Brown Spider Venom: The Identification and Biotechnological Potential of Venom Toxins

6

Daniele Chaves-Moreira, Dilza Trevisan-Silva, Luiza Helena Gremski, and Silvio Sanches Veiga

Contents

Abstract

Brown spiders (*Loxosceles* genus) are venomous arthropods that use venom for predation and defense. These spiders have also been associated with human accidents, and the primary clinical manifestations are dermonecrosis with gravitational lesion spreading, hematological disturbances, and acute renal failure. *Loxosceles* venom comprises a complex mixture of toxins enriched in low molecular mass proteins (5–40 kDa). Characterization of this venom revealed three highly expressed protein classes: phospholipase-D family proteins, astacin-like proteases, and inhibitor cystine knot (ICK) peptides. A recent

D. Chaves-Moreira • D. Trevisan-Silva • S.S. Veiga (✉)
Department of Cell Biology, Federal University of Paraná, Curitiba, Paraná, Brazil
e-mail: dani_chaves@ufpr.br; dilzatrevisan@gmail.com; veigass@ufpr.br

L.H. Gremski
Department of Cell Biology, Federal University of Paraná, Curitiba, Paraná, Brazil

Departament of Medical Pathology, Clinical Hospital, Federal University of Paraná, Curitiba, Paraná, Brazil
e-mail: luiza_hg@yahoo.com.br

P. Gopalakrishnakone, J.J. Calvete (eds.), *Venom Genomics and Proteomics*, Toxinology,
DOI 10.1007/978-94-007-6416-3_28

study also showed the presence of several other venom proteins, such as serine proteases, protease inhibitors, hyaluronidases, allergen-like toxins, and translationally controlled tumor protein (TCTP), expressed at low levels in *Loxosceles* venom. The Brown spider phospholipase-D family proteins have been well studied, and these toxins alone induce inflammatory responses, dermonecrosis, hemolysis, thrombocytopenia, and renal failure. In addition, the functional role of hyaluronidases as spreading factors in loxoscelism has been demonstrated. However, the biological characterization of other toxins has not been reported. Nevertheless, the mechanism by which *Loxosceles* toxins exert noxious effects is not fully elucidated. The aim of this chapter is to provide insights into Brown spider toxins, including the identification of novel toxins using molecular and proteomics approaches, and the biological characterization and structural description of toxins using X-ray crystallography. Putative biotechnological uses of *Loxosceles* toxins and future perspectives in this field will also be discussed.

Introduction

Spiders of the *Loxosceles* genus are one of the four groups of venom-producing spiders that cause significant clinical manifestations or human fatalities following envenomation (Palagi et al. 2013). This condition, referred to as loxoscelism, is commonly observed after accidents involving *Loxosceles* spiders and is characterized by several reactions. Although most bites are benign, local and systemic symptoms can emerge. Local reactions manifest as dark blue-violet necrotic wounds with gravitational spread that eventually become indurated, generating scar tissue (Chaim et al. 2011). In approximately half of cases, cutaneous lesions have been associated with nonspecific systemic symptoms, including fever, fatigue, headache, vomiting, pruritic reactions, and rash (Chaim et al. 2011; Isbister and Fan 2011). Systemic loxoscelism is a less frequent complication more often observed in children, with manifestations such as renal failure and hematological disturbances, i.e., disseminated intravascular coagulation and intravascular hemolysis (Isbister and Fan 2011).

Cutaneous loxoscelism has been described in several studies examining rabbit skin exposed to *Loxosceles* venom (Chatzaki et al. 2012; Silvestre et al. 2005). These studies describe the massive infiltration and aggregation of inflammatory cells, interstitial edema, increased vascular permeability, intravascular fibrin network deposition, thrombus formation inside the capillaries, and vessel degeneration. Longer exposure times lead to the degeneration and necrosis of the skin muscle and leukocyte infiltration into the skeletal muscle. The massive hemorrhage and destruction of epidermal integrity and necrosis of surrounding collagen near the epidermis have also been observed (Chatzaki et al. 2012; Gremski et al. 2014; Silvestre et al. 2005).

Viscerocutaneous loxoscelism is the systemic injury resulting from *Loxosceles* envenomation, and this condition occurs in up to 13 % of cases (Hogan et al. 2004).

Patients might develop acute kidney injury, which is responsible for most lethal cases of *Loxosceles* envenomation (Abdulkader et al. 2008). The analysis of mice renal tissue following the treatment of *Loxosceles intermedia* venom showed acute tubular necrosis in several nephrons, glomerular collapse, tubular epithelial cell cytotoxicity, eosinophilic material deposition in the tubular lumen, and basement membrane alterations (Luciano et al. 2004; Tambourgi et al. 1998). A direct effect of *L. intermedia* venom on kidney structures was also demonstrated in mice (Luciano et al. 2004). Using rats as models to examine *Loxosceles gaucho* venom nephrotoxicity, Lucato and coworkers (2011) revealed that this venom produces renal injury and rhabdomyolysis, independently from dermonecrotic injury or blood pressure changes. These authors attributed the observed renal injury to renal vasoconstriction and myoglobinuria because no direct effect of venom on isolated proximal tubules was demonstrated.

The severity of loxoscelism has been associated with the venom content. Several factors associated with the spider specimen, such as size, sex, species, nutritional state, and age, might influence the amount and content of venom. Evidence suggests that female venom exhibits the highest biological activity, positively correlated with higher sphingomyelinase activity (de Oliveira et al. 2005). *Loxosceles* venom primarily comprises a complex mixture of protein-based biologically active compounds with toxic and/or enzymatic activities (Gremski et al. 2014). Nonprotein compounds include sulfated guanosine derivatives, the major small-molecule components of the venom of *Loxosceles reclusa* (Schroeder et al. 2008). However, no correlation between loxoscelism and the presence of these molecules in the venom has been described. Additionally, the histamine content detected in *Loxosceles intermedia* venom is high enough to induce inflammatory responses (Paludo et al. 2009).

As previously mentioned, *Loxosceles* venom is enriched in protein-based molecules. The 1D and 2D electrophoresis profiles of the components from *L. intermedia* venom revealed two main groups of proteins at 20–40 kDa and 2–5 kDa (Fig. 1). The 2D map also showed that most spots were localized between pI 6 and 10, but the more intense spots were localized at neutral pI (Fig. 1). The 2D protein *Loxosceles* venom profile is similar among distinct species, such as *L. intermedia*, *L. adelaida*, *L. similis*, *L. gaucho*, and *L. laeta* (Barbaro et al. 2005; Luciano et al. 2004; Machado et al. 2005; Trevisan-Silva et al. 2013). Many protein and peptide toxins present in *Loxosceles* venom have been described, and the biological and biochemical properties of these compounds have been reported, providing insight into the pathophysiology of envenomation (Chaim et al. 2011; Hogan et al. 2004). Toxins, such as metalloproteases (da Silveira et al. 2007a; Feitosa et al. 1998), serine proteases (Veiga et al. 2000b), phospholipase-D family proteins (Chaim et al. 2006; da Silveira et al. 2007b; Tambourgi et al. 2002), hyaluronidases (Barbaro et al. 2005; da Silveira et al. 2007c), and insecticidal peptides (de Castro et al. 2004), have been detected in *Loxosceles* venom. Proteome analysis also revealed metalloproteases, serine proteases, phospholipases-D, and hyaluronidases in *L. intermedia* venom (dos Santos et al. 2009).

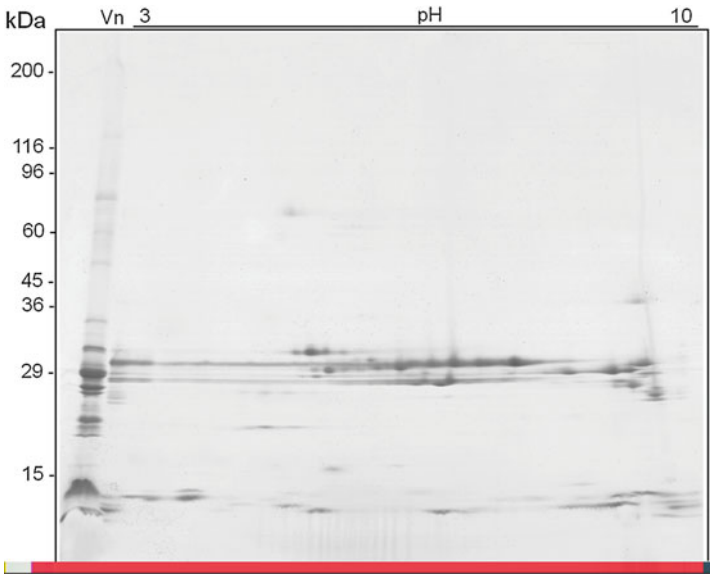

Fig. 1 Protein profiles from 1D and 2D gel electrophoresis analyses of *L. intermedia* crude venom. For 2D electrophoresis, samples of 150 μg of *L. intermedia* venom were subjected to isoelectric focusing (IEF) on 13-cm immobilized pH gradient gel strips with a pH linear range of 3–10. The second dimension analysis was performed on a linear SDS-PAGE gradient of 7.5–20 % under reducing conditions. The second dimension analysis was performed using isoelectric-focused venom and a sample of 20 μg of *L. intermedia* venom without IEF. The gels were stained with colloidal Coomassie Brilliant Blue. Vn indicates the 1D protein profile of *L. intermedia* venom. 3 pH 10 indicates the 2D protein profile of *L. intermedia* venom. Protein molecular mass markers are indicated on the left of the figure

