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Original Article

Partial Proteomics Analysis of *Montivipera raddei* Venom

Zohreh Jafari[a], Mojgan Bandehpour[a, b]*, Mohammad Kaboli[c], Bahram Kazemi[b]

[a] *Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[b] *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[c] *Department of Environmental Science, Faculty of Natural Resources, University of Tehran, Tehran, Iran.*

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HIGHLIGHTS

- Identification of several proteins from *Montivipera raddei* venom.
- Characterization of the six high concentrated proteins by MALDI-TOF/TOF spectroscopy in venom.
- Zinc metalloproteinase-disintegrin-like ecarin, L-amino-acid oxidase, metalloproteinase kistomin, Thiamine-monophosphate kinase, Ancrod, Acidic phospholipase A2 were identified in venom of *Montivipera raddei*.

ABSTRACT

The snake venom is a potent source of a variety of drugs and therapeutic components. This study aimed to isolate and characterize some of proteins in *Montivipera raddei* venom. The protein bands and spots obtained by SDS-PAGE and two-dimensional electrophoresis were analyzed. The separated protein spots based on isoelectric point and molecular weight were scattered in the 15 to 66 kDa ranges and pI from 5 to 8. Six proteins was more extensively characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF) analysis. These characterized proteins included Zinc metalloproteinase-disintegrin-like ecarin, L-amino-acid oxidase, metalloproteinase kistomin, Thiamine-monophosphate kinase, Ancrod, Acidic phospholipase A2.

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Introduction

Snake venom is a mixture of proteins and its various components can be classified according to their mechanism of action, for example, serine proteases, metalloproteinases, Kunitz-type protease inhibitors, phospholipases A2, L-amino acid oxidases, C-type lectin(-like) proteins (CLP), disintegrins, vascular endothelial growth factors, three-finger toxins, and cysteine-rich secretory proteins (Tasoulis et al., 2017; Munawar et al., 2018). Snake venoms typically consist of various pharmacologically active ingredients with important medical applications. *Montivipera raddei* is a mountain viper that its venom composes a mixture of proteins and other components that affect the blood coagulation cascade. Some of these proteins, called pro-

Zohreh Jafari : https://orcid.org/0000-0002-1532-576X
Mojgan Bandehpour : https://orcid.org/0000-0002-5479-2846

*Corresponding Author:
Email: m.Bandehpour@sbmu.ac.ir (M. Bandehpour)

coagulation factors, that promote coagulation while others named the anticoagulant factor stops the coagulation process (Kini, 2005). Snake venom metalloproteinases (SVMPs) are major components of viper venoms and affect the hemostatic system through coagulation and anticoagulant activity (Tasoulis et al., 2017).

Serine proteinases present in snake venom are also divided into two groups, serine proteases that need only Ca^{2+} ions and phospholipids, such as Oscutarin, and serine proteases that stimulated by the addition phospholipids, Ca^{2+} ions and FVa, such as hopsarin D, trocarin D, notenarin D and Notecarin (Rao et al, 2005). Numerous studies on venom proteomics profiles of snakes have been performed using two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) techniques (Fox et al, 2008; Chen et al, 2013; Vejayan et al, 2014). Due to the limitations of the separation techniques applied for different types of proteins (hydrophobic, acidic, alkaline and membrane proteins), the MALDI-TOF mass spectrometry technique is used as a potent approach to study the proteomic profile of biological samples after gel electrophoresis (Bunai et al, 2005). Proteome analysis with this method has performed on more than 55 snake species venom (Serrano et al, 2005).

The proteome analysis of *Montivipera raddei* venom has not been reported for the species lived in Iran. Since the *Montivipera raddei* venom contains blood-clotting substances and other therapeutic components that can be used in drug designing. Therefore, this study was designed to identify the high concentrated proteins of *Montivipera raddei* venom, which may be related to its oharmacologial activities. The venom proteins was analyzed using SDS-PAGE, 2D-electrophoresis, and MALDI-TOF mass spectroscopy.

Materials and Methods

Venom samples and chemicals

The venoms of *Montivipera raddei* resident in the laboratory of live animals of the College of Agriculture and Natural Resources at Tehran University were obtained based on the National Institutes of Health guide for the care and use of laboratory animals (NIH). The venoms stored at -80 °C at the laboratory of the Cellular and Molecular Biology Research Center at the Shahid Beheshti University of Medical Sciences. Chemicals for electrophoresis, including acrylamide, bisacrylamide, and Tris-HCl (pH 8.8 and 6.8) were purchased from Merck Co., Germany. Sodium dodecyl sulfate (SDS), Ammonium persulfate (APS), Tetramethylethylenediamine (TEMED), Dithiothreitol (DTT), and iodoacetamide (IAM) were purchased from Sigma Aldrich, UK.

