Kütahya Dumlupınar University
Institute of Graduate Studies

Journal of Scientific
Reports-A
E-ISSN: 2687-6167
Number 54, September 2023

RESEARCH ARTICLE

EFFECT OF TEMPERATURE ON THE PROTEIN PROFILE OF *MACROVIPERA LEBETINUS* (BLUNT-NOSED VIPER) VENOM: A PRELIMINARY STUDY

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Receive Date: 06.07.2023 *Accepted Date: 26.09.2023*

ABSTRACT

Snake venom is a complex biological fluid consisting mainly of proteins and peptides possessing diverse biological activities. Snake venoms draw attention due to their bioactive proteins/peptides with therapeutic and diagnostic potential. Testing the stability of snake venom proteins under different conditions including temperature provides useful data for venom research. *Macrovipera lebetinus*, blunt-nosed viper, is the biggest viper species of Türkiye distributed mainly in eastern and southeastern Anatolia. Although its venom components were investigated before, there is limited data regarding the effect of temperature on its venom proteins. The present study aimed to investigate the effect of temperature on the venom proteins of *M. lebetinus*. For this purpose, venom samples were incubated at 25, 37, and 50°C. Thereafter, venom proteins were separated by two-dimensional gel electrophoresis (2D-PAGE) method. Some qualitative and quantitative differences in the protein profile indicating structural changes and degradation were observed especially after 50°C treatment. It has been found that the protein spots most affected by temperature will most likely contain metalloproteinase, phospholipase A_2 and L-amino acid oxidase enzymes, by comparing the experimental molecular weight and pI values with those in the literature. Detailed studies including enzyme activities and toxicity assays will provide more data on the stability of *M. lebetinus* venom under different conditions.

Keywords: *2D-PAGE, Macrovipera lebetinus obtusa, Phospholipase A_2, Protease, Snake venom.*

1. INTRODUCTION

Snake venom is a proteinaceous secretion of the cells in the venom gland. Viperid venoms, rich in proteases, interfere with the hemostatic system and cause tissue damage [1]. Snakebite is one of the important health problems especially in Africa and Asia [2]. But snake venom is also a source for the discovery of new bioactive proteins and peptides that can be used for therapeutic and diagnostic purposes [3]. Proteins and peptides found in snake venoms can be grouped into major protein families, however, the composition of snake venom shows intra- and inter-specific variation at different degrees [4].

Macrovipera lebetinus (Linnaeus, 1758) (= *Macrovipera lebetina*, *Vipera lebetina*), blunt-nosed viper, is the biggest viper species distributed in Türkiye and Cyprus. The range of this medicinally important viper reaches to Kashmir region towards the east and Kazakhstan and Dagestan (southwest of Russia) to the north [5]. Its bite affects blood coagulation, and causes symptoms like hemorrhage, tissue damage, and swelling [6]. The following protein families have been identified in the venom of *M. lebetinus* up to date: snake venom metalloproteinase (SVMP), snake venom serine proteinase (SVSP), phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO), hyaluronidase, 5´-nucleotidase, phosphodiesterase (PDE), C-type lectin-like protein (CLP), cysteine-rich secretory protein (CRISP), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), disintegrin, Kunitz-type serine protease inhibitor, bradykinin-potentiating peptide (BPP), and natriuretic peptide [7,8]. These proteins are responsible for the biological activities of *M. lebetinus* venom such as cytotoxic, antimicrobial, antiaggregant, anticancer, anti/procoagulant, necrotic and hemorrhagic activities [9-12].

Effects of the temperature on the biological activities and proteomic profiles of some snake venoms were investigated before. Some of these studies reported the presence of the heat-resistant toxins (even after incubation at 100°C). However, activity losses were also reported in some venoms after heat treatment. These studies have indicated that changes in the activities and structures of snake venom proteins may vary between different protein classes and should be studied for each venom separately [13].

