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VARIATION OF PROTEIN PROFILE AMONG CONSECUTIVE STINGS OF THE SCORPION *PARABUTHUS LEIOSOMA* (FAMILY: BUTHIDAE) FROM EGYPT, SUPPORTS THE VENOM-METERING HYPOTHESIS IN SCORPIONS.

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Abstract

Scorpions use their venom to immobilize prey items and defend themselves against predators. Scorpion venom is a complex mixture composed of a wide array of substances such as salts, small molecules, peptides, and proteins. During analysis of the prey capture behavior of the scorpion *Parabuthus leiosoma,* we noticed that the scorpion stings the prey several successive stings in different specific regions. The stings started with ventral surface of the abdomen followed by thorax, shoulders and finally the head. This finding raised the possibility that the scorpion might use the venom with different composition in different contexts. To check this possibility, venom was collected as sequential drops (stings) by electrical stimulation (20 Volt) of the telson of the *Parabuthus leiosoma*. Protein content was determined by spectrophotometer using total protein KIT. The results showed marked variation of the total protein among the consecutive stings of each of the investigated scorpions. To go further, the protein profiles for all the collected stings were analyzed separately using 10% SDS-polyacrylamide electrophoresis (SDS-PAGE). Similarly, differences were observed among the stings, not only in the number of the protein bands, but also in the intensity of the bands. Thus, these results indicated that different venom components are unequally represented among consecutive stings supporting the venom-metering hypothesis.

Key words: Scorpion, Venom, Stinging, Consecutive stings, Electrophoresis, *Parabuthus leiosoma*

Introduction

Scorpions are predatory arthropod animals, feeding on small arthropods. Scorpions are common and ecologically important arthropods in arid and semi-arid ecosystems throughout the world (Polis, 2001; Brown, 2004). Egypt has four scorpion families; Buthidae, Diplocentridae, Scorpionidae and Euscorpiidae (EL-Hennawy, 2002). Genus *Parabuthus* is the most dangerous and medically important species of the family Buthidae. The genus *Parabuthus* currently comprises 28 species, 20 of which occur in southern Africa, and is restricted to arid and semi-arid regions (Dyason *et al*., 2002). *Parabuthus leiosoma* is a rare scorpion species recorded only in the Elba protected area, Red sea of Egypt (EL-Hennawy, 2008). Adults usually prey on large arthropods. This species has also invaded man-made structures including houses. The envenomation by these scorpion is of significant medical importance, particularly in western regions of southern Africa (Newlands, 1978; Bergman, 1997). The primary effects of a sting are neuromuscular, with significant parasympathetic nervous system and cardiac involvement (Bergman, 1997).

Scorpions use their venom to immobilize prey items, defend themselves against aggressors and mating. Their venom is a unique weapon composed of water, salts, small molecules, peptides, and proteins (Zlotkin *et al*., 1978; Yahel-Niv and Zlotkin, 1979; Polis and Sissom, 1990; Simard and Watt, 1990; Tallarovic *et al*., 2000). Among the peptides present in venom, short-chain neurotoxins (SCNs) act on potassium and chloride channels, whereas long-chain neurotoxins (LCNs) primarily act on sodium channels (Possani *et al*., 1999; De La Vega and Possani, 2004; Du Plessis *et al*., 2008). Production and storage of protein-rich venom is undoubtedly an expensive metabolic investment, especially for species adapted to survive in extreme ecosystems on scarce resources (Inceoglu *et al*., 2003). Thus, according to the venom-metering (Hayes *et al*., 2002; Hayes, 2008), or venom optimization (Wigger *et al*., 2002), hypothesis, venomous animals should use their venom as economically as possible. Venom can be viewed as a limited commodity due to storage constraints, metabolic costs of production and ecological costs of depletion (Hayes *et al*., 2002; McCue, 2006; Nisani *et al*., 2007; Hayes, 2008; Nisani, 2008). Indeed, many studies have shown that venomous animals regulate their venom expenditure during predatory or defensive situations (Boeve *et al*., 1995; Malli *et al*., 1999; Hayes *et al*., 2002; Stewart and Gilly, 2005; Hayes, 2008). So that the conservative use of venom by many species of scorpions suggests that venom secretion is also regulated. A study on the sting use of *Parabuthus* species is a good example of the regulation of sting use, demonstrating the conservative use of venom (Rein, 1993). When collecting venom from some scorpions the appearance of the initial venom tends to be transparent and, over successive stings, the venom becomes opalescent and finally assumes a milky-viscous appearance (Zlotkin and Shulov, 1969; Yahel-Niv and Zlotkin, 1979). Yahel-Niv and Zlotkin (1979) demonstrated that both composition and toxicity of the secretion varied among consecutive stings. Recent studies indicate that *Parabuthus transvaalicus* similarly secretes a small quantity of transparent venom termed ‘prevenom’ with initial stings, followed by milky ‘venom’ in subsequent stings (Inceoglu *et al*., 2003). Their characterization indicated that each one of the forms has different protein bands which coincide with the depletion of venom from the venom glands of scorpions. The venom of *P. transvaalicus* contains fewer than 100 major peptides (Possan*i et al*., 1999; Inceogl*u et al*., 2001). This venom exhibits high specificity toward both insects and mammals (Possan*i et al*., 1999). Its dual specificity could be attributed to a diet that presumably consists largely of insects (Polis, 1979) and the susceptibility of these large scorpions to mammalian predators (i.e., they offer a high caloric yield as prey).

