ELSEVIER

Comparative Biochemistry and Physiology Part B 135 (2003) 25–33

CBP

www.elsevier.com/locate/cbpb

The phylogenetic distribution of sphingomyelinase D activity in venoms of Haplogyne spiders

Greta J. Binford*, Michael A. Wells

Department of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ 85721, USA

Received 6 October 2002; received in revised form 8 February 2003; accepted 10 February 2003

Abstract

The venoms of *Loxosceles* spiders cause severe dermonecrotic lesions in human tissues. The venom component sphingomyelinase D (SMD) is a contributor to lesion formation and is unknown elsewhere in the animal kingdom. This study reports comparative analyses of SMD activity and venom composition of select *Loxosceles* species and representatives of closely related Haplogyne genera. The goal was to identify the phylogenetic group of spiders with SMD and infer the timing of evolutionary origin of this toxin. We also preliminarily characterized variation in molecular masses of venom components in the size range of SMD. SMD activity was detected in all (10) *Loxosceles* species sampled and two species representing their sister taxon, *Sicarius*, but not in any other venoms or tissues surveyed. Mass spectrometry analyses indicated that all *Loxosceles* and *Sicarius* species surveyed had multiple (at least four to six) molecules in the size range corresponding to known SMD proteins (31–35 kDa), whereas other Haplogynes analyzed had no molecules in this mass range in their venom. This suggests SMD originated in the ancestors of the *Loxosceles*/*Sicarius* lineage. These groups of proteins varied in molecular mass across species with North American *Loxosceles* having 31–32 kDa, African *Loxosceles* having 32–33.5 kDa and *Sicarius* having 32–33 kDa molecules.

Keywords: Evolutionary origin; *Loxosceles*; *Sicarius*; Toxin; Diversity; Variation; Comparative; Proteins

1. Introduction

Bites of spiders in the genus *Loxosceles,* the recluse or brown spiders, cause severe necrotic lesions and systemic effects in humans throughout the world (Atkins et al., 1958; recent reviews in White et al., 1995; Boyer et al., 2001). The genus *Loxosceles* includes nearly 100 described species whose native distribution is the Americas, Mediterranean Europe and Africa (Gertsch and Ennik, 1983). These spiders build small tangled webs in crevices, under rocks and debris, and in caves, and vary in their propensity toward mobility and association with people. For most *Loxosceles* species, venom characteristics have not been described. Thus, it is not known whether bites of all *Loxosceles* species are capable of causing dermonecrosis. Medically relevant bites have been reported from a few *Loxosceles* species from each of the continents where these spiders are native (White et al., 1995; Boyer et al. 2001). Furthermore, there is clinical and experimental evidence of dermonecrosis similar to loxoscelism resulting from bites from six-eyed crab spiders in the genus *Sicarius* (Newlands, 1982; Newlands and Atkinson, 1988; Van Aswegen et al., 1997), members of which are

*Corresponding author. Department of Biology, Lewis and Clark College, 0615 S.W. Palatine Hill Rd., Portland, OR, 97219, USA. Tel.: +1-503-768-7653; fax: +1-503-768-7658.
E-mail address: binford@lclark.edu (G.J. Binford).

1096-4959/03/$ - see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S1096-4959(03)00045-9

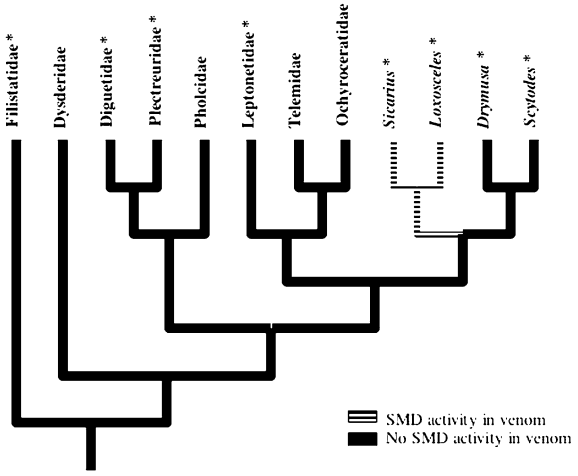

Fig. 1. The phylogenetic relationships of Haplogyne spiders based on Platnick et al. (1991). The presence of sphingomyelinase D in venoms is mapped onto the tree. Venoms and/or tissues were sampled for SMD activity from representatives of taxa marked with an asterisk.

thought to be the closest relatives of *Loxosceles* (Platnick et al., 1991) (Fig. 1). This suggests that dermonecrotic activity in venoms is found throughout *Loxosceles* and is present in close relatives outside of the genus.

