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Enzymatic analysis of *Hemiscorpius lepturus* scorpion venom using zymography and venom-specific antivenin

Ramin Seyedian [a], Mohammad Hassan Pipelzadeh [b,*], Amir Jalali [c], Euikyung Kim [d,**], Hyunkyoung Lee [d], Changkeun Kang [d], Mijin Cha [d], Eun-tae Sohn [d], Eun-Sun Jung [d], Ali Hassan Rahmani [e], Abbas Zare Mirakabady [f]

[a] *Department of Pharmacology and Toxicology, Jundishapur University of Medical Sciences, Ahvaz; and Department of Pharmacology, Blusher University of Medical Sciences, Iran*
[b] *Department of Pharmacology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[c] *Department of Pharmacology and Toxicology, Jundishapur University of Medical Sciences, Ahvaz, Iran*
[d] *College of Veterinary Medicine, Gyeongsang National University, Jinju, 660-701, South Korea*
[e] *Poisoning Centre, Razi Hospital, Jundishapur University of Medical Sciences, Ahvaz, Iran*
[f] *Iranian Razi Institute of Poisonous Animals, Karaj, Iran*

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ABSTRACT

Hemiscorpius lepturus envenomation exhibits various pathological changes in the affected tissues, including skin, blood cells, cardiovascular and central nervous systems. The enzymatic activity and protein component of the venom have not been described previously. In the present study, the electrophoretic profile of *H. lepturus* venom was determined by SDS-PAGE (12 and 15%), resulting in major protein bands at 3.5–5, 30–35 and 50–60 kDa. The enzymatic activities of the venom was, for the first time, investigated using various zymography techniques, which showed the gelatinolytic, caseinolytic, and hyaluronidase activities mainly at around 50–60 kDa, 30–40 kDa, and 40–50 kDa, respectively. Among these, the proteolytic activities was almost completely disappeared in the presence of a matrix metalloproteinase inhibitor, 1, 10-phenanthroline. Antigen-antibody interactions between the venom and its Iranian antivenin was observed by Western blotting, and it showed several antigenic proteins in the range of 30–160 kDa. This strong antigen–antibody reaction was also demonstrated through an enzyme-linked immunosorbent assay (ELISA). The gelatinase activity of the venom was suppressed by Razi institute polyvalent antivenin, suggesting the inhibitory effect of the antivenin against *H. lepturus* venom protease activities. Prudently, more extensive clinical studies are necessary for validation of its use in envenomed patients.

1. Introduction

Scorpion stings are a major public health problem in many tropical countries, including Iran, where scorpion sting mortality is largely attributable to *Hemiscorpius lepturus* (*H. lepturus*) (Prendini, 2000). Unlike other scorpions studied so far, the venom of *H. lepturus* is highly cytotoxic for its highest mortality rate among the scorpion-associated envenomations in Iran (Radmanesh, 1998; Pipelzadeh et al., 2006, 2007). The clinical manifestations observed from *H. lepturus* envenomation include hemolysis, dermonecrotic reactions, renal failure, cardiovascular disease and central nervous system disorders (Radmanesh, 1990; Jalali et al., 2010). Envenomed patients are usually treated under close monitoring with intramuscular

* Corresponding author. Tel.: +986114458849; fax: +986113738283.
** Corresponding author.
E-mail addresses: mhpipelzadeh@yahoo.com (M.H. Pipelzadeh), ekim@gnu.ac.kr (E. Kim).

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injection of pepsin digested Razi institute polyvalent antivenin raised against 6 common Iranian scorpions. Until now, some clinicians are still sceptical about the efficacy and usefulness of this polyvalent antivenin for the treatment of envenomed patients. The scope of our study was to determine the protein components of this venom, the polyvalent antivenin, and in addition to investigate its protease-like enzymatic activities using SDS-PAGE zymography and to assess the binding activity of polyvalent antivenin by Western blotting and ELISA method.

2. Materials and methods

2.1. Reagents

Gelatin from porcine skin; hyaluronic acid sodium salt from rooster comb; α casein from bovine milk; bovine serum albumin (BSA); calcium chloride; Tris base; alcian blue 8GX; 1,10-phenanthroline and sodium chloride were purchased from Sigma-Aldrich Co (St Louis CO, USA). Molecular weight size marker for electrophoresis was obtained from Invitrogen Co. (USA). All other reagents were analytical grade from commercial sources.

