

Fractionation and enzymes activity measurement of the scorpion *Androctonus crassicauda* (Scorpionida: Buthidae) venom

Fatemeh Salabi^{1*}, Mohamad Nemati¹ and Mahsa Lari Baghal²

¹ Assistant Professor, Department of Venomous Animals and Anti-Venom Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Ahvaz, Iran

² Expert, Department of Venomous Animals and Anti-Venom Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Ahvaz, Iran

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Abstract

The scorpion of *Androctonus crassicauda* is one of the Buthida family members whose neurotoxic venom is deadly to humans. Enzymes are abundant components of animal venom that play roles in local and systemic symptoms following envenoming. The venom of this scorpions contains various pathological enzymes, among which studies have reported the toxic effects of venom's hyaluronidase and phospholipase A2 enzymes. This study aimed to measure the activity of venom enzymes of hyaluronidase and phospholipase A2 from scorpion of *A. crassicauda* and its fractions. For this purpose, the venom from scorpion of *A. crassicauda* were collected and lyophilized. The whole venom was fractionated on a gel filtration (Sephadex G-50) column. Then protein concentration, hyaluronidase and phospholipase A2 activity of the crude venom and its fractions were determined. Gel filtration on Sephadex G-50 and SDS-PAGE electrophoresis of *A. crassicauda* crude venom revealed four protein picks and 15 protein bands with the majority of molecular masses between 10.0 and 15.0 kDa, respectively. The supernatant obtained from crude venom clarification showed protein content 71.80%, high hyaluronidase (92.40%) and low PLA2 (302±2.1 mg/U) enzyme activity. Fraction 1 showed highest hyaluronidase and phospholipase A2 activities. We found that pH 5 is the optimum pH for hyaluronidase activity and phospholipase A2 activity increases with increasing incubation time. In conclusion, venom of *A. crassicauda* displayed the hyaluronidase and phospholipase A2 enzymes activity, which were separated by gel filtration chromatography in fraction I. Such information is important both for predicting the biological activity of venom components and useful for developing effective antivenoms based on these venom components.

Key words: *Androctonus crassicauda*, Hyaluronidase, Phospholipase A2, Gel filtration chromatography, SDS-PAGE chromatography

Introduction

With over 2,000 discovered species, scorpions are distributed in many terrestrial ecosystems (Cala-Riquelme and Colombo, 2011). Among these known species of scorpions, only a few threaten humans. However, scorpion stings reach about 50000 cases yearly (Firoozian et al, 2020)

and are considered a significant health problem in tropical and subtropical regions, including Iran (Dehgahni et al, 2010). Every year, many cases of scorpion stings and some cases of death due to scorpion stings have been reported from different parts of Iran (Shahi et al, 2019).

* **Corresponding Author:** Fatemeh Salabi, Assistant Professor, Department of Venomous Animals and Anti-Venom Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Ahvaz, Iran
E-mail: f.salabi@rvsri.ac.ir



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According to the latest available information, 64 species of scorpions have been identified in Iran, which are classified into 20 genera and three families: Buthidae, Scorpionidae and Hemiscorpidae (Firoozfar et al, 2019; Jafari et al, 2020; Salabi and Jafari, 2021). About 25 species in the family of Buthidae have venom deadly to humans, among which there are reports of death due to sting of the *Androctonus crassicauda* scorpion (Ruppert et al, 2004; Salabi and Jafari, 2022). This is one of the most venomous scorpions that is distributed in most areas of Iran, including Bushehr, Razavi and South Khorasan, Ardabil, Khuzestan and Sistan and Baluchistan provinces (Sharifinia et al, 2017). It is estimated that 35 to 40% of recorded cases of scorpion stings in Khuzestan province are related to *A. crassicauda* scorpion (Firoozian et al, 2020). The venom of this scorpion is neurotoxic and affects most vital organs of the human body (Radmanesh, 1990). Previous studies have shown that the venom of scorpions is composed of varying concentrations of toxic proteins including neurotoxin, cardiotoxin, nephrotoxin, hemolytic toxin, relatively low molecular weight molecules, and enzymes such as phosphodiesterases, phospholipases, hyaluronidases, and glycosaminoglycans (Senthilkumaran et al, 2015).

Venom of the Buthidae family exhibits significant hyaluronidase activity (Salabi and Jafari, 2023). Interestingly, among this family, the venom of *A. crassicauda* is a rich source of toxic compounds of which hyaluronidase and phospholipase A2 enzymes are important components (Salabi and Jafari, 2023; Morey et al, 2006; Díaz-García et al, 2015). Hyaluronidase enzyme is an acid-active enzyme and glycosidase belonging to the glycoside hydrolase family (Salabi and Jafari, 2023). Due to its catalytic role in the polymerization of larger molecules of hyaluronic acid (hyaluronan) to low molecular weight fragments of hyaluronan, this enzyme plays a role in

local and systemic symptoms such as severe and fatal anaphylactic reactions during envenoming (Kodkhodaei Eliadrani et al, 2006; Salabi and Jafari, 2023).