Sphingomyelinase D (SMD) has been identified as an active venom component in *L. reclusa* venoms that is sufficient for causing lesion formation in mammalian tissues (Forrester et al. 1978; Kurpiewski et al., 1981; Tambourgi et al., 1998b; Pedrosa et al., 2002). SMD causes cell lysis by hydrolysis of the membrane phospholipid sphingomyelin (Geren et al., 1976; Forrester et al., 1978; Merchant et al., 1998) and complement-dependent hemolysis (Tambourgi et al., 1995, 1998b). Sphingomyelinases of comparable size have also been identified in the venoms of the South American *Loxosceles* species *L. intermedia*, *L. gaucho*, and *L. laeta* (Barbaro et al., 1996; Tambourgi et al., 1998a,b; Guilherme et al., 2001). In *L. reclusa*, four active sphingomyelinase forms of comparable size have been identified (Kurpiewski et al., 1981). In *L. intermedia* two functional sphingomyelinases and two homologs without sphingomyelinase D activity have been identified (Barbaro et al., 1996; Tambourgi et al., 1998b). Considerable N-terminal amino acid sequence homology has been noted among proteins derived from *L. intermedia*, *L. laeta*, *L. gaucho*, *L. reclusa*, *L. deserta* and *L. arizonica* (Cisar et al., 1989; Barbaro et al., 1996; Tambourgi et al., 1998b; Gomez et al., 2001; Pedrosa et al., 2002; Binford et al., unpublished data).

While cleavage of phospholipids is a common and necessary housekeeping phenomenon, cleavage at the D site (between the choline and phosphate) of these molecules is rare. Currently SMD activity is unknown elsewhere in the animal kingdom (Truett and King, 1993). Therefore the origin of SMD in venoms of the *Loxosceles* lineage represents a unique evolutionary event, the timing of which is unknown. The goals of this study were to: (1) survey for the presence of SMD activity in venoms and other tissues from a diverse group of *Loxosceles* and other closely related Haplogyne spiders; (2) estimate the timing of evolutionary origin of SMD within this clade and thereby infer the range of extant species with this enzyme in their venoms; (3) preliminarily examine patterns of variability of proteins in the molecular weight range of SMD across taxa that have SMD activity in their venoms. We found SMD activity only in venoms of the *Loxosceles* and *Sicarius* species surveyed. From this we infer that SMD originated once in the ancestors of the lineage including *Loxosceles* and *Sicarius.* Given the central role of SMD in dermonecrosis, this suggests that venoms of all species in this lineage are capable of causing dermonecrosis in mammalian tissues.

2. Materials and methods

2.1. Spider collection

All spiders used in this study were collected from the field by visually searching. Species names and collecting localities are listed in Table 1. Specific details of collecting localities are available from the author by request. Voucher specimens are maintained in the personal collection of G.J.B.

2.2. Venom collection

Spiders were anesthetized with CO_2, and venom was extracted by electrical stimulation. To avoid contamination, fangs were rinsed with distilled water, and regurgitate was collected by a vacuum attached to a blunt syringe needle held on spiders' mouths. Venom from the tips of the fangs was drawn into microcapillary tubes, pooled among individuals, and stored frozen at −80 °C until analysis. Spiders were fed 1 day after milking, and

Table 1
Species included in this study, their collection localities, and estimates of total venom protein per individual (average and standard deviation). Sample sizes (n) refer to the number of samples for which venom was quantified. Venom samples were pooled venoms collected from multiple female individuals from the same collecting site (four to 20 individuals), except for *P. tristis*, *K. arizonica* and *C. captiosus*, which were each single samples from one individual. Venom amounts per individual spider were estimated by dividing total venom protein per sample by the number of spiders milked per sample. There were no estimates of total venom protein per individual for *L. oasa* or *S. perfecta* because samples of venoms from these species were from tissue homogenates

