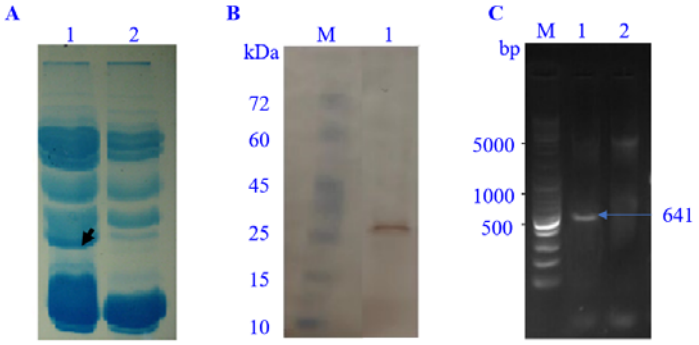

Fig. 3. Isolation of the purified protein. (A) Diethylaminoethyl (DEAE)-cellulose chromatography. Lane 1, fraction 1; lane 2, fraction 2; (B) cloning of encoding gene. Lane M, protein marker; lane 1, fraction 1; (C) gel electrophoresis of the recombinant pEX-A2-H vector containing the met gene. Lane M, DNA marker; lane 1, amplification of the met gene with specific primers of the ecarin gene; lane 2, negative control; fraction 1, purified metalloprotease exposed by anti-ecarin serum.

Table 1. Proteins matching the same set of venom peptides.

Unique marker (m/z)	Protein score	Peptide sequence	Protein name (PDB ID)	Biological process
1190.57	73	KNNKDDPFWRN	Phospholipase B like (T1DLW3)	Lipid degradation, lipid metabolism
1464.72	87	KDFIQKQDEWTRQ	Phospholipase (T1DLW3)	Lipid degradation, lipid metabolism
1138.53	68	SVDFDSESPR	Cysteine-rich venom protein (P86537)	Calcium channel impairing neurotoxin
1206.56	66	MASAEDDLIARY	Thiamine-monophosphate kinase	Magnesium ion binding, thiamine-phosphate kinase activity, phosphorylation

MALDI-TOF/TOF MS and database search

The protein was identified by MALDI-TOF/TOF MS as a metalloproteinase-like protein with a molecular weight of 23794.35 Da. Table 1 shows proteins PDB ID matching the same set of venom peptides along with m/z markers and higher scores compared to the others.

In silico confirmation of the isolated metalloproteinase sequence

For the achievement of a cDNA library from the venom glands of an *M. raddei* by PCR, a pair of specific primers of the original ecarin gene was used. The amplified DNA fragment was cloned into the pEX-A2-H plasmid by homologous recombination process in the Dam$^-$ strain of *E. coli*. For further validation, the recombinant fragment was confirmed using the sequencing (Fig. 3C). The sequencing results showed that the met cDNA (deposited in NCBI GenBank under accession number LC599329) contained 641 bp of the coding sequence, encoding a protein with 207 amino acid residues (the ORF section has been submitted in NCBI). It showed 100% similarity with Met active site domain isolated from *Echis carinatus* and *Echis pyramidum leakeyi*. The protein had an instability index of 23.794 kDa, classifying it as a stable protein. The predicted isoelectric point was 6.13. The total number of negatively (Asp + Glu) and positively (Arg + Lys) charged residues were 24 and 20, respectively (9).

Among the 5 structure models generated by the I-TASSER server, the significant model was selected with confidence score (C-score) = 0.274, template measure score (TM-score) = 0.731, root mean square deviation (RMSD) = 2.69 Å, and coverage (Cov) = 0.949. The Met protein was structurally similar to the target protein in the PDB ID. 2e3x called the regulatory subunit of the blood coagulation factor X- and IX-activating enzyme (Fig. 4A). The identity score of the Zmet protein to target protein was 0.279. As shown in Fig. 4, the isolated metalloproteinase structure (ribbon protein) significantly covered (nearly 100%) the coagulation factor X-activating enzyme light chain 1 active site domain (Q4PRD1).

The multiple alignment of the 3 amino acid sequence fragments (*Echis carinatus* (Q90495), *Echis pyramidum leakeyi* (E9JGJ0), and Met protein using the COBALT tool showed a nearly conserved domain with 60 amino acids in all of them and displayed a similar binding region in protein structures (Fig .5).

Recombinant expression of Met protein in CHO-S cell line

For the expression of the met gene and functional analysis, the CHO-S cell line was transfected with the pEX-A2- met expression vector. The met gene was inserted into the genome through homologous recombination, ensuring stable protein expression. The recombinant Met protein was purified using S-protein agarose beads. Protein expression was analyzed using SDS-PAGE (Fig. 4B) and confirmed by Western blotting (Fig. 4C) using the anti-ecarin mouse serum and HRP-conjugated anti-mouse antibody.

