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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **07.032.811**

Solicitud de patente a nombre de **UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO Y LABORATORIOS SILANES S.A. DE C.V.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO Y LABORATORIOS SILANES S.A. DE C.V.**, domiciliadas en México D.F., México, solicitante de la patente de la referencia, en respuesta al oficio No. **04460** notificado por fijación en lista el 21 de marzo de 2012, nos permitimos informar lo siguiente:

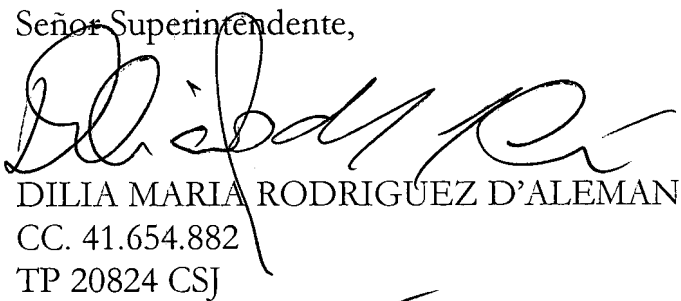
Que atentamente presentamos ante ese Despacho copia informal de los siguientes documentos:

- BARBARO K.C. ET AL.: "Compared Chemical Properties of Dermonecrotic and Lethal Toxins from Spiders of the genus *Loxosceles* (Araneae)" J. PROTEIN CHEM. vol. 15, no. 4, May 1996, pages 337-343, XP008072142.
- TAMBOURGI D.V. ET AL: "Molecular cloning, expression, function and immunoreactivities of members of a gene family of phingomyelinases from *Loxosceles* venom glands" MOLECULAR IMMUNOLOGY vol. 41, no. 8, July 2004, pages 831-840.
- PEDROSA M.D. ET AL.; "Molecular cloning and expression of a functional dermonecrotic and haemolytic factor from *Loxosceles laeta* venom" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol, 298, no. 5, 15 November 2002, pages 638-645.
- KALAPOTHAKIS E. ET AL.: "Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of *Loxosceles intermedia* spider venom" TOXICON vol. 40, no. 12, December 2002, pages 1691-1699.

- RAMOS-CERRILLO B. ET AL.: "Genetic and enzymatic characterization of sphingomyelinase D isoforms from the North American fiddleback spiders *Loxosceles boneti* and *Loxosceles reclusa*" TOXICON vol. 44, no. 5, October 2004, pages 507-514

Con la presentación de estos documentos damos cabal cumplimiento al requerimiento realizado por esa oficina mediante el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina.

Señor Superintendente,



DILIA MARIA RODRIGUEZ D'ALEMAN
CC. 41.654.882
TP 20824 CSJ

Compared Chemical Properties of Dermonecrotic and Lethal Toxins from Spiders of the Genus *Loxosceles* (Araneae)

Katia C. Barbaro,^{1,2} Marcelo V. Sousa,³ Lauro Morhy,³ Vera R. D. Eickstedt,⁴ and Ivan Mota¹

Received January 30, 1996

Loxosceles spider venom usually causes a typical dermonecrotic lesion in bitten patients, but it may also cause systemic effects that may be lethal. Gel filtration on Sephadex G-100 of *Loxosceles gaucho*, *L. laeta*, or *L. intermedia* spider venoms resulted in three fractions (A, containing higher molecular mass components, B containing intermediate molecular mass components, and C with lower molecular mass components). The dermonecrotic and lethal activities were detected exclusively in fraction A of all three species. Analysis by SDS-PAGE showed that the major protein contained in fraction A has molecular weight approximately 35 kDa in *L. gaucho* and *L. intermedia*, but 32 kDa in *L. laeta* venom. These toxins were isolated from venoms of *L. gaucho*, *L. laeta*, and *L. intermedia* by SDS-PAGE followed by blotting to PVDF membrane and sequencing. A database search showed a high level of identity between each toxin and a fragment of the *L. reclusa* (North American spider) toxin. A multiple sequence alignment of the *Loxosceles* toxins showed many common identical residues in their N-terminal sequences. Identities ranged from 50.0% (*L. gaucho* and *L. reclusa*) to 61.1% (*L. intermedia* and *L. reclusa*). The purified toxins were also submitted to capillary electrophoresis peptide mapping after *in situ* partial hydrolysis of the blotted samples. The results obtained suggest that *L. intermedia* protein is more similar to *L. laeta* toxin than *L. gaucho* toxin and revealed a smaller homology between *L. intermedia* and *L. gaucho*. Altogether these findings suggest that the toxins responsible for most important activities of venoms of *Loxosceles* species have a molecular mass of 32–35 kDa and are probably homologous proteins.

KEY WORDS: *Loxosceles*; spider; dermonecrotic toxin; lethal toxin; amino acid sequence.

1. INTRODUCTION

Spider venoms consist of mixtures of numerous proteins with enzymatic and/or toxic activities. In

Brazil, the genus *Loxosceles* is widely distributed, with seven species ranging from northern to southern regions (Cardoso *et al.*, 1988). Loxoscelism has been recognized as a health problem in Brazil since 1957 (Rosenfeld *et al.*, 1957) and is considered a very important form of araneism, based on the fact that hundreds of accidents are reported to the Ministry of Health of Brazil (1990) as well as the high rate of intradomiciliar infestation by these spiders (Ribeiro *et al.*, 1993). Three species of genus *Loxosceles* are most common in the south of Brazil: *L. gaucho*, *L. laeta*, and *L. intermedia*.

¹ Laboratório de Imunopatologia, Instituto Butantan, 05503-900, São Paulo, SP, Brazil.

² To whom correspondence should be addressed; e-mail: kbarbaro@usp.br.

³ Centro Brasileiro de Sequenciamento de Proteínas, Departamento de Biologia Celular, Universidade de Brasília, 70910-900, Brasília, DF, Brazil.

⁴ Laboratório de Artrópodos Peçonhentos, Instituto Butantan, 05503-900, São Paulo, SP, Brazil.

The most common finding after the spider's bite is a dermonecrotic lesion, but it may also cause systemic effects that may be lethal (Cardoso *et al.*, 1988). The bite is initially painless. Pain from mild to intense starts after 2-6 hr and is followed by erythema, itching, swelling, and tenderness. Three days to 1 week later an eschar may form and drop off leaving an ulcer that heals within 1-2 months. Histologically the lesion resembles a cutaneous Arthus reaction with edema, polymorphonuclear infiltration, fibrinoid necrosis, vacuolization of arteriole walls, and intense hemorrhage into the dermis. In addition to leucocyte infiltration, the complement system is also involved (Futrell, 1992). The systemic effects are much less common: fever, malaise, chills, weakness, nausea, and vomiting may occur within 24 hr. The hematologic disturbances may include hemolytic anemia, thrombocytopenia, and intravascular coagulation (Futrell, 1992).

We report here the results of fractionation of *Loxosceles* venoms by Sephadex G-100 column chromatography and SDS-polyacrylamide electrophoresis, the determination of the N-terminal sequence, and a comparison of capillary electrophoresis peptide mapping profiles of 35 and 32 kDa *Loxosceles* toxins.

2. MATERIALS AND METHODS

2.1. Animals and Venoms

Outbred Swiss mice weighing from 20 to 22 g and adult white rabbits weighing from 3 to 4 kg were provided by the Butantan Institute animal house. Specimens of *L. gaucho* were collected in the state of São Paulo and those of *L. intermedia* and *L. laeta* in the states of Paraná and Santa Catarina, Brazil. The spiders were kept in quarantine for 1 week without food before venom collection. The venom was obtained as previously described (Barbaro *et al.*, 1992). A pool of venoms collected from approximately 800 spiders of each species was used. The protein content of the venom pool was determined according to the method of Lowry *et al.* (1951).

2.2. Gel Filtration

A column (1.8 × 70.0 cm) of Sephadex G-100 (Pharmacia Fine Chemicals Ltd, Sweden) equilibrated with 0.02 M ammonium bicarbonate buffer,

pH 6.0, was used. Six mg of venom of each species of *Loxosceles* were applied to the column and 1.5-ml samples were collected with a flow rate of 20 ml/hr. Elution was monitored by absorbance at 280 nm. Fractions corresponding to each protein peak were then pooled, lyophilized, and stored at -20°C.