Species	Collection locality	n	Total venom protein (μg/spider)
Loxosceles arizonica	Sta. Catalina Mtn Foothills, Tucson, Pima Co., Arizona, USA	4	28.1±5.8
Loxosceles deserta	Granite Mtns., Riverside Co., California, USA	3	45.1±11.9
Loxosceles apachea	Portal, Cochise Co., Arizona, USA	2	38.2±1.1
Loxosceles alamosa	Alamos, Sonora, Mexico	1	65
Loxosceles reclusa	Oxford, Lafayette Co., Mississippi, USA	3	30.0±5.8
Loxosceles rufescens KY	University of Kentucky, Lexington, Fayette Co., Kentucky (native to Europe)	3	25.0±11.5
Loxosceles rufescens IN	Indiana State House, Indianapolis, Marion, Co., Indiana (native to Europe)	3	34.6±3.3
Loxosceles laeta	Downtown Los Angeles, Los Angeles Co., California, USA (Native to S. America)	1	215.6
Loxosceles speluncarum	Cave, Groenkloof Nature Reserve, Fountain Valley, Gauteng, South Africa	3	29.4±7.6
Loxosceles sp. Hoogenoeg	Cave, Hoogenoeg, Strydpoort Mtns, Northern Province, South Africa	1	23.7
Loxosceles spinulosa	Ndumo Park, Kwazulu-Natal, South Africa	2	36.7±5.4
Sicarius hahni	Hoogenoeg, Strydpoort Mtns, Northern Province, South Africa	3	193.2±67.6
Sicarius testaceus	Oorloogskloop Nature Reserve, Northern Cape, South Africa	2	331.4±151.5
Drymusa capensis	Paradise Stream, Newland's Forest, Capetown, South Africa	3	265.1±118.0
Diguetia canites	Brown Canyon, Baboquivari Mtns., Pima Co., Arizona, USA	2	143.71±17.5
Plectreurus tristis	Burns Piñon Ridge Reserve, San Bernadino Co., California, USA	1	301.85
Kukulkania arizonica	Sta. Catalina Mtn Foothills, Pima Co., Tucson, Arizona, USA	1	72.2
Ctenus captiosus	Gainsville, Alachua Co., Florida, USA	1	604.25
Scytodes perfecta	University of Arizona, Pima Co., Tucson, Arizona, USA	2	–
Leptoneta oasa	Anrdeas Canyon, Palm Springs, Riverside Co., California, USA	1	–

were not milked again for over 1 month. The length of each spider from the dorsal, anterior tip of the cephalothorax to the posterior tip of the abdomen was measured after milking.

Only venoms from adult females were used in order to avoid potential confounding effects of ontogenetic or intersexual variation (Atkinson and Walker, 1985; Malli et al., 1993; de Andrade et al., 1999; Binford, 2001).

2.3. Protein quantification

Total protein concentration of venom samples was estimated following a modification of the Lowry method (Hartree, 1972). The average amount of total venom protein yielded per individual was estimated by dividing total protein per sample by the number of individuals milked per sample.

2.4. SMD enzyme analysis

All SMD activity assays in this study were done using an Amplex™ Red Phospholipase D assay kit (Molecular Probes A-12219) using sphingomyelin as the substrate in place of lysophosphatidylcholine. This assay specifically monitors SMD activity by using choline oxidase to oxidize free choline released after cleavage of the phosphodiester bond of sphingomyelin. Released hydrogen peroxide binds to Amplex™ Red resulting in a fluorescent molecule (resorufin). Reactions volumes were 200 μl and were incubated for 30 min at 37 °C before measuring fluorescence absorbance/emission at 563/587 nm (Perkin Elmer luminescence spectrometer LS50 B). Control assays were run with 1× Amplex™ Red buffer in place of venom each time SMD assays were done and were used to establish the baseline.

2.4.1. Crude venom

Crude venoms were diluted with 1× Amplex™ Red buffer to standard concentrations. Optimization of this assay indicated that concentrations of total venom protein greater than 0.5 μg resulted in inner filtering effects that confounded the ability to quantitatively compare concentration dependent SMD activity levels across samples in which this enzyme was present. Control assays of 1.4 μg *L. arizonica* venom using lysophosphatidylcholine as the substrate did not result in fluorescence intensity greater than the baseline. Controls using sphingomyelin as the substrate and 20 μg of bovine serum albumin also did not result in fluorescence intensity greater than the baseline.

2.4.2. Tissue homogenates

SMD activity was assayed from homogenates of venom glands of *Scytodes perfecta* and from whole cephalothorax homogenates of *Leptoneta oasa*. Crude venom was not easily obtainable from these species because *Scytodes* eject viscous glue when electrostimulated, and *L. oasa* are very small (total body length <2 mm) making venom milking or dissection of venom glands unfeasible. Homogenates of venom glands from *L. arizonica* and *Kukulkania arizonica* were used as positive and negative controls, respectively.

SMD activity in tissues other than venom glands was analyzed for *L. arizonica*, *S. perfecta*, and *K. arizonica*. For each of these species homogenates were made of the venom glands, the cephalothorax excluding the venom gland, and the abdomen. Tissues were homogenized in 1× Amplex™ Red reaction buffer with 25 ng/μl of protease inhibitor cocktail containing equal amounts of pepstatin A, antipain, chymostatin and leupeptin. Control reactions were done excluding sphingomyelin for each tissue sample.