Most likely, hyaluronidases present in scorpion venom are not toxic by themselves, but they act as venom dispersal agents because they facilitate the diffusion of venom into the prey tissues by catalyzing the hydrolysis of glycosaminoglycans in the connective tissue, thereby causing systemic envenoming (Salabi and Jafari, 2023; Morey et al, 2006; Pessini et al, 2001; Tibballs et al, 2011). In tissue damage caused by scorpion stings, the abundance or biological activity of hyaluronidases increases; as a result, it leads to the accumulation of hyaluronic acid (hyaluronan), which in turn stimulates the immune system and creates an inflammatory response (Nevalainen et al, 2004).

In addition, scorpions from the family Buthidae show venom phospholipase A2 (PLA2) activity (Kodkhodaei Eliadrani et al, 2006). The PLA2 is a large family of secretory enzymes that catalyzes the hydrolysis of the ester bond of glycerophospholipids at the SN-2- position and causes the release of the corresponding fatty acid and the lysis of phospholipids (Nevalainen et al, 2004; Salabi and Jafari, 2024). The PLA2 of venom is toxic and possesses a wide spectrum of pharmacological effects including neurotoxicity, cardiotoxicity, systemic or local myotoxicity, platelet aggregation inhibition, anticoagulant and oedema inducing activities (Kodkhodaei Eliadrani et al, 2006). This enzyme via catalyzing the hydrolysis of phospholipids to produce lysophospholipids and free fatty acids, leads to cause mitochondrial membrane disruption in the respiratory muscle (Kodkhodaei Eliadrani et al, 2006). Irritation of the skin after Jellyfish envenomation has been reported to be due to high levels of this enzyme in the stings area (Tibballs et al, 2011). The

phospholipase activity of the venom of this marine species is responsible for toxic effects such as myotoxicity or neurotoxicity, as well as cell membrane instability and changes in cell permeability in the damaged tissue, and some inflammatory reactions such as pain and swelling, which are caused by the accumulation of active fatty acids (Nevalainen et al, 2004). Furthermore, direct hemolytic effect of phospholipase A2 enzyme from *Anuroctonus phaiodactylus* scorpion venom on the red blood cell (RBC) has been reported (Valdez-Cruz et al, 2004).

Although a few studies have been conducted on the activity of hyaluronidase enzymes (Salabi and Jafari, 2023; Morey et al, 2006; Díaz-García et al, 2015) and phospholipase enzymes (Kodkhodaei Eliadrani et al, 2006; Valdez-Cruz et al, 2004; DíazGarcía et al, 2015) in scorpion venom, no studies were found on the activity of these enzymes in *A. crassicauda* scorpion venom.

In this study, we attempted to separate the venom fractions of this scorpion venom by gel chromatography. The gel filtration chromatography is an approach commonly used particularly for gaining information about venom composition and comparing scorpion venoms (Khataminia et al, 2020; Shirmardi et al, 2010). The purpose of this study was to identify and measure the activity of phospholipase A2 and hyaluronidase enzymes in *A. crassicauda* scorpion crude venom and its extracted fractions.

Materials and Methods

Scorpions of *A. crassicauda* were collected from Baghmalek city of Khuzestan province (Figure 1) in summer and autumn 2023, and were kept alive until the time of venom milking in the arthropod laboratory, Southwest branch of Razi Vaccine and Serum Research Institute, Iran, Ahvaz. Scorpion venom milking was carried out using electroshock method (Salabi and Jafari, 2022; Salabi and Jafari,

2024). Then the venoms were lyophilized by freeze drying at -75°C . The dried venom was kept at -20°C until the time of the experiment.

The whole venom fractionation was performed using gel filtration chromatography, also known as, Size Exclusion Chromatography (SEC). The SEC separates the mixtures of molecules from venom based on their size. Separation of scorpion venom was performed using gel filtration medium of Sephadex G-50, with a column of 75cm in length, 4cm in inner diameter, 0.2 M of ammonium acetate as a mobile phase, and flow rate of 0.5 mL/min.

Briefly, 100 mg of lyophilized crude venom were dissolved in 2 ml of 0.2 molar ammonium acetate buffer. Then, the resulting solution was centrifuged at $15,000\times g$ for 20 minutes at 4°C , and the soluble part (supernatant) was separated and passed through a column of gel filtration medium of Sephadex G-50 in equilibrium with 0.2 molar ammonium acetate buffer (pH 8.5). The column was washed with the same buffer at a flow rate of 0.5 mL/min and the different fractions of protein elution were collected in separate tubes (3 ml per each) by an automatic collection device. The absorption of the samples was monitored using a UV detector at 280 nm (Nevalainen et al, 2004) and dried in a lyophilization method.