Snake venom proteomics process

The venom protein concentration was measured by Bradford technique (Bradford et al, 1976), using bovine serum albumin (BSA) as standard. In brief, an aqueous dilution of venom and a serial dilution of BSA (0-100 µg/mL) were prepared, then, 20 µL of each samples was mixed with 1 mL of Bradford reagents (Takara, Japan), after 15 min incubation at room temperature, the absorption of samples was measured at 595 nm. Venom concentration was calculated based on the BSA standard calibration curve.

One-dimensional gel electrophoresis (SDS-PAGE)

A serial dilutions of pooled venom sample was prepared and their concentrations were measured by Bradford method. Then, 1 to 20 diluted venom (equal to 2.5 µg) was separated on gradient SDS-PAGE (5-12%) (polyacrylamide gradient gels electrophoresis) at 70 mA, 100 V for 3 h.

Two-dimensional gel electrophoresis (2DE)

For total protein separation, 50 µg of diluted pooled venom was loaded on the IPG strips (pH 3-10, 17 cm, Bio-Rad), which was rehydrated passively for 1 h. The voltage settings for isoelectric focusing (IEF) was 14000 Vh. After IEF and equilibration, the second dimension SDS-PAGE gel of 12% run using constant voltage (100 V) for 1.5 h. The gels were visualized by colloidal Coomassie brilliant blue staining. Then, 20 of the sharpest spots were taken out from the gel and homogenized using 300µL sterilized PBS and protein purification performed after 15 min using a Pierce SDS-PAGE Sample Prep Kit (Thermo Scientific, USA). Protein concentration measured by Bradford technique, using bovine serum albumin (BSA) as standard.

Protein identification by MALDI-TOF/TOF mass spectroscopy and Mascot analysis

MS-MALDI-TOF/TOF spectroscopy of purified proteins spots were performed at Proteomics International Pty Ltd Company, Australia. Results were analyzed using Mascot sequence matching software which searches the sequence databases and identifies the best matches. Then the peptide sequences are compared and characterized with the most similarity by the Mascot server. It is a potent search engine which applies mass spectrometry data to discern proteins from DNA, RNA and protein sequence databases as well as spectral libraries (http://www.matrixscience.com/server.html).

Results and Discussion

In this study, several protein agents present in *Montivipera raddei* venom were isolated. The venom of the Viperidae family, including the *Montivipera raddei*, is a rich source of new compounds that have applications in medicine, pharmacy, and biochemistry. The protein content of venom in this study was calculated between 60 and 90% of total mass. Generally, snake venom protein content has been previously reported from 49 to 96% (Mackessy et al, 2006; Vejayan et al, 2010).

One-dimensional gel electrophoresis

SDS-PAGE pattern of prepared *Montivipera raddei* pooled venom proteins is shown in Fig. 1. Four condensed bands were observed in the areas 15 to 66 kDa. Comparison of SDS-PAGE and 2DE results showed that bands between 30 and 70 kDa were composed of several protein aggregates. Using the 2DE technique, these protein mixtures were separated based on isoelectric pH (pI) and molecular weight (MW) (Vejayan et al, 2010).

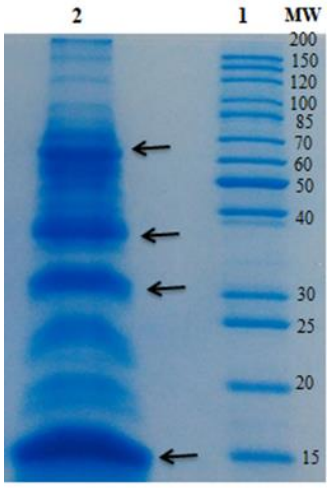

Figure 1. SDS-PAGE analysis of the proteins profile of *Montivipera raddei* venom. Whole venom proteins (50µg) were resolved on a linear gradient (12%) SDS gel and visualized by coomassie brilliant blue staining. Arrows show the most condensed bands. Lane 1) molecular mass markers (kDa); lane 2) *Montivipera raddei* venom sample. As seen, more than 15 bands were separated.

Two-dimensional gel electrophoresis

Fig. 2 shows a reference 2-DE map of *Montivipera raddei* venom, in which a lot of spots were detected. The pIs of protein spots ranged from 3 to 10; in which most of them had a pI < 8. The molecular masses of most proteins were between 14 kDa and 69 kDa. These results are in agreement with studies showing that enzymes of the Viperidae family venom have been observed at the neutral pI and in higher molecular mass ranges (Sakurai et al, 2003). In this study, the 2-DE gel displayed vertical bands that hindered the identification of some proteins. Vertical bands were also observed in other studies (Vejayan et al, 2014).