2.5. Comparative protein mass determination

2.5.1. SDS-PAGE

Venom components were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide mini-gels. All analyses used 10 μg of crude venom per lane. All gels included 10 lanes with low range standards (2.75–43 kDa) (Gibco) on the far left lane and high range standards (14.3–200 kDa) (Gibco) on the far right lane, and were run using a tall, mini-gel apparatus (9×5×0.1 cm) (Hoeffer). Gels were stained with silver stain (Silver Staining basic protocol, *Current Protocols in Molecular Biology*, 1999).

2.5.2. Mass spectrometry

Comparative mass spectrometry analyses were done using Ciphergen protein chip surface-enhanced laser desorption/ionization (SELDI) mass spectrometry. Total venom protein (0.5 μg) from different species was loaded onto every other spot of a 24-spot H4 ProteinChip (Ciphergen) and coated with EAM1 (Ciphergen). These conditions

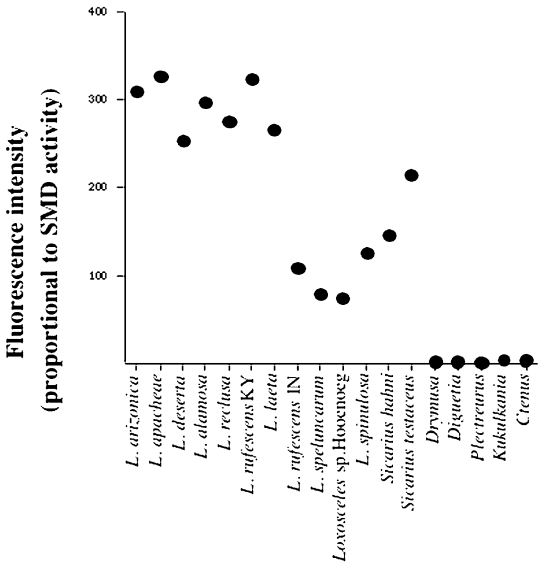

Fig. 2. Fluorescence intensities (correspond to SMD activity) resulting from Amplex Red assays of crude venoms. Each assay used 0.2 μg of total venom protein.

optimized detection of molecules corresponding to the size of SMD.

3. Results

3.1. Venom concentrations

Estimates of venom amounts obtained per milking per spider are listed in Table 1.

3.2. Sphingomyelinase D activity

The distribution of taxa from which representative individuals were screened for SMD activity in crude venoms or tissue homogenates is indicated in Table 1 and Fig. 1. All 10 *Loxosceles* species surveyed and both *Sicarius* species had SMD activity in their venom (Figs. 1 and 2). SMD assays of no other crude venoms resulted in fluorescence intensities above the baseline (Fig. 2). Assays of up to 2.0 μg of venom from *Drymusa capensis*, the closest relatives of *Loxosceles* and *Sicarius* for whom crude venom was analyzed, did not detect SMD activity. All SMD assays of tissue homogenates, venom gland tissue homogenates, and cephalothorax homogenates had fluorescence intensities above the baseline. Therefore, control assays were repeated without sphingomyelin substrate for each of these samples, and assays were done with 10 μg total protein. For every sample except *L. arizonica* venom gland extract, fluorescence intensities for reactions without sphingomyelin were greater than samples that included sphingomyelin (Fig. 3).

3.3. SDS-PAGE

SDS-PAGE separations of crude venoms of *Loxosceles* species, *Sicarius*, *Drymusa* and *Diguetia* are represented in Fig. 4. Proteins corresponding to the molecular weights of known sphingomyelinase D samples are clearly visible in all *Loxosceles* species and *Sicarius.* Neither *Drymusa* nor *Diguetia* have molecules of this molecular weight in their venoms.

3.4. SELDI mass spectrometry

Comparative mass spectrometry analysis detected proteins of molecular mass between 31 and 35 kDa in *Loxosceles* and *Sicarius* species (Fig. 5). There were no proteins in this mass range in venoms of any other species sampled (Fig. 5). The resolution was not sufficient to determine with confidence the number of molecules in this mass

