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Research paper

Astacin-like metalloproteases are a gene family of toxins present in the venom of different species of the brown spider (genus *Loxosceles*)☆

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ABSTRACT

Brown spiders have a worldwide distribution, and their venom has a complex composition containing many different molecules. Herein, we report the existence of a family of astacin-like metalloprotease toxins in *Loxosceles intermedia* venom, as well as in the venom of different species of *Loxosceles*. Using a cDNA library from the *L. intermedia* venom gland, we cloned two novel cDNAs encoding astacin-like metalloprotease toxins, LALP2 and LALP3. Using an anti-serum against the previously described astacin-like toxin in *L. intermedia* venom (LALP1), we detected the presence of immunologically-related toxins in the venoms of *L. intermedia*, *Loxosceles laeta*, and *Loxosceles gaucho*. Zymographic experiments showed gelatinolytic activity of crude venoms of *L. intermedia*, *L. laeta*, and *L. gaucho* (which could be inhibited by the divalent metal chelator 1,10-phenanthroline) at electrophoretic mobilities identical to those reported for immunological cross-reactivity. Moreover, mRNAs extracted from *L. laeta* and *L. gaucho* venom glands were screened for astacin-like metalloproteases, and cDNAs obtained using LALP1-specific primers were sequenced, and their deduced amino acid sequences confirmed they were members of the astacin family with the family signatures (HEXXHXXGXXHE and MXY), LALP4 and LALP5, respectively. Sequence comparison of deduced amino acid sequences revealed that LALP2, LALP3, LALP4, and LALP5 are related to the astacin family. This study identified the existence of gene family of astacin-like toxins in the venoms of brown spiders and raises the possibility that these molecules are involved in the deleterious effects triggered by the venom.

1. Introduction

The astacin family of metalloproteases is formed by zinc endopeptidases. The family was named following the description of the prototypical digestive enzyme astacin from crayfish, *Astacus astacus* [1,2]. The astacin family comprises structurally-related digestive, extracellular, or cell surface-bound proteases that are involved either in digestive functions or peptide processing and play a role in the activation of growth factors, degradation of polypeptides, and processing of extracellular molecules [1,3,4]. Astacin family members are multifunctional proteases characterized by the consensus sequence HEXXHXXGXXHE, which is the zinc-binding domain, and the Met-turn MXY, both of which are key elements for this family [3]. To date more than 200 astacin-like enzymes have been identified in different organisms, such as humans, mice, rats, amphibians, fishes, sea urchins, insects, mollusks, and even bacteria [4,5], providing support for their biological importance. In the human and mouse genomes, there are six astacin family genes (two meprins, three BMP1/tolloid-like, one ovastacin), but in *Caenorhabditis elegans* there are 40 [6]. Recently, the identification of an astacin-like protease in the venom of the brown spider *Loxosceles intermedia* was described [7]. This molecule, named LALP (*Loxosceles* Astacin-Like Protease), was cloned and expressed as a recombinant protein. This is the first

☆ The nucleotide sequence data reported for *Loxosceles* astacin-like metalloprotease toxins will appear in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers: Full length - LALP2-GQ227490; LALP3-GQ227491. ESTs - LALP4-GR277667; LALP5-GR277668.

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astacin family member described in venom and it may exhibit deleterious activity following brown spider envenomation.

Loxoscelism, gangrenous arachnidism, or necrotic arachnidism are terms used to describe lesions and signals induced by bites from *Loxosceles* genus spiders. Loxoscelism is characterized by a dermonecrotic lesion with gravitational spreading and some involvement at the systemic level, including intravascular hemolysis, acute renal failure, and disseminated intravascular coagulation [8,9].

The genus *Loxosceles*, Sicariidae family, comprises 100 described species, the majority of which can be found in the Americas, West Indies, and Africa [10,11]. In Brazil, there are three species with medical importance, which are *L. intermedia*, *Loxosceles laeta* and *Loxosceles gaucho* [12].

Loxosceles venom contains several protein toxins and is enriched with molecules of low molecular mass (5–40 kDa) [8,9]. Toxins including alkaline phosphatase, hyaluronidase, metalloproteases, phospholipases-D, and low molecular mass (5.6–7.9 kDa) insecticidal peptides have been identified [7,8,13–18]. Among these venom toxins, phospholipase-D family members (30–35 kDa; also called dermonecrotic toxins) play an essential role on the pathogenesis of loxoscelism [19–22].