2.2. Venom and Iranian polyvalent antivenin preparation

H. lepturus venom and its polyvalent antivenin were purchased from Razi institute of Iran (Hessarak, Karaj). Briefly, *H. lepturus* scorpions were obtained from Khuzestan province in Iran and their raw venoms were collected by applying electrical shock (15 V) on their telsons. The collected venom was pooled, lyophilized and stored at −20 °C before use. For experiment, a sample of the lyophilized venom was reconstituted by the addition of phosphate buffered saline (PBS) or distilled water. The protein concentrations of the venom and the polvalent antivenin were determined by Bradford method (Bradford, 1976), with bovine serum albumin as a standard.

2.3. SDS-PAGE & Western blotting

The protein components of *H. lepturus* venom (15, 30 and 60 μg) and Iranian polyvalent antivenin (12.5, 25 and 50 μg) were analyzed using SDS-PAGE separately with 15 and 12% acrylamide gels under non-reducing conditions, respectively by the method of Laemmli (Laemmeli, 1970) and the gels were stained after electrophoresis by Coomassie Blue G-250 for identification of protein bands. Molecular mass markers were included in all runs. For immunoblotting, the proteins on the gel were transferred electrophoretically into a polyvinylidene difluoride membrane, and stained with Ponceau S to check transfer efficiency; the blot was then probed at 4 °C with Razi institute polyvalent antivenin overnight. The membrane was washed for 10 min (3×) with fresh TBST, and incubated for 60 min with a purified rabbit anti-horse secondary antibody, diluted (1:5000) in TBST containing 5% non-fat dry milk. After gentle washing with TBST, the blots were developed with substrate solution (PowerOpti-ECL Western blotting detection reagent) and exposed with blue light-sensitive film (Fujifilm Corporation, Tokyo, Japan).

2.4. Zymography assays

In order to study the enzymatic activities of the venom, SDS-PAGE (12%) was prepared and polymerized with either gelatin or casein (0.1%). Aliquots of venom (25 and 33 μg) were prepared with Tris–Glycine SDS sample buffer (2×) and left to stand for 10 min at room temperature. Electrophoresis was carried out using 15 mA. The gels were washed in Triton X-100 for 30 min to remove SDS and were incubated overnight at 37 °C in reaction buffer (Tris base: 1.2 g, Tris-HCl: 6.3 g, NaCl: 11.7 g, $CaCl_2$: 0.74 g dissolved in 1 liter of distilled water for gelatin zymography (Liota and Stetler-Stevenson, 1990), or Tris-HCl buffer (30 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$) for casein zymography (Choi et al., 2001). The gels were then stained for 30 min with 0.125% Coomassie blue G-250 dye, followed by destaining for 30 min in 7.5% acetic acid and 5% methanol. The clear zone of substrate indicated the presence of either gelatin or casein degrading activities, depending on the substrate used in the assay. 1, 10-phenanthroline, a metal chelating agent, was used for the negative control samples.

2.5. Hyaluronidase assay

Hyaluronidase activity of the venom was examined by the method of Barbaro et al. (2005) with a little modification. Briefly, SDS-PAGE gels were prepared with hyaluronic acid, which was incorporated into gels as a hyaluronidase substrate in 12% resolving gel at the final concentration of 340 μg/ml. Venom samples (33 μg), dissolved in Laemmli buffer under non-reducing conditions, were electrophoresed at 15 mA and 4 °C. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 min to remove SDS and incubated overnight at 37 °C in the incubation buffer (20 mM Tris, 0.5 mM calcium chloride, pH 7.4) for hyaluronidase reaction. The gels were then stained with alcian blue in 7% acetic acid (pH 2.5) for one hour and destained in 7% acetic acid with counter staining by Coomassie blue. To verify the presence of metalloproteases, the procedure was repeated in the presence of 1, 10-phenanthroline (3 mM, final concentration), a protease inhibitor, which was added to all the gel washing and incubation buffers. Clear areas in the gel indicate the regions of enzyme activity.