More recently, transcriptome analyses were conducted to examine the protein content of *Loxosceles* venom obtained from the venom gland of two spider species: *L. laeta* and *L. intermedia* (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The results showed the high expression of phospholipase-D and metalloprotease toxin families, and for *L. intermedia*, transcripts encoding ICK peptides (insecticidal peptides) were also highly expressed. Other toxin families, such as serine proteases, hyaluronidases, venom allergens, TCTP (translationally controlled tumor protein), enzymatic inhibitors, and C-type lectins, showed low levels of expression (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). These cDNA libraries provided an overview of the *Loxosceles* venom and facilitated the description of new molecules of biotechnological interest. Figure 2 displays the expression profile of the toxin families produced by *L. intermedia* venom gland, highlighting the main groups of venom components. Since then, several components, i.e., TCTP, hyaluronidase, *Loxosceles intermedia* recombinant dermonecrotic toxin 7 (LiRecDT7), and U2-SCRTX-Li1b, have been further explored and produced as recombinant molecules (Ferrer et al. 2013; Matsubara et al. 2013; Sade et al. 2011; Vuitika et al. 2013). Together with recombinant phospholipase-D and

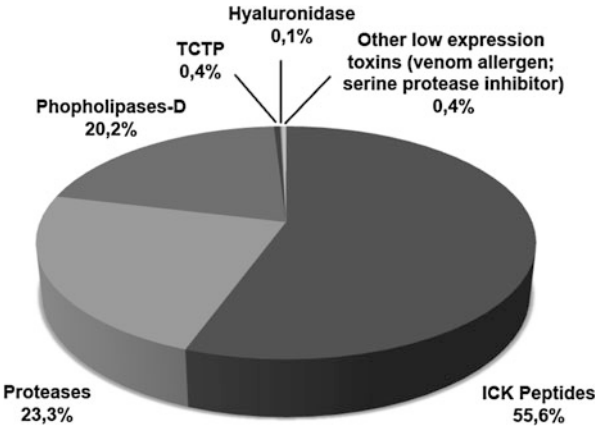

Fig. 2 Expression profile of *L. intermedia* venom toxin families over the total toxin-encoding sequences

metalloprotease LALP1 proteins, these recombinant toxins have been used as tools to provide further knowledge concerning venom activity and loxoscelism.

In this chapter, the relevance of these toxins to loxoscelism and the potential biotechnological application of these molecules are discussed.

Phospholipases-D

The phospholipase-D (PLD) family of toxins is the most well-studied and well-characterized component in the *Loxosceles* species venoms. These molecules have been reported to play an important role in the development of clinical sign and symptoms in loxoscelism. As these toxins induce dermonecrosis in vivo, the Brown spider PLDs are also known as dermonecrotic toxins (Chaim et al. 2011).

Gremski and coworkers (2010) revealed that 20.2 % of all the toxin-encoding ESTs from the *L. intermedia* venom gland correspond to PLDs, representing a significant proportion of the toxins present in the Brown spider venom (Fig. 2). Consistent with these data, Wille and coworkers (2013) conducted 2D electrophoresis analyses, showing at least 25 spots immunologically related to PLD toxins in the *L. intermedia* crude venom. Indeed, Machado and coworkers (2005) also identified at least 11 PLD isoforms, termed Loxonecrogin, in the venom of *L. gaucho*, suggesting the presence of several different PLDs in the Brown spider venom. Moreover, seven isoforms of PLD isoforms were generated as recombinant proteins, named LiRecDT (***L****oxosceles* ***i****ntermedia* ***rec****ombinant* ***d****ermonecrotic* ***t****oxin 1–7*), based on the *L. intermedia* venom gland transcriptome (Gremski et al. 2014), and these proteins have been classified as members of the Loxtox family (Kalapothakis et al. 2007). These recombinant PLDs were further examined to determine their roles in the inflammatory reaction, dermonecrosis, nephrotoxicity, and hemolysis observed in loxoscelism (Chaim et al. 2006; Chaves-Moreira et al. 2011; Luciano et al. 2004; Paludo et al. 2009).

Several PLD isoforms have also been identified in the venom of other *Loxosceles* species. In *L. reclusa* venom, the native PLDs were present at a molecular mass of approximately 32 kDa, and these four active isoforms induced dermonecrotic lesions, hemolysis, and platelet aggregation. Two PLD isoforms, at 32 and 35 kDa, were also described in *L. laeta* venom, and these proteins exhibited complement-dependent hemolysis, dermonecrosis, and sphingomyelin hydrolysis. In *L. laeta* venom two new PLD isoforms were reported: rLIPLD1, a dermonecrotic toxin with sphingomyelin hydrolysis activity, and rLIPLD2, a seemingly inactive protein, lacking the PLD region (i.e., rLIPLD2 lacked the initial amino acids of the catalytic site, such as His12). Several other PLD isoforms from the venom of other *Loxosceles* species, such as *L. gaucho*, have also been well characterized. It has been shown that recombinant isoforms of PLD from different species are able to reproduce most of the toxic effects observed after Brown spider bite and antigenic properties of the venom and can reverse venom effects with serum against them (Chaim et al. 2011; Gremski et al. 2014).

The PLDs are responsible for a large variety of disturbances in loxoscelism. Both native and recombinant PLDs trigger dermonecrotic lesions, increase vascular permeability, induce an intense inflammatory response at the inoculation site and at a systemic level, and promote platelet aggregation, hemolysis, nephrotoxicity, and even lethality in controlled experiments (Chaim et al. 2011; Gremski et al. 2014). In addition, recombinant *L. intermedia* phospholipase-D stimulates channel-mediated calcium influx into the cells via L-type calcium channels (Chaves-Moreira et al. 2011; Wille et al. 2013).

Initially, it was suggested that Loxtox protein family toxins played a role in the exclusive cleavage of the head-groups of sphingomyelin, the so-called sphingomyelinases. Further studies have shown that other substrates, such as glycerophospholipids and lysophospholipids, are also susceptible to PLD catalysis. Thus, the term phospholipase-D is more suitable for Brown spider dermonecrotic toxins (Chaim et al. 2010; Chaves-Moreira et al. 2011; Wille et al. 2013).

Studies comparing recombinant isoforms with distinct capacities for degrading substrates have shown differences in the intensity of the effects of these proteins (Chaim et al. 2010). Several recombinants of PLD isoforms from the *Loxosceles* genus have been heterologously produced in *E. coli*, and large amounts of the soluble and enzymatically active forms of these proteins are easily obtained. In addition, structural analysis of the catalytic site provided important insights into the enzymatic activities of each isoform (de Giuseppe et al. 2011; Murakami et al. 2005). Recently, de Giuseppe and coworkers (2011) published the crystal structure of LiRecDT1 from *L. intermedia*, indicating that this toxin contained an additional disulfide bond in the PLD structure catalytic loop compared with the previously described PLD from *L. laeta*. The structural details of PLD molecules reflect the distinct enzymatic behaviors of the venom from different species. PLDs with different structures could have different substrate affinities or enzymatic activities; therefore, these differences could explain the clinical symptoms or severity observed at the local bite site or the systemic effects during envenomation by different species of the *Loxosceles* genus.

Comparisons of the amino acid sequences of spider venom PLDs indicate that these proteins contain either 284 or 285 amino acids and display a significant degree of homology (de Santi Ferrara et al. 2009). The single polypeptide chain folds to form a distorted barrel, and the inner barrel surface is lined with eight parallel **β**-strands (termed A–H) linked by short flexible loops to eight **α**-helices (termed helices 1–8) that form the outer surface of the barrel (Murakami et al. 2005). The interconnecting loops are primarily hydrophilic and hydrophobic in the upper and lower sections, respectively. A short **β**-strand is inserted between strand B and helix 2, and two short helices are inserted between helix 3 and strand D and helix 4 and strand E. The catalytic loop is stabilized by a disulfide bridge (Cys51 and Cys57), and a second disulfide bridge (Cys53 and Cys201), which links the catalytic loop to the flexible loop to significantly reduce the flexibility of the latter loop, is present only in the class II enzymes (de Giuseppe et al. 2011). Because the **α**-helices, **β**-strands, and loops vary in length and character, the barrel is significantly distorted. The interior of the barrel is densely packed with hydrophobic amino acids, and in the short N-terminal section and the C-terminal extension, which contains a short helix, a **β**-strand and a random-coiled region cap the torus of the far side of the barrel. The surface loops forming the near side of the barrel are primarily hydrophobic, and a narrow cavity provides access to the catalytic site, characterized by a ring of negatively charged amino acids (de Giuseppe et al. 2011; Murakami et al. 2005). The catalytic and Mg^{2+} binding sites are located in a shallow depression and contain His12, Glu32, Asp34, Asp91, His47, Asp52, Trp230, Asp233, and Asn252, which are fully conserved in *Loxosceles* PLD isoforms (de Giuseppe et al. 2011; Murakami et al. 2005). Mutagenesis studies (de Giuseppe et al. 2011) and the crystal structure of PLDs (Murakami et al. 2005) indicate the involvement of two histidines in close proximity to the metal ion-binding site in the acid–base catalytic mechanism. Based on the structural results, His12 and His47 of PLD are key residues for catalysis and are assisted by a hydrogen bond network that involves Asp52, Asn252, and Asp233. Glu32, Asp34, Asp91, and solvent molecules coordinate the metal ion, which is essential for the catalytic activity of PLDs.