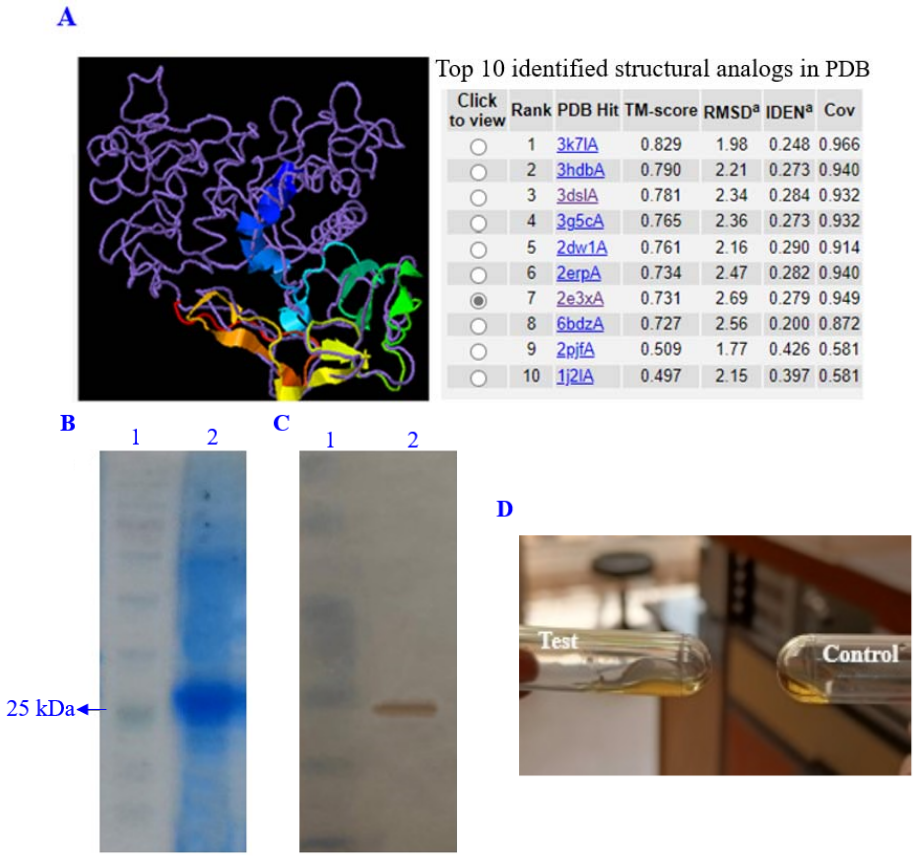

Fig. 4. Identified metalloproteinase structure and activity confirmation. (A) Met protein modeling by the I-TASSER program. The structure of Met was shown in ribbon form, and the structural analog (PDB ID. 2e3x) was displayed using a purple backbone trace (TM-score = 0.731, RMSD = 2.69 Å, and Cov = 0.949). (B) SDS-PAGE of the purified Met protein expressed in CHO-S. Lane 1, protein marker; lane 2, purified protein band. (C) Interaction of the purified protein with anti-ecarin mouse serum. Lane 1, Prestained protein marker; lane 2, confirmed Met band. (D) Bioactivity of expressed Met in blood plasma prothrombin time test after 3 s. Met, Metalloprotease; C-score, confidence score; TM-score, template measure score; RMSD, root mean square deviation; Cov, coverage; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CHO-S, Chinese hamster ovary-S; treat, clot formation in plasma; control, no clot formation in negative control.

Table 2. PT test for 0.5 mg/mL concentration of recombinant purified expressed protein. Data were expressed as mean ± SD.

Protein concentration	PT (s)	Description
Metalloprotease 0.5 mg/mL	10 ± 3	The clot is fully formed.
Normal PT (without protein)	13 ± 21	The clot is fully formed.

PT, Prothrombin time.

Coagulation activity of recombinant Met protein by PT test

The purified recombinant protein concentration measured using a nano-drop spectrophotometer was 0.5 mg/mL. The results showed that active Met protein could initiate the coagulation by creating proteolytic incisions in plasma prothrombin molecules, converting them to thrombin (Fig. 4D). The normal PT without recombinant protein was about 13 s. In comparison, the plasma coagulation time was successfully reduced to 3 s using the recombinant Met protein (Table 2).

Fig. 5. Graphical overview of the Met protein alignment with *Echis carinatus* and *Echis pyramidum leakeyi* metalloproteinase sequence fragments. It displayed a nearly conserved domain (red color) with 60 amino acids in all of them and displayed a similar binding region in protein structures. Met, metalloprotease.