2.6. Development of ELISA for H. lepturus venom

Antigen-antibody interaction between *H. lepturus* venom and Razi institute polyvalent antivenin was assessed by an ELISA method developed in our laboratory. The venom samples were dissolved in 0.05 M carbonate bicarbonate buffer (pH 9.6) to a final volume 100 μl and at a 10 x concentration ranging from 1 ng/ml to 1 mg/ml and were applied as a coating antigen into the wells of microtiter plate, which was incubated at 4 °C for overnight. On the next morning, the dilute venom (coating antigen) was removed and the plate was washed three times with 0.4 M sodium acetate buffer (0.02 M Tris base, 0.4 M sodium acetate, 0.05% Tween 20, pH 7.3). The remaining protein-binding sites in the coated wells were blocked by the

incubation with 100 μl of blocking buffer (5% non-fatty skim milk in PBS) for 1 h at room temperature. The microplate was washed twice with 0.4 M sodium acetate salt buffer and incubated with 100 μl of peroxidase-conjugated primary antibody [Razi Institute polyvalent antivenin diluted (1:500) in TBST containing 5% non-fatty dry milk] for 2 h. After washing four times with 0.5 M sodium acetate salt buffer (0.02 M Tris base, 0.5 M sodium acetate, 0.1% Tween 20, pH 7.7), the microplate was incubated with 100 μl of 3′3′5′5′-tetramethylbenidine dihydrochloride solution for 10 min at room temperature. The reaction was stopped by adding stop solution (2 N H_2SO_4), and the optical density was measured at 450 nm by Biotek (PowerWave XS).

2.7. Neutralizing capacity of the polyclonal antivenin against H. lepturus venom gelatinase

Gelatin zymography was carried out to evaluate anti-gelatinase effects of escalating doses of our antivenin. For this, zymography was carried out as previously described. Briefly, porcine gelatin was dissolved in distilled water (0.12%, w/v) and used for the preparation of the running gel (12.5%). Four percent stacking gel was polymerized in the absence of substrate. In the experiment, venom (33 μg) alone and polyvalent antivenin (125 μg) alone were prepared as positive control and as negative control, respectively. For the treatment samples, equal amounts of venom (33 μg) were individually incubated for an hour at room temperature (Liota and Stetler-Stevenson, 1990) with the polyvalent antivenin at indicated concentrations (10, 50, and 100 μg). The samples were then mixed with Tris–Glycine SDS sample buffer (2×) and left for 1 h at room temperature after vortexing. Then, they were loaded and run on an electrophoresis at a constant current (15 mV) in ice cold temperature. Upon completion of electrophoresis, SDS of the gels was removed by washing gels with 2.5% Triton X-100 twice for 30 min. For the reaction of enzymatic activity of the venom, the gels were incubated overnight at 37 °C in developing buffer (Tris base: 1.2 g, Tris-HCl: 6.3 g, NaCl: 11.7 g, $CaCl_2$: 0.74 g, distilled water to 1 liter) (Liota and Stetler-Stevenson, 1990). The gels were stained with 0.125% Coomassie blue dye, and destained for 30 min in destaining solution (7.5% acetic acid, 5% methanol). Eventually, an observation of clear zones demonstrated the lysis of gelatin or collagen by the enzymatic activities of venom.

3. Results

3.1. SDS-PAGE analysis of H. lepturus venom and Razi institute polyclonal antivenin

The electrophoretic profiles, with 15% acrylamide gel, of *H. lepturus* venom showed at least 10 different protein components, which were widely distributed in the range of molecular mass between 3.5 kDa and 260 kDa (Fig. 1A). There were several major bands, being located at the approximate molecular weights of 4 kDa, 30 kDa and 50 kDa as well as many other small components around these regions. Additionally, Razi institute polyvalent antivenin was also examined by 12% SDS-PAGE for characterizing its electrophoretic patterns (Fig. 1B). From this, at least, 6 major bands could be observed at the molecular weight between 10 and 110 kDa. Among these the protein bands at 30 kDa as well as 10–15 kDa were the most predominant.