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The venoms and its fractions were analyzed by SDS-PAGE (15% acrylamide gels) under non-reducing conditions, using the method of Laemmli (1970). Prior to electrophoresis, venom samples (30 µg) were mixed 1:1 (v/v) with sample buffer [125 mM Tris-HCl, pH 6.8, SDS (sodium dodecyl sulfate) 2.5%, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM TLCK (N- α -p-tosyl-L-lysine chloromethyl ketone), 4 mM EDTA (ethylenediaminetetraacetic acid), glycerol 20%, and bromophenol blue 0.05%], boiled for 3 min, and placed on the gel. Gel electrophoresis was carried out at 25 mA for 4 hr, and the gels were stained with 0.1% amido black. Bovine serum albumin (67 kDa), hen egg albumin (43 kDa), trypsinogen (24 kDa), and lysozyme (14 kDa) (Sigma Chemical Company, St. Louis, MO) were used as molecular weight markers.

2.4. Dermonecrotic Activity

Dermonecrotic activity was assayed by i.d. injections of 0.2 ml of 0.15 M NaCl containing 3 µg of venom or its fractions into a shaved area in the rabbit dorsum as previously described (Barbaro *et al.*, 1992). The area of skin necrosis was inspected 48 hr after injection and is expressed as the mean of four areas (cm² ± SD) of skin necrosis.

2.5. Lethal Activity

Groups of eight mice were injected i.d. with 0.2 ml of 0.15 M NaCl containing 2 LD₅₀ of each venom and its fractions and the lethal activity was determined 48 hr after injection.

2.6. N-Terminal Sequencing

The venom of each *Loxosceles* species was submitted to further purification by SDS-PAGE followed by electroblotting to PVDF membrane.

2.7. Database Search and Analysis

The N-terminal sequence obtained was searched against the NR (Non-Redundant) Sequence Database kept at NCBI (National Center for Biotechnology Information, Bethesda, MD), using the BLAST Network Service (Altschul *et al.*, 1990). Multiple sequence alignment was then carried out by using the program CLUSTAL (Higgins and Sharp, 1989).

2.8. Capillary Electrophoresis Peptide Mapping

The *Loxosceles* toxins (35 and 32 kDa) were submitted to capillary electrophoresis peptide mapping in a 270A-HT CE System (Applied Biosystems) after *in situ* partial hydrolysis of the blotted samples with 3 N HCl at 80°C during 1 week.

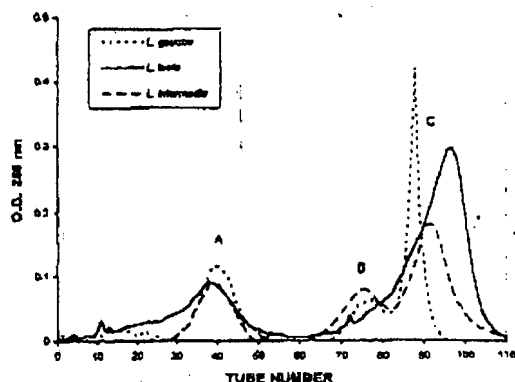


Fig. 1. Fractionation of *Loxosceles gaucho*, *L. laeta*, and *L. intermedia* spider venom by Sephadex G-100 column chromatography. Samples containing 6 mg of venom were loaded onto the column and eluted with 0.02 M NH_4HCO_3 at a flow rate of 20 ml/hr. Samples of 1.5 ml/tube were collected and their protein content monitored by absorbance at 280 nm.

protein of each fraction was injected i.d. in rabbits. Fraction A, containing the higher molecular weight components, showed dermonecrotic activity, while fractions B and C had no activity (Table I). The venom and fraction A produced large dermonecrotic lesions in rabbits, with necrosis spreading by gravity to the skin of the lateral body wall. Lethal activity of the venom fractions was also located exclusively in fraction A (Table I).

The results of SDS-PAGE analysis of the venoms and their fractions are shown in Fig. 2. At least nine components were revealed in all three venoms. The major component was localized around the 35 kDa region in *L. gaucho* and *L. intermedia* venoms and at approximately 32 kDa in *L. laeta* venom. Fractions A of *L. gaucho* and *L.*

Table I. Determination of the Dermonecrotic and Lethal Activities of *L. gaucho*, *L. laeta*, and *L. intermedia* Venom and their Fractions

Venom	Dermonecrotic activity ^a	Percent survival ^b
<i>L. gaucho</i>	5.4 ± 3.6	0
Fraction A	14.1 ± 6.9	12.5
Fraction B	0	100
Fraction C	0	100
<i>L. laeta</i>	1.8 ± 0.8	0
Fraction A	10.2 ± 4.1	0
Fraction B	0	100
Fraction C	0	100
<i>L. intermedia</i>	2.7 ± 1.2	12.5
Fraction A	8.9 ± 2.4	12.5
Fraction B	0	100
Fraction C	0	100

^a The dermonecrotic activity (cm^2) is expressed as the mean of four areas ± SD determined 48 hr after venom or fraction injection.

^b Percent survival 48 hr after venom or fraction injection.

Toxins of *Loxosceles* Venom

The main protein bands (32 kDa for *L. laeta* and 35 kDa for *L. gaucho* and *L. intermedia*) were excised and kept at -20°C until sequencing. The blotted sample was sequenced in an Applied Biosystems 477A Pulsed-Liquid Phase Automatic Sequencer fitted on line with an Applied Biosystems 120A PTH-Amino Acid Analyzer. The equipment was programmed and operated according to the manufacturer's instructions.

2.7. Database Search and Sequence Alignment

The N-terminal sequence obtained was searched against the NR (Non-Redundant) Sequence Database kept at NCBI (National Center for Biotechnology Information, Bethesda, MD), using the BLAST Network Service (Altschul *et al.*, 1990). Multiple sequence alignment was then carried out by using the program CLUSTAL (Higgins and Sharp, 1989).

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The *Loxosceles* toxins (35 and 32 kDa) were submitted to capillary electrophoresis peptide mapping in a 270A-HT CE System (Applied Biosystems) after *in situ* partial hydrolysis of the blotted samples with 3 N HCl at 80°C during 1 week.

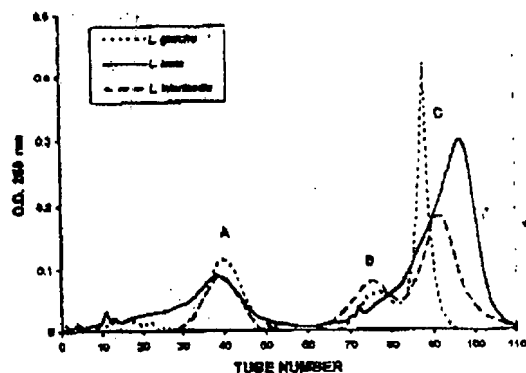


Fig. 1. Fractionation of *Loxosceles gaucho*, *L. laeta*, and *L. intermedia* spider venom by Sephadex G-100 column chromatography. Samples containing 6 mg of venom were loaded onto the column and eluted with 0.02 M NH_4HCO_3 at a flow rate of 20 ml/hr. Samples of 1.5 ml/tube were collected and their protein content monitored by absorbance at 280 nm.

3. RESULTS

Gel filtration on a Sephadex G-100 column of venoms of *L. gaucho*, *L. laeta*, and *L. intermedia* resulted in very similar elution patterns with three major fractions designated A, B, and C (Fig. 1). Fraction A contained components of higher molecular weight, whereas fractions B and C contained components of smaller molecular mass of the venom. These fractions were tested for their dermonecrotic and lethal activities.

To locate the dermonecrotic activity, 3 μg of protein of each fraction was injected i.d. in rabbits. Fraction A, containing the higher molecular weight components, showed dermonecrotic activity, while fractions B and C had no activity (Table I). The venom and fraction A produced large dermonecrotic lesions in rabbits, with necrosis spreading by gravity to the skin of the lateral body wall. Lethal activity of the venom fractions was also located exclusively in fraction A (Table I).

The results of SDS-PAGE analysis of the venoms and their fractions are shown in Fig. 2. At least nine components were revealed in all three venoms. The major component was localized around the 35 kDa region in *L. gaucho* and *L. intermedia* venoms and at approximately 32 kDa in *L. laeta* venom. Fractions A of *L. gaucho* and *L.*

Table I. Determination of the Dermonecrotic and Lethal Activities of *L. gaucho*, *L. laeta*, and *L. intermedia* Venom and their Fractions

Venom	Dermonecrotic activity ^a	Percent survival ^b
<i>L. gaucho</i>	5.4 ± 3.6	0
Fraction A	14.1 ± 6.9	12.5
Fraction B	0	100
Fraction C	0	100
<i>L. laeta</i>	1.8 ± 0.8	0
Fraction A	10.2 ± 4.1	0
Fraction B	0	100
Fraction C	0	100
<i>L. intermedia</i>	2.7 ± 1.2	12.5
Fraction A	8.9 ± 2.4	12.5
Fraction B	0	100
Fraction C	0	100

^a The dermonecrotic activity (cm^2) is expressed as the mean of four areas $\pm$ SD determined 48 hr after venom or fraction injection.