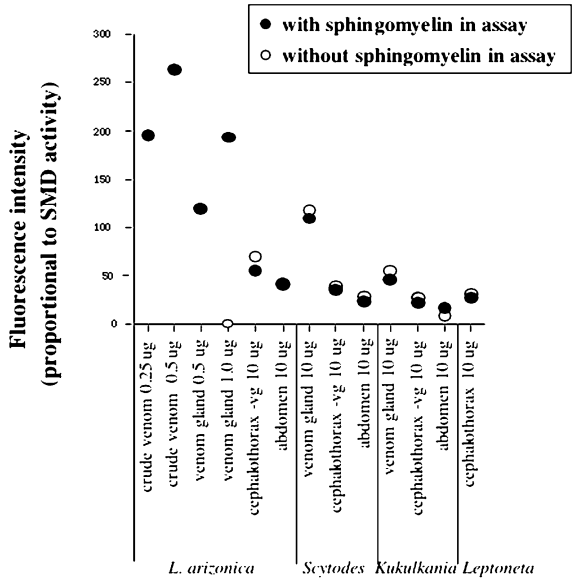

Fig. 3. Fluorescence intensities (correspond to SMD activity) resulting from Amplex Red assays of crude venom (*L. arizonica*) and tissue homogenates. 'Cephalothorax–vg' refers to homogenates of the cephalothorax after removal of the venom gland. The amount of total protein loaded is included for each sample.

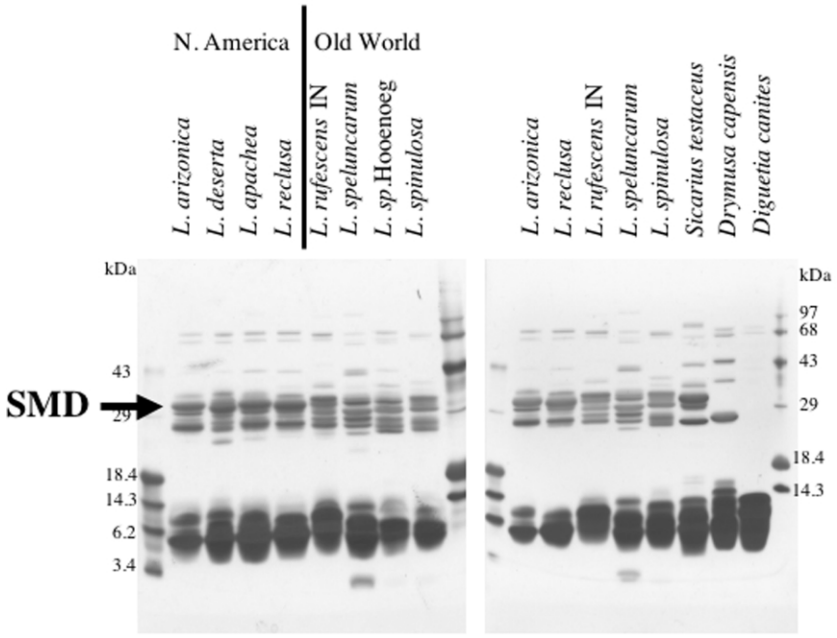

Fig. 4. SDS-PAGE (15% acrylamide) separations of crude venoms stained with silver. Ten micrograms of crude venom were loaded per lane.

range for any species. However, it is clear that all species with SMD activity in their venom had more than one molecule in this mass range (Fig. 5b). *Loxosceles* species in the *reclusa* and *deserta* species groups (North America) had proteins ranging in mass from 31 to 32 kDa. *L. rufescens* had proteins of 31–33.5 kDa. *Loxosceles* species native to Southern Africa and *Sicarius* had proteins ranging between 32 and 33.5 kDa (Fig. 5b).

4. Discussion

The enzyme sphingomyelinase D from *Loxosceles* venoms has received much research attention because of its causative effects in dermonecrosis. Because SMD is only known from *Loxosceles* venoms and a few species of pathogenic bacteria (Truett and King, 1993), the evolutionary origin of this enzyme presents an interesting puzzle. A central clue in deciphering this puzzle is to know when, or how many times, SMD originated in the lineage that includes *Loxosceles*. The data presented here indicate that within the Haplogyne lineage SMD activity is only present in venoms of *Loxosceles* and their sister genus *Sicarius.* Furthermore, molecules corresponding to the size of SMD in venoms were only present in *Loxosceles* and *Sicarius*. Given this distribution of data, the most parsimonious evolutionary scenario for the origin of this enzyme is a single origin in the most recent common ancestor of the *Loxosceles*/*Sicarius* clade (Fig. 1).

Although we have not done an exhaustive survey of all *Loxosceles* and *Sicarius* species, the taxa in our study include representatives of the four geographic regions to which *Loxosceles* are native (Mediterranean Europe—where *L. rufescens* are native, Africa, North and South America). Phylogenetic relationships among species have not been resolved, but the geographic diversity of our sampling makes it likely to represent phylogenetically disparate species. Given that all species analyzed from this clade had clear SMD activity in their venom, it is likely that SMD originated once in this lineage and has been retained in all extant species. More complete taxonomic surveys and mammalian bioassays are necessary to verify this; however, it is conservative from a medical perspective to conclude that all species in this clade have SMD in their venom and their bites are capable of causing dermonecrotic lesions.