To the best of our knowledge, no studies of the protein profile of *P. leiosoma* venom have been reported in the literature. Thus, the aim of this study is to investigate the protein profile of *P. leiosoma* venom across consecutive stings as a model to understand how scorpions appear to optimize venom expenditure.

Material and Methods

Animal maintenance and venom collection

Three Individuals of the rare scorpion *P. leiosoma* were collected from Elba protected area, Egypt and kept individually in plastic containers at 25°C - 28°C. Venom was collected as sequential stings by electrical stimulation (20 Volt) in the articulation of the telson. The stings were collected on a glass slide; each drop was dissolved in 50 µl double distilled water and then centrifuged at 14,000 rpm for 15 min at 4ºC. Supernatant was collected separately and finally stored at - 20ºC until use.

Protein content determination

Colorimetric determination was performed by using spectrophotometer (Jasco-V530). Commercial diagnostic kit from Vitro Scient chemicals was used for the biochemical assay. Equal amount of 20 µl venom extracts was used for determination of total protein (mg/dl). Data obtained were analyzed and figured by software system (Microsoft Excel).

Venom electrophoresis

Scorpion venom was analyzed on 10% SDS-PAGE according to Laemmli (1970). Gels containing the separated proteins were stained with 0.1% Coomassie Blue R-250. Protein marker (Precision plus protein ™ dual color standards #161-0374, BioRAD) was run in parallel in order to calculate the molecular weights of the venom proteins. Then, the photograph of the gel was taken and analyzed by Gel pro analyzer software (3.1).

Results

Protein content determination revealed variation of the venom protein among consecutive stings of the scorpion *P. leiosoma*

From each scorpion the venom was collected as nine sequential stings. At the beginning of electrical stimulation, scorpions first secrete a ‘‘transparent’’ version of their venom, then they secrete an ‘‘opaque’’ venom, which is white in color and subsequent stings became viscous. The protein concentrations of consecutive stings of *P. leiosoma* venom are shown in (Fig. 1). Results showed that, the venom is rich in protein. Concentration ranged from 1.4 to 4.9 mg/dl but varied and fluctuated considerably among the sequence of the following stings. Figure (1) illustrated that, the lowest total protein concentration was recorded in 1st sting, then the concentration starts to increase to reach the peak at 4th sting while the protein content of the other subsequent stings go to be fluctuated.