^b Percent survival 48 hr after venom or fraction injection.

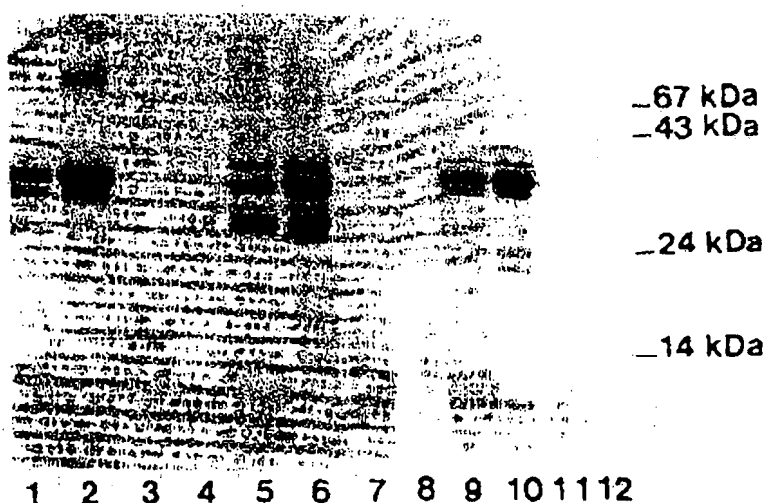


Fig. 2. SDS-polyacrylamide electrophoretic separation of *L. gaucho*, *L. laeta*, and *L. intermedia* total venom and their A, B, and C fractions. The fractions were prepared as described in the text. (1) *L. gaucho* whole venom, (2) fraction A, (3) fraction B, (4) fraction C; (5) *L. laeta* whole venom, (6) fraction A, (7) fraction B, (8) fraction C; and (9) *L. intermedia* whole venom, (10) fraction A, (11) fraction B, (12) fraction C. Numbers at the right correspond to positions of molecular weight markers.

intermedia were resolved into major bands with molecular weights around 35 kDa plus other minor bands, whereas fraction A of *L. laeta* venom showed a major band around 32 kDa. Fraction B of all three venoms showed a major component at around 10–15 kDa. No protein band was detected by this technique in fraction C in all three toxocelic venoms. Note that the profile of fractions A of *L. laeta* and *L. intermedia* venom obtained after filtration in Sephadex G-100 was more complex, showing many other contaminating proteins that were not present in fraction A of *L. gaucho*. In order to be sure that the 32 and 35 kDa components of the venoms were responsible for the dermonecrotic activity, we submitted the three venoms to SDS-PAGE and then cut each gel in many pieces (keeping a parallel strip for staining) from which we eluted the venom component located in each piece by agitating each one in 0.5 ml of PBS (0.15 M phosphate buffer saline, pH 7.2) for 18 hr at room temperature. Two hundred microliters of each eluted sample was then injected into rabbit skin to test its activity. Dermonecrotic activity was detected in the cut pieces corresponding to the 32 kDa component of *L. laeta* venom and to the 35 kDa component of *L. gaucho* and *L.*

intermedia venoms. The eluates obtained from the other pieces showed no dermonecrotic activity.

The determination of the N-terminal sequences of the major components of the venoms (35 and 32 kDa) of *L. gaucho*, *L. intermedia*, and *L. laeta* responsible for dermonecrotic and lethal activities was carried out. A BLAST search of the N-terminal sequences of the toxins against the NR sequence database at NCBI showed similarity between each one of them and a fragment of the North American spider *L. reclusa* (Cisar *et al.*, 1989). A multiple sequence alignment of the sequences of the toxins of *L. gaucho*, *L. intermedia*, *L. laeta*, and *L. reclusa* (Fig. 3) showed many identical residues among them. A calculation of the percentage of identity among the toxins was performed (Table II). Identities above 50% (*L. gaucho* and *L. reclusa*) were found. *L. intermedia* and *L. reclusa* showed the highest score of identity (61.1%).

Peptide mapping by capillary electrophoresis (Fig. 4) showed great similarity of peak profiles from *L. laeta* and *L. intermedia* toxin digests between 3.9 and 6.1 min (group 1 of peaks) and between 8.5 and 10.5 min (groups 3 and 4 of peaks). However, between 9.6 and 10.5 min (group 4 of peaks) there was similarity among peak profiles

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      5    10   15   20   25   30   35
L. gaucho  ADNKRPIWMGGMVNSLAQIKEFVGLGLNSEKDNKWYKQ
L. intermedia AGNKRPIWIMGAMVNAIKDYDFVNLGA-NNIX--K----
L. laeta      ADNRRIWNLGHMVNALKQIPTFLXDGA-NA-----
L. reclusa   AN-KR.VWIMGHMVNAVYQI-EFVNLGA-NSI-DIDVS--
      +---+ +---+ +---+ +---+ +---+ +---+
  
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Fig. 3. Multiple sequence alignment of the N-terminal sequences of *L. gaucho*, *L. laeta*, *L. intermedia*, and *L. reclusa* toxins. An equal sign means identical residues across all sequences; a plus sign indicates conservative amino acid residue substitutions. Gaps (—) were placed for maximum alignment.

of all three species, although *L. gaucho* venom presented higher peaks in this region and *L. laeta* and *L. intermedia* peaks were shifted to the right side of the electrophoregram. Between 6.9 and 8.5 min (group 2 of peaks), *L. gaucho* and *L. intermedia* presented some minor peaks with similar profiles. Some peaks between 6.2 and 6.9 min could be seen only in *L. gaucho* toxin digest. These results suggested that *L. intermedia* and *L. laeta* are more similar to each other than to *L. gaucho*.

4. DISCUSSION

Separation of *L. gaucho*, *L. laeta*, and *L. intermedia* venoms by Sephadex G-100 gel filtration produced three major fractions (Fig. 1). The dermonecrotic and lethal activities were found only in the first peak containing the higher molecular mass components (fraction A), whereas the other fractions (B and C) were devoid of these activities.

Analysis by SDS-PAGE of the three venoms showed at least nine components that were stained in each one (Fig. 2). The proteins responsible for the dermonecrotic and lethal activities present in fraction A showed a molecular mass of about 35 kDa in *L. gaucho* and *L. intermedia* venoms, but only approximately 32 kDa in *L. laeta* venom. This difference may be explained by the fact that whereas *L. gaucho* and *L. intermedia* are native to Brazil, *L. laeta* is native to Chile (Gertsch and Ennick, 1983). Geren *et al.* (1973, 1975, 1976) working with *L. reclusa* reported that the venom

components responsible for lethal and dermonecrotic activities were two toxins of about 34 kDa. Components with lower molecular weight showed no toxic activity. More recently, Rees *et al.* (1984) detected two major components in *L. reclusa* venom, one of 34 kDa and another of 68 kDa. In agreement with results obtained with other *Loxosceles* venoms, only the 34 kDa component showed toxic activity.

The high percentage identity in the N-terminal sequences of *L. gaucho*, *L. intermedia*, and *L. laeta* toxins when compared to *L. reclusa* toxin (Cisar *et al.*, 1989) (Fig. 3 and Table II) strongly suggests that they are all similar proteins.

The results obtained after submitting these venoms to capillary electrophoresis peptide mapping (Fig. 4) suggest that *L. intermedia* toxin is more similar to *L. laeta* toxin than to *L. gaucho* toxin and reveal a smaller homology between *L. intermedia* and *L. gaucho*. As the N-terminal regions showed similar sequences and close identity percentages for all the three newly sequenced toxins, probably some internal sequence differences may exist.

There are many reports describing the multiple components of *Loxosceles* venom and their various biological properties (Mota and Barbaro, 1995). However, it is difficult to make meaningful comparisons or relate specific biological effects to one venom component because there are large differences in venom preparation, scheme of purification, and methods used to study the mechanisms of action of the venom components.