DISCUSSION

Every year, numerous individuals suffer trauma and severe injuries due to accidents, and in many cases, the inability to control bleeding from these incidents leads to fatalities (11). Furthermore, bleeding disorders present significant challenges for individuals with coagulation issues, as these patients often lack certain coagulation factors, limiting their emergency treatment options. Effective bleeding control is crucial for these patients. Over the years, various methods to control bleeding have been extensively studied (12).

On the other hand, the use of blood coagulation drugs is particularly challenging for patients with special conditions, such as cardiovascular disease, due to the risk of clot formation, embolisms, and stroke (13). Additionally, individuals with coagulation disorders, like hemophiliacs, require continuous administration of coagulation factors. These patients face severe difficulties during surgeries, traffic accidents, and other events causing significant bleeding (12-14).

Snake venom is a natural genesis of pharmacologically active compounds, comprising a complex mixture of proteins and enzymes that affect physiological processes such as thrombosis and hemostasis (15). Several snake venom-derived peptides are used in the pharmaceutical industry, including captopril, enalapril, tirofiban, eptifibatide, batroxobin, and cobratide (16). The proteolytic enzymes of snake venom can disrupt blood hemostasis by either activating or inhibiting the coagulation cascade (17). Snake venom metalloproteinases (SVMPs) and serine proteinases (SVSPs) act as procoagulants and/or anticoagulants. The procoagulant pharmacological properties of SVMPs offer the potential for developing new drugs to treat various bleeding disorders (18). In this study, a procoagulant protein in Caucasus viper venom named Met from Zanjan Province was isolated, characterized, and expressed in recombinant form.

The SDS-PAGE analysis showed that the apparent molecular mass of viper venom proteins ranged from 10 to 180 kDa. Firstly,

Kornalik *et al*. introduced ecarin in *Echis carinatus* venom as a prothrombin activator in 1975 (19). Ecarin activates prothrombin without cofactors, converting prothrombin II to thrombin or prothrombin to meziothrombin, which subsequently activates thrombin (20). Similar to Kornalik's findings, the current study observed that the further purification of Zanjani viper venom increased its coagulant effect.

Williams *et al*. reported that snake venom comprises proteins with diverse molecular weights and post-translational modifications, suggesting that multiple genes are involved in venom synthesis (21). Venom proteins are encoded by genes classified into over 10 gene families (22). Taherian *et al*. evaluated the venom of the Latifi viper using SDS-PAGE to isolate strong protein bands, which were then examined by the partial thromboplastin time test to assess the intrinsic coagulant system. They found that a 26 kDa band could coagulate plasma in less than one second, identifying it as a serine protease through MS analysis (23).

Several metalloproteinases have been isolated and characterized from the venom of various species, including *Gloydius halys* (24), *Cerberus rynchops* (25), *Crotalus adamanteus* (17), *Buthus martensi* (26), and *Cryptelytrops albolabris* (27). The metalloproteinase identified in the present research was structurally similar to the active site of the regulatory subunit of the blood coagulation factor X- and IX-activating enzyme (Q4PRD1) and other snake metalloproteinases in the P-I classes (28). It reduced PT to 3 s compared to the normal 13 s. Commercial Ecarin is a highly purified Met isolated from the venom of the saw-scaled viper *Echis carinatus* and a specific activator of prothrombin commercially. In comparison with our recombinant Met protein PT, Ecarin PT or echis clotting time (ECT) is been reported at 22.6 - 29 s (29).

Mammalian cell lines, particularly CHO cells, are widely used for the industrial production of therapeutic proteins due to their ability to correctly fold and assemble proteins and perform complex post-translational modifications (30-32). Currently, one-third of FDA-approved drugs are derived from natural products and their derivatives. Discovering novel specialized compounds in unexplored organisms is a promising approach for developing new drugs (33,34).

Overall, ongoing research across various countries aims to identify the best and most accessible coagulation factors from natural sources like animal venoms. Each study presents unique challenges and complexities, making direct comparison of laboratory results difficult. Ultimately, the efficacy of these factors must be evaluated under *in vivo* conditions to determine their potential therapeutic applications.

CONCLUSION

In this study, we confirmed that the Met isolated from *M. raddei* venom, with a molecular mass of approximately 25 kDa, exhibited up to 90% coverage of the coagulation factor X-activating enzyme light chain 1 active domain. The Met protein structure possessed a binding domain similar to that of the metalloproteinases from *E. carinatus* and *E. pyramidum leakeyi*, with approximately 60 amino acid fragments. The thrombin time parameter for the produced Met protein was 3 s, suggesting its potential as a candidate for the development of an efficient drug for treating coagulation disorders.

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Conflicts of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

Z. Jafari and M. Bandehpour designed and performed experiments, and co-wrote the paper; B. Sedaghati performed bioinformatics analyses; M. Bandehpour and B. Kazemi supervised the research; M. Kaboli contributed to the editing of the paper. All authors read and approved the final manuscript version.

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