Furthermore, there are clear differences in the hydrolytic ability of PLD isoforms within the *Loxosceles* genus (Chaim et al. 2010; Chaim et al. 2011). Studies of LiRecDTs (isoforms 1–7) showed dermonecrosis at different levels in rabbit skin, consistent with the results of the spectrofluorimetric analysis of sphingomyelin hydrolysis (Chaim et al. 2011; Gremski et al. 2014). Ribeiro and coworkers (2007) reported that LiRecDT1 and LiRecDT2 were similar in all functional tests, including in vivo edema or cytotoxicity, while the effects of LiRecDT3 were significantly less intense. The observed amino acid alignment paralleled these results: LiRecDT1 and LiRecDT2 were markedly similar, but LiRecDT3 was slightly different. LiRecDT3 showed some important differences in hydrophobicity at the boundaries of the catalytic site, reflecting the differential performance of this enzyme (Ribeiro et al. 2007). In addition, site-directed mutagenesis of His12 in LiRecDT1, predicted to play a central role in catalysis, was not sufficient to completely abolish the catalytic activity of this enzyme. Moreover, the LiRecDT1H12A mutant isoform shows a dramatic reduction in enzymatic activity, but with no change in the secondary

structure, compared with LiRecDT1. Interestingly, although the mutant isoform did not induce the same level of any activity examined, attachment to the cell surface or to mobilized lipids remained unaltered (Chaim et al. 2010; Paludo et al. 2009; Wille et al. 2013). It is likely that other protein domains besides the catalytic cleft were preserved, and these domains might be relevant for interactions with the cell membrane or lipid substrates. PLD catalysis has primarily been associated with the release of lipid metabolites, which could modulate a wide range of biological events, such as the cell cycle, cell proliferation, cell differentiation processes, and cell death (Chaim et al. 2011; Gremski et al. 2014).

Dermonecrotic toxins are enzymes produced and secreted from Brown spider venom glands. These molecules catalyze the hydrolysis of phospholipids, such as sphingomyelin (SM), at a terminal phosphodiester bond to release choline and produce ceramide 1-phosphate (C1P) (Chaim et al. 2010; Chaves-Moreira et al. 2011; Wille et al. 2013). PLDs also mediate the Mg^{+2}-dependent hydrolysis of lysophosphatidylcholine (LPC) and release lysophosphatidic acid (LPA) (Chaves-Moreira et al. 2011; Horta et al. 2013; van Meeteren et al. 2004). These toxins are proteins that vary in molecular mass from 30 to 35 kDa and include a signal peptide followed by a propeptide. The amino acid sequences of PLDs are highly conserved (55–99 %), particularly in the residues around the catalytic cleft.

Studies have shown the upregulated expression of proinflammatory cytokines/chemokines after human fibroblasts are exposed to *L. reclusa* PLD (Dragulev et al. 2007), which hydrolyzes cell membrane SM into C1P, generating a receptor-dependent inflammatory response. This idea challenged the hypothesis that instead of C1P, LPA is a preferential product and bioactive metabolite, reflecting the relative abundance of LPC in the plasma as a substrate (van Meeteren et al. 2004). Recently, Horta and coworkers (2013) showed that cell death was induced through *L. similis* whole venom (LsV), and this effect was particularly observed with the recombinant isoform of *L. intermedia* PLD, recLiD1 (Kalapothakis et al. 2002). LPA released through the PLD activity of LsV and recLiD1 did not activate LPA receptors in the presence of an LPA1/LPA3 antagonist. This effect was indirectly observed through ELISA assays for IL-6, IL-8, CXCL1, and CXCL2. Moreover, these authors did not detect a role for LPA in the apoptosis induced by LsV or recLiD1 in fibroblasts and endothelial cells in vitro, which might be associated with other LPA-independent stimuli or the effect of C1P on the cell membrane receptors, as previously described. The variety of molecular mechanisms triggered through *Loxosceles* PLDs and their lipid metabolites should be further investigated as a complex event dependent on the types of cells involved, the abundance and availability of the lipid substrate, and intracellular signaling cascades. PLDs can serve as biotools to examine cell–cell communication via cell membranes in the context of inflammation. PLD isoforms have been proposed as potential models for designer drugs or other biotechnological applications (Chaim et al. 2011; Gremski et al. 2014). The production of more stable PLD isoforms with enhanced enzymatic activity would greatly contribute to many areas of toxinology and provide information to further the current understanding of the biochemical features, biological implications, and their related molecular mechanisms of PLDs.

Moreover, recombinant PLDs from *Loxosceles arizonica* also catalyze the transphosphatidylation of SM to generate cyclic ceramide phosphate (CC(1,3)P) and LPC to generate palmitoyl cyclic phosphatidic acid (CPA). The biological properties of cyclic phosphates are vastly different from their monoester counterparts, and these molecules might be relevant to the pathology of Brown spider envenomation (Lajoie et al. 2013).

Proteases

Proteases were first identified as components of *Loxosceles* venom in 1976 (Eskafi and Norment 1976). This study described the protease activity of *L. reclusa* crude venom against insect larvae using histochemical techniques. In addition, *L. reclusa* venom showed protease activity on distinct L-aminoacyl-β-naphthylamide derivatives, and L-leucyl-β-naphthylamide was more susceptible to the action of *L. reclusa* proteases. Subsequently, caseinolytic activity was reported in the venom of three *Loxosceles* species: *L. gaucho*, *L. laeta*, and *L. intermedia* (Chaim et al. 2011; Gremski et al. 2014). Two classes of proteases, metalloproteases and serine proteases, have been identified in *L. intermedia* venom (Feitosa et al. 1998; Veiga et al. 2000a).

Loxosceles metalloproteases were first described as two molecules of distinct molecular masses that degrade gelatin and exhibit clear substrate preferences (Feitosa et al. 1998). These metalloproteases were named Loxolysin A, a 20–28-kDa fibronectinolytic protease that is also responsible for the partial hydrolysis of fibrinogen (Aα and Bβ chains), and Loxolysin B, a 30–32-kDa gelatinolytic protease (Feitosa et al. 1998). Serine proteases have been detected in *L. intermedia* venom using zymography assays showing two gelatinolytic signals after trypsin activation (Veiga et al. 2000a). The biochemical nature of these proteases was characterized based on the total inhibition of gelatin hydrolysis using distinct serine-protease inhibitors, such as aprotinin, benzamidine, leupeptin, PMSF, and soybean-trypsin inhibitor (Veiga et al. 2000a). The biological functions of the proteases in *Loxosceles* venom remain unknown, but it has been suggested that these molecules might play a role in prey digestion and the deleterious effects observed after Brown spider envenomation. *Loxosceles* proteases might promote the diffusion of other venom toxins through the permeabilization of tissues and propagation of the toxic effects of other biologically active venom toxins. Moreover, these proteases could be important for processing other toxic proteins that become active after proteolysis (Barbaro et al. 2005; Chaim et al. 2011; da Silveira et al. 2002; Feitosa et al. 1998; Gremski et al. 2014). Notably, studies on the proteases in *Loxosceles* venom are scarce, and there have been more reports on the identification of metalloproteases than serine proteases.

Complementary data from *Loxosceles* serine proteases was obtained from transcriptome and proteome analyses (dos Santos et al. 2009; Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The transcriptome analysis of the *L. laeta* venom gland revealed fourteen ESTs that displayed similarity to serine-protease sequences

from the database (Fernandes-Pedrosa et al. 2008). In addition, the transcriptome analysis of the *L. intermedia* venom gland uncovered five transcripts with similarity to distinct members of serine-protease families (Gremski et al. 2010). These toxins are poorly expressed in *L. intermedia* venom, representing 0.3 % of the toxin transcripts (Gremski et al. 2010). These findings are consistent with the results of a proteome study of *L. intermedia* venom, in which five peptide sequences with similarities to snake venom proteases were identified (dos Santos et al. 2009). A previous analysis of the bidimensional protein profile of *L. intermedia*, *L. laeta*, and *L. gaucho* venoms showed spots with molecular masses corresponding to the identified serine-protease activities (Machado et al. 2005). However, the biological functions of *Loxosceles* serine proteases remain unknown. It has been suggested that this class of toxins are important not only for prey digestion but also for local tissue toxicity and hematological disturbances, such as blood coagulation and fibrinolysis, during Brown spider envenomation (Gremski et al. 2014; Veiga et al. 2000a). Indeed, the serine proteases in snake venom possess thrombin-like fibrinogenase and plasminogen-activating activities (Gremski et al. 2014). Because serine proteases present diverse structures, functions, and modes of action, further studies on *Loxosceles* serine proteases are imperative to obtain a better understanding of loxoscelism pathology and to develop potential novel therapeutic agents. Indeed, the potential therapeutic use of snake venom serine proteases in vascular diseases, such as in myocardial infarction, ischemic stroke, and thrombotic diseases, has been previously studied. Reptilase®, used to determine fibrinogen levels in the presence of heparin, is a thrombin-like serine protease purified from *Bothrops atrox* snake venom. Additionally, recombinant serine proteases have been studied in both basic and clinical research. A thrombin-like enzyme from the venom of an *Agkistrodon rhodostoma* viper improves the outcomes after cerebral stroke in humans, and Defibrase, an enzyme from the venom of *Bothrops*, is clinically beneficial in ischemic stroke (Chaim et al. 2011; Gremski et al. 2014).