Table II. Percentage of Identity Between N-Terminal Sequences of *Loxosceles* Toxins

Species	<i>L. gaucho</i>	<i>L. intermedia</i>	<i>L. laeta</i>	<i>L. reclusa</i>
<i>L. gaucho</i>	—	57.6% (35 aa)	53.3% (30 aa)	50.0% (34 aa)
<i>L. intermedia</i>	57.6% (35 aa)	—	56.7% (30 aa)	61.1% (34 aa)
<i>L. laeta</i>	53.3% (30 aa)	56.7% (30 aa)	—	53.3% (34 aa)
<i>L. reclusa</i>	50.0% (34 aa)	61.1% (34 aa)	53.3% (34 aa)	—

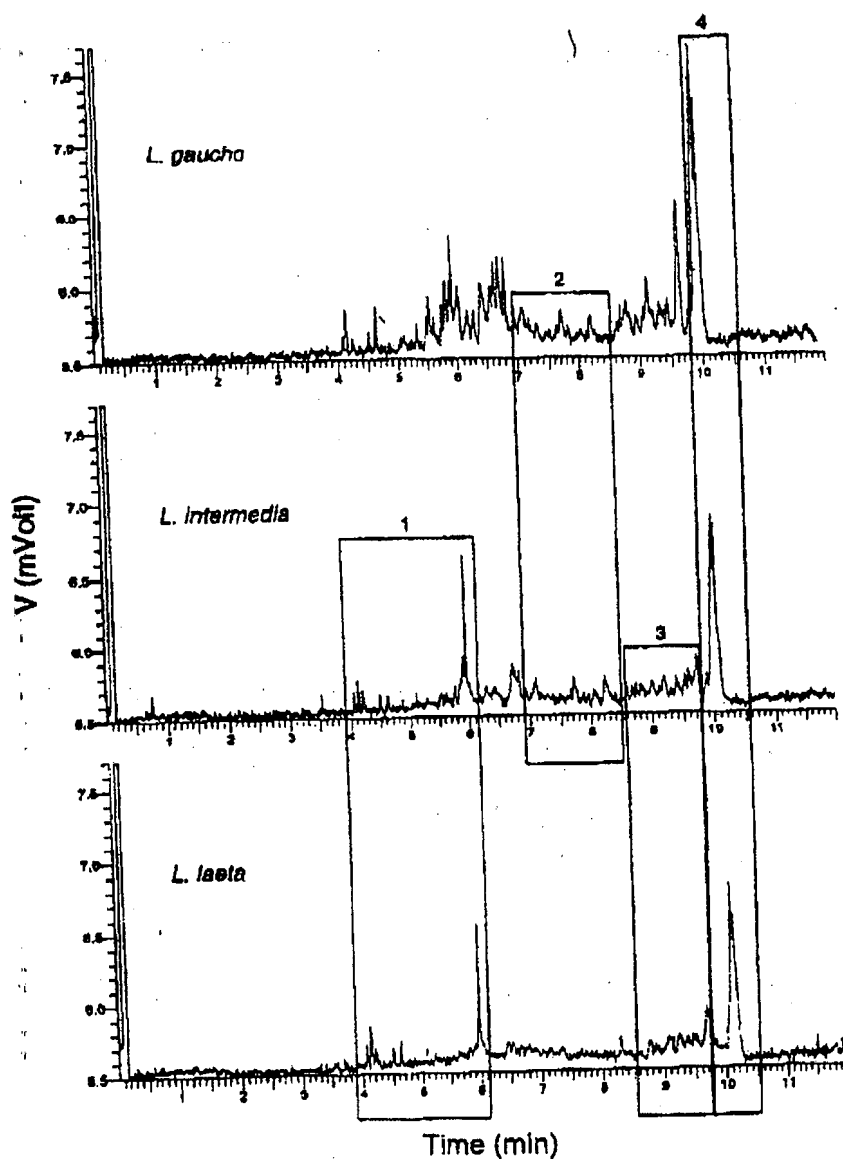


Fig. 4. Comparison of capillary electrophoresis peptide mapping profiles from *L. gaucho*, *L. lutea*, and *L. inermis* venoms. The *Loxosceles* toxins were submitted in capillary electrophoresis peptide mapping in a 270A-HT CE System (Applied Biosystems) after *in situ* partial hydrolysis of the blotted samples with 3 N HCl at 80°C during 1 week. Groups of similar peaks are boxed and numbered 1-4.

However, it seems that dermonecrotic and lethal activities are common characteristics of the *Loxosceles* genus and that the active components

from all species have a similar molecular mass, between 32 and 35 kDa. In fact, intense antigenic cross-reactivity between components of venoms

from *L. gauchoi*, *L. laeta*, and *L. intermedia* has been observed (Barbaro *et al.*, 1994). Altogether our findings suggest that toxins from *Loxosceles* species are probably homologous. Amino acid composition analysis is under way for further confirmation and quantitation of this homology.

ACKNOWLEDGMENTS

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Molecular cloning, expression, function and immunoreactivities of members of a gene family of sphingomyelinases from *Loxosceles* venom glands[☆]

Denise V. Tambourgi^{a,*}, Matheus de F. Fernandes Pedrosa^a, Carmen W. van den Berg^b,
Rute M. Gonçalves-de-Andrade^a, Matheus Ferracini^a, Danielle Paixão-Cavalcante^a,
B. Paul Morgan^c, Neil K. Rushmere^c

^a Laboratório de Imunoquímica, Instituto Butantan, Av. Prof. Vital Brazil, 1500, CEP 05503-900 São Paulo, SP, Brazil

^b Department of Pharmacology, Therapeutics and Toxicology, University of Wales, College of Medicine, Cardiff, UK

^c Department of Medical Biochemistry and Immunology, University of Wales, College of Medicine, Cardiff, UK

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Abstract

Loxoscelism is the clinical condition produced by the venom of spiders belonging to the genus *Loxosceles*, which can be observed as two well-defined clinical variants: cutaneous loxoscelism and systemic or viscerocutaneous loxoscelism. We have recently identified, purified and characterised the toxins (sphingomyelinases) from *Loxosceles intermedia* venom that are responsible for all the local (dermonecrosis) and systemic effects (complement dependent haemolysis) induced by whole venom. In the present study, we have cloned and expressed the two functional sphingomyelinases isoforms, P1 and P2, and shown that the recombinant proteins display all the functional characteristics of whole *L. intermedia* venom, e.g., dermonecrotic and complement-dependent hemolytic activities and ability of hydrolyzing sphingomyelin. We have also compared the cross-reactivities of antisera raised against the toxins from different *Loxosceles* species and show here that the cross-reactivity is high when toxins are from the same species (P1 and P2 from *L. intermedia*) but low when the toxins are from different species (*L. intermedia* versus *L. laeta*). These data suggest that in order to obtain a suitable comprehensive neutralizing antiserum using the recombinant toxin as an immunogen, a mixture of the recombinant toxins from the different species has to be used. The use of anti-recombinant toxin antisera may have clinical benefits to those individuals displaying acute loxoscelic lesions.

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1. Introduction

Envenomation by spiders belonging to the genus *Loxosceles* (Araneae, Sicariidae), found in temperate and tropical regions of America, Africa and Europe, commonly results in impressive local necrotic skin lesions and more rarely causes systemic effects, including profound intravascular hemolysis (Barretto et al., 1985; Schenone et al., 1989; Ginsburg and Weinberg, 1988; Sezerino et al., 1998; Gendron, 1990;

Futrell, 1992; White et al., 1995; Bey et al., 1997). The predominant clinical sign is a cutaneous reaction characterized by the appearance of necrosis around the bite, resulting in ulceration. Healing of the ulcer often requires months. The scale of these lesions is remarkable considering that the spider injects only a few tenths of a microliter of venom, containing no more than 30 µg of protein. Mild systemic effects induced by envenomation, such as fever, malaise, pruritus and exanthema are common, while intravascular hemolysis and coagulation, sometimes accompanied by thrombocytopenia and renal failure, occur in approximately 16% of the victims (Barretto et al., 1985; Schenone et al., 1989; Ginsburg and Weinberg, 1988; Sezerino et al., 1998; Gendron, 1990; Futrell, 1992; Bey et al., 1997).

Loxosceles is the most poisonous spider in Brazil and children who develop the more severe systemic effects after

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* Corresponding author. Tel.: +55 11 3726 7222x2231; fax: +55 11 3726 1505.

E-mail address: dvtambourgi@butantan.gov.br (D.V. Tambourgi).

envenomation nearly always die. At least three different *Loxosceles* species of medical importance are known in Brazil (*L. intermedia*, *L. gaucho*, *L. laeta*) and more than 2000 cases of envenomation by *L. intermedia* alone are reported each year. In South Africa, *L. parrami* and *L. spinulosa* are responsible for cutaneous loxoscelism (Newlands et al., 1982). In Australia, a cosmopolitan species, *L. rufescens*, is capable of causing ulceration in humans. In the USA, at least five *Loxosceles* species, including *L. reclusa* (brown recluse), *L. apachea*, *L. arizonica*, *L. unicolor* and *L. deserta* are known to cause numerous incidents (Ginsburg and Weinberg, 1988; Gendron, 1990; Bey et al., 1997; Desai et al., 2000).

