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Antarease-like Zn-metalloproteases are ubiquitous in the venom of different scorpion genera

Ernesto Ortiz [a], Martha Rendón-Anaya [b], Solange Cristina Rego [c], Elisabeth Ferroni Schwartz [c], Lourival Domingos Possani [a,*]

[a] Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autonóma de México, Avenida Universidad 2001, Cuernavaca 62210, Mexico
[b] Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Km 9.6 Libramiento Norte Carretera León, Irapuato, Guanajuato 36821, Mexico
[c] Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília, DF 70910-900, Brazil

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ABSTRACT

Background: The venoms of several scorpion species have long been associated with pancreatitis in animal models and humans. Antarease, a Zn-metalloprotease from *Tityus serrulatus*, is able to penetrate intact pancreatic tissue and disrupts the normal vesicular traffic necessary for secretion, so it could play a relevant role in the onset of acute pancreatitis.
Methods: The cDNA libraries from five different scorpion species were screened for antarease homologs with specific primers. The amplified PCR products were cloned and sequenced. A structural model was constructed to assess the functionality of the putative metalloproteases. A phylogenetic analysis was performed to identify clustering patterns of these venom components.
Results: Antarease-like sequences were amplified from all the screened cDNA libraries. The complete sequence of the antarease from *T. serrulatus* was obtained. The structural model of the putative antarease from *Tityus trivittatus* shows that it may adopt a catalytically active conformation, sharing relevant structural elements with previously reported metalloproteases of the ADAM family. The phylogenetic analysis reveals that the reported sequences cluster in groups that correlate with the geographical localization of the respective species.
Conclusions: Antareases are ubiquitous to a broad range of scorpion species, where they could be catalytically active enzymes. These molecules can be used to describe the evolution of scorpion venoms under different ecogeographic constrains.
General significance: For the first time the complete sequence of the antareases is reported. It is demonstrated that antareases are common in the venom of different scorpion species. They are now proposed as targets for antivenom therapies.

1. Introduction

It is well known that certain scorpion stings are associated with acute pancreatitis in humans. In particular, the stings from the *Tityus* species produce abdominal pain, nausea and vomiting and in many cases development of acute pancreatitis. After the first clinical cases were reported by Waterman in 1938 [1] for accidents involving the Trinidadian scorpion *Tityus trinitatis*, other reports confirmed the linkage between scorpion poisoning and acute pancreatitis for this species [2,3]. The venoms from other species of the *Tityus* genus, as well as some of their fractions and isolated components have been shown to produce pancreatitis or morphological changes associated with it. That is the case for *Tityus asthenes* from Colombia, which induced acute pancreatitis in children [4], *Tityus serrulatus* from Brazil as tested in dogs, rats and guinea pigs [5–8] and *Tityus discrepans* from Venezuela in mice [9]. Light microscopy of pancreas from *Tityus zulianus*-envenomed mice revealed interstitial edema and vacuolization of acinar cells [10]. Acute pancreatitis has also been documented in children stung by *Leiurus quinquestriatus* from Israel [11,12]. It has been shown that the *Mesobuthus tamulus* venom acts directly on exocrine pancreas to cause acute pancreatitis to anesthetized dogs and rabbits [13].

Paul Fletcher's group, working at East Carolina University, showed that venoms from *Tityus* scorpions are potent secretagogues that can elicit the release of secretory proteins from the exocrine pancreas [5,8,14,15] and neurotransmitters from synaptosomes [15]. Purified toxins or whole venoms from *T. serrulatus*, *Tityus bahiensis* and *Tityus stigmurus* trigger exocytosis in a dose-dependent manner [5,8,15]. Morphological studies by microscopy of pancreatic acinar cells after

Abbreviations: ADAM, a disintegrin and metalloproteinase; BLAST, basic local alignment search tool; HMM, hidden Markov model; MD, molecular dynamics; NPT, constant-temperature, constant-pressure ensemble; PDB, protein data bank; PSI-BLAST, position-specific iterative BLAST; Rgyr, radius of gyration; SVMP, snake venom protease, SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; TsTX, tityustoxin

* Corresponding author. Tel.: +52 777 3291647.
E-mail address: possani@ibt.unam.mx (L.D. Possani).

