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Influence of post-starvation extraction time and prey-specific diet in *Tityus serrulatus* scorpion venom composition and hyaluronidase activity

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ABSTRACT

The role of diet in venom composition has been a topic of intense research interest. This work presents evidence that the variation in the venom composition from the scorpion *Tityus serrulatus* (Ts) is closely associated with post-starvation extraction time and prey-specific diet. The scorpions were fed with cockroach, cricket, peanut beetle or giant Tenebrio. The venoms demonstrated a pronounced difference in the total protein and toxins composition, which was evaluated by electrophoresis, reversed-phase chromatography, densitometry, hyaluronidase activity and N-terminal sequencing. Indeed, many toxins and peptides, such as Ts1, Ts2, Ts4, Ts5, Ts6, Ts15, Ts19 frag. II, hypotensins 1 and 3, PAPE peptide and peptide 9797 (first described in Ts venom), were all identified in different proportions in the analyzed Ts venoms. This study is pioneer on assessing the influence of the starvation time and the prey diet on hyaluronidase activity as well as to describe a modification of Tricine-gel-electrophoresis to evaluate this enzyme activity. Altogether, this study reveal a large contribution of the extraction time and diet on Ts venom variability as well as present a background to recommend the cockroach diet to obtain higher protein content and the cricket diet to obtain higher hyaluronidase specific activity.

1. Introduction

The variation of venoms composition and toxicity is ubiquitous in many venomous animals (Abdel-Rahman et al., 2009; Chippaux et al., 1991; Colinet et al., 2013; Cologna et al., 2013; Pimenta et al., 2003; Richards et al., 2012; Saad et al., 2012). Concerning intraspecies, venom variability can be influenced by geographical location and climate, age, genetics, sexual dimorphism, diet, extraction procedure, but the interaction of these factors is poorly understood (Boldrini-Franca et al., 2010; Halassy et al., 2011; Oliveira et al., 2013; Oukkache et al., 2013; Pucca et al., 2011).

In Brazil, scorpion stings are responsible for most accidents related to venomous animals, being considered *Tityus serrulatus* the main dangerous scorpion species (Cologna et al., 2009). It is also responsible for the greatest number of accidents due to its high proliferation by

Abbreviations: Ts, *Tityus serrulatus*; RP-FPLC, reversed-phase fast protein liquid chromatography; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; NaScTxs, Na^+ channels scorpion toxins; KTxs, potassium channel scorpion toxins; BPP, bradykinin-potentiating peptide.

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parthenogenesis (Schneider and Cella, 2010). As the primary function of the scorpion venom is to immobilize the prey, the venom composition reflects natural selection on the scorpion feeding. Thus, prey-specific diet has been implicated as the most potential cause of venom variability and is considered a topic of intense research interest (Barlow et al., 2009; Daltry et al., 1996; Richards et al., 2012; Sasa, 1999).

T. serrulatus venom is composed of a great variety of molecules in a highly complex mixture, mainly neurotoxins, enzymes (proteinases and hyaluronidase), components with antimicrobial activities, bradykinin-potentiating peptides (BPP), a natriuretic peptide recently described (Alves et al., 2013; Oukkache et al., 2013) and other peptides whose biological functions have not been clarified yet. Although all these molecules are found in all *T. serrulatus* venom, previous researches described relevant qualitative and quantitative composition variability by different methods: ELISA (Kalapothakis and ChavezOlortegui, 1997), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Pimenta et al., 2003) and fractal dimension analysis/mass fingerprint (Oliveira et al., 2013). Moreover, variations in the venom composition of scorpions are also related to the symptoms disparity in the envenomed victims (Freire-Maia et al., 1994).

Symptoms and signs observed in scorpion envenoming have been attributed to the effects of the neurotoxins from venom interacting with ionic channels of excitable cell membranes, inducing a massive release of neurotransmitters (Possani et al., 1999). Additionally, the venom contains hyaluronidase which catalyzes the breakdown of hyaluronan of the interstitial matrix. This enzyme is considered a "spreading factor", since it increases the diffusion of venom toxins through the tissues and potentiates the toxic action of the venom, contributing to local and systemic envenoming (Bordon et al., 2012; Kreil, 1995; Possani et al., 1999).