We recently have purified and characterized the toxins from *L. intermedia* venom that are responsible for all the local and systemic effects induced by whole venom (Tambourgi et al., 1995, 1998a). Two highly homologous proteins, termed P1 and P2, with Mr 35 kDa were purified to homogeneity and shown to be endowed with sphingomyelinase activity, were both able to induce dermonecrosis in rabbits, and rendered human erythrocytes susceptible to lysis by complement (C) (Tambourgi et al., 1998a). In a mouse model of *Loxosceles* envenomation, we showed that the toxins also induced intravascular hemolysis and provoked a cytokine response, which resembled that seen in endotoxic shock (Tambourgi et al., 1998b). We subsequently elucidated the mechanism of C-susceptibility and showed that the toxins facilitate activation of the alternative pathway of C on human erythrocytes by removal of glycoporphins as a consequence of activation of an endogenous metalloproteinase (Tambourgi et al., 2000) and activation of the classical pathway of C, possibly by disruption of the membrane asymmetry (Tambourgi et al., 2002).

The difficulty in obtaining large amounts of venom and purified venom components is one of the limiting factors in studying the mechanisms involved in loxoscelism and raising an effective therapeutic antiserum. Cloning and expression of the *Loxosceles* toxins would enable us to further dissect the mechanism of *Loxosceles* sphingomyelinase action on human blood cells and tissues and establish if antisera raised against the main toxic component in the venom are able to prevent development of loxoscelism. We have recently cloned and expressed one of the sphingomyelinases from *L. laeta* venom (Smase I), which displayed all the biological activities of whole venom (Fernandes Pedrosa et al., 2002). An antiserum raised in rabbits against this recombinant protein effectively neutralized *L. laeta* venom toxicity. The N-terminal sequence of this *L. laeta* toxin displayed a significant similarity with the first 25 amino acids of the purified toxins P1 and P2 from *L. intermedia*. The venoms of various *Loxosceles* species contain several functionally active isoforms of the toxins. While we found an identity at the amino acid level of 40–81% between sphingomyelinase and sphingomyelinase like cDNAs cloned from the *L. laeta* gland (Fernandes Pedrosa et al., 2002) the level of similarity of the toxins between different species is not known. It is often the case that the species of *Loxosceles* that has

bitten the patient is not known. Hence, it is important to raise a neutralizing antiserum which is effective against all *Loxosceles* species and to do this it is essential to know whether antibodies against the different toxin isoforms and toxins from different *Loxosceles* species are cross-reactive. The aims of the present study were to clone and express the two functional toxin isoforms P1 and P2 from the spider gland of *L. intermedia*, to compare the functional activities of the recombinant toxins with whole venom and to investigate the inter- and intra-species cross-reactivities of antibodies raised against the purified recombinant proteins.

2. Materials and methods

2.1. Chemicals, reagents and buffers

Tween 20, bovine serum albumin (BSA) and paraformaldehyde were purchased from Sigma (St. Louis, MO, USA). 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and Nitroblue Tetrazolium (NBT) were from Promega Corp. (Madison, WI, USA). Buffers were: Veronal Buffered Saline (VBS²⁺), pH 7.4, containing 10 mM Na Barbitone, 0.15 mM CaCl₂ and 0.5 mM MgCl₂; PBS, pH 7.2, 10 mM Na Phosphate, 150 mM NaCl; FACS buffer (PBS, 1% BSA, 0.01% sodium azide).

2.2. Antibodies

Monoclonal antibodies against Glycophorin A (GPA: Bric 256) and Glycophorin C (GPC: Bric 4) were from the International Blood Group Reference Laboratory (IBGRL, Bristol, UK). Rabbit anti-mouse IgG-FITC (RAM-FITC), and goat anti-rabbit IgG-FITC (GAR-FITC), goat anti-rabbit-IgG-alkaline phosphatase (GAR-AP) and goat anti-rabbit IgG horseradish peroxidase (GAR-HRPO) were from Amersham Pharmacia Biotech (Buckinghamshire, England, UK).

2.3. Spiders and venoms

Loxosceles intermedia, *L. laeta* and *L. gaucho* spiders were provided by Laboratório de Imunoquímica, Instituto Butantan, Brazil. The venoms were obtained by electrostimulation by the method of Bucherl (1969) with slight modifications. Briefly, 15–20 V electrical stimuli were repeatedly applied to the spider sternum and the venom drops were collected with a micropipette, vacuum dried and stored at –20 °C. Stock solutions were prepared in PBS at 1.0 mg/ml. The protein content of the samples was evaluated by the Lowry method (1951).

2.4. Normal human serum and erythrocytes

Human blood was obtained from healthy donors. Blood samples drawn to obtain sera were collected without

anticoagulant and allowed to clot for 2 h at RT, the normal human serum (NHS) was stored at -80°C . Blood samples drawn to obtain erythrocytes (E) for subsequent use as target cells were collected in anticoagulant (Alsever's old solution: 114 mM citrate, 27 mM glucose, 72 mM NaCl, pH 6.1).

2.5. cDNA generation

Twenty *L. intermedia* spiders were milked to stimulate the production of mRNAs in the venom glands. After 5 days, the spider venom gland/sacks were collected and total RNA was extracted from the glands using the Ultraspec RNA isolation system, using the method as recommended by the manufacturer (AMS, Abingdon Oxon, UK). The rapid amplification of cDNA ends (RACE) method of Frohman et al. (1988) was used to obtain full-length cDNAs encoding P1 and P2 toxins.

2.6. Derivation of the 3' end of the *Loxosceles* spider proteins P1 and P2 cDNA

Purified RNA (3 μg) was reverse transcribed by incubation with Superscript RNase H-reverse transcriptase and a poly(dT) adaptor primer Q_T (Frohman et al., 1988; 28 pmol). The 3' cDNA ends of *Loxosceles* spider proteins P1 and P2 were obtained by using the followed degenerate primers: Spider 1 (TGGATHATGGGNCAYATGGTNA) corresponding to amino acid residues 8–15: WIMGHMVN, and Spider 2 (CARATHGAYGARTTYGTNA) corresponding to amino acid residues 19–25: QIDEFVN of the mature purified toxins P1 and P2 (Tambourgi et al., 1998a). Primers Spider 1 and Q₀ (Frohman et al., 1988) were used in a PCR reaction with the Q_T generated spider cDNA mixture to amplify the 3' end of the cDNAs of P1 and P2. A touchdown PCR reaction was carried out using a starting annealing temperature of 54°C , which was decreased every second cycle by 2°C down to 40°C , at which temperature 25 cycles were conducted. PCR fragments were subjected to agarose gel electrophoresis, Southern blotted and probed using radiolabeled Spider 2 primer. A single positive 900 bp PCR DNA fragment was purified from agarose gel and cloned into pGEM-T cloning vector (Promega). Colonies were sequenced using an ABI 373A automated sequencer and Prism labeling kit (Applied Biosystems, Warrington, UK). Two DNA sequences correlating with the N-terminal protein sequences of P1 and P2 were obtained (Protein sequence accession numbers: P83045 and P83046; Tambourgi et al., 1998a).

2.7. Derivation of the 5' end of P1 and P2 *Loxosceles* spider protein cDNA

The 5' ends of the cDNA encoding *Loxosceles* spider P1 and P2 proteins were obtained using the rapid amplification of cDNA ends (RACE) method (Frohman et al., 1988).

Common anti-sense spider primers were designed based on the sequence obtained for the 3' cDNA ends. Initial primer spider AS1 was used to drive the gene-specific reverse transcription of the 5' end of both P1 and P2 mRNAs and two subsequent primers, spider AS2, AS3, were used in a nested PCR reaction with primers Q₀ and Q₁, respectively using similar conditions as described (Frohman et al., 1988). A single 350 bp PCR DNA fragment was isolated, purified, cloned into the pGEM-T vector and sequenced. Two sequences were obtained corresponding to the N-terminal protein sequences of P1 and P2.

2.8. Isolation of full-length cDNA sequences

New primers corresponding to the 5' and 3' untranslated cDNA regions of P1 and P2 were used to PCR full-length cDNA. PCR products (1200 bp) generated from fresh Q_T reverse transcribed primed spider salivary gland mRNA were ligated into pGEM-T. Sequences obtained were identical to those obtained for the individually cloned 3' and 5' cDNAs. The complete cDNA sequences have been deposited in Genbank as AY304471 for P1 and AY304472 for P2.