Two metalloproteases have been identified in *L. rufescens* venom, a 23-kDa fibrinogenolytic protease and a 27.5-kDa gelatinolytic protease, and enzymatic activity was inhibited using 1,10-phenanthroline, thereby confirming the metalloprotease activity of these proteins (Young and Pincus 2001). The degradation of Aα and Bβ fibrinogen chains has been reported in the venom of both *L. reclusa* and *L. laeta*, and this activity was also inhibited with 1,10-phenanthroline. Other extracellular matrix molecules have been demonstrated as substrates of *Loxosceles* metalloproteases, such as entactin and heparan sulfate proteoglycans (Veiga et al. 2000b). It has been recently suggested that the *Loxosceles* metalloproteases identified in these studies were derived from gastric contents during venom extraction and not components of the venom. Nevertheless, the venom collected through electrostimulation and macerated venom glands were compared, and the protein profile and the protease activity of both samples were similar (da Silveira et al. 2002). Moreover, the distinct proteolytic profiles of the gastric content of *L. intermedia* and the venom obtained through both electrostimulation and macerated glands have been examined, and the results confirmed that metalloproteases are venom components (da Silveira et al. 2002). Consistently, the

proteolytic effect of *L. rufescens* venom was observed in venom obtained from the microdissection of the venom glands, a procedure that ensures the absence of gastric contaminants (Young and Pincus 2001). Evidence of *Loxosceles* metalloproteases has continued to increase, and *L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta*, and *L. reclusa* venoms showed in vitro protease activities primarily between 18.1 and 31.8 kDa (Barbaro et al. 2005). Thus far, all *Loxosceles* venoms have exhibited gelatinolytic, caseinolytic, and fibrinogenolytic activities, and most of these activities were abolished with 1,10-phenanthroline (Barbaro et al. 2005).

The analysis of a cDNA library of *L. intermedia* venom glands revealed a sequence encoding a metalloprotease presenting the hallmarks of astacin family enzymes: a catalytic domain of 18 amino acids (HEXXHXXGXXHEXXRXDR) and a conserved methionine involved in a sequence turn, met-turn (MXY) (Bond and Beynon 1995; da Silveira et al. 2007a). The zinc-dependent astacin (Merops M12A family) proteases have been described in most organisms from yeasts to humans. Although astacin family members are structurally related to metalloproteases, these enzymes present extremely diverse biological functions, including peptide hydrolysis, growth factor activation, and extracellular matrix component processing (Bond and Beynon 1995). In addition, these enzymes carry the name astacin based on the prototype enzyme isolated from the gastric fluid of the crayfish *Astacus astacus*, which played a role in digestion (Bond and Beynon 1995). The *L. intermedia* astacin LALP (*Loxosceles* astacin-like protease) was the first astacin family member identified as a constituent of animal venom. Two new astacin protease isoforms have recently been identified as toxins in *L. intermedia* venom, namely, LALP2 and LALP3. In addition, astacins have also been identified in *L. laeta* (LALP4) and *L. gaucho* (LALP5) venoms, suggesting the existence of an interspecies toxin family and revealing the importance of these metalloproteases as components of *Loxosceles* venom (Chaim et al. 2011; Gremski et al. 2014). Interestingly, the transcriptome analysis of *L. intermedia* and *L. laeta* venom glands revealed that astacin metalloproteases are highly expressed toxins (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). In the *L. intermedia* venom gland, astacin transcripts comprise 22.6 % of the toxin-encoding transcripts and 8 % of the total transcripts in the *L. laeta* venom gland (Chaim et al. 2010; Fernandes-Pedrosa et al. 2008). Thus, *Loxosceles* proteases (metalloproteases and serine proteases) account for 23.1 % of the total toxin-encoding transcripts in the *L. intermedia* venom gland, second only to insecticidal peptide sequences, which comprise the majority of expressed toxins (Fig. 2). In addition, the analysis of astacin content in *L. intermedia*, *L. laeta*, and *L. gaucho* venoms, using two-dimensional Western blotting and zymography, demonstrated a high content of active astacins among the three analyzed venoms, consistent with the high mRNA expression of astacins reported in the transcriptome analysis (Trevisan-Silva et al. 2013). Moreover, this study showed a distinct and complex proteolytic profile of *Loxosceles* astacin-like proteases, highlighting the importance of this toxin family to Brown spider biology and loxoscelism toxicity (Trevisan-Silva et al. 2013).

The functional characterization of isolated *Loxosceles* astacins has only been described for the first recombinant astacin isoform from *L. intermedia* venom

(da Silveira et al. 2007a). LALP hydrolyzes fibrinogen, fibronectin, and gelatin, consistent with previous data from distinct *Loxosceles* crude venoms (da Silveira et al. 2007a). Moreover, rabbit subendothelial cells exposed to LALP showed morphological alterations, such as the loss of substrate adhesion and cells that were fully rounded up and lysed, demonstrating the cytotoxic effect induced by LALP (da Silveira et al. 2007a). These results further suggest that metalloproteases could play a role in the toxic effects of Brown spider bites and also indicate the potential of these toxins for biotechnological applications (Chaim et al. 2011; da Silveira et al. 2007a; Gremski et al. 2014). Therefore, additional studies are required to explore the use of *Loxosceles* astacins as tools for the diagnosis of loxoscelism, basic research protocols, and the design of new drugs as agonist and/or inhibitor molecules. Additionally, astacins could be used as thrombolytic agents, and astacin inhibitors could be developed for use in the prevention of atherosclerosis.

Hydrolases (Hyaluronidases)

Hyaluronidases are a class of enzymes widely distributed throughout the animal kingdom. Hyaluronidases primarily degrade hyaluronic acid (HA), a ubiquitous component of the vertebrate extracellular matrix, and might also present hydrolytic activity upon chondroitin, chondroitin sulfate (CS), and dermatan sulfate (DS). Studies on several animal venoms, such as snakes, scorpions, spiders, bees, caterpillars, wasps, cone snails, fish, lizards, and stingrays, have described hyaluronidase activity. These matrix-degrading enzymes have been proposed to act as "spreading factors" in venom, as these enzymes promote tissue disorganization, facilitating the increased diffusion of other toxins throughout the tissues of the bite victim (Chaim et al. 2011; Gremski et al. 2014).

Cutaneous loxoscelism is characterized by edema, erythema, and necrosis, indicating extracellular matrix disturbances potentially associated with the actions of venom proteases and hydrolases. Indeed, a previous study of *L. reclusa* venom demonstrated hyaluronidase activity upon HA and CS types A, B, and C (Wright et al. 1973). The estimated molecular masses of the purified hyaluronidases were 33 and 63 kDa. Additionally, it has been demonstrated that rabbit antivenom serum inhibited both hyaluronidase activity in vitro and the spreading effect induced by the whole venom in vivo (Wright et al. 1973). HA-substrate SDS-PAGE was used to describe the hyaluronidase activity of a 32.5-kDa protein identified in *L. rufescens* venom (Young and Pincus 2001). Moreover, zymography assays revealed a 44-kDa hyaluronidase responsible for HA degradation in the venom obtained from five *Loxosceles* species of medical importance in the Americas (*L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta*, and *L. reclusa*) (Barbaro et al. 2005). These data suggest the biological conservation and significance of *Loxosceles* hyaluronidases (Barbaro et al. 2005). Two hyaluronidase molecules of 41 and 43 kDa were characterized as pH-dependent endo-β-*N*-acetyl-d-hexosaminidases in *L. intermedia* venom (da Silveira et al. 2007a). These enzymes degraded

HA and CS in vitro and HA in rabbit skin (da Silveira et al. 2007a). Consistently, a proteomics study also described the presence of hyaluronidases in *Loxosceles* venoms (dos Santos et al. 2009). *Loxosceles* hyaluronidases exhibit high activity, requiring few micrograms of the total venom to identify the enzymatic activity (Barbaro et al. 2005; da Silveira et al. 2007a). The transcriptome analysis of *L. laeta* and *L. intermedia* venom glands showed that this class of toxins is expressed at low levels (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). In the *L. laeta* venom gland, hyaluronidases represent only 0.13 % of the total expressed sequences (Fernandes-Pedrosa et al. 2008). The transcriptome of *L. intermedia* identified a transcript with similarity to hyaluronoglucosaminidase 1 from *Rattus norvegicus* (gbjEDL77243.1), which represents 0.1 % of the total toxin-encoding sequences (Gremski et al. 2010). The low expression of hyaluronidase transcripts might reflect the difficulties associated with the purification of this native enzyme from *Loxosceles* venom, leading to the lack of the biological and biochemical characterization of this protein. Using molecular biology techniques, Ferrer and coworkers (2013) generated a recombinant *L. intermedia* venom hyaluronidase, presenting a calculated molecular mass of 46.1 kDa. The active enzyme, named Dietrich's hyaluronidase, was obtained after refolding in vitro and was able to degrade HA and CS. These results are consistent with previous reports of native hyaluronidases that degrade glycosaminoglycans, demonstrating that the recombinant hyaluronidase can also be considered a chondroitinase (Ferrer et al. 2013). The biological characterization of Dietrich's hyaluronidase showed an increase in the erythema, ecchymosis, and dermonecrotic effect induced with the recombinant dermonecrotic toxin (LiRecDT1) in rabbit skin (Ferrer et al. 2013). This study greatly contributed to the understanding of loxoscelism pathology, showing that hyaluronidase is a spreading factor in *Loxosceles* venom (Ferrer et al. 2013). Additionally, active Dietrich's hyaluronidase can be used in further studies to examine the physiological and pathological role of hyaluronidases in HA degradation. The degradation of HA promotes tissue permeabilization and is involved in a great number of events, such as bacterial pathogenesis, fertilization, and cancer progression (Girish and Kemparaju 2007). Consequently, there are potential applications of hyaluronidases, including distinct surgical procedures, drug delivery, research oncology, and in vitro fertilization protocols (Chaim et al. 2011; Girish and Kemparaju 2007).