The lyophilized venom and its fractions were reconstituted on a day before testing by dissolving 1 mg of each in 1 mL of deionized water, activated overnight in 4°C , and centrifuged at $15,000\text{ g}$ for 20 min to precipitate the non-soluble materials. The supernatant obtained from crude venom and its fractions were clarified on testing day using $0.2\text{ }\mu\text{m}$ filters and stored at 4°C until use.

The concentration of protein in the clarified supernatant of crude venom and its fractions were determined by the colorimetric technique of Bradford protein assay with bovine serum albumin (BSA) at a concentration of 1 mg/mL as standard,

based on a six-point calibration curve constructed using 6 concentrations of BSA (Bradford, 1976). One calibration blank sample (buffer only) was used to calibrate the photometer, so it was set to "zero". Finally, Bradford reagent was added to all samples and, after 20 min, absorbance of samples was measured at 595nm. The standard curve was prepared in duplicate and the samples in triplicate.

Identification and characterization of venom hyaluronidase from *A. crassicauda* have previously been reported (Salabi and Jafari, 2023). Here, the activity of hyaluronidase enzyme was assayed based on the turbidimetric method using hyaluronic acid as substrate (Pukrittayakamee et al, 1988). First, 1 mg of crude venom and its fractions were dissolved in 1 mL of sodium acetate buffer (pH 6) containing 0.2 M sodium acetate and acetic acid and 0.15 M NaCl, shaken for 2 hours at room temperature, centrifuged at 15,000 g for 20 minutes at 4°C, clarified using 0.2 µm filters, and were stored at 4 °C until use. Then, 100 µl of clarified supernatant of samples with a concentration of 1 mg/ml was poured into a series of test tubes. The volume of each tube was adjusted to 400 µl with sodium acetate buffer. Then, 100 µl of hyaluronic acid (0.5 mg/ml in sodium acetate buffer) was added to all the tubes, the test tubes were shaken and incubated for 15 minutes at 37°C. The reaction was then stopped by adding 1 mL of cetyltrimethylammonium bromide (Sigma-Aldrich, H5882) (2.5% w/v). Two tubes: one called calibration blank tube containing only buffer to set the photometer to zero and one called standard tube containing buffer and hyaluronic acid were considered 100% turbidity. The absorbance of samples and blanks was measured in 10 min using a spectrophotometer (model: M501 Single Beam Scanning) at 400nm. The amount of hyaluronidase activity was calculated using the following formula as the percentage of hydrolyzed hyaluronate compared to the absorption of the standard

sample venom (buffer and hyaluronic acid only) as a percentage of turbidity.

$$100 - ((\text{Absorption of the sample} / \text{absorption of the standard})) \times 100$$

The pH optimum for the hyaluronidase activity was investigated by incubating hyaluronic acid in 0.2 M sodium acetate buffers at pH 4.0 to 6.0 and, 300 mM Tris-HCl buffer at pH 8.0 to 9.0 for 15 minutes in the presence of NaCl; then, hyaluronidase activity was assayed immediately (Amoozgari et al, 2016). The clarified supernatant of crude venom and its fractions prepared for measuring the activity of hyaluronidase enzyme were used to assay the relationship of hyaluronidase activity to pH. The pH of buffers was measured at room temperature (Test was repeated twice).

To prepare the venom and its fractions samples, lyophilized venom and its fractions were reconstituted on the day of testing by dissolving 1 mg of each in 1 mL of deionized water, shaken for 2 hours at room temperature, centrifuged at 15,000 g for 20 min, and clarified using 0.2 µm filters.

The method of Shanaki et al. (2009) was used to assay the activity of phospholipase A2 enzyme in crude venom and its extracted fractions. In this test, egg yolk was used as a source of lecithin. To prepare the egg yolk suspension, one egg yolk was dissolved in physiological serum (0.9% sodium chloride) with a final volume of 100 ml. Egg yolk suspension was diluted 10 times to prepare the working suspension. In a series of test tubes, 300 microliters of dissolved crude venom and fractions of venom (1 mg/ml) were added to 1 ml of egg yolk work suspension. Furthermore, a calibration blank sample (egg yolk suspension only) was used to set the photometer to zero. All samples were incubated at 37°C for 30 min. Then, 1 ml of 0.9% sodium chloride solution was added to each tube to stop the reaction. The absorbance of samples was measured at 925nm (Gorham et al, 1975). The difference

between the absorbance of the blank tube and the samples was considered as a value of phospholipase of the venom. Every 1% difference in absorption corresponds to one unit per mg.