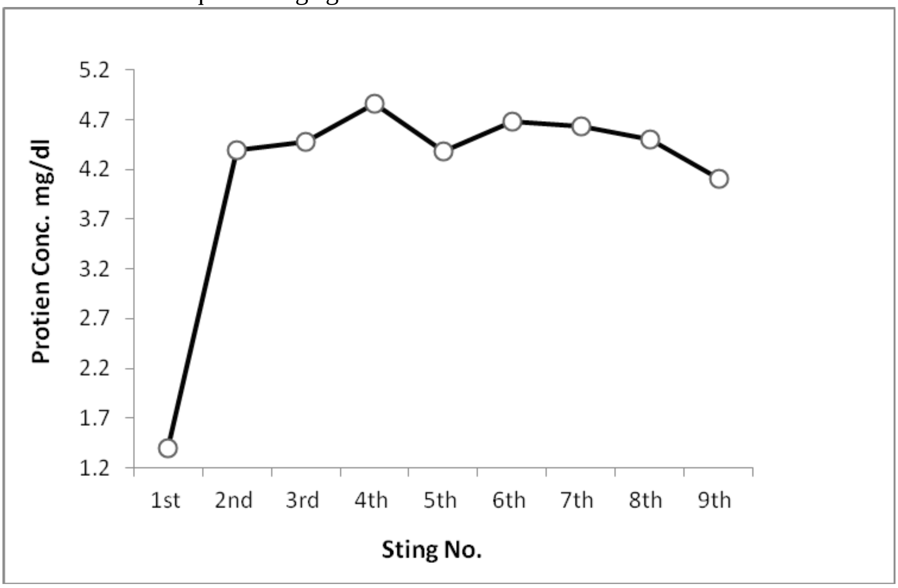

Fig. (1): Protein concentration varies among consecutive stings of the scorpion *Parabuthus leiosoma.*

SDS-PAGE analysis of the venom showed variation in protein composition among consecutive stings of the scorpion *P. leiosoma*

SDS-PAGE of consecutive stings of *P. leiosoma* venom (Fig. 2) showed the presence of different protein bands ranged between 200 to 12 kDa. In this analysis, 18 different protein bands (Table 1), with different molecular weight and intensity, were detected. There was also a variation in the number of bands between all stings except sting no. 8 and 9; with similar pattern. The number of the protein bands for the investigated sequential stings ranged from 6 to 12 bands. The protein profile of the first sting showed only 6 bands ranged from 67 to 14 kDa. Also the band at 67 kDa was recorded only in the first three stings; 1^{st}, 2^{nd} and 3^{rd} stings. While the bands no. 11 (35 kDa), 13 (27 kDa) and 18 (12 kDa) were consistently appeared in all consecutive stings. Interestingly, bands no. 11 and 13 showed a clear inverse relationship regarding the intensity throughout the consecutive stings. At the 1^{st} sting, the band no. 11 showed higher intensity than that of band no. 13. The opposite situation was observed in 2^{nd}, 3^{rd} and 4^{th} sting but the bands returned to the original case in 5^{th}, 6^{th}, 7^{th}, 8^{th} and 9^{th} stings, i.e. as in 1^{st} sting. It worth to mention that at 3^{rd} sting, the band no. 11 reached the maximum intensity but the band no. 13 reached its minimum intensity as shown in the figure 2 and 3. However, the third consistently band no. 18 (12 kDa) appeared as the highest band intensity among all consecutive stings except in case of the 1^{st} sting.

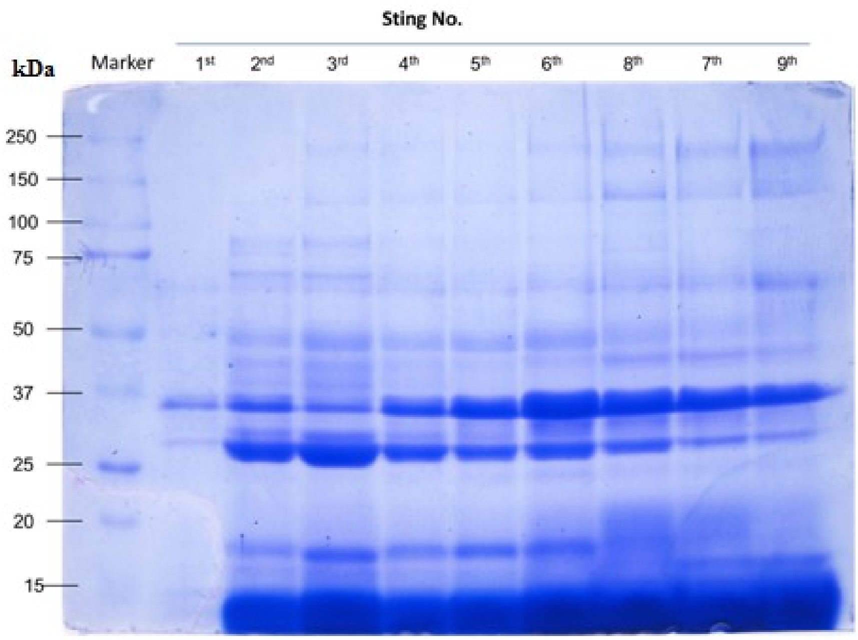

Fig. (2): SDS-PAGE of *Parabuthus leiosoma* venom among consecutive stings, gel was stained with Coomassie blue. Numbers at the left correspond to the mobility of the protein marker.