Inhibitor Cystine Knot (ICK) Peptides

Spiders are one of the most successful terrestrial predators, and the production of highly toxic venom to subdue prey and detain predators is one of the main features that contribute to the overall success of spiders (Windley et al. 2012). Most spider venoms contain small disulfide-rich peptide neurotoxins as major components, and these proteins are the largest and most extensively studied group of spider toxins (Windley et al. 2012).

Nearly 60 % of all spider venom toxins have three disulfide bridges, and these peptides primarily target voltage-activated ion channels. These peptides typically

contain a "disulfide pseudo-knot" and can be classified among toxins and inhibitory polypeptides with an "inhibitor cystine knot" (ICK) motif (Windley et al. 2012). The cystine knot is a structural motif with an embedded ring formed by two disulfide bonds that, together with their connecting backbone, are threaded by a third disulfide bond. This motif forms a structure comprising three antiparallel β-sheets (Daly and Craik 2011). In addition to ICK peptides, this same structural motif is present in two other families: the cyclic cystine knot (CCK) or cyclotide family and growth factor cystine knot (GFCK) family (Iyer and Acharya 2011). The ICK scaffold renders these peptides highly resistant to extremes of pH, organic solvents, high temperatures, and protease activities, which makes these molecules adequate targets for the design of drugs, molecular tools, and bioinsecticides (Daly and Craik 2011). The homology between the amino acid sequences of ICK peptides is typically low, but the distribution of cysteine residues is generally conserved. The consensus sequence of peptides exhibiting the ICK motif is $CX_{3–7}CX_{3–8}CX_{0–7}CX_{1–4}CX_{4–13}C$ – where X is any amino acid. The biological activities of spider ICK peptides are quite diverse, showing activity at voltage-activated sodium, calcium, and potassium channels, acid-sensing ion channels, transient receptor potential channels, and mechanosensitive channels (Windley et al. 2012).

Castro and coworkers (2004) were the first to describe ICK peptides in *Loxosceles* venom, and these authors isolated three peptides, LiTx1–3, with insecticidal activity from the venom of *L. intermedia*. The amino acid sequences of these toxins were demonstrated to contain an ICK motif, structurally defining these proteins as ICK peptides. These components are polypeptides with molecular masses ranging from 5.6 to 7.9 kDa, presenting insecticidal activity against highly destructive pests, such as *Spodoptera frugiperda* and *Spodoptera cosmioides*. Further analysis of the sequences revealed potential posttranslational modification regions, such as *N*-myristoylation, amidation, and casein kinase II phosphorylation sites, in the sequences of LiTx1–3. Based on the sequences of these toxins, the authors proposed that LiTx3 might act on NaV channels and both LiTx2 and LiTx3 might act on NaV or CaV channels (de Castro et al. 2004). Subsequently, these authors also described the sequence of another ICK peptide from *L. intermedia*, LiTx4. Analysis of the *L. intermedia* venom gland transcriptome showed that ICK peptides comprise 55,6 % of toxin-encoding mRNAs (Fig. 2). The most abundant toxin transcripts were similar to LiTx3 (32 %), followed by LiTx2 (11.4 %), LiTx1 (6.2 %), and LiTx4 (3.7 %) (Gremski et al. 2010). Additionally, 2.3 % of the toxin-encoding mRNAs represent transcripts similar to the ICK neurotoxin Magi3 from *Macrothele gigas*, which binds to site 3 of NaV channels. The transcriptome analysis of the *L. laeta* venom gland showed that 0,2 % of all toxin transcripts were similar to Magi3 (Fernandes-Pedrosa et al. 2008). Further analysis of the ICK peptide mRNAs from the *L. intermedia* venom gland revealed several sequences similar to LiTx3 that have not yet been described, potentially representing novel ICK neurotoxins (Gremski et al. 2010). Indeed, the cloning and production of a recombinant peptide from *L. intermedia* venom with high similarity to the ICK family of peptides, particularly LiTx3, has been described (Matsubara et al. 2013).

The recombinant peptide, named U2-sicaritoxin-Li1b (U2-SCRTX-Li1b), was used to demonstrate the antigenic cross-reactivity of antisera raised against the crude venom of *L. intermedia*, *L. gaucho*, and *L. laeta* with U2-SCRTX-Li1b. This cross-reactivity confirms the presence of ICK-like toxin members in these *Loxosceles* venoms, consistent with the idea that this toxin family is widespread throughout the genus (Matsubara et al. 2013).

Currently, there is no evidence of the involvement of Brown spider ICKs in the pathogenesis of spider bites (Gremski et al. 2014). There is increasing interest in toxins presenting the ICK motif, as the molecules exhibit marked stability and target ion channels. These features suggest that these molecules might play an important role in drug design and therapeutic applications and the development of bioinsecticides and pharmacological tools to study the pharmacology and biophysics of ion channels (Windley et al. 2012).

Translationally Controlled Tumor Protein (TCTP)

The *L. intermedia* venom gland transcriptome analysis described the sequence of a protein identified as a member of the TCTP family and revealed that this TCTP is present at relatively low levels in the venom, i.e., only 0,4 % of the toxin-encoding transcripts (Gremski et al. 2010). *Translationally controlled tumor proteins (TCTPs)* were first described as proteins that were regulated at the translational level. The *tumor* is derived from the first TCTP cDNA sequence described, which was obtained from a human mammary tumor (Bommer 2012). This protein has also been demonstrated as a histamine-releasing factor (HRF) and a fortilin (Bommer 2012). The *Loxosceles intermedia* TCTP was cloned and expressed as a heterologous protein in an *E. coli* expression system. The functional characterization of the recombinant protein, LiTCTP, showed that this toxin caused edema and enhanced vascular permeability (Sade et al. 2011). The cutaneous symptoms of envenomation with *Loxosceles* venom include erythema, itching, and pain. In some cases, *Loxosceles* spider bites can cause hypersensitivity or even allergic reactions. These responses could be associated with histaminergic events, such as an increase in vascular permeability and vasodilatation. LiTCTP could be associated with these deleterious venom activities, as this protein was identified in *L. intermedia* venom (Sade et al. 2011). Another *Loxosceles* TCTP has been described in the venom gland of *L. laeta* using transcriptome analysis (Fernandes-Pedrosa et al. 2008). A recent transcriptome analysis revealed a TCTP protein (named GTx-TCTP) in the venom gland and pereopodal muscle of the tarantula *Grammostola rosea* (Kimura et al. 2012).

Proteins of the TCTP superfamily have previously been described in the gland secretions of ixodid ticks and distinct spider species. In spiders, TCTP was first identified as the main pharmacological toxin in the venom gland of the wolf spider (*Lycosa godeffroyi*). The tarantula *Grammostola rosea* transcriptome analysis also identified the GTx-TCTP. Recently, TCTP proteins were identified in the venom gland of the spit spider (*Scytodes thoracica*). The three TCTP cDNA sequences identified in *S. thoracica* were 86 % identical to LiTCTP (Zobel-Thropp et al. 2014).

TCTP family members have been described as extracellular HRFs and are associated with the allergic reactions of parasites. The TCTPs are completely conserved among species from the same genus. The LiTCTP phylogeny tree demonstrates the similarities with the TCTPs from ixodid ticks, which were also characterized as HRFs (Sade et al. 2011). Ultrastructural studies of the *L. intermedia* venom gland revealed that TCTPs and other constituents of the whole venom are secreted through holocrine secretion (Gremski et al. 2010). TCTP secretion proceeds via an ER-/Golgi-independent or nonclassical pathway, most likely mediated through secreted vesicles, known as exosomes (Hinojosa-Moya et al. 2008). TCTP mRNAs do not encode a signal sequence, and no precursor protein has been described; however, a TCTP protein has been identified in the biological fluid of asthmatic or parasitized patients, the saliva of ticks (Hinojosa-Moya et al. 2008), and the crude venom of spiders. TCTPs represent a large, highly conserved, and ubiquitous protein family in eukaryotes, and these proteins are widely expressed in various tissues and cell types. TCTP protein levels are highly regulated in response to a wide range of extracellular signals and cellular conditions, suggesting that these proteins participate in various biological functions at diverse biochemical and signaling pathways. Indeed, multiple functions and biochemical roles for TCTPs have previously been examined (Chaim et al. 2011; Gremski et al. 2014).