It is striking that venoms of *L. laeta* and *Sicarius* yielded an order of magnitude more total venom protein upon electrostimulation than the other *Lox-*

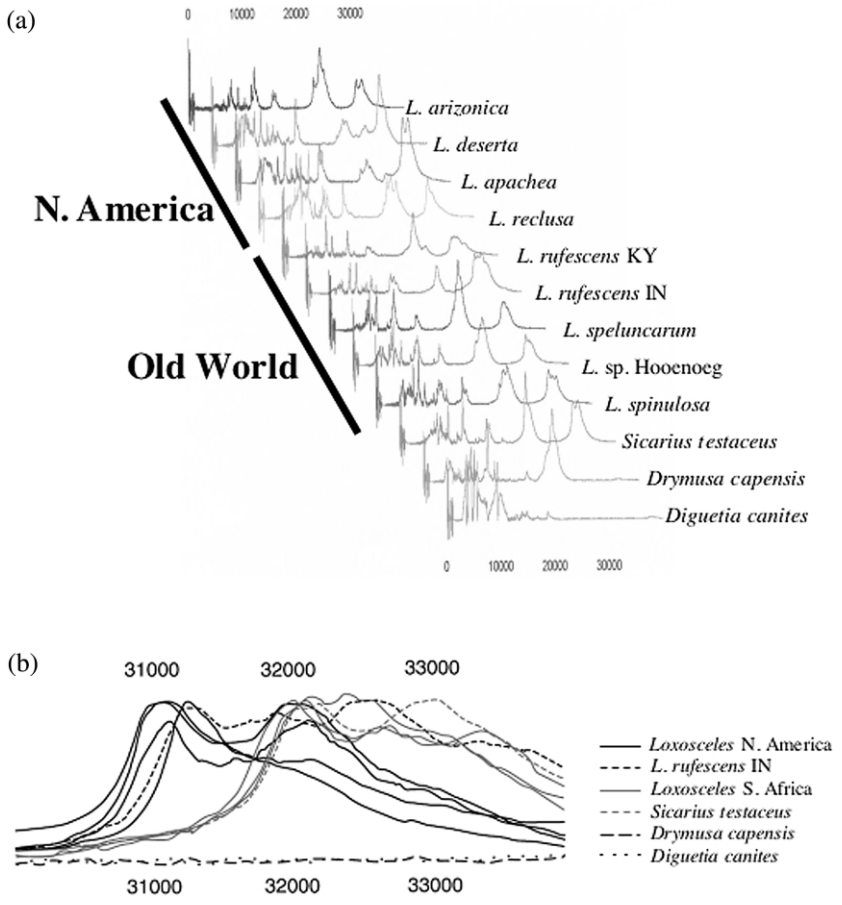

Fig. 5. Aligned spectra of molecular weights of venom proteins from SELDI mass spectrometry. Aliquots of 0.5 μg of total venom protein were loaded per sample. (a) Whole venom; (b) size region corresponding to known SMD samples.

osceles species surveyed (Table 1). Furthermore, SMD activity per unit total venom protein was comparable between these species and the *Loxosceles* species that have well-documented and serious dermonecrotic effects on human tissues. If the severity of lesion formation is positively correlated with absolute amounts of SMD, bites from *L. laeta* and *Sicarius* may be capable of inducing more severe reactions than other species. Analyses of the effects of *Sicarius* venoms in rabbits indicate that dermenecrotic lesions develop more rapidly after *Sicarius* bites than after bites of South African *Loxosceles* (Newlands, 1982; Newlands and Atkinson, 1990a). In fact, some African researchers have touted *Sicarius* as the most dangerous spider known; however, the biology of these animals makes human envenomation events rare (Newlands and Atkinson, 1990b).

While SMD activity was present in all *Loxosceles*/*Sicarius* species we surveyed, it is clear from this study and previous work (Kurpiewski et al., 1981; Tambourgi et al., 1998b, Alagón, personal communication) that there is variation in the number and molecular weights of SMD molecules across species. While our sampling was limited, variation among species appeared to have some extent of geographic signature (Fig. 5b). Venoms of North American species had proteins in the size range of SMD that were generally smaller (31–32 kDa) than those in venoms of Old World species (32–33.5 kDa). Evidence from protein characterization of venoms of South American *Loxosceles* suggests the presence of multiple active forms of SMD ranging from 32 to 35 kDa (Barbaro et al., 1996; Tambourgi et al., 1998b). There is evidence suggesting that both post-translational modification

and the expression of paralogous genes contribute to within-venom variation in SMD molecules, however, the relative contribution of these processes to variation is unknown. Post-translational modification is expected to contribute because glycosylation is reported as necessary for activity of *Loxosceles* venom components (Viega et al., 1999). Furthermore, there is mounting evidence that multiple paralogous genes are expressing SMD proteins in venoms as well as related proteins that do not have SMD activity (Tambourgi et al., 1988b; Pedrosa et al., 2002, personal observation). The extent to which molecular variation in SMD and related molecules affects the activity and or antigenic cross-reactivity of venoms across species is currently unknown.