Phospholipase (units per mg) = (absorption of the blank - absorption of the sample) $\times 1000$

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% polyacrylamide concentration as previously described by Dorce et al. (2018) to analyze the crude venom and its fractions from SEC. To prepare the samples, 5 mg of lyophilized crude venom and its fractions were dissolved in 100 μ l of distilled water, shaken for 2 hours and centrifuged at 15,000 g for 20 min. Then, the samples were mixed with an equal volume of sample buffer and applied (20 μ l) on a 12% separating polyacrylamide gel. The output voltage was 80 V for 5 hours. After the electrophoresis, the gel was removed from the device, stained with Coomassie blue and photographed. Protein molecular weight marker (Prestained Protein Ladder 10-250, SINACLON, Cat.No.SL7001) containing bands ranging from 5-245kDa was used for estimation of the molecular weight of the crude venom fractions.

The standard curves of the BSA and calculations were performed in Microsoft Excel 2013 software. According to the Bradford assay, a linear trend line was determined by the standard curve graph. The results of different experiments were expressed as mean \pm standard deviation with 3 repetitions. The $P \leq 0.05$ is considered statistically significant.

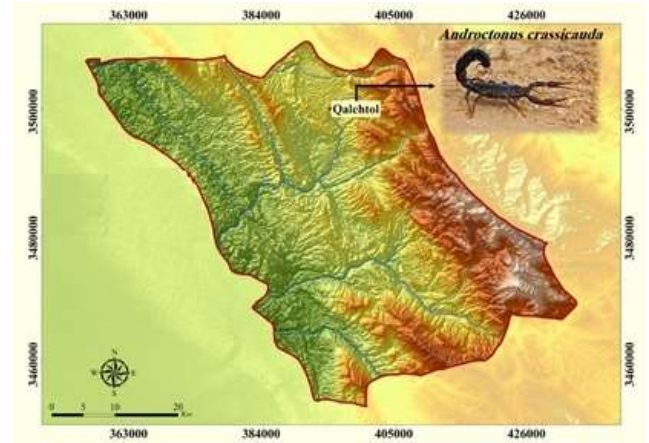


Figure 1: Location map of sampling point

Results

Gel filtration chromatography of venom

In SES technique the separation of the venom component of *A. crassicauda* was performed. In this study, the crude venom of *A. crassicauda* was separated into four fractions (F1-F4) by gel filtration chromatography on a Sephadex G-50 (Figure 2). After the chromatography, the extracted fractions were lyophilized and stored at -20°C until the assay.

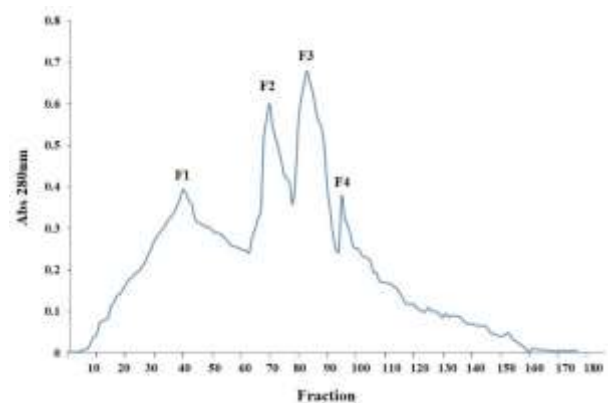


Figure 2: Output of gel chromatography of *A. crassicauda* venom on sephadex G50 size exclusion column

Determination of protein values

The amount of protein was measured in crude venom and four fractions obtained by gel filtration chromatography using the Bradford method. After creating the BSA standard curve, linear regression of BSA values was calculated (Figure 3). To determine the concentration of the unknown protein solution using the standard curve, instead of y, we put the optical absorption value of the protein samples. According to the standard curve diagram, the amounts of protein of crude venom and its fractions are presented in Table 1. As a result, the crude venom contains 71.80 % protein. According to the results, the highest amount of protein observed in peak 1 (90.13%). Among all

fractions obtained from SES, only FI showed a significant increase of protein value compared to crude venom and other fractions ($p < 0.05$) (Table 1).

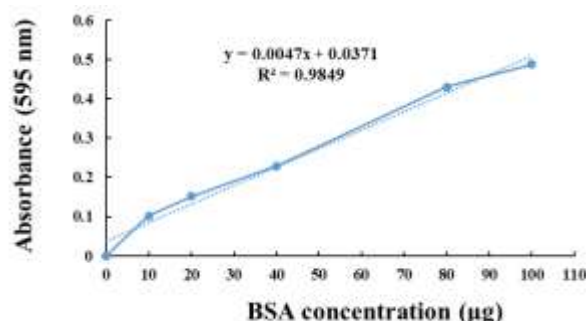


Figure 3: The standard curve obtained from measuring different concentrations of BSA standard solution

Table 1: Protein content, hyaluronidase and phospholipase A2 activity in crude venom and its fractions from *A. crassicauda*. Protein content of samples determined using Bradford assay, performed in triplicates in each test sample (n=3).