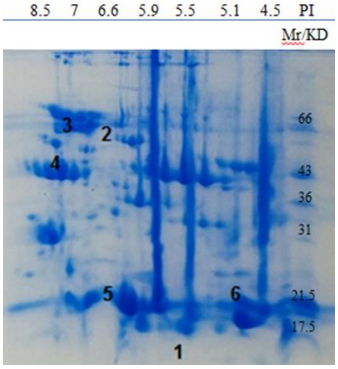

Figure 2. Image of two-dimensional gel electrophoresis of *Montivipera raddei* venom. 50 µg/µL venom subjected to IEF (pH 3-10) and 12% SDS-PAGE, followed by Coomassie staining. Spots indicated by numbers 1 to 6, are those were extracted and more analyzed by MOLDI-TOF Mass Spectroscopy. Related data are reported in Table 1.

Mass spectrometry and database search

To identify proteins derived from 2D-PAGE, six spots were manually picked from Coomassie blue-stained gels and analyzed by Mass Spectroscopy. These spots were sent to Proteomics International Pty Ltd Company for analysis using the MOLDI-TOF method. Table 1 shows the results of the MS analysis that has been sorted by molecular weight amounts. These results indicate that spot 3 with a molecular weight of 66000 Da belongs to Zinc metalloproteinase-disintegrin-like ecarin and the lowest molecular weight of 15000 Da belongs to Acidic phospholipase A2.

Table 1. Identified proteins by MOLDI-TOF Mass Spectroscopy in *Montivipera raddei* venom, sorted based on molecular weight.

Spot number	Identified protein	Accession number	MW(kDa)	PI
1	Acidic phospholipase A2	Q9DF33	15	5.5
6	Ancrod	P26324	26	5
5	Thiamine-monophosphate kinase	D6V9W7	33	6.5
4	metalloproteinase kistomin	P0CB14	47	8
2	L-amino-acid oxidase	P81382	58	6.5
3	Zinc metalloproteinase-disintegrin-like ecarin	Q90495	66	7

Some proteins that showed higher protein concentrations, identified in MALDI-TOF mass spectrometry, which may induce the hemotoxic properties of the venom snake (Mackessy et al, 2006). The isolated and identified proteins present different biochemical effects and each one can applied as a natural drug. L-amino-acid oxidases (LAAO) derived from *Montivipera raddei* venom can have an anticoagulant effect by affecting coagulation factors. Studies have shown that LAAO exerts its anticoagulant effect by reducing the factor IX effect (Sakurai et al, 2003). Ancrod is an anti-fibrinogen drug and is used in the treatment of many diseases, such as stroke, thrombosis, sickle cell disease (Qin et al, 2013) and induces fibrinolytic responses by activating plasminogen (Dempfle et al, 2001). Phospholipase A2 (PLA2) is a multifunctional enzyme and its anticoagulant, inflammatory, myotoxic, neurotoxic and enzymatic functions have been reported frequently (Harris et al, 2013; Casais-e-Silva et al, 2016; Costa et al, 2017).

Thiamine pyrophosphate (TPP) is the active form of thiamin (B1 vitamin) and is a cofactor of enzymes such as pyruvate dehydrogenase, transketolase and alpha-ketoglutarate dehydrogenase that are required for ATP synthesis. Thiamine monophosphate kinase (ThiL) performs ATP-dependent phosphorylation of thiamine monophosphate to convert to thiamine pyrophosphate (Settembre et al, 2003).

Kistomin, such as ecarin, is a zinc metalloproteinase that belongs to the SVMP family, but unlike the ecarin, it cleaves the glycoproteinVI membrane receptor (GPIb) and inhibits platelet aggregation (Slagboom et al, 2020). Ecarin is a glycoprotein with molecular mass range of around 55-86 kDa, due to post-translational modifications (Nishida et al, 1995). It acts as a hirudin inhibitor in the blood. Ecarin can create thrombin from anomalous de-carboxy thrombin without the need for calcium ions or cofactors and causes platelet aggregation and blood coagulation (Schieck et al, 1972). Therefore it widely used to detect low serum prothrombin levels, to study coagulation disorders, in patients with vitamin K deficiency or patients with hepatic diseases (Rosing 1992) and to confirm the presence of prothrombotic antibody in patients blood (Moore, 2007; Kini et al, 2016).

Conclusion

In this study, *Montivipera raddei* venom was evaluated due to its valuable components with pharmaceutical applications. Different proteins including zinc metalloproteinase-disintegrin-like ecarin, L-amino-acid oxidase, metalloproteinase kistomin, thiamine-monophosphate kinase, Ancrod, Acidic phospholipase A2 were identified in this venom through 2DE analysis, which were in the range of 15-66 kDa molecular weight and pIs of 5 to 8. Some of the main bioactivities reported for *Montivipera raddei* venom may related to these dominant proteins.

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Competing Interests

The authors have declared that they have no competing interests.

Ethical Statement

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. 1401.860].

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Authors Contribution

Zohreh Jafari, Mojgan Bandehpour, and Bahram Kazemi designed and performed experiments and bioinformatics analyses. Mohammad Kaboli provided the venom. Mojgan Bandehpour and Bahram Kazemi supervised the research. All authors wrote, read and approved the final manuscript.

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