Snake venom is an important natural source for the discovery of peptides and proteins with therapeutic and diagnostic value [3]. Many proteins and peptides were purified and characterized from snake venoms up to date, including *M. lebetinus* venom [7]. Investigating the stability and activity of the venom proteins under different conditions (eg. temperature, pH) is important for venom research, biotechnological product development, antiserum production processes, and treatment of snakebite. In the present study, it was aimed to investigate the effect of temperature on the venom proteins of *M. lebetinus*, a medicinally important viper that causes snakebite cases resulting in hospital care [6,14], by using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

2. MATERIAL AND METHODS

2.1. Venom and Reagents

Pooled venom extracted from two adult *M. lebetinus obtusa* (Figure 1) collected from Diyarbakır and Şanlıurfa provinces (southeastern Türkiye) was lyophilized as described before [8,9]. Ethical permission was obtained from Ege University Animal Ethics Committee when the study was conducted (permission no. 2010-43). All the reagents were molecular biology grade and deionized water was obtained from the Milli-Q system (Millipore, Billerica, USA). Agarose was purchased from Sigma-Aldrich (St. Louis, USA), ampholyte and dithiothreitol (DTT) from Fluka (St. Gallen, Switzerland); 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and bovine serum albumin (BSA) were from Amresco (OH, USA), protein ladder was from Fermentas (Vilnius, Lithuania). All the other reagents (e.g., acrylamide/bis 30% solution, bromophenol blue, iodoacetamide, Bradford reagent, urea, tris, sodium dodecyl sulfate) were purchased from Bio-Rad (CA, USA).

Figure 1. In situ photograph of *Macrovipera lebetinus 355btuse* from Şanlıurfa province.

2.2. Treatments
After reconstitution of the venom sample by deionized water, the sample was then aliquoted into three tubes (100 µL) and incubated at 25, 37, and 50°C for one hour (one tube for each temperature). After the treatment, the samples were immediately mixed with 2D-PAGE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% ampholytes) and prepared for isoelectric focusing.

2.3. Determination of the Protein Concentration
The protein amount of the reconstituted venom was determined using Bradford's method modified for a 96-well microplate as described before [8,9]. BSA was used as a calibration standard. All the measurements were carried out in triplicate and the mean values were used for calculations.

2.4. 2-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)
After treatments, venom samples were immediately mixed with 2D-PAGE rehydration buffer to a total volume of 300 µL and pipetted into isoelectric focusing wells for active rehydration at 50 V constant voltage overnight. The 2D-PAGE protocol was applied as described in detail before [8]. Protein amount was determined using the reconstituted venom sample and the same volume (175 µg protein) of each treatment was loaded.

3. RESULTS

Venom proteins of *M. lebetinus* were separated between pH 4-8 isoelectric points and approximately 10-150 kDa molecular weights (Figure 2). Chains of spots were observed, possibly due to the presence of protein isoforms. The profiles of 25 and 37°C samples were similar in general. But some qualitative and quantitative differences were observed after incubation at 50°C (Figure 2). Intense protein spots at around 100-120 kDa and pI 5,8 pH disappeared, or the intensity decreased significantly in the 50°C sample, whereas four spots became visible at ~ 40 kDa and 6.3 pI. Spots at

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around 13-14 kDa and 4,4 pI were significantly changed in the 50°C sample with a decrease in the intensities of two spots while the intensities of two lower spots were increased (Figure 2C). The intensity of one spot below the 15 kDa marker and at pH 7 was also altered between samples.