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treatment with purified toxins or the whole venom from *T. serrulatus* reveal not only a dramatic decrease in the number of zymogen granules due to the induced discharge, but also other cellular changes such as vacuolization, interstitial swelling, partial effacement of the acinar lumen, loss of microvilli, and the appearance of secretory material and cellular debris in the lumen, suggesting the loss of structural integrity. These events have been associated to acinar cell damage and proposed to be possible early events ultimately progressing to the cellular necrosis of acute pancreatitis observed in vivo after *T. serrulatus* intoxication [8,14]. The effects of "tityustoxin" ("TsTX"; [16]), a heterogeneous fraction from *T. serrulatus* venom [17], on the pancreatic secretion of rats were investigated by Novaes et al. [7]. TsTX did not change the serum levels of lactate dehydrogenase, thus suggesting that this particular alteration induced by the whole venom is due to other component(s) of the venom, acting alone or synergistically with TsTX. Though isolated toxins are capable of eliciting the secretory response of the acinar cells, it has been speculated that the clinical manifestations of acute pancreatitis observed with the whole venom could be the result of the synergistic action of several components of the venom [7,8] as the neurotoxins that act indirectly on the pancreatic function by stimulating the liberation of acetylcholine in the pancreatic nerve terminals [18,19].

The most significant venom components in terms of their abundance, physiological effects and medical consequences, are ion channel-modulating toxins. But scorpion venoms are a rich source of other biologically active molecules. These include some enzymes. To date, several enzymatic activities have been characterized in scorpion venoms, including phospholipase A [20,21], hyaluronidase [22], sphingomyelinase D [23], lysozyme [24] and gelatinolytic/caseinolytic proteases, mostly serine-proteases [25]. Two mRNA sequences coding for putative Zn-metalloproteases from the Asian scorpion *Mesobuthus eupeus* have been submitted to GenBank (Accession numbers EF442045.1 and EF442046.1). The presence of proteases in the scorpion venoms has been associated with the facilitation of venom permeation into tissues [25] and the posttranslational processing of toxin precursors [26].

It was very recently that a novel zinc-metalloprotease, named antarease, was purified from the venom of *T. serrulatus* and characterized [27]. It constitutes the first example of a scorpion protease with a potentially pathogenic mechanism of action. Antarease appears to penetrate intact tissue and specifically cleave the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) involved in pancreatic secretion, thus disrupting the normal vesicular traffic. Due to its activity, antarease might be one of the components responsible for the onset of acute pancreatitis observed after *T. serrulatus* intoxication. If this proves to be the case, this protease should be considered as a therapeutic target for the generation of antivenoms able to neutralize the toxic effect of the whole venom, an effort that has until now focused on the ion-channel modifying toxins [28,29]. It was recently shown that the venoms of the Brazilian scorpions *T. serrulatus*, *T. stigmurus* and *T. bahiensis* are able to cleave dynorphin 1–13, an activity associated with metalloproteinases. The finding that the two Brazilian antivenoms used for human therapy poorly neutralize the dynorphin 1–13 cleavage activity of those venoms reinforces the idea that better antivenoms are still needed [30].

The reported sequence of the antarease was determined by direct Edman sequencing of the N-terminus and of peptides derived from several proteolytic cleavages. Despite the fact that this finding was quite original, describing important structural features and possible functions of an enzyme that might play a crucial role on human pancreatitis caused by scorpion stings [27], the sequence reported is nevertheless incomplete, with about 10% of its amino acid sequence not determined. In addition, small sequence differences were found when analyzing the sequence reported in the original paper and the one in the UniProt Database (accession number P86392). Given the importance that this enzyme might have on acute pancreatitis, we consider relevant to determine its complete precise amino acid sequence. The total amount of this enzyme present in *T. serrulatus* soluble venom, based on chromatographic separation and mass spectrometry analysis, indicates that this enzyme is around 0.5% of the whole soluble venom. For sequencing analysis we used a molecular biology approach, looking for the sequence of this protein-coding mRNA.