Today, to fractionate *T. serrulatus* venom, our group uses a CM-Cellulose-52 column (Arantes et al., 1989) followed by rechromatography (Cologna et al., 2011; Pessini et al., 2001), which requires great quantities of *T. serrulatus* venom. However, an alternative procedure employing a reversed-phase fast protein liquid chromatography (RP-FPLC) on a C18 column can be also used. Nevertheless, intraspecies composition variability of *T. serrulatus* venom using chromatography after different post-starvation extraction times and prey-specific diets has not been studied so far.

Since *T. serrulatus* reproduces by parthenogenesis and it tends to stay in a restricted area, influence of sexual and geographical variations in venom could be discarded (Pimenta et al., 2003). However, a recently published study showed a significant regional variation in toxicity of Ts venom (Oliveira et al., 2013), which can raise the hypothesis that the diet with different preys can directly influence the Ts venom composition. Moreover, in 2003, Pimenta et al. showed through mass spectrometry that the extraction time after starvation influences the venom composition (Pimenta et al., 2003), but only one type of prey was used in the feeding and the diet type was not informed.

Based on that, this study represents an advance toward comprehension of the variability in *T. serrulatus* venom composition after different starvation periods as well as different prey-specific diets.

2. Material and methods

2.1. T. serrulatus

T. serrulatus specimens were obtained from the region of Ribeirão Preto and were maintained in the serpentarium of Faculdade de Medicina de Ribeirão Preto (FMRP-USP, University of São Paulo at School of Medicine of Ribeirão Preto), Brazil, in accordance with the guidelines of Ibama, Brazilian Institute of Environment. The adult scorpions were randomly selected by body length to minimize age differences. We also tried to exclude the pregnant scorpions. All animals were submitted to a previous electrical extraction process (Lowe and Farrell, 2011) in order to prevent pre-existent compounds interfering with individual concentration variability in the venom and leave the venom glands in their active state (Rendon-Anaya et al., 2012). Thereafter, each scorpion was kept in separated boxes with water *ad libitum* during 5 days to enable new venom storage in the gland.

2.2. Post-starvation extraction times and diets with different preys

Each adult scorpion was individually fed with one cockroach (*Nauphoeta cinerea*, about 2.0 cm long, weighing 500 mg), 5 days after previous electrical stimulation. Initially, groups of 20 scorpions were used; however, it was necessary to exclude some specimens which did not eat or ate only a part of the prey. Thus, groups of 10 scorpions that surely ate the entire prey were used. The venom was extracted using electrical stimulation method (with the minimal voltage required − 12 V) during different times after feeding: 24 h, 10, 20 and 30 days (10 d, 20 d, 30 d). The venoms of each time group were pooled and desiccated. Further, the pooled venoms were dispersed in 0.2 mL of ultrapure water, centrifuged at 10,015 × *g*, 4 °C, for 10 min. The precipitate was resuspended twice under the same conditions and the supernatants were pooled, resulting in the crude soluble venom without mucus. The protein concentration of crude soluble venoms was estimated by NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) using the extinction coefficient shown below, previously determined for the soluble venom.

$$\varepsilon_{280\ \mathrm{nm}}^{1\ \mathrm{mg/mL}} = 1.65.$$

Another group of adult scorpions ($n = 10$) was individually fed with one cricket (*Grilus* sp, about 1.5 cm long, weighing 500 mg), under the same conditions of the diet with cockroaches. The venom was extracted 30 days after feeding the scorpion with cricket. Other different diets with peanut beetle (*Palembus dermestoides*, about 0.5 cm long, weighing 50–100 mg) and giant Tenebrio (*Zophobas morio*, about 5 cm long, weighing 400–600 mg) were also tested.