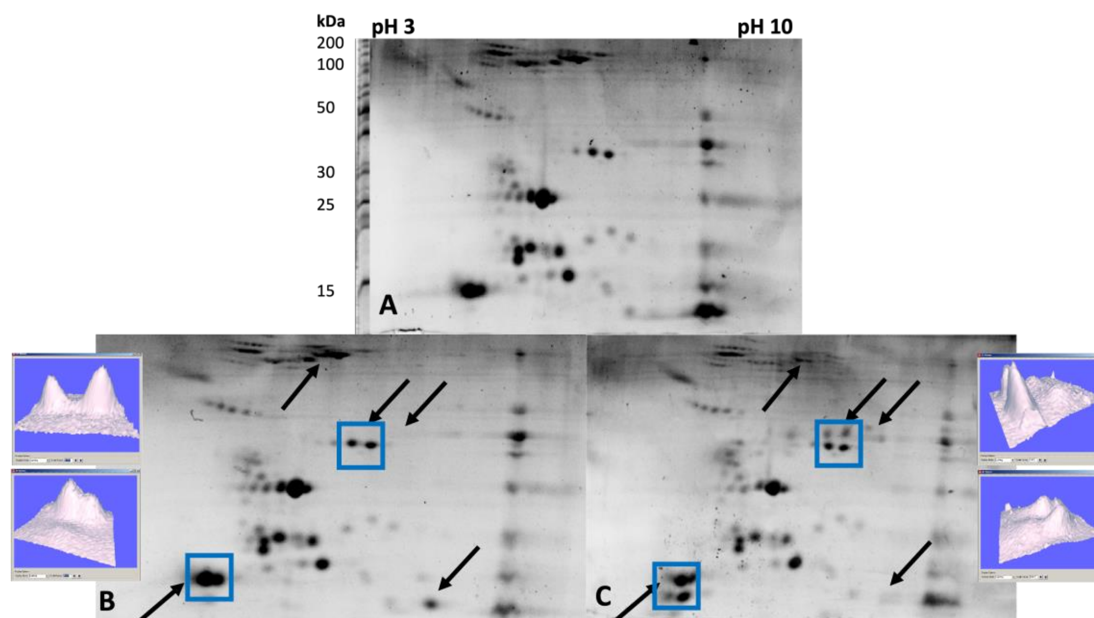

Figure 2. Protein profiles of *Macrovipera lebetinus* venom obtained by 2D-PAGE after incubation at 25 (A), 37 (B), and 50°C (C). Arrows indicate the prominent differences in protein spot patterns. Representative density graphics obtained from the gel analysis software PDQuest were demonstrated for two regions indicated with blue boxes.

4. DISCUSSION

The results of the present study showed that treating venom at 50°C temperature causes some changes in the protein structure, while 25 and 37°C temperatures did not differ significantly. As the main prey of blunt-nosed vipers are mammals and birds, a 25-37°C temperature can be considered within the optimal activity range of the venom [15]. Hence, bioactivity studies using snake venoms are generally carried out at these degrees. But of course, maximum activity temperatures of purified enzymes may change. For example, optimum working temperature can be high up to 75°C for PLA_2s in *Daboia russelli* [16], whereas it can be around 25°C for PLA_2s from *Vipera ammodytes* [17]. A dimeric metalloproteinase in the venom of *Cerastes vipera* was reported to have optimum of 60°C [18].

The effect of temperature on the overall activity and protein profiles of different snake venoms was also investigated by various researchers. The stability of hemorrhagic toxins in different viper species belonging to the genera *Agkistrodon*, *Bothrops*, *Crotalus*, and *Sistrurus* was investigated under

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different temperature conditions [19]. Even with incubation at 100°C for 5 min, *in vitro* proteolytic activity and *in vivo* hemorrhagic activity of some species were retained. However, activity loss was observed for some of the species tested. Heat treatment resulted in the denaturation of some proteins but most of the bands remained similar In many species, as assessed by SDS-PAGE. The effect of temperature and different storage conditions on the protein profile, enzyme activity and toxicity of *Crotalus molossus molossus* (a rattlesnake) crude venom was investigated and it was found that Incubating the venom sample at 37°C even for a week had little effect on the protein profile and activities while LAAO activity showed greater variation [13]. In another study, effect of the temperature on a new LAAO purifed from *Crotalus durissus collilineatus* venom was investigated and significant activity loss was observed after treatment at 37 and 100°C temperatures [20]. Changes in PLA_2 and protease activities were reported in historical venom samples of *Bothrops asper* and *B. atrox* that had been stored by dessication at room temperature, indicating the effects of the storage conditions on the venom bioactivity [21]. These previous reports show that some proteins are more prone to show variation in their activities dependent to the storage conditions such as temperature change while some of them are more stable.