Since acute pancreatitis has also been observed for other scorpion venoms, we set to look for similar sequences in cDNA libraries of other species of the *Tityus* and *Centruroides* genera to which we have access. Additional to the findings described here concerning the primary structure of antarease and antarease-like enzymes from different species of the genus *Tityus* it is worth mentioning that analysis of the evolutive role of these enzymes in scorpion venoms is also an original part of this communication.

2. Experimental

2.1. cDNA library construction

The cDNA library from the venom glands of *Tityus trivittatus*, constructed with the SMART cDNA Library Construction Kit (Clontech) was described before [31].

For this work new cDNA libraries were constructed from the telsons of individual specimens of *T. serrulatus*, *Tityus fasciolatus*, *Tityus pachyurus* and *Tityus* sp. This last is yet an undescribed *Tityus* species from Colombia, collected in Popayán (Cauca), Colombia (2°26′39″N, 76°36′18″W). For total RNA extraction the ZR RNA MiniPrep Kit (Zymo Research) was used following the instructions from the manufacturer. The total RNA was quantified by absorbance with a NanoVue Spectrophotometer (GE Healthcare, Life Sciences). Further, the In-Fusion SMARTer cDNA Library Construction Kit (Clontech) was used to construct the cDNA library. For first strand amplification, 0.5 μg of total RNA was employed. The double-stranded cDNA was amplified (RT-PCR) from 2 μl of the previous reaction, fractioned by size through a CHROMA-1000 column and quantified by absorbance. Nine hundred nanograms of ds-cDNA were ligated to 300 ng linearized pSMART2IF vector and electrotransformed into electrocompetent DH5α cells.

2.2. Primer design, PCR amplification and cloning

The available protein sequence of the antarease from *T. serrulatus* (SwissProt P86392.1) was backtranslated with full degeneracy. Within the generated sequence a search was performed to identify specific regions with the lowest possible degeneracy that could function as primers. Two regions were identified close to the 5′ and 3′ ends of the degenerated sequence and for them primers were designed: AntUp1, 5′-GAYGAYTGYATHGTNGTNGARTAYTAYAT-3′ (29-mer, tm = 52.6–65.7 °C, degeneracy = 3072, all calculations made with Oligo v7.54) and AntLw2: 5′-RTCNSWRCAYTGYTGRAADATRCA-3′ (24-mer, tm = 51.7–63.3 °C, degeneracy = 3072). Y = G/C, H = A/T/C, N = A/T/G/C, R = A/G, S = G/C, W = A/T, D = A/G/T.

The *Tityus trivittatus* cDNA library was constructed with the pDNR-LIB vector (Clontech), so a combination of the above degenerated primers and T7DIR (5′-GTAATACGACTCACTATAGGG-3′) or M13REV (5′-AACAGCTATGACCATGTTCAC-3′) specific for the vector, were used for amplification. The PCR conditions were 5 min 94 °C, followed by 30 cycles of 30 s 94 °C, 1 min 50 °C, 1 min 72 °C, plus an extension step of 5 min, 72 °C. *Pfu* Polimerase was used (Fermentas). The product was purified by PAGE/QIAquick Gel Extraction Kit (QIAGEN), blunt cloned into pBluescriptKS(+) and electrotransformed into competent DH5alpha cells. Positive clones were selected and sequenced. A partial sequence was obtained for the cDNA that was lacking the 5′ region of the mRNA. From this sequence, the primer AntTtrLw3 (5′-ATTCCCAG CTCCAACGTATCCATC-3′) was designed and used in combination with the T7DIR primer to amplify the remaining sequence from the *T. trivittatus* library. The PCR conditions and cloning procedure were the same as above.