Table. 1. Variation of electrophoretic protein pattern in consecutive stings of *Parabuthus leiosoma* venom as determined by SDS-PAGE analysis.

Band No.	Protein band (kDa)	Sting no.								
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th
1	200			+	+	+	+	+	+	+
2	125		+	+	+	+	+	+	+	+
3	82		+	+						
4	75		+					+		
5	67	+	+	+						
6	62		+	+	+	+	+	+	+	+
7	50								+	+
8	48		+	+	+	+	+	+		
9	45		+	+	+	+	+	+	+	+
10	37		+	+						
11	35	+	+	+	+	+	+	+	+	+
12	30	+	+	+	+				+	+
13	27	+	+	+	+	+	+	+	+	+
14	25						+	+	+	+
15	17		+	+	+	+	+		+	+
16	15		+	+	+	+				
17	14	+						+	+	+
18	12	+	+	+	+	+	+	+	+	+

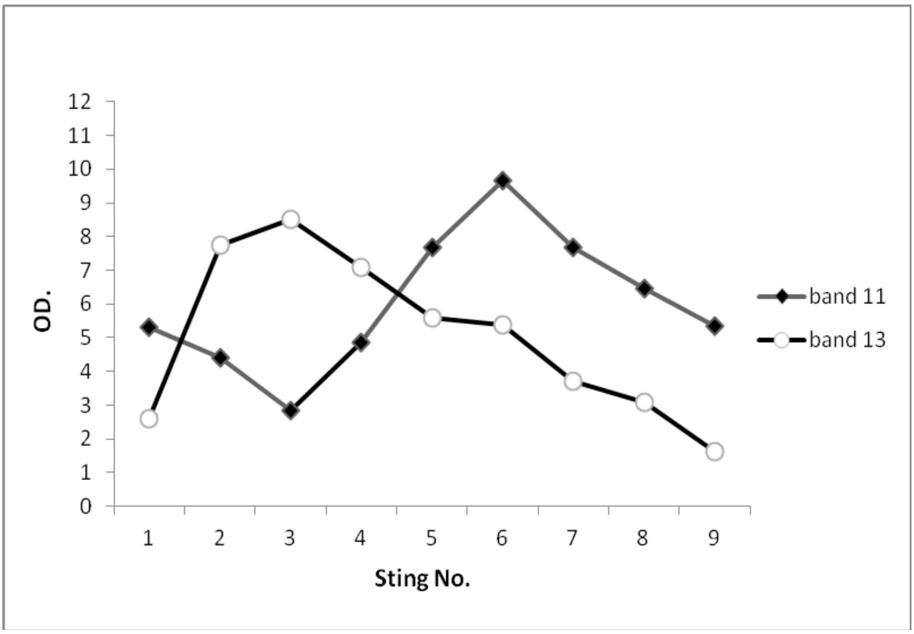

Fig. (3): Gradual increase and decrease of protein concentration in a reverse relationship between two proteins; 35 kDa (band no.11) and 27 kDa (band no. 13) in the sequential stings of *Parabuthus leiosoma.*