2.9. Subcloning in expression vector

The cDNAs corresponding to mature P1 and P2 (Fig. 1), were amplified from plasmids containing full length sequence using primer Spider 3 (CGGAGCTCGAGAGCAG-GTAATCGTCGGCCT; corresponding to a *Xho*I restriction site (italics) and the N-terminal amino acids AGNRRPI (bold) of P1)) and Spider 4AS (GCGAAGCTTGCAGAATT-TATTCTTGAATGTC; containing the DNA sequences corresponding to the C-terminal amino acids TFKK (bold) and *Hind*III restriction site (italics)). Similarly, the cDNA encoding mature P2 was amplified with Spider 4 primer (CGAGCTCGAGAGCGGATAAACGTCGGCCC; containing a *Xho*I restriction site (italics) and the N-terminal amino acid sequences ADKRRP (bold)) and spider 4AS. PCR fragments (850 bp) were digested with *Xho*I and *Hind*III and cloned into the corresponding sites in pRSET-B bacterial expression vector (Invitrogen). The use of the pRSET-B bacterial expression system results in the expression of a recombinant fusion protein, including a 6xHis-tag at the N-terminus and a 26 amino acid linker followed by the mature proteins P1 and P2 (N-terminal amino acid sequence before the coding sequence of the mature protein: 'MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDP-SSR').

2.10. Recombinant protein expression

Recombinant proteins were produced as described previously (Fernandes Pedrosa et al., 2002). In brief: pRSETB-L. *intermedia* P1 or P2 cDNAs transformed *E. coli* BL21 (DE3) (Invitrogen) cells were inoculated in 50 mL of 2YT/amp and grown overnight at 37°C and induced with

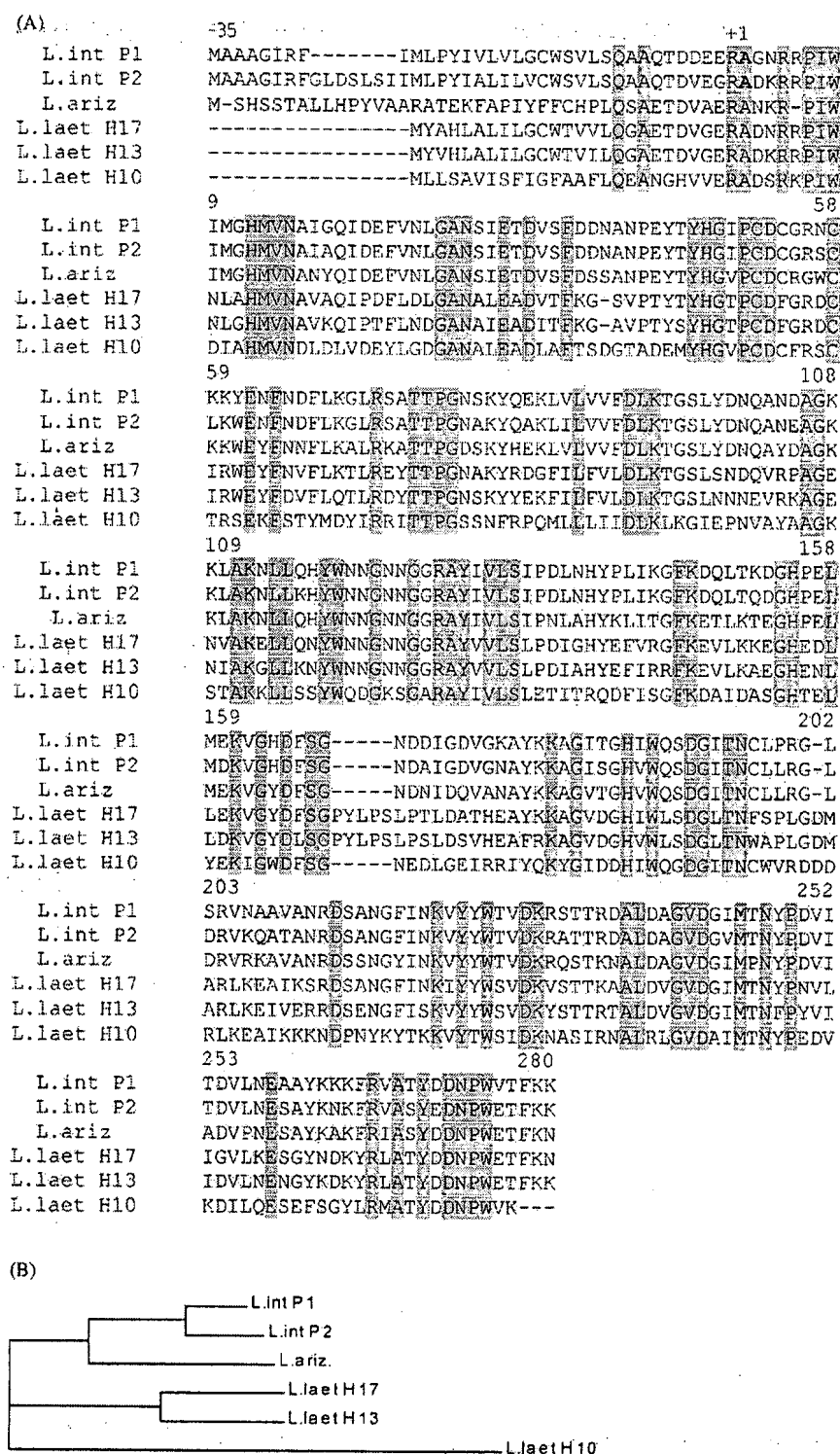


Fig. 1. (A) Alignment of the complete deduced amino acid sequences of *Loxosceles intermedia* (L. int), *arizonica* (L. ariz) and *laeta* (L. laet) toxins. The N-terminal amino acid position (in bold; Tambourgi et al., 1998a) of the mature toxins is located at position +1. (B) Dendrogram based on deduced amino acid sequences of *L. intermedia* Smases P1 and P2; *L. laeta* Smase I (H17), H10 and H13 (accession numbers: AY093599, AY093600 and AY093601, respectively (Fernandes Pedrosa et al., 2002); and "sphingomyelinase D dermonecrotic enzyme precursor" from *L. arizonica* (accession number: AF512953; unpublished data).

IPTG. Recombinant proteins (rP1 and rP2) were harvested from the pellet by French pressure and purified on a Ni (II) Chelating Sepharose Fast Flow column (Pharmacia, Sweden, 1.0 × 6.4 cm). Smase I of *L. laeta* was obtained as previously described (Fernandes Pedrosa et al., 2002).

2.11. Sphingomyelinase activity

Sphingomyelinase activity of *L. intermedia* venom and of the recombinant proteins was assayed by measuring the enzyme-catalyzed hydrolysis of TNPAL-sphingomyelin (Sigma) as described (Gatt et al., 1980; Tambourgi et al., 2002).

2.12. Antiserum

Adult rabbits were injected intradermally with 500 ng of F35 (unfractionated P1, P2, P3 purified from *L. intermedia* venom) (Tambourgi et al., 1995) or with 3 µg of the recombinant proteins (rP1, rP2 or rSmase I of *L. laeta*) adsorbed to Al(OH)₃. The injections were repeated five times with 15 days intervals. Blood samples were collected 1 week after the last injection and sera stored at –20 °C.

2.13. Treatment of erythrocytes with the recombinant sphingomyelinases P1 and P2

Erythrocytes were washed and resuspended at 2% in VBS²⁺ and incubated with increasing concentrations of the recombinant toxins or *L. intermedia* venom for 30 min at 37 °C. Control samples were incubated with VBS²⁺. The cells were washed five times, resuspended to the original volume in VBS²⁺, and analyzed in a hemolysis assay or prepared for flow cytometry analysis.

2.14. Hemolysis assays

One hundred microliter of 2% E pre-incubated for 30 min with recombinant toxins, venom or VBS²⁺ were mixed with 100 µl of NHS (1/2 in VBS²⁺). Background or total cell lysis was evaluated by incubation of E with VBS²⁺ or H₂O, respectively. After incubation for 1 h at 37 °C, unlysed cells were spun down; the absorbance of the supernatant was measured at 414 nm and expressed as percentage of lysis. Mean and standard deviations were determined from duplicate samples. E and NHS were always from the same donor.

2.15. Flow cytometry

Human erythrocytes (25 µl of 2%) pre-treated or not with venom/recombinant proteins were incubated for 30 min with 25 µl of anti-GPA, anti-GPC (both 1 µg/ml) or with rabbit serum against F35 (diluted 1:250 in FACS buffer). After washing, cells were incubated with the appropriate FITC-labeled secondary antibodies for 30 min. The cells

were washed and fixed in FACS buffer containing 1% paraformaldehyde and analyzed by flow cytometry (FAC-Scalibur, Becton Dickinson, CA, USA).