TCTP proteins have already been described as calcium-binding proteins and as proteins that interact with the cytoskeleton through the binding and stabilization of microtubules. The involvement of TCTPs in the mitotic spindle has also been shown, and TCTPs are now considered as regulators of mitosis. A crucial role for TCTPs in early development has also been described. The loss of TCTP expression in mice leads to increased spontaneous apoptosis during embryogenesis and causes lethality. TCTP can be described as a multifunctional protein, reflecting the high number of protein partners and several areas/pathways of cell metabolism involving this protein (Amson et al. 2012). TCTPs are also a therapeutic target in cancers. The downregulation of TCTP has been implicated in biological models of tumor reversion, and these proteins are the targets of various anticancer drugs (Amson et al. 2012).

Studying LiTCTPs can elucidate the biological aspects of loxoscelism, particularly those aspects associated with histaminergic symptoms. Moreover, the analysis of LiTCTP can provide new insights into the functional characterization of the TCTP family. LiTCTP represents a promising tool for studies in toxinology and immunological and allergenic and experimental oncology.

Putative Toxins

The overall composition of *Loxosceles* venoms has been recently studied using proteome and transcriptome analyses (dos Santos et al. 2009; Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The results of these studies have not only uncovered the sequences of several toxins previously implicated in the pathophysiology of loxoscelism, such as phospholipases-D and metalloproteases, but sequences that

might represent proteins with putative roles in envenomation, i.e., putative toxins, have also been revealed.

Sequences with significant similarities to allergen-like toxins from other venoms were detected in the transcriptome studies of *L. laeta* and *L. intermedia* venom glands (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). In both cases, these transcripts are poorly expressed. The sequences identified in the *L. intermedia* transcriptome encode cysteine-rich venom allergens that show significant similarities to allergens from another spider genus (*Lycosa singoriensis*), as well as scorpions and mite allergens (Gremski et al. 2010). The amino acid sequence of a putative allergen from *L. laeta* venom is similar to venom allergen III (sp|P35779|VA3_SOLRI) and includes the presence of conserved cysteine residues (Fernandes-Pedrosa et al. 2008). In addition, the *L. intermedia* proteome analysis revealed a putative allergenic protein similar to a mite allergen (dos Santos et al. 2009). Indeed, allergic reactions following *Loxosceles* bites have been described in a few cases, as reviewed by Gremski and coworkers (2014). A fine macular or papular eruption appeared over the entire body in approximately 25 % of the published loxoscelism cases (Pippirs et al. 2009). Additionally, cases of AGEP (acute generalized exanthematous pustulosis) after accidents with *L. reclusa* and *L. rufescens* have been reported (Lane et al. 2011; Makris et al. 2009). A recombinant allergen from *L. intermedia* venom has previously been cloned, with a calculated molecular mass of 46.2 kDa and a predicted hydrophobic import signal to the endoplasmic reticulum (Ferrer VP and de Mari TL, 2014, personal communication). This recombinant protein might facilitate the investigation of the mechanisms underlying the allergic responses observed in loxoscelism cases and might be used for biomedical purposes in this field (Gremski et al. 2014).

Putative enzymatic inhibitors represented by transcripts with significant similarity to sequences encoding this group of proteins have been described in *Loxosceles* venom gland transcriptome analyses (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The sequences identified in the *L. laeta* transcriptome showed significant similarities with serine (or cysteine)-protease inhibitors of diverse species with different functions and activities associated with the inhibition of factor Xa (Fernandes-Pedrosa et al. 2008). These studies also revealed transcripts with sequences similar to the serpin Bl-Spn-1, which inhibits the proprotein-processing proteases PC1/3 and furin. The *L. intermedia* transcriptome analysis revealed an EST similar to protease inhibitors from the serpin family, as described in the *L. intermedia* proteome, which also showed sequences similar to inhibitors from the cystatin family and Kunitz-type inhibitors (dos Santos et al. 2009; Gremski et al. 2010). Thus, *Loxosceles* venom might contain protease inhibitors belonging to different groups. It has been suggested that venom protease inhibitors might protect the integrity of the toxins through resistance to prey proteases (dos Santos et al. 2009; Zhao et al. 2011). Venom protease inhibitors are potential candidates to mediate certain biological processes, because proteases are involved in several physiological processes and are excellent therapeutic targets.

The transcriptome analysis of the *L. laeta* venom gland revealed 15 clones (0,5 % of the total) with sequence similarities to C-type lectins from different

species, i.e., *Bos taurus* (mammal) and *Tachypleus tridentatus* (arthropod), among others (Fernandes-Pedrosa et al. 2008). These clones were classified as "possible toxins." Indeed, a recent study showed that a C-type lectin isolated from *Bothrops atrox* snake venom exhibited glycan-binding characteristics similar to endogenous mammalian glycan-binding proteins and induced significant changes in leukocyte migration and activation that might contribute to host inflammation following *B. atrox* envenomation (Sartim et al. 2014). The authors point that previous studies have also implicated venom C-type lectins in the inflammatory response. In addition, venom C-type lectins have been suggested as prospects for potential use in cancer therapy because these factors promote antiangiogenesis and interfere with tumor cell proliferation (Calderon et al. 2014).

Transcripts with similarity to a salivary protein from *Ixodes scapularis* ticks have also been identified in the *L. laeta* transcriptome and included in the group of "possible toxins" (Fernandes-Pedrosa et al. 2008). The authors suggested that these sequences might be associated with substances that prevent blood clotting in the saliva of this tick for feeding functions. In addition, sequences similar to chitinolytic enzymes, which catalyze the hydrolysis of chitin, a polysaccharide in the exoskeleton and gut lining of arthropods and fungi, have also been identified in the *L. laeta* transcriptome and classified as "possible toxins" (Fernandes-Pedrosa et al. 2008).

A group of transcripts similar to "5′-nucleotidase" was also detected in the *L. laeta* venom glands (Fernandes-Pedrosa et al. 2008). The authors pointed that this group of enzymes might affect hemostasis through the inhibition of platelet aggregation, as these enzymes deplete the ADP present in the plasma. Although these transcripts were classified as encoding "possible toxins," Sales and Santoro (2008) showed that the venom of *Loxosceles gaucho* lacks most nucleotidase and DNase activities.

The presence of phosphate-releasing enzymes has been reported in *L. gaucho* venom (Sales and Santoro 2008). This study showed that alkaline phosphatases are primarily involved in the degradation of nucleotides in *L. gaucho* venom. Indeed, the authors point that previous studies demonstrated that the venom of *L. reclusa* shows ATPase and alkaline phosphatase activities. However, the role of alkaline phosphatase in *Loxosceles* venoms remains unknown.

Conclusion and Future Directions

Studies on *Loxosceles* toxins are continuously increasing. In recent years, several new toxins have been identified in *Loxosceles* venom through data from molecular biology techniques, proteomic studies, and the characterization of recombinant toxins. Indeed, the identification, biological characterization, and structural analysis of *Loxosceles* toxins improved the current knowledge of the venom composition and participation of these toxins in loxoscelism. However, several molecules remain unidentified, without biological characterization and/or unknown mechanisms of action, particularly the toxins with low expression levels.

Therefore, studies focusing on the recombinant production of novel toxins or the production of increased amounts of some known toxins are imperative to characterize the influence of these toxins on the toxic effects of Brown spider venoms and to explore the putative biotechnological applications of these molecules. Additionally, the design of inhibitor molecules for different toxins could be used as a tool to elucidate the underlying mechanisms and elaborate basic and clinical research protocols.

Cross-References

► Shotgun Approaches for Venom Analysis

References

Abdulkader RC, Barbaro KC, Barros EJ, Burdmann EA. Nephrotoxicity of insect and spider venoms in Latin America. Semin Nephrol. 2008;28(4):373–82.

Amson R, Pece S, Marine JC, Di Fiore PP, Telerman A. TPT1/ TCTP-regulated pathways in phenotypic reprogramming. Trends Cell Biol. 2012;23(1):37–46.

Barbaro KC, Knysak I, Martins R, Hogan C, Winkel K. Enzymatic characterization, antigenic cross-reactivity and neutralization of dermonecrotic activity of five Loxosceles spider venoms of medical importance in the Americas. Toxicon. 2005;45(4):489–99.

Bommer U. Cellular function and regulation of the translationally controlled tumour protein TCTP. Open Allergy J. 2012;5:19–32.

Bond JS, Beynon RJ. The Astacin family of metalloendopeptidases. Protein Sci. 1995;4(7): 1247–61.

Calderon LA, Sobrinho JC, Zaqueo KD, de Moura AA, Grabner AN, Mazzi MV, Marcussi S, Nomizo A, Fernandes CF, Zuliani JP, Carvalho BM, da Silva SL, Stabeli RG, Soares AM. Antitumoral activity of snake venom proteins: new trends in cancer therapy. Biomed Res Int. 2014;2014:203639.