The identification of proteases in the brown spider venom comes from previous descriptions by Eskafi and Norment [23], who reported protease activity against *Heliothis virescens* and *Musca domestica* larvae in *Loxosceles reclusa* venom. Using the same model, Jong et al. [24] determined that the venom hydrolyzes L-aminoacyl-β-naphthylamide derivatives. Additionally, it was reported that metalloproteases in the venom of *L. intermedia* degrade gelatin, fibronectin, and fibrinogen [13]. Veiga et al. [25] described the hydrolytic activity of the venom on Engelbreth-Holm-Swarm tumor (EHS)-basement membrane structure, purified entactin, and the protein core of heparan sulfate. Also, *L. intermedia* venom triggers proteolytic activity on the sub-endothelial blood vessel basement membrane and extracellular matrix of endothelial cells in culture [26]. By studying *L. rufescens* venom, Young and Pincus [27] described metalloproteases with caseinolytic, gelatinolytic, and fibrinogenolytic activities. Zanetti et al. [28] described a fibrinogenolytic and metalloprotease-dependent activity in the venom from *L. gaucho* and *L. laeta* and purified a 30-kDa molecule with fibrinogenolytic activity from *L. intermedia* venom. Using venom gland extract from *L. intermedia*, da Silveira et al. [29] reported the presence of metalloproteases and proved those molecules were components of brown spider venoms. The presence of metalloproteases in the venom of different *Loxosceles* species, including *L. intermedia*, *L. gaucho*, *Loxosceles deserta*, *L. laeta*, and *L. reclusa*, provides evidence for a conserved feature and biological significance of these molecules [15]. Finally, the identification, cloning, expression, purification, and functional characterization of a metalloprotease characterized as an astacin-like toxin in the *L. intermedia* venom gland corroborated the previous data describing metalloproteases in *Loxosceles* venoms and was the first report of the presence of an astacin-like enzyme as a constituent toxin in animal venoms [7]. Studies on astacin-like proteases from *Loxosceles* venoms generate additional knowledge not only restricted to loxocelism but also for astacin family, moreover these molecules show putative biotechnological applications as biotolls for research and laboratory protocols and as novel pharmaceuticals (thrombolityc agents for example) [30].

Herein, we report the identification and cloning of two novel metalloproteases (LALP2 and LALP3), which we characterized as astacin-like toxins in the *L. intermedia* venom gland, thereby identifying a gene family of toxins. We also characterized this gene family of astacin-like toxins in the *Loxosceles* genus by immunological cross-reactivity of antibodies against LALP1 with different *Loxosceles* spider venoms. Supporting these findings, LALP1-related molecules (named LALP4 and LALP5) from other *Loxosceles* species were identified by cDNA cloning and sequencing. The results corroborate previous data that described metalloproteases in *Loxosceles* venoms and demonstrate for the first time that astacins are a gene family present in the venoms of the three analyzed *Loxosceles* species (*L. intermedia*, *L. laeta* and *L. gaucho*) and probably will be found widespread in the genus.

2. Materials and methods

2.1. Reagents

Polyclonal antibodies against *L. intermedia* crude venom toxins and recombinant toxin (LALP1) were produced in rabbits as previously described [31,32]. Crude venom from *L. intermedia* was extracted from wild-caught spiders according to Feitosa et al. [13]. Polyclonal antibodies against *L. laeta* and *L. gaucho* crude venom toxins were a kind gift from Instituto Butantan (São Paulo, São Paulo, Brazil) by Dr. Katia C. Barbaro. Adult *L. laeta* and *L. gaucho* spiders were obtained from CPPI (Curitiba, Paraná, Brazil).

2.2. cDNA library construction

The venom gland cDNA library was previously constructed by our group [33]. Briefly, venom gland mRNAs from adult *L. intermedia* spiders were purified using the FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, USA). cDNAs were synthesized using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen), cloned into the *Not* I/*Sal* I pre-cut pSPORT1 vector, and transformed into DH5α *Escherichia coli* cells.

2.3. cDNA library screening

Randomly selected colonies (approximately 100 clones) were inoculated in LB containing 100 μg/ml ampicillin and grown overnight at 37 °C (with aeration), and recombinant plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN, Valencia, USA). The cloned cDNAs were sequenced on both strands using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK). Reactions were analyzed by an ABI 377 automatic sequencer (Applied Biosystems). The T7 and SP6 promoter regions were used to prime the sequencing reactions. The cDNA sequences were analyzed and used to search the NCBI GenBank protein databases [34].

2.4. Cloning of cDNAs encoding LALP2 and LALP3

cDNAs encoding the putative mature metalloprotease toxins LALP2 and LALP3 were amplified by PCR using *Pfu* DNA Polymerase (Fermentas, Burlington, Canada). The forward primers contained the *Xho* I restriction site for LALP2 and *Nde* I restriction site for LALP3 at the 5′ end, and the reverse primers contained the *Bam*H I restriction site and the native stop codon of the cDNA for the both isoforms (Fig. 1). The PCR products were digested with *Xho* I or *Nde* I and *Bam*H I and gel purified using PerfectPrep Gel Cleanup Kit (Eppendorf, Hamburg, Germany). Purified DNAs were subcloned into pET-14b (Novagen, Madison, USA) digested with *Xho* I or *Nde* I and *Bam*H I. The correct construct was confirmed by PCR using T7 as the forward primer and SP6 as the reverse primer and sequencing.

2.5. LALP1 expression and purification

LALP1 was obtained as described by da Silveira et al. [7] The final construct of the mature protein LALP1/pET-14b was transformed