Samples	Protein content (%)	Hyaluronidase activity (%)	Mean phospholipase activity (mg/U) ± SD
Crude venom	71.80	92.40	302±2.1
FI	90.13	99.66	993±1.7
FII	26.40	10.68	198±2.4
FIII	15.62	0	144±3.6
FIV	24.19	17.69	285±2.9

Hyaluronidase activity

The hyaluronidase activity in crude venom and its fractions were assessed via turbidimetric assay (Table 1). Based on the results, fraction 1 showed an increase of hyaluronidase activity (99.66%) compared to crude venom (92.40%). Among all fractions, only FI showed a high absorbance. The remaining fractions had no absorbance (F3) or significantly low absorbance (FII and FIV) (Table 1).

Effect of pH on hyaluronidase activity

Due to the fact that the activity of the enzyme has its maximum value at the optimal pH, in this study, we investigated the optimal pH for the hyaluronidase

enzyme (Figure 4). The assay was performed at least in duplicate and the enzyme assays were averaged. By comparing the activity of hyaluronidase enzyme in crude venom and its first fraction at different pH conditions, it was found that the highest enzyme activity in both crude venom and first fraction was measured at pH 5 and the lowest level of activity was related to pH 8. At pH 4, the values were not recorded due to the destruction of hyaluronic acid. Our results showed that the optimum pH for hyaluronidase hydrolytic activity occurred at pH 5 (Figure 4). Similar to the results shown in figure 3, the enzyme activity in FI is higher than that of the crude venom.

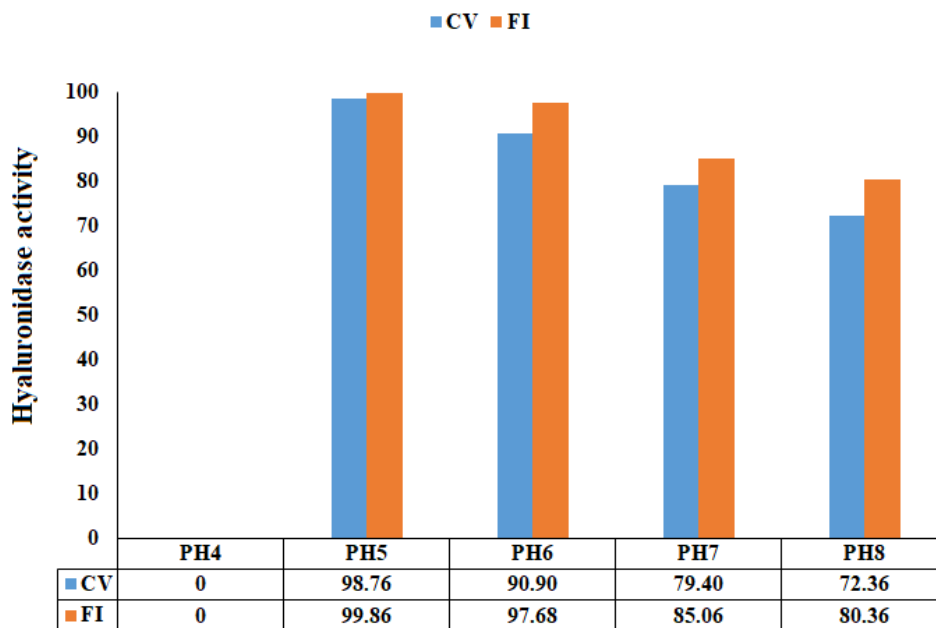


Figure 4: Optimum pH chart of venom hyaluronidase using hyaluronic acid as a substrate in the presence of NaCl and over a 15 min incubation period. CV; crude venom, FI; first fraction of venom

Phospholipase A2 activity

The activity of the phospholipase in *A. crassicauda* crude venom and its fractions was assessed using the egg yolk suspension. The results of activity of this enzyme in crude venom and different fractions are listed in table 1. As shown, the activity value of the crude venom was 302 ± 2.1 (mg/U). Based on the obtained results, the highest level of activity of this enzyme was observed in FI and the lowest level of activity was observed in FIII. According to all the results listed in table 1, the activity of hyaluronidase and phospholipase enzymes increase with the increase of protein value.

Here, the effect of incubation time on the PLA2 enzyme activity was also measured (Figure 5). The results showed that by increase of incubation time, the activity of phospholipase enzyme increased both in crude venom and its different fractions. By one hour incubation, the activity of this enzyme increased significantly ($P > 0.05$). Based on the results of table 1 and figure 5, similar to hyaluronidase activity, the highest phospholipase A2 activity was observed in FI.