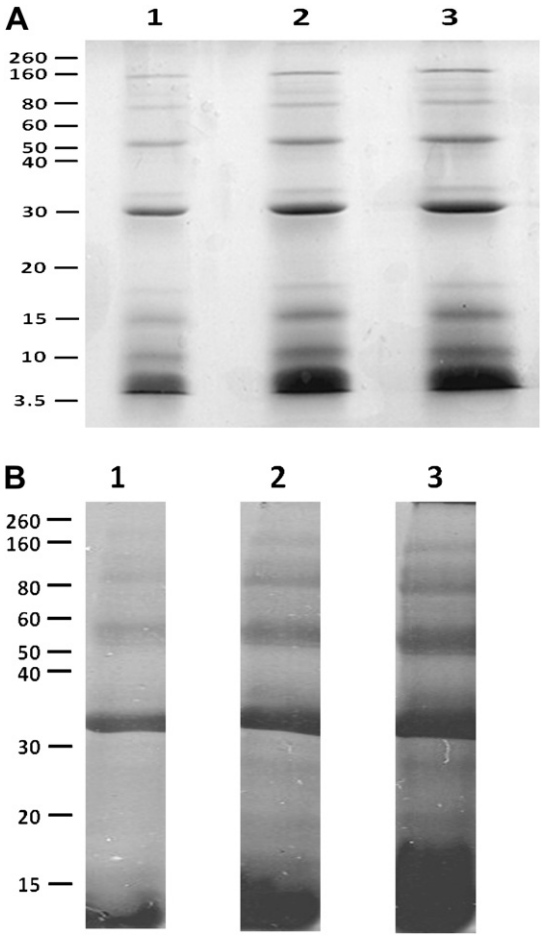

Fig. 1. Protein components of *H. lepturus* venom and Razi institute polyvalent antivenin. (A) The protein components of *H. lepturus* venom were separated by SDS-PAGE (15% acrylamide) and stained with Comassie blue dye. (lane 1: 15, lane 2: 30 and lane 3: 60 μg). (B) Razi institute polyvalent antivenin was run on a 12% SDS-PAGE and stained with Comassie blue dye. (lane 1: 10, lane 2: 25 and lane 3: 50 μg). Numbers on the left indicate the molecular weight of size markers.

3.2. Gelatinase, caseinase and hyaluronidase activities of H. lepturus venom

H. lepturus venom showed significant amounts of enzymatic activities against casein; gelatin, or hyaluronic acid by zympgraphy method. This venom presented a weak caseinolytic band around 30 kDa, (Fig. 2, lane 1) which appears to be a metalloproteinase, since its activity was abolished after the incubation of the gel in the metal chelator, 1, 10-phenanthroline. Gelatinolytic activity was similarly observed in this venom; with one weak band placed around 30 kDa, and a stronger band near 50 kDa

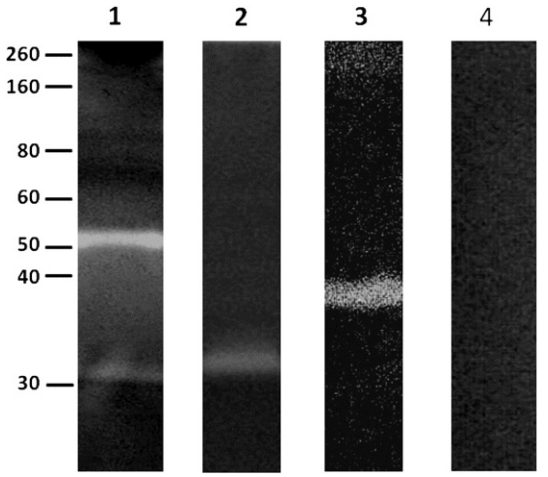

Fig. 2. Enzymatic activities of *H. lepturus* venom. Zymographies were performed for gelatinase activity (1), caseinase activity (2), and hyaluronidase activity (3). The venoms (lanes 1 and 3: 33 μg, lane 2: 25 μg) were run on 12% SDS-PAGEs containing respective substrate and analyzed by using zymography assays as described in Materials and Methods. For negative control, the venom was pre-incubated in the presence of 5 mM 1,10 phenantroline, a metalloprotease inhibitor (lane 4).