Discussion

During collecting the venom of the scorpion *P. leiosoma* as consecutive stings, we observed that the first droplet of venom has different physical properties than the rest of the venom. Inceoglu *et al*, (2003) reported that the first droplet refers as ‘‘prevenom’’. This phenomenon was also observed by Yahel-Niv and Zlotkin (1979) in the scorpion *Leiurus quinquestriatus*. These authors reported that the appearance of venom changes from transparent to opalescent and to viscous secretions in successive stings. Surprisingly, each one of the three forms has different protein bands, and that these coincide with the depletion of venom from the venom glands of scorpions. Also Inceoglu *et al*, (2003) reported that the prevenom is a particular type of venom with unique properties and with a different molecular mechanism of action than that of the venom. Its use helps conserve the more valuable venom, whereas on the other hand, it provides superb toxicity. This finding agrees with our results where the protein profile of first sting revealed that the first droplet has a small number of protein bands. Only 6 bands were recorded ranged from 67 to 14 kDa. The previous chemical analysis of the prevenom showed that it contains less protein and higher concentration of K^+ salt than that in the venom. It is noteworthy to mention that this pattern is in agreement with the general evolutionary formula of ‘‘from simple to more complex’’ (Trifonov *et al*., 2001). It may be advantageous for a scorpion to use the prevenom to deter a predator and/or make an impression by causing intense pain. This may be enough to immobilize a small arthropod and initiate feeding on it. For example, prevenom may distract a mammal because of its pain causing and ‘‘hyperactivating’’ abilities, giving the scorpion an opportunity to escape. On the other hand, in cases where its life is in danger, the scorpion may need to defend itself with the utmost urgency, thus justifying the presence of a more deadly mixture in the venom (Inceoglu *et al*., 2003). In general, it seems that prevenom may be sufficient for many routine encounters (Yahel-Niv and Zlotkin, 1979). This strategy shows that scorpion posses prevenom not only for conservation of venom but also for achieving increased biological activity.

The results showed marked variation of the total protein concentration among the consecutive stings of *P. leiosoma*. Similarly, differences were observed among the stings, not only in the number of the protein bands, but also in the intensity of the bands. These results suggest that *P. leiosoma* regulates venom expenditure not only at the level of protein concentration but also for protein composition. Accordingly, Nisani and Hayes (2011) demonstrated that the composition of venom injected covaries with venom volume, such that regulation of volume leads to regulation of composition. Also they noticed that *P. transvaalicus* can deliver variable quantities of venom in a single sting depending on level of threat, and appears to squirt defensively a larger volume of venom that is often expended upon stinging (Nisani, 2008).

Results showed a changeable reverse relationship between 35 and 27 kDa among consecutive stings of *P.leiosoma*. The interplay between these two proteins reflect how much precise that scorpion can control the venom mixture in each sting. The important question arises, how these proteins are regulated and secreted. As a trial to understand the mechanism of venom secretion Nisani and Hayes (2011) suggested that, the venom storage is heterogeneous; that is, peptides are not evenly distributed within the duct or lumen of the venom gland of *P. transvaalicus,* as the clear prevenom always comes before the milky venom and the order is never reversed. Whether different venom products are regionally secreted and stored within the gland without mixing or secretion is homogenous but involves inactivation-degradation and/or reabsorption for venom residing in the lumen or a portion of it, remains to be determined.

Also our results support the venom-metering (Hayes *et al*., 2002; Hayes, 2008), or venom optimization (Wigger *et al*., 2002), hypothesis. This hypothesis proposes that venomous animals use their venom judiciously, and make cognitive decisions about how much venom to inject. That may be because venom can be an expensive commodity (McCue, 2006; Nisani *et al*., 2007; Nisani, 2008; Pintor *et al*., 2010), and many venomous animals have been shown to be judicious in their venom expenditure (Boeve *et al*., 1995; Malli *et al*., 1999; Hayes *et al*., 2002; Hayes, 2008). There are several reasons why scorpions should be judicious their venom reserves. Venom regeneration in scorpions has measurable metabolic costs (Nisani *et al*., 2007; Nisani, 2008). Scorpion with insufficient venom may be unable to capture additional prey or defend itself against attack until its supply of venom has been at least partially restored. In addition to the scorpion's need for conserving a valuable commodity, the optimal amount of venom injected may vary with the context of use (Nisani and Hayes, 2011). This study addressed the latter issue and has shown that *P. leiosoma* meters the amount of venom injected. Moreover, scorpions in general, and buthids in particular, demonstrate a strong preference for retreat when threatened (Newlands, 1969), which is another indicator of venom conservation. In conclusion, this study provides evidence that scorpions regulate venom expenditure also give the capacity to make decisions regarding usage quantity, and, indirectly, the composition (prevenom or venom) of venom injected provides further support for the venom metering hypothesis. However, the elucidation of the mechanism involved in stinging and venom composition of different proteins requires further study

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