2.16. Dermonecrotic activity

Two hundred microliters of recombinant toxins or *L. intermedia* venom in PBS (25 µg/ml) were injected intradermally in the shaved back of adult rabbits. Control sites were injected with equal volume of PBS. The size of the lesions was measured during a 24 h period after injection.

2.17. ELISA

Microtitre plates were coated with 100 µl of *Loxosceles* venoms or recombinant proteins (10 µg/ml; overnight at 4 °C). Plates were blocked with 5% BSA in PBS and dilutions of rabbit anti-rP1, rP2 or rSmase I sera made in PBS were added. After 1 h incubation at room temperature (RT), plates were washed with PBS/0.1% Tween 20 and incubated with GAR-HRPO (1/1000; 1 h, RT). Plates were washed and developed with OPD substrate according to the manufacturers conditions (Sigma, St. Louis, USA).

2.18. Electrophoresis and Western blotting

Recombinant proteins (rP1, rP2 and rSmase I of *L. laeta*) or *L. intermedia*, *L. gaucho* and *L. laeta* venoms were solubilised in non-reducing sample buffer and run on 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue or were blotted onto nitrocellulose. After transfer, the membranes were blocked with PBS containing 5% BSA and incubated with rabbit sera against F35 fraction or anti-recombinant proteins (diluted 1:250) for 1 h at room temperature. Membranes were washed three times for 10 min each with PBS/0.05% Tween 20, and incubated with GAR-IgG-AP (1/3000) in PBS/1% BSA for 1 h at room temperature. After washing three times for 10 min each with PBS/0.05% Tween 20, blots were developed using NBT/BCIP according to the manufacturer's instructions (Promega).

3. Results

3.1. Cloning of *L. intermedia* spider venom sphingomyelinases P1 and P2

5' and 3' rapid amplification (RACE) of cDNA generated from mRNA extracted from the *L. intermedia* gland yielded DNA fragments of 1166 bp and 1131 bp in length, corresponding to P1 and P2 proteins (accession numbers: AY304471 and AY304472), respectively. Translation of the cDNA open reading frames for P1 and P2 revealed that both proteins comprised two putative signal peptides (P1, 35 and

26; P2, 42 and 26 amino acids in length; inferred from the N-terminal sequence data (Tambourgi et al., 1998a)), which upon cleavage results in a mature protein of 280 amino acids with a predicted mass of 31,236 Da for P1 and 31,277 Da for P2 (Fig. 1). Additionally, cDNA sequence data confirmed the N-terminal amino acid sequences previously obtained by Edman degradation for P1 and P2 (Tambourgi et al., 1998a). Comparative DNA sequence analysis of the regions containing the two Methionine start codons with the consensus sequences for initiation of eukaryotic translation (Kozak, 1984) revealed that the initiation sites of the longer signal peptides for P1 and P2 exhibited a greater level of homology with the consensus compared to the shorter signal peptides. This suggests that the longer signal peptides in all probability represent the N-termini of the precursor proteins.

Comparative analysis of P1 and P2 reveals approximately 85% identity at the level of amino acid and DNA sequence (Fig. 1). Additional comparative analysis between *L. intermedia* P1 and P2 proteins with the recently cloned 'Sphingomyelinase I (H17)' from *Loxosceles laeta* spider (accession number: AY093599; Fernandes Pedrosa et al., 2002) and 'sphingomyelinase like proteins H13 and H10' (accession numbers: AY093600 and AY093601; Fernandes Pedrosa et al., 2002) and 'sphingomyelinase D dermonecrotic enzyme precursor' from *L. arizonica* (accession number: AF512953; unpublished) revealed percentages of identity at the amino acid level of 40–85% between and within species (Fig. 1). Such levels of similarities can be seen in the dendrogram (Fig. 1B) generated by using Vector NTI Suite software (Informax) based on the sphingomyelinase amino acid sequences.

We have previously shown that the phospholipases D (PLD) of *Corynebacterium pseudotuberculosis* (accession number L16586; McNamara et al., 1995) displays 24–40% homology with the first 30 amino acids of *L. intermedia* toxins (Tambourgi et al., 1998a) and we have also demonstrated that, similar to P1 and P2, this PLD, can induce dermonecrosis and complement dependent hemolysis of autologous erythrocytes (Tambourgi et al., 2002). Further-

more, bacterial PLD, similar to P1 and P2, contain four cysteines in the mature protein suggesting that the prerequisite of correct eukaryotic folding involving these residues may be unnecessary for the activity of the spider proteins.

3.2. Production of recombinant mature *L. intermedia* toxins

The cDNA fragments encoding the mature *L. intermedia* P1 and P2 toxins were subcloned in bacterial expression vector pRSET B and expressed in *E. coli* BL21(DE3) with a polyhistidine tag, the anti-X pressTM epitope, an enterokinase cleavage site followed by the mature *Loxosceles* P1 and P2 proteins, respectively. Expression of P1 and P2 resulted in 37 and 36 kDa recombinant proteins, respectively, clearly visible by SDS-PAGE in the bacterial cell extracts (Fig. 2). The intensity of the bands was amplified by further incubation with IPTG. No band was observed in cells transformed with the vector only (data not shown).

The recombinant P1 (rP1) and P2 (rP2) proteins were purified from the soluble fraction of cell lysates by Ni(II)-chelating chromatography and eluted from the resin in extraction buffer containing 0.8 M imidazole at greater than 95% purity (Fig. 2A). Rabbit polyclonal anti-serum raised against the native purified *L. intermedia* venom sphingomyelinases (anti-F35) recognized purified recombinant P1 and P2 as well as a band of approximately 33 kDa in the spider venom by western blotting (Fig. 2B). The slightly higher Mr of the recombinant proteins was due to the extra N-terminal tag, which increased the size of the recombinant proteins by approximately 3 kDa.

3.3. Functional activities of recombinant *L. intermedia* P1 and P2

In order to assess whether the recombinant proteins P1 and P2 displayed all the functional activities of whole venom, several functional assays were carried out.

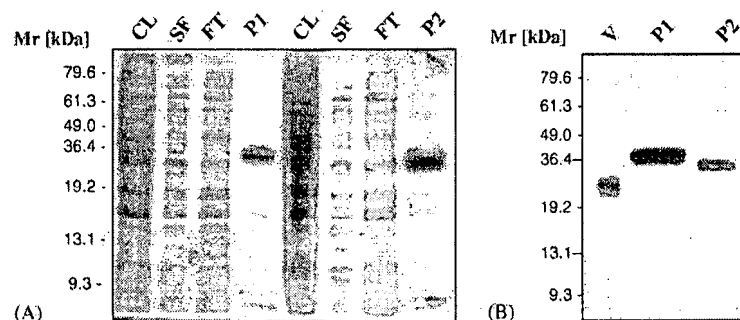


Fig. 2. SDS-PAGE and Western blot analysis of recombinant mature P1 and P2 toxins. (A) Extracts of *E. coli* BL21(DE3) transformed with the pRSETB-*L. intermedia* P1 or P2 cDNAs were induced with IPTG for 2 h. The cells were collected by centrifugation (CL), resuspended in extraction buffer and disrupted by french-pressure. The supernatant (SF) was loaded onto a Ni(II) Chelating Sepharose Fast Flow column, the flow through collected (FT), the column washed and the recombinant protein (P1 and P2) eluted with the elution buffer. Samples were separated by SDS-PAGE (12% gel) under non-reducing conditions and stained with Coomassie blue. (B) Purified recombinant proteins P1 and P2 and *L. intermedia* venom (V) were run on 12% SDS-PAGE gel under non-reducing conditions and western blotted using anti-serum raised against *L. intermedia* sphingomyelinases (anti-F35 serum).