(Fig. 2, lane 2). Similarly, these gelatinolytic activities were almost completely disappeared after the incubation with 5 mM of 1, 10-phenanthroline, suggesting them to be metalloproteinases (Fig. 2, lane 4). In addition, hyaluronidase activity was detected at around 40 kDa (Fig. 2, lane 3).

3.3. Western blotting and the optimization of ELISA using Razi institute antivenin

Immunoblot analysis using this antivenin as a primary antibody revealed that there was strong antibody-antigen reactivity at apparent molecular weight masses of 30–35 kDa, and a few other antigenic proteins (50–60 kDa, and 60–260 kDa) (Fig. 3A and B). These results suggest that the antivenin binds to the venom proteins with high affinity, under *in vitro* conditions, thereby neutralizes the venom activity. The assay conditions were optimized in our laboratory for the ELISA experiment. Razi institute polyvalent antivenin was diluted in 5% non-fat dry milk in TBST and used as a primary antibody for ELISA. As shown in Fig. 3C, this Iranian antivenin shows a high cross reactivity against *H. lepturus* venom, indicating a strong interaction between the antigen (venom) and antibody (antivenin).

3.4. Antigelatinase activities of Razi institute antivenin

From the above results, it is presumed that the Iranian antivenin neutralizes *H. lepturus* venom activity. This was tested using gelatin zymography assay as previously described. The results showed that the gelatinolytic activity of *H. lepturus* scorpion venom was suppressed by the treatment with Razi institute polyvalent antivenin in a concentration-dependent manner (Fig. 3D).

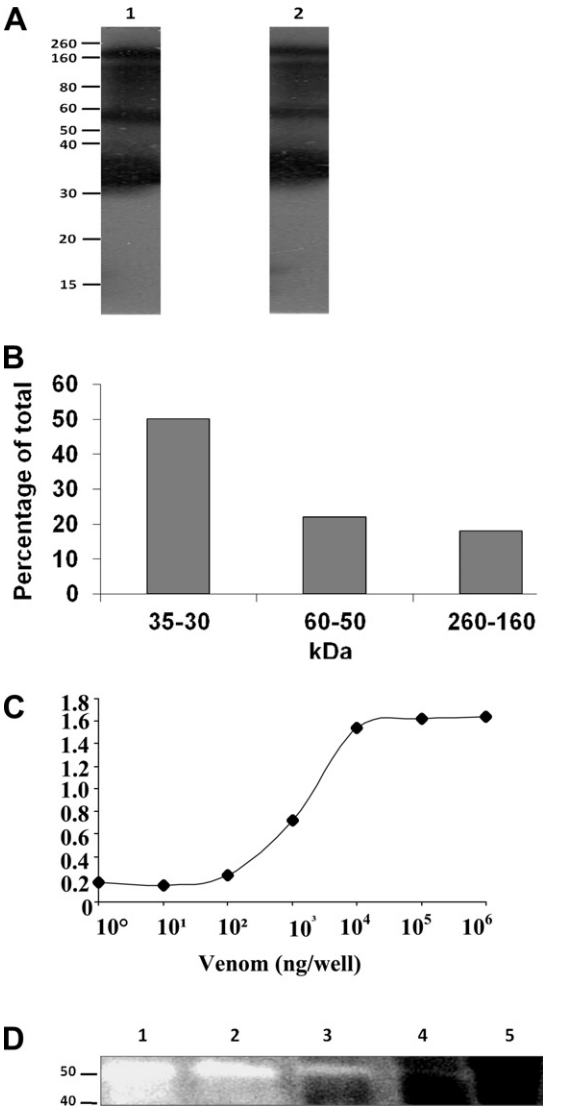

Fig. 3. Antigen-antibody reaction and its neutralizing effect of *H. lepturus* gelatinase activity. (A) Western blotting analysis of *H. lepturus* venom using Iranian antivenin. The crude venom samples (lane 1: 8 μg, lane 2: 6 μg) were immunoblotted using Razi institute multivalent antivenin as a primary antibody. (B) The signal intensities of the major immunoblotting bands for *H. lepturus* venom were analyzed using Image-J software and plotted graphically as % of total. (C) ELISA assay results for mixtures of varying concentrations of the venom in the presence of peroxidase-conjugated antivenin (1:500). (D) Neutralization of the venom gelatinase activity using the antivenin. (lane 1: Venom alone, 33 μg, lane 2: Venom, 33 μg + antivenin (AV), 10 μg, lane 3: Venom, 33 μg + AV, 50 μg, lane 4: Venom, 33 μg + AV, 100 μg, lane 5: AV alone, 125 mg).