Chaim OM, Sade YB, da Silveira RB, Toma L, Kalapothakis E, Chavez-Olortegui C, Mangili OC, Gremski W, von Dietrich CP, Nader HB, Sanches VS. Brown spider dermonecrotic toxin directly induces nephrotoxicity. Toxicol Appl Pharmacol. 2006;211(1):64–77.

Chaim OM, da Silveira RB, Trevisan-Silva D, Ferrer VP, Sade YB, Boia-Ferreira M, Gremski LH, Gremski W, Senff-Ribeiro A, Takahashi HK, Toledo MS, Nader HB, Veiga SS. Phospholipase-D activity and inflammatory response induced by Brown spider dermonecrotic toxin: endothelial cell membrane phospholipids as targets for toxicity. Biochim Biophys Acta. 2010;1811(2):84–96.

Chaim OM, Trevisan-Silva D, Chaves-Moreira D, Wille AC, Ferrer VP, Matsubara FH, Mangili OC, da Silveira RB, Gremski LH, Gremski W, Senff-Ribeiro A, Veiga SS. Brown spider (Loxosceles genus) venom toxins: tools for biological purposes. Toxins (Basel). 2011;3(3): 309–44.

Chatzaki M, Horta CC, Almeida MO, Pereira NB, Mendes TM, Dias-Lopes C, Guimaraes G, Moro L, Chavez-Olortegui C, Horta MC, Kalapothakis E. Cutaneous loxoscelism caused by *Loxosceles similis* venom and neutralization capacity of its specific antivenom. Toxicon. 2012;60(1):21–30.

Chaves-Moreira D, Souza FN, Fogaca RT, Mangili OC, Gremski W, Senff-Ribeiro A, Chaim OM, Veiga SS. The relationship between calcium and the metabolism of plasma membrane phospholipids in hemolysis induced by Brown spider venom Phospholipase-D toxin. J Cell Biochem. 2011;112(9):2529–40.

da Silveira RB, dos Santos Filho JF, Mangili OC, Veiga SS, Gremski W, Nader HB, von Dietrich CP. Identification of proteases in the extract of venom glands from Brown spiders. Toxicon. 2002;40(6):815–22.

da Silveira RB, Wille AC, Chaim OM, Appel MH, Silva DT, Franco CR, Toma L, Mangili OC, Gremski W, Dietrich CP, Nader HB, Veiga SS. Identification, cloning, expression and functional characterization of an Astacin-like metalloprotease toxin from *Loxosceles intermedia* (Brown spider) venom. Biochem J. 2007a;406(2):355–63.

da Silveira RB, Pigozzo RB, Chaim OM, Appel MH, Silva DT, Dreyfuss JL, Toma L, Dietrich CP, Nader HB, Veiga SS, Gremski W. Two novel dermonecrotic toxins LiRecDT4 and LiRecDT5 from Brown spider (*Loxosceles intermedia*) venom: from cloning to functional characterization. Biochimie. 2007b;89(3):289–300.

da Silveira RB, Chaim OM, Mangili OC, Gremski W, Dietrich CP, Nader HB, Veiga SS. Hyaluronidases in *Loxosceles intermedia* (Brown spider) venom are endo-beta-*N*-acetyl-d-hexosaminidases hydrolases. Toxicon. 2007c;49(6):758–68.

Daly NL, Craik DJ. Bioactive cystine knot proteins. Curr Opin Chem Biol. 2011;15(3):362–8.

de Castro CS, Silvestre FG, Araujo SC, de Gabriel MY, Mangili OC, Cruz I, Chavez-Olortegui C, Kalapothakis E. Identification and molecular cloning of insecticidal toxins from the venom of the Brown spider *Loxosceles intermedia*. Toxicon. 2004;44(3):273–80.

de Giuseppe PO, Ullah A, Silva DT, Gremski LH, Wille AC, Chaves Moreira D, Ribeiro AS, Chaim OM, Murakami MT, Veiga SS, Arni RK. Structure of a novel class II phospholipase D: catalytic cleft is modified by a disulphide bridge. Biochem Biophys Res Commun. 2011;409(4):622–7.

de Oliveira KC, Goncalves de Andrade RM, Piazza RM, Ferreira Jr JM, van den Berg CW, Tambourgi DV. Variations in Loxosceles spider venom composition and toxicity contribute to the severity of envenomation. Toxicon. 2005;45(4):421–9.

de Santi Ferrara GI, Fernandes-Pedrosa Mde F, Junqueira-de-Azevedo Ide L, Goncalves-de-Andrade RM, Portaro FC, Manzoni-de-Almeida D, Murakami MT, Arni RK, van den Berg CW, Ho PL, Tambourgi DV. SMase II, a new sphingomyelinase D from *Loxosceles laeta* venom gland: molecular cloning, expression, function and structural analysis. Toxicon. 2009;53(7–8):743–53.

dos Santos LD, Dias NB, Roberto J, Pinto AS, Palma MS. Brown recluse spider venom: proteomic analysis and proposal of a putative mechanism of action. Protein Pept Lett. 2009;16(8):933–43.

Dragulev B, Bao Y, Ramos-Cerrillo B, Vazquez H, Olvera A, Stock R, Algaron A, Fox JW. Upregulation of IL-6, IL-8, CXCL1, and CXCL2 dominates gene expression in human fibroblast cells exposed to *Loxosceles reclusa* sphingomyelinase D: insights into spider venom dermonecrosis. J Invest Dermatol. 2007;127(5):1264–6.

Eskafi FM, Norment BR. Physiological action of *Loxosceles reclusa* (G&M) venom on insect larvae. Toxicon. 1976;14(1):7–13.

Feitosa L, Gremski W, Veiga SS, Elias MC, Graner E, Mangili OC, Brentani RR. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in Brown spider (*Loxosceles intermedia*) venom. Toxicon. 1998;36(7):1039–51.

Fernandes-Pedrosa F, Junqueira-de-Azevedo Ide L, Goncalves-de-Andrade RM, Kobashi LS, Almeida DD, Ho PL, Tambourgi DV. Transcriptome analysis of *Loxosceles laeta* (Araneae, Sicariidae) spider venomous gland using expressed sequence tags. BMC Genomics. 2008;9:279.

Ferrer VP, de Mari TL, Gremski LH, Trevisan Silva D, da Silveira RB, Gremski W, Chaim OM, Senff-Ribeiro A, Nader HB, Veiga SS. A novel Hyaluronidase from Brown spider (*Loxosceles intermedia*) venom (Dietrich's Hyaluronidase): from cloning to functional characterization. PLoS Negl Trop Dis. 2013;7(5):e2206.

Girish KS, Kemparaju K. The magic glue hyaluronan and its eraser Hyaluronidase: a biological overview. Life Sci. 2007;80(21):1921–43.

Gremski LH, da Silveira RB, Chaim OM, Probst CM, Ferrer VP, Nowatzki J, Weinschutz HC, Madeira HM, Gremski W, Nader HB, Senff-Ribeiro A, Veiga SS. A novel expression profile of

the *Loxosceles intermedia* spider venomous gland revealed by transcriptome analysis. Mol Biosyst. 2010;6:2403–16.

Gremski LH, Trevisan-Silva D, Ferrer VP, Matsubara FH, Meissner GO, Wille AC, Vuitika L, Dias-Lopes C, Ullah A, de Moraes FR, Chavez-Olortegui C, Barbaro KC, Murakami MT, Arni RK, Senff-Ribeiro A, Chaim OM, Veiga SS. Recent advances in the understanding of Brown spider venoms: from the biology of spiders to the molecular mechanisms of toxins. Toxicon. 2014;83:91–120.

Hinojosa-Moya J, Xoconostle-Cazares B, Piedra-Ibarra E, Mendez-Tenorio A, Lucas WJ, Ruiz-Medrano R. Phylogenetic and structural analysis of translationally controlled tumor proteins. J Mol Evol. 2008;66(5):472–83.

Hogan CJ, Barbaro KC, Winkel K. Loxoscelism: old obstacles, new directions. Ann Emerg Med. 2004;44(6):608–24.

Horta CC, Oliveira-Mendes BB, do Carmo AO, Siqueira FF, Barroca TM, dos Santos Nassif Lacerda SM, De Almeida Campos Jr PH, de Franca LR, Ferreira RL, Kalapothakis E. Lysophosphatidic acid mediates the release of cytokines and chemokines by human fibroblasts treated with loxosceles spider venom. J Invest Dermatol. 2013;133(6):1682–5.

Isbister GK, Fan HW. Spider bite. Lancet. 2011;378(9808):2039–47.

Iyer S, Acharya KR. Tying the knot: the cystine signature and molecular-recognition processes of the vascular endothelial growth factor family of angiogenic cytokines. FEBS J. 2011;278 (22):4304–22.

Kalapothakis E, Araujo SC, de Castro CS, Mendes TM, Gomez MV, Mangili OC, Gubert IC, Chavez-Olortegui C. Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of *Loxosceles intermedia* spider venom. Toxicon. 2002;40(12):1691–9.

Kalapothakis E, Chatzaki M, Goncalves-Dornelas H, de Castro CS, Silvestre FG, Laborne FV, de Moura JF, Veiga SS, Chavez-Olortegui C, Granier C, Barbaro KC. The Loxtox protein family in *Loxosceles intermedia* (Mello-Leitao) venom. Toxicon. 2007;50(7):938–46.