Knowing when SMD originated in Haplogyne venoms not only facilitates a better understanding of the risks associated with bites of species in this lineage, it also creates a framework for studying the mechanism of evolutionary origin of this unique and toxic enzyme. One possible mechanism for the evolutionary origin of SMD in *Loxosceles*/*Sicarius* is via a change in expression pattern of a gene that was present in the lineage before SMD was expressed in venom gland tissues. The detection of SMD activity in tissues other than venom glands in close relatives of *Loxosceles* that do not have SMD activity in their venoms would be evidence supporting this hypothesis. Our lack of detection of SMD in tissues other than venom glands in either *Loxosceles* or close relatives is evidence against evolutionary origin by a change in expression pattern. This result coupled with evidence that SMD is a member of a gene family (Tambourgi et al., 1998b; Pedrosa et al., 2002 personal observation) and is likely a derived member of the glycerophosphoryl diester phosphodiesterase protein domain family (unpublished data), supports the hypothesis that SMD originated de novo in *Loxosceles*/*Sicarius* venoms.

Acknowledgments

Rupert Harris, Wessel Pretorius, Norman Larson, and Astri and John Leroy helped with collection of South African spiders. Rick Vetter collected many of the *L. deserta*, Gail Stratton collected the *L. reclusa,* and G.B. Edwards collected the *Ctenus captiosus.* Ian Jangaard helped with the Ciphergen mass spectrometry analysis. Leon Lotz helped with identification of South African species. This work was supported by a NIH training grant to Center for Insect Sciences (L. Tolbert P.I.) and NIH NRSA fellowship F32 GM20874-01 to GJB and MAW.

References

Atkins, J.A., Wingo, C.W., Sodeman, W.A., Flynn, J.E., 1958. Necrotic arachnidism. Am. J. Trop. Med. Hyg. 7, 165–184.

Atkinson, R.K., Walker, P., 1985. The effects of season of collection, feeding, maturation and gender on the potency of funnel-web spider (*Atrax infestus*) venom. Aust. J. Exp. Biol. Med. Sci. 63, 555–561.

Barbaro, K.C., Sousa, M.V., Morhy, L., Eickstedt, V.R.D., Mota, I., 1996. Compared chemical properties of dermonecrotic and lethal toxins from spiders of the genus *Loxosceles* (Araneae). J. Prot. Chem. 15, 337–343.

Binford, G.J., 2001. An analysis of geographic and intersexual variation in venoms of the spider *Tegenaria agrestis* (Agelenidae). Toxicon 39, 955–968.

Boyer, L.V., McNally, J.T., Binford, G.J., 2001. Spider bites. In: Auerbach, P.S (Ed.), Wilderness Medicine: Management of Wilderness and Environmental Emergencies. Mosby, St. Louis, MO, pp. 807–838.

Cisar, C.R., Fox, J.W., Geren, C.R., 1989. Screening a brown recluse spider genomic library for the gene coding for the mammalian toxin using an oligonucleotide probe. Toxicon 27, 37–38.

de Andrade, R.M.G., de Oliveira, K.C., Giusti, A.L., da Silva, W.D., Tambourgi, D.V., 1999. Ontogenetic development of *Loxosceles intermedia* spider venom. Toxicon 37, 627–632.

Forrester, L.J., Barrett, J.T., Campbell, B.J., 1978. Red Blood-cell lysis induced by venom of brown recluse spider—role of sphingomyelinase-D. Arch. Biochem. Biophys. 187, 355–365.

Geren, C.R., Chan, T.K., Howell, D.E., Odell, G.V., 1976. Isolation and characterization of toxins from brown recluse spider venom. Arch. Biochem. Biophys. 174, 90–99.

Gertsch, W.J., Ennik, F., 1983. The spider genus *Loxosceles* in North America, Central America, and the West Indies (Araneae, Loxoscelidae). Bull. Am. Mus. Nat. History 175, 264–360.

Gomez, H.F., Miller, M.J., Waggener, M.W., Lankford, H.A., Warren, J.S., 2001. Antigenic cross-reactivity of venoms from medically important North American *Loxosceles* spider species. Toxicon 39, 817–824.