4. Discussion

In the present work, the proteolytic enzymatic activities (gelatinase and caseinase) of *H. lepturus* venom were examined, for the first time, by SDS-PAGE zymogaphy. These activities disappeared or decreased significantly by the incubation of the gels in the presence of a MMP inhibitor, 1, 10-phenanthroline. The hyaluronidase activity of the venom was located at the approximate molecular

weight of 40 kDa. The hyaluronidase of this scorpion venom may affect the stability of blood vessel walls (Veiga et al., 2001), and increase the spreading of venom toxins. Systemic disturbances, such as renal failure, hemolysis and other clinical manifestations, in the envenomed patients by this scorpion may be attributable to the enzymatic components. Interestingly, these enzymatic entities are quite similar by their molecular weights to those of *Loxosceles desserta* (Barbaro et al., 2005). Hence, it is suspected that the proteases of *H. lepturus* venom may play an indirect role in the activation of complement system, which participates in dermonecrosis and hemolysis of the envenomed patients as observed in Loxocelism (Espino-Solis et al., 2009).

The common remedy of the patients envenomed by *H. lepturus* is the intramuscular injection of one 5 ml vial of the antivenin to neutralize the undesirable venom effects and other symptomatic treatments (Jalali et al., 2010). The protein components of Razi institute polyvalent antivenin were resolved by SDS-PAGE (12%) under non-reducing conditions. The protein bands were distributed in the wide range of approximate molecular masses from 5 kDa to 150 kDa. The Razi institute antivenin appeared to have some impurities; especially below 30 kDa (67% by Image-J software), that can be associated with some adverse allergic reactions upon the intravenous injection of the antivenin for the treatment of envenomed patients. From our J image analysis, approximately 18% of the antivenin appears to be composed of $F(ab')_2$ (results not shown) with molecular weight of around 96 kDa. These findings propose that more efforts must be carried out on the refinement and concentration processes for preparation of higher qualities of this antivenin.

An ELISA assay was developed using the venom and the antivenin in our laboratory, since it has many advantages, such as rapidity, sensitivity and low cost. Our results showed that Razi institute polyvalent antivenin has a high affinity to *H. lepturus* venom, suggesting that the antivenin has specificity for detection and inhibition of the enzymatic activities of this venom. However, in order to assess the significance of these results under *in vivo* conditions further studies as those recommended by WHO are needed. Furthermore, the sensitivity and the validity of this ELISA method for detecting *H. lepturus* venom in the sera of humans envenomed by the scorpion need to be assessed in future studies.

The antigen-antibody reactivity was also examined using Western blotting with *H. lepturus* venom and Razi institute polyvalent antivenin, respectively. From the study, at least four antigenic components were verified with the greatest intensity in 30–35 kDa (approximately 50% of the total intensity by Image-J software analysis). There is a great similarity in that the presence of 30 kDa bands between *H. lepturus* venom of the present study and *Loxosceles intermedia* venom, which has been previously observed by Western blotting (Felicori et al., 2006). This can be due to the presence of sphingomyelinase D (SMaseD) in the scorpion venom, as identified from the brown spider venom. SMases I and II can induce dermonecrotic reaction and are able to bind to erythrocyte surface that promote hemolysis (Ribeiro et al., 2007). Both of these clinical characteristics are well known symptoms in *H. lepturus* envenomation. The molecular mechanisms of *H. lepturus*–associated envenomation are currently under investigation in our laboratory for the skin injuries and other systemic disturbances.

In conclusion, these data suggest that the venom of this scorpion, whose envenomation are common in the warm months of the year in Iran, presents different biological, enzymatic and immunological characteristics from those of other scorpions and it is rather similar to brown spider venom.

Conflict of interest

None.

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