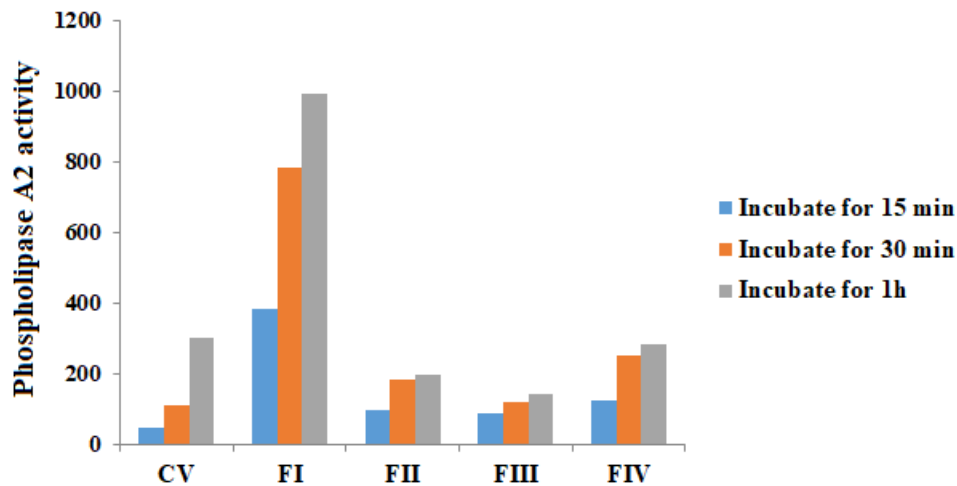


Figure 5: Effect of incubation time on the PLA2 activity of crude venom and its fractions. Over 1 h of observation; each point on the graph corresponds to the PLA2 enzyme activity rate measured in the 15 min, 30 min and 1 h of incubation, respectively

SDS-PAGE of *A. crassicauda* venom

The electrophoretic profile of *A. crassicauda* scorpion venom by SDS-PAGE is shown in figure 6. The SDS-PAGE chromatography densitometric analysis showed that the *A. crassicauda* crude venom presented a profile consisting of 15 bands with the majority of molecular masses between 10 and 17kDa, so that the observed bands below 17kDa presented higher concentration of proteins and more prominent among other bands (Figure 6). According to PLA2 and hyaluronidase activities results, only fraction 1 was used to SDS-PAGE electrophoresis. In FI, two bands were observed with a molecular weight of about 35-40kDa.

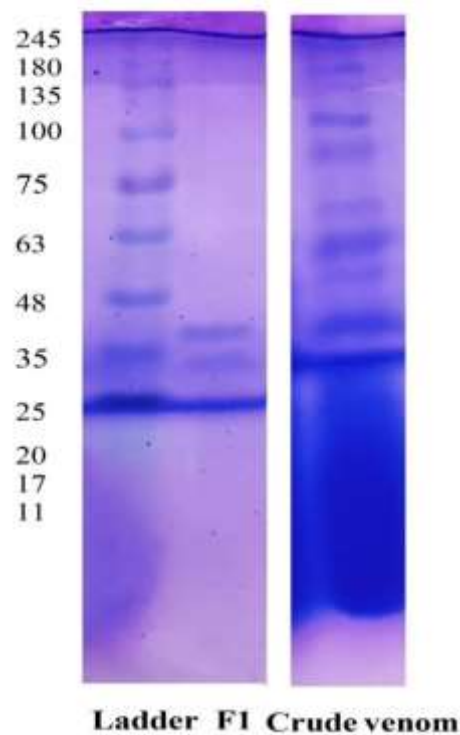


Figure 6: *A. crassicauda* venom separated by SDS-PAGE (12% T, 4% C, 1-mm thick) and stained in Coomassie blue. A colored protein ladder (10-250kDa) was used to monitoring protein separation during SDS-polyacrylamide gel electrophoresis.

Discussion

Scorpion venom enzymes have a broad range of toxicity and induce a wide spectrum of pharmacological effects that play a pivotal role in scorpion bite envenoming damage, among which hyaluronidase and phospholipases are outstanding (Nazari et al, 2023; Salabi and Jafari, 2023). The presence of hyaluronidase enzyme in the venom of some venomous animal had been previously reported (Amoozgari et al, 2016; Sutti et al, 2014; Topchiyeva et al, 2016; Morey et al, 2006; Díaz-García et al, 2015). The venom PLA2 is also an enzyme that belongs to a family of lipolytic enzyme esterase ubiquitously found in the venom of many venomous species, including snakes, bees, and scorpions, that contains a variety of secreted phospholipases A2 (Kodkhodaei Eliadrani et al, 2006). Studies have been conducted to identify, isolate and measure the activity of PLA2 and hyaluronidase in the venom of scorpions and other venomous species such as snakes and bees (Ketelhut et al., 2003; Nazari et al., 2023); however, the identification and measurement of the activity of these enzymes in the venom of *A. crassicauda* have not been performed so far. As the toxicity of PLA2 and hyaluronidase of scorpion venoms is a well-known subject, the aim of the present study is to demonstrate for the first time that the venom of the *A. crassicauda* also exhibited these enzymes activity in vitro. Analysis of *A. crassicauda* venom on gel filtration chromatography revealed similar results with Iranian *Mesobuthus Eupeus* venom reported previously, where four fractions (F1-F4) were collected through Sephadex G-50 gel chromatography (Shirmardi et al, 2010). In contrast, venom of *H. lepturus* was separated into six fractions using SES method (Khataminia et al, 2020).