Effect of the temperature on *Macrovipera lebetinus* venom was assessed by 2D-PAGE method in the present study. Venoms of the two subspecies of *M. lebetinus* (*M. l. obtusa* and *M. l. lebetina*) were compared by 2D-PAGE and protein identification from *M. l. obtusa* venom was achieved using MALDI-TOF mass spectrometry-based bottom-up proteomic analysis in a previous study [8]. Additionally, some other proteomic investigations were carried out on *M. lebetinus* venom from different subspecies using the venomics approach [22-24] and many of its major venom proteins were purified [7]. The aim of the present study was to investigate the effect of temperature on the venom proteome of *M. lebetinus*. Therefore, mass spectrometry-based protein identification was not performed again in this study, rather the previous studies were referenced to have a clue on the protein classes. SDS-PAGE was used commonly to compare protein profiles in studies investigating the effect of temperature on snake venoms [19,13]. But 2D-PAGE is a more powerful technique that separates more proteoforms allowing to make more detailed comparisons [25,26]. The protein profile of *M. lebetinus* venom as obtained by 2D-PAGE in the present study is consistent with a previous study by Igci and Demiralp [8]. Earlier studies have shown that the most abundant proteins classes found in *M. lebetinus* venom are SVMP, SVSP, PLA_2, CLP, LAAO, and disintegrin [8,22-24], which are responsible for Its pathogenesis and biological activities [9-12].

According to the results, one of the altered protein spot regions is around 100-120 kDa and 5.8 pI, in which the intensity of two spots was decreased significantly. Based on the previous studies, it can be concluded that SVMP, LAAO, PDE, and 5′-nucleotidase can be found between this molecular weight in *M. lebetinus* venom and SVMP is more abundant than the others [7,15]. SVMP and LAAO can be found at the same molecular weight and pI in *M. lebetinus* venom [27]. Therefore, it is difficult to separate these proteins by 2D-PAGE. Although reducing conditions were used for 2D-PAGE, subunits, monomeric forms, and/or native multimeric proteins that remained unreduced may present in these spots. Earlier studies have shown that high-molecular-weight snake venom proteins are more affected by heat than low-MW proteins and peptides [28-30]. LAAO isolated from *Macrovipera lebetinus* venom was also found to be stable from 4 to 25°C while heating at 70°C inactivated the enzyme in 15 min [27]. Hence, heat-resistant low-mw toxins/proteins, rather than high-mw ones were

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detected and identified in various viperid and elapid venoms [19,26-28]. This conclusion in the literature is also valid for *M. lebetinus* venom, as the results revealed that structural changes/degradation occurred especially in high-MW proteins.

Another group of altered spots was observed at ~ 13 kDa and 4.4 pI. Acidic PLA_2 type II enzymes with aggregation inhibiting and anti-cancer activities were purified from *M. lebetinus* venom, having molecular weights between 13-14 kDa and pI between 4,0-4,6 pH [31,32]. *M. lebetinus* venom also contains heterodimeric disintegrins with a similar molecular weight as PLA_2 enzymes have [7]. Although experimentally determined pI is not available in the literature, theoretical pI calculation (performed through UniProt) of a disintegrin lebein-2-alpha (UniProt: Q3BK13) gives the result as 7,5, which is not consistent with the experimentally observed pI value of the spot of interest. There is another spot that matches these values (~ 12 kDa, 7,8 pI) on 2D-PAGE gels. Moreover, PLA_2 was identified from the same 2D-PAGE spot in a previous study [8]. Although exact identification can be achieved using mass spectrometry or N-terminal sequencing, results of the earlier studies indicate that these protein spots in 2D-PAGE gel possibly contain PLA_2 enzymes. Decreasing PLA_2 activity was also reported for *Crotalus durissus terrificus* venom after heat treatment at 56°C [29]. In the aforementioned study, it was found that heating *Crotalus durissus terrificus* rattlesnake venom at 56, 70, and 100°C gradually decreased the toxic activity (LD_{50}) and PLA_2 activity, which indicated possible changes in protein structures. The results of the present study also indicated a structural change in PLA_2 of *M. lebetinus* venom. Four spots appeared after 50°C heat treatment may originate from the degradation of proteins with higher molecular weight, which was visible on 2D-PAGE images. Altered protein spots below 15 kDa and at pI 7 may contain other types of PLA_2 enzymes or disintegrin.