Guilherme, P., Fernandes, I., Barbaro, K.C., 2001. Neutralization of dermonecrotic and lethal activities and differences among 32–35 kDa toxins of medically important *Loxosceles* spider venoms in Brazil revealed by monoclonal antibodies. Toxicon 39, 1333–1342.

Hartree, E.F., 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48, 422–427.

Kurpiewski, G., Forrester, L.J., Barrett, J.T., Campbell, B.J., 1981. Platelet aggregation and sphingomyelinase D activity of a purified toxin from the venom of Loxosceles reclusa. Biochim. Biophys. Acta 678, 467–476.

Malli, H., Vapenik, Z., Nentwig, W., 1993. Ontogenetic changes in the toxicity of the venom of the spider *Cupiennius salei* (Araneae, Ctenidae). Zool. Jb. Physiol. 97, 113–122.

Merchant, M.L., Hinton, J.F., Geren, C.R., 1998. Shingomyelinase D activity of the brown recluse spider (*Loxosceles reclusa*) venom as studied by 31P-NMR: effects on the time-course of sphingomyelin hydrolysis. Toxicon 36, 537–545.

Newlands, G., 1982. Preliminary report on the medical importance of *Sicarius* (Araneae: Sicariidae) and the action of its venom. Mem. Inst. Butantan. 46, 293–304.

Newlands, G., Atkinson, P., 1988. Review of southern African spiders of medical importance, with notes on the signs and symptoms of envenomation. S. Afr. Med. J. 73, 235–239.

Newlands, G., Atkinson, P., 1990a. Behavioural and epidemiological considerations pertaining to necrotic araneism in southern Africa. S. Afr. Med. J. 77, 92–95.

Newlands, G., Atkinson, P., 1990b. A key for the clinical diagnosis of araneism in Africa south of the equator. S. Afr. Med. J. 77, 96–97.

Pedrosa, M.F.F., Azevedo, I.L.M.J., Goncalves-de-Andrade, R.M., van den Berg, C.W., Ramos, C.R.R., Ho, P.L., Tambourgi, D.V., 2002. Molecular cloning and expression of a functional dermonecrotic and haemolytic factor from *Loxosceles laeta* venom. Biochem. Biophys. Res. Commun. 298, 638–645.

Platnick, N.I., Coddington, J.A., Forster, R.R., Griswold, C.E., 1991. Spinneret morphology and the phylogeny of haplogyne spiders (Araneae, Araneomorphae). Am. Mus. Novit. 3016, 1–73.

Tambourgi, D.V., Magnoli, F.C., Von Eickstedt, V.R.D., Benedetti, Z.C., Petricevich, V.L., da Silva, W.D., 1995. Incorporation of a 35-kilodalton purified protein from *Loxosceles* intermedia spider venom transforms human erythrocytes into activators of autologous complement alternative pathway. J. Immunol. 155, 4459–4466.

Tambourgi, D.V., Petricevich, V.L., Magnoli, S.L.M.R., Jancar, S., Dias Da Silva, W., 1998a. Entotoxemic-like shock induced by *Loxosceles* spider venoms: pathological changes and putative cytokine mediators. Toxicon 36, 391–403.

Tambourgi, D.V., Magnoli, F.C., van den Berg, C.W., Morgan, B.P., de Araujo, P.S., Alves, E.W., et al., 1998b. Sphingomyelinases in the venom of the spider *Loxosceles* intermedia are responsible for both dermonecrosis and complement-dependent hemolysis. Biochem. Biophys. Res. Commun. 251, 366–373.

Truett, A.P. III, King, J.L.E., 1993. Sphingomyelinase D: a pathogenic agent produced by bacteria and arthropods. Adv. Lipid Res. 26, 275–291.

Van Aswegen, G., Rooyen, J.M., Van Der Nest, D.G., Veldman, F.J., De Villers, T.H., Oberholzer, G., 1997. Venom of a six-eyed crab spider, *Sicarius testaceus* (Purcell, 1908), causes necrotic and haemorrhagic lesions in the rabbit. Toxicon 35, 1149–1152.

Viega, S.S., Gremski, W., Santos, V.L.P., Feitosa, L., Mangili, O.C., Nader, N.B., Dietrich, C.P., Brentani, R.R., 1999. Oligosaccharide residues of *Loxosceles intermedia* (brown spiders) venom proteins: dependence on glycosylation for dermonecrotic activity. Toxocon 37, 587–607.

White, J., Cardosa, J.L., Wen Fan, H., 1995. Clinical toxicology of spider bites. In: Meier, J., White, J. (Eds.), Handbook of Clinical Toxicology of Animal Venoms and Poisons. CRC Press, New York, pp. 259–329.