In this study using the turbidity method, we assayed the hyaluronidase activity of crude venom and its fractions. The results clearly indicated that F1 showed more hyaluronidase activity than crude venom,

which was due to its lower quantities in crude venom compared to F1 (Based on a protein assay). Among the four fractions, fraction 3 did not show any hyaluronidase activity. This may be due to our experimental conditions or may be due to the absence of hyaluronidase enzyme in this molecular weight range related to this fraction. In general, the results indicated the presence of hyaluronidase enzyme in *A. crassicauda* venom. We also found that hyaluronidase from *A. crassicauda* venom presents optimal activity at pH 5.

There are some studies which identify a variety of hyaluronidase activities in venom of *Lebetina* snake (Amoozgari et al, 2016), *Vitalius dubius* spider (Sutti et al, 2014), *Apis mellifera L. caucasica* (Topchiyeva et al, 2016), *Mesobotus apius* scorpion (Kodkhodaei Eliadrani et al, 2006), *Palamneus gravimanus* scorpion (Morey et al, 2006), and *Rhopalurus junceus* scorpion (Díaz-García et al, 2015). Similar to our study, Díaz-García et al. (2015) investigated the activity of the hyaluronidase enzyme in scorpion *R. junceus* crude venom and fractions. They reported that the FI fraction (extracted by gel filtration chromatography) had hyaluronidase enzyme activity. Pessini et al. (2001) had suggested pH 6 as the optimal pH for maximum activity of the isolated hyaluronidase in *Tityus serrulatus* scorpion venom. Studies on the effect of pH upon the activity of venom hyaluronidase have shown that the pH optimum for the hydrolysis activity occurs at 5. 2 in the presence of, and at 6. 0 in the absence of NaCl (Gorham et al, 1975). In our previous bioinformatics work, different isoforms for hyaluronidase were identified in the venom of *Hemiscorpius lepturus* and *A. crassicauda*, and pH between 5.82 and 9.24 was predicted as the optimal PH. (Salabi and Jafari, 2023). The optimal PH of 5 to 8 was also reported for other venom hyaluronidase enzymes found in the snake and bee (Salabi and Jafari, 2023).

Previously, the presence of PLA2 activity was detected in scorpion venom of *Heterometrus fulvipes* (Ramanaiah et al, 1990), *Mesobotus apius* (Kodkhodaei Eliadrani et al, 2006) and *Anuroctonus phaiodactylus* (Valdez-Cruz et al, 2004). To prove the presence of PLA2 in *A. crassicauda* venom, the crude venom and its fractions were assayed for PLD2 activity through Shanaki et al. (2009) method. Here, we confirmed that the crude venom of *A. crassicauda* showed low enzymatic PLA2 activity while high enzymatic PLA2 activity was measured for FI. In contrast, Díaz-García et al. (2015) reported that the activity of PLA2 enzyme in *R. junceus* venom was not detected even at the highest concentration of venom used in the study (10mg/ml) and there was no hemolytic effect in red blood cells (2mg/ml) suggesting the lack of PLA2 enzymes. Kodkhodaei et al. (2006) studied the venom of the *Mesobotus apius* for phospholipase and hyaluronidase activities and demonstrated both enzymatic activity in crude venom and its first fraction. They also mentioned that it seems that the hyaluronidase enzyme is the only enzyme that is present in a significant amount in the venom of scorpions of the Butide family.

In this study, we report an electrophoretic profile of *A. crassicauda* crude venom that has many similarities with electrophoretic profiles of other scorpion venom species previously reported, in which the number of bands and the high content of low molecular weight proteins below 17kDa are described

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Conflict of Interest

The authors declare that they have no conflict of interest.

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(Díaz-García et al, 2015; Dorce et al, 2018). Here, the hyaluronidase activity was reported in the FI fraction (from gel filtration chromatography), which, according to the SDS-PAGE result, had a molecular weight of about 35-45 kDa. This range is within the range of previously reported hyaluronidase molecular weight; 44.8kDa for hyaluronidase from the Brazilian scorpion *Tityus stigmurus* (Batista et al, 2007) and 45kDa for hyaluronidase from the *R. junceus* venom (Rodríguez-Ravelo et al, 2013).