Kimura T, Ono S, Kubo T. Molecular cloning and sequence analysis of the cDNAs encoding Toxin-Like peptides from the venom glands of Tarantula Grammostola rosea. Int J Pept. 2012;2012:731293.

Lajoie DM, Zobel-Thropp PA, Kumirov VK, Bandarian V, Binford GJ, Cordes MH. Phospholipase D toxins of Brown spider venom convert lysophosphatidylcholine and sphingomyelin to cyclic phosphates. PLoS One. 2013;8(8):e72372.

Lane L, McCoppin HH, Dyer J. Acute generalized exanthematous pustulosis and Coombs-positive hemolytic anemia in a child following *Loxosceles reclusa* envenomation. Pediatr Dermatol. 2011;28(6):685–8.

Lucato Jr RV, Abdulkader RC, Barbaro KC, Mendes GE, Castro I, Baptista MA, Cury PM, Malheiros DM, Schor N, Yu L, Burdmann EA. *Loxosceles gaucho* venom-induced acute kidney injury–in vivo and in vitro studies. PLoS Negl Trop Dis. 2011;5(5):e1182.

Luciano MN, da Silva PH, Chaim OM, dos Santos VL, Franco CR, Soares MF, Zanata SM, Mangili OC, Gremski W, Veiga SS. Experimental evidence for a direct cytotoxicity of *Loxosceles intermedia* (Brown spider) venom in renal tissue. J Histochem Cytochem. 2004;52(4):455–67.

Machado LF, Laugesen S, Botelho ED, Ricart CA, Fontes W, Barbaro KC, Roepstorff P, Sousa MV. Proteome analysis of Brown spider venom: identification of loxnecrogin isoforms in *Loxosceles gaucho* venom. Proteomics. 2005;5(8):2167–76.

Makris M, Spanoudaki N, Giannoula F, Chliva C, Antoniadou A, Kalogeromitros D. Acute generalized Exanthematous Pustulosis (AGEP) triggered by a spider bite. Allergol Int. 2009;58(2):301–3.

Matsubara FH, Gremski LH, Meissner GO, Constantino Lopes ES, Gremski W, Senff-Ribeiro A, Chaim OM, Veiga SS. A novel ICK peptide from the *Loxosceles intermedia* (Brown spider) venom gland: cloning, heterologous expression and immunological cross-reactivity approaches. Toxicon. 2013;71:147–58.

Murakami MT, Fernandes-Pedrosa MF, Tambourgi DV, Arni RK. Structural basis for metal ion coordination and the catalytic mechanism of sphingomyelinases D. J Biol Chem. 2005;280 (14):13658–64.

Palagi A, Koh JM, Leblanc M, Wilson D, Dutertre S, King GF, Nicholson GM, Escoubas P. Unravelling the complex venom landscapes of lethal Australian funnel-web spiders (Hexathelidae: Atracinae) using LC-MALDI-TOF mass spectrometry. J Proteomics. 2013;80:292–310.

Paludo KS, Biscaia SM, Chaim OM, Otuki MF, Naliwaiko K, Dombrowski PA, Franco CR, Veiga SS. Inflammatory events induced by Brown spider venom and its recombinant dermonecrotic toxin: a pharmacological investigation. Comp Biochem Physiol C Toxicol Pharmacol. 2009;149(3):323–33.

Pippirs U, Mehlhorn H, Antal AS, Schulte KW, Homey B. Acute generalized exanthematous pustulosis following a Loxosceles spider bite in Great Britain. Br J Dermatol. 2009;161:208–9.

Ribeiro RO, Chaim OM, da Silveira RB, Gremski LH, Sade YB, Paludo KS, Senff-Ribeiro A, de Moura J, Chavez-Olortegui C, Gremski W, Nader HB, Veiga SS. Biological and structural comparison of recombinant phospholipase D toxins from *Loxosceles intermedia* (Brown spider) venom. Toxicon. 2007;50(8):1162–74.

Sade YB, Boia-Ferreira M, Gremski LH, da Silveira RB, Gremski W, Senff-Ribeiro A, Chaim OM, Veiga SS. Molecular cloning, heterologous expression and functional characterization of a novel translationally-controlled tumor protein (TCTP) family member from *Loxosceles intermedia* (Brown spider) venom. Int J Biochem Cell Biol. 2011;44(1):170–7.

Sales PB, Santoro ML. Nucleotidase and DNase activities in Brazilian snake venoms. Comp Biochem Physiol C Toxicol Pharmacol. 2008;147(1):85–95.

Sartim MA, Riul TB, Del Cistia-Andrade C, Stowell SR, Arthur CM, Sorgi CA, Faccioli LH, Cummings RD, Dias-Baruffi M, Sampaio SV. Galatrox, a C-type lectin in Bothrops atrox snake venom, selectively binds LacNAc-terminated glycans and possesses the capacity to induce acute inflammation. Glycobiology. 2014.

Schroeder FC, Taggi AE, Gronquist M, Malik RU, Grant JB, Eisner T, Meinwald J. NMR-spectroscopic screening of spider venom reveals sulfated nucleosides as major components for the brown recluse and related species. Proc Natl Acad Sci U S A. 2008;105(38):14283–7.

Silvestre FG, de Castro CS, de Moura JF, Giusta MS, De Maria M, Alvares ES, Lobato FC, Assis RA, Goncalves LA, Gubert IC, Chavez-Olortegui C, Kalapothakis E. Characterization of the venom from the Brazilian Brown spider *Loxosceles similis* Moenkhaus, 1898 (Araneae, Sicariidae). Toxicon. 2005;46(8):927–36.

Tambourgi DV, Magnoli FC, van den Berg CW, Morgan BP, de Araujo PS, Alves EW, Da Silva WD. Sphingomyelinases in the venom of the spider *Loxosceles intermedia* are responsible for both dermonecrosis and complement-dependent hemolysis. Biochem Biophys Res Commun. 1998;251(1):366–73.

Tambourgi DV, De Sousa Da Silva M, Billington SJ, Goncalves De Andrade RM, Magnoli FC, Songer JG, Van Den Berg CW. Mechanism of induction of complement susceptibility of erythrocytes by spider and bacterial sphingomyelinases. Immunology. 2002;107(1): 93–101.

Trevisan-Silva D, Bednaski AV, Gremski LH, Chaim OM, Veiga SS, Senff-Ribeiro A. Differential metalloprotease content and activity of three Loxosceles spider venoms revealed using two-dimensional electrophoresis approaches. Toxicon. 2013;76:11–22.

van Meeteren LA, Frederiks F, Giepmans BN, Pedrosa MF, Billington SJ, Jost BH, Tambourgi DV, Moolenaar WH. Spider and bacterial sphingomyelinases D target cellular lysophosphatidic acid receptors by hydrolyzing lysophosphatidylcholine. J Biol Chem. 2004;279(12):10833–6.

Veiga SS, da Silveira RB, Dreyfus JL, Haoach J, Pereira AM, Mangili OC, Gremski W. Identification of high molecular weight serine-proteases in *Loxosceles intermedia* (Brown spider) venom. Toxicon. 2000a;38(6):825–39.

Veiga SS, Feitosa L, dos Santos VL, de Souza GA, Ribeiro AS, Mangili OC, Porcionatto MA, Nader HB, Dietrich CP, Brentani RR, Gremski W. Effect of Brown spider venom on basement membrane structures. Histochem J. 2000b;32(7):397–408.

Vuitika L, Gremski LH, Belisario-Ferrari MR, Chaves-Moreira D, Ferrer VP, Senff-Ribeiro A, Chaim OM, Veiga SS. Brown spider Phospholipase-D containing a conservative mutation (D233E) in the catalytic site: identification and functional characterization. J Cell Biochem. 2013;114(11):2479–92.

Wille AC, Chaves-Moreira D, Trevisan-Silva D, Magnoni MG, Boia-Ferreira M, Gremski LH, Gremski W, Chaim OM, Senff-Ribeiro A, Veiga SS. Modulation of membrane phospholipids, the cytosolic calcium influx and cell proliferation following treatment of B16-F10 cells with recombinant Phospholipase-D from *Loxosceles intermedia* (Brown spider) venom. Toxicon. 2013;67:17–30.

Windley MJ, Herzig V, Dziemborowicz SA, Hardy MC, King GF, Nicholson GM. Spider-venom peptides as bioinsecticides. Toxins (Basel). 2012;4(3):191–227.

Wright RP, Elgert KD, Campbell BJ, Barrett JT. Hyaluronidase and esterase activities of the venom of the poisonous brown recluse spider. Arch Biochem Biophys. 1973;159(1):415–26.

Young AR, Pincus SJ. Comparison of enzymatic activity from three species of necrotising arachnids in Australia: *Loxosceles rufescens*, *Badumna insignis* and *Lampona cylindrata*. Toxicon. 2001;39(2–3):391–400.

Zhao H, Kong Y, Wang H, Yan T, Feng F, Bian J, Yang Y, Yu H. A defensin-like antimicrobial peptide from the venoms of spider, Ornithoctonus hainana. J Pept Sci. 2011;17(7):540–4.

Zobel-Thropp PA, Correa SM, Garb JE, Binford GJ. Spit and venom from scytodes spiders: a diverse and distinct cocktail. J Proteome Res. 2014;13(2):817–35.