In conclusion, the present preliminary study showed that 50°C heat treatment led to the structural changes and degradation of some proteins in *M. lebetinus* venom, while most of the protein spots were not affected. Although similar studies were carried out with the venoms of different studies, there is limited data available in the literature about *M. lebetinus* crude venom in this regard. Venom proteins of *M. lebetinus* affected by heat are among the most abundant proteins based on the spot intensities on 2D-PAGE gel. These proteins are possibly PLA_2, SVMP, and LAAO, which play important roles in the pathology of *M. lebetinus* venom [7,9]. Moreover, these proteins have the potential for therapeutic and diagnostic use. For example, metalloproteinases purified from *M. lebetinus* venom interfere with the coagulation cascade by having factor X activating and fibrin(ogen)olytic activities [7]. Snake venom samples are routinely stored in the freezer (preferably –80°C) and freeze-dried. However, investigations on the thermal stability and storage conditions of venom proteins could provide useful data for venom research, antivenom production, and the evaluation of possible treatment methods for snakebites. The study should be extended to include higher temperatures, enzyme activities, *in vivo* and *in vitro* toxicity assays, and protein identification.

ACKNOWLEDGMENTS

I thank Prof. Dr. Bayram GÖÇMEN who passed away on 22 March 2019 and Prof. Dr. Mehmet Zülfü YILDIZ, for their help in obtaining the venom samples. I am grateful to Ankara University Biotechnology Institute for providing laboratory infrastructure.

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APPENDIX

EGE ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU

SAYI: 2010-043 25/02/2011
KONU: Onay

Etik kurulumuza yapmış olduğunuz başvuru doğrultusunda **"TÜRKİYE'DE YAYILIŞ GÖSTEREN BAZI ENGEREK ZEHİRLERİNİN MOLEKÜLER İÇERİKLERİ, SİTOTOKSİSİTELERİ VE KOAGÜLASYON ÜZERİNDEKİ ETKİLERİNİN ARAŞTIRILMASI VE BİYOTEKNOLOJİK KULLANIM POTANSİYELİNİN DEĞERLENDİRİLMESİ"** isimli araştırma projeniz değerlendirilmiştir.

Yürütücü: **Yrd. Doç. Dr. Fatma Duygu ÖZEL DEMİRALP, Ankara Üniversitesi Biyoteknoloji Enstitüsü**
Prof. Dr. Bayram GÖÇMEN, Ege Üniversitesi Fen Fakültesi
Doç. Dr. Ahmet Rüçhan AKAR, Ankara Üniversitesi Tıp Fakültesi
Op. Dr. Serkan DURDU, Ankara Üniversitesi Tıp Fakültesi
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Proje başvuru formunuzda belirtildiği koşullarda deney hayvanı kullanarak araştırmayı gerçekleştirmeniz kurulumuz tarafından uygun bulunmuştur. Saygılarımla bilgilerinizi rica ederim.

Prof. Dr. Rasih YILMAZ
(E.Ü. Hayvan Deneyleri Yerel Etik Kurulu Başkanı)

Prof. Dr. Süleyman AKKAN | Prof. Dr. Hüseyin TEZEL
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Prof. Dr. Gülcihan Mehtap KÖKSAL | Doç. Dr. Tansel TANRIKUL
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