In conclusion, the present study used Size Exclusion Chromatography as a protein separation technique to separate venom fractions, in which SEC was well able to separate venom fractions. It is an easy-to-do and reliable strategy that can be used to separate different venom fractions. So, with this technique, the venom fractions can be evaluated *in vitro* and *in vivo* in addition to isolation. Furthermore, in this study, the activity of two important venom enzymes, hyaluronidase and PLA2, were evaluated by turbidimetric method. Our data indicated that *A. crassicauda* crude venom did contain hyaluronidase and PLA2 activities, and these features help to best understand the content and toxic potential of this species' venom. We found the optimum pH for hyaluronidase activity of *A. crassicauda* venom (pH 5). Finally, SDS-PAGE was performed to confirm the fractionation of crude venom and its fractions by SEC technique.

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جداسازی فراکسیون‌ها و اندازه‌گیری فعالیت آنزیم‌های زهر عقرب (Scorpionida: Buthidae) *Androctinus crassicauda*

فاطمه ثعلبی^{۱*}، محمد نعمتی^۱ و مهسا لاری‌بقال^۲

^۱ استادیار گروه جانوران سمی و تولید آنتی‌ونوم، موسسه تحقیقات واکسن و سرم‌سازی رازی، سازمان تحقیقات، آموزش و ترویج کشاورزی (AREEO)، اهواز، ایران
^۲ کارشناس گروه جانوران سمی و تولید آنتی‌ونوم، موسسه تحقیقات واکسن و سرم‌سازی رازی، سازمان تحقیقات، آموزش و ترویج کشاورزی (AREEO)، اهواز، ایران

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چکیده

عقرب *Androctinus crassicauda* یکی از اعضای خانواده بوتیده است که سم عصبی آن برای انسان کشنده است. آنزیم‌ها اجزای فراوان زهر حیوانی هستند که در ایجاد علائم موضعی و سیستمیک ناشی از مسمومیت نقش دارند. زهر این عقرب حاوی آنزیم‌های پاتولوژیک مختلفی است که از بین آن‌ها، مطالعات اثرات سمی آنزیم‌های هیالورونیداز و فسفولیپاز A2 زهر را گزارش کرده‌اند. این مطالعه با هدف اندازه‌گیری فعالیت آنزیم‌های هیالورونیداز و فسفولیپاز A2 زهر عقرب *A. crassicauda* و فراکسیون‌های آن انجام شد. برای این منظور، سم عقرب *A. crassicauda* جمع‌آوری و لیوفیلیز شد. اجزای کل زهر روی یک ستون فیلتراسیون ژل (Sephadex G-50) جدا شدند. سپس غلظت پروتئین، فعالیت هیالورونیداز و فسفولیپاز A2 زهر خام و فراکسیون آن تعیین شد. فیلتراسیون ژل روی Sephadex G-50 و الکتروفورز SDS-PAGE سم خام *A. crassicauda* به ترتیب چهار فرکشن پروتئینی و ۱۵ باند پروتئینی با اکثریت جرم‌های مولکولی بین ۱۰/۰ تا ۱۵/۰ کیلو دالتون را نشان دادند. مایع رویی به دست آمده از شفاف‌سازی زهر خام، محتوای پروتئین ۷۱/۸۰ درصد، فعالیت آنزیم هیالورونیداز بالا (۹۲/۴۰ درصد) و فعالیت آنزیم PLA2 پایین ($30.2 \pm 2/1$ میلی‌گرم در واحد) را نشان داد. فراکسیون ۱ بیشترین فعالیت هیالورونیداز و فسفولیپاز A2 را نشان داد. ما دریافتیم که pH 5، pH بهینه برای فعالیت آنزیم هیالورونیداز است و فعالیت آنزیم فسفولیپاز A2 با افزایش زمان انکوباسیون افزایش می‌یابد. در نتیجه، فعالیت آنزیم‌های هیالورونیداز و فسفولیپاز A2 در سم *A. crassicauda* یافت شد و کروماتوگرافی فیلتراسیون ژل این آنزیم‌ها را در فرکشن ۱ جدا کرد. چنین اطلاعاتی هم برای پیش‌بینی فعالیت بیولوژیکی اجزای زهر مهم است و هم برای توسعه پادزهرهای مؤثر مفید است.

کلمات کلیدی: *Androctinus crassicauda*، هیالورونیداز، فسفولیپاز A2، کروماتوگرافی ژل فیلتراسیون، کروماتوگرافی SDS-PAGE

* نویسنده مسئول: فاطمه ثعلبی، استادیار گروه جانوران سمی و تولید آنتی‌ونوم، موسسه تحقیقات واکسن و سرم‌سازی رازی، سازمان تحقیقات، آموزش و ترویج کشاورزی (AREEO)، اهواز، ایران

E-mail: f.salabi@rvsri.ac.ir



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