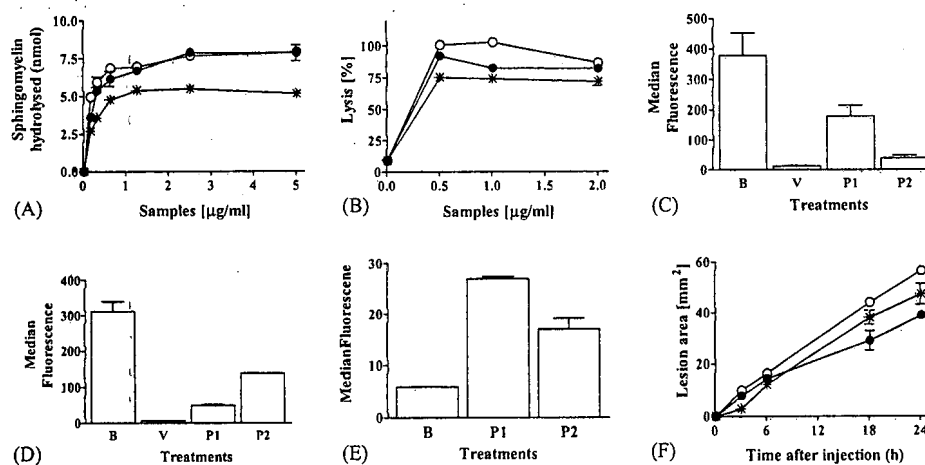


Fig. 3. Functional activities of recombinant *L. intermedia* P1 and P2. (A) *Smase* activity: TNPAL-sphingomyelin was incubated with different amounts of buffer, *L. intermedia* recombinant proteins P1 (●), P2 (○) or venom (*). After 2 h of incubation at 37 °C, the reaction was stopped, the absorbance determined and the sphingomyelinase activity expressed as nmol of TNPAL-sphingomyelin hydrolysed. (B) *Hemolysis*: E pre-treated with different amounts of purified recombinant proteins P1 (●), P2 (○), venom (*) or VBS²⁺ (B) were incubated with NHS. After incubation for 1 h at 37 °C, unlysed cells were spun down; the absorbance of the supernatants was measured at 414 nm and expressed as percentage of lysis. Alternatively, pre-treated E were analyzed for the expression of (C) GPA and (D) GPC, by flow cytometry using the antibodies Bric 256 and Bric 4, respectively. (E) *Toxin binding*: the ability of the toxins rP1 and rP2 to bind to the E surface was tested by flow cytometry using polyclonal rabbit serum against native purified Smases (anti-F35). (F) *Dermonecrosis*: adult rabbits were injected intradermally with 5 µg of rP1 (●), rP2 (○) or crude venom (*). The animals received buffer for negative control reactions. The areas of the dermonecrotic lesions were determined during a 24 h period after injection.

Sphingomyelinase activity, as exhibited by native P1 and P2, was preserved in the corresponding cloned toxins and was assessed by the ability of these recombinant proteins to hydrolyze TNPAL-sphingomyelin, a synthetic colored derivative of sphingomyelin. Recombinant proteins were more active than the whole venom (Fig. 3A).

We have previously shown that sphingomyelinases isolated from *L. intermedia* spider venom transform human erythrocytes (E) into activators of the complement system (Tambourgi et al., 1995, 1998a, 2000, 2002). In order to assess whether the recombinant toxins could also induce C-dependent hemolysis, E were incubated with increasing amounts of rP1 and rP2 or crude venom. As shown in Fig. 3B, the recombinant proteins as well as *L. intermedia* venom were able to render E susceptible to lysis by autologous C in a dose dependent manner.

Removal of glycoporphins (GPs) is partially responsible for the increased C-susceptibility of erythrocytes treated with P1 or P2 toxins purified from *L. intermedia* venom as we have previously shown (Tambourgi et al., 2000). To investigate if the recombinant sphingomyelinases induced a similar response, E were incubated with the toxins, venom or buffer and analyzed for the expression of glycoporphin A (GPA) and C (GPC) by flow cytometry. A significant reduction in binding of anti-GPA and GPC antibodies, recognizing extracellular epitopes close to the membrane, was observed after treatment of E with rP1 and rP2 or crude venom (Fig. 3C and D), suggesting that the recombinant proteins induced the cleavage of the glycoporphins. In this assay, whole venom showed more potency than the recombinant proteins. The

disappearance of GPA and GPC epitopes was associated with the incorporation of the toxins into the E cell surface as detected by anti-F35 serum (Fig. 3E).

The ability of rP1 and rP2 to induce dermonecrotic lesions was tested by injecting rabbits with 5 µg of the proteins. The animals received either buffer or *L. intermedia* venom (5 µg) as negative and positive controls, respectively. A typical loxoscelic lesion, as revealed by the presence of edema, erythema and mild tenderness, developed in the skin area injected with rP1 and rP2 or venom within a few hours of injection and approximately 24 h post injection, necrosis with gravitational spread and scar were observed at the inoculation site (Fig. 3F). Recombinant proteins and whole venom were equally potent.

3.4. Immunochemical cross-reactivity

Comparison of the amino acid sequences of the recombinant toxins from *L. intermedia* (rP1 and rP2) and *L. laeta* (SmaseI), demonstrated as expected a high level of identity between P1 and P2 (85%), but a much lower level of identity between P1 or P2 and Smase I (around 56%). Monospecific polyclonal anti-sera were produced in rabbits by injection with purified recombinant proteins. These anti-sera were able to neutralize the dermonecrotic activity of the recombinant proteins and the whole venoms (Fernandes Pedrosa et al., 2002; unpublished). In an ELISA, using recombinant proteins rP1, rP2 and rSmase I and *L. intermedia*, *L. laeta* and *L. gaucha* venoms as antigens, the antisera were tested for cross-reactivities. As

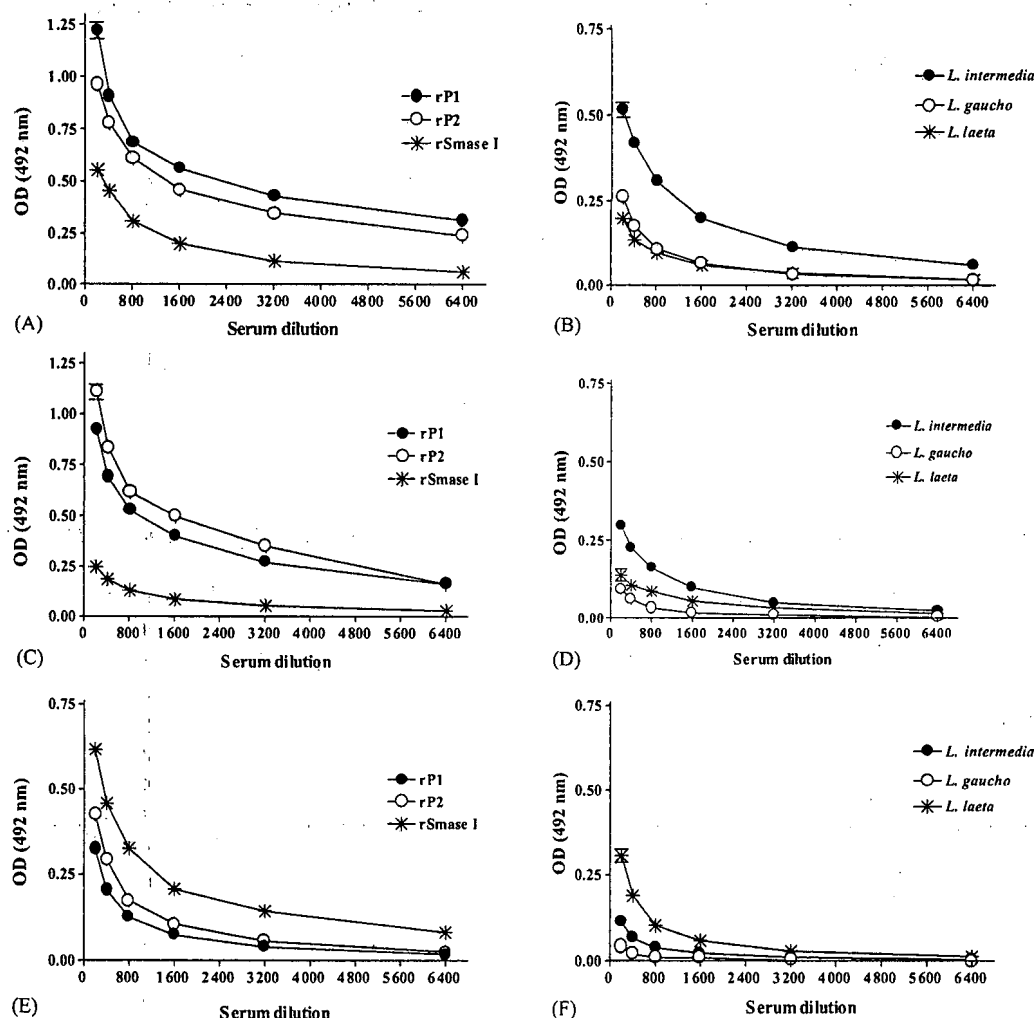


Fig. 4. Cross-reactivities of rabbit anti-recombinant *Loxosceles* sera. Smases measured by ELISA. ELISA plates were coated with 100 ng of *L. intermedia* recombinant P1 (rP1) and P2 (rP2), and *L. laeta* recombinant Smase I (rSmase I; A, C, E) or with *Loxosceles* venoms (*L. intermedia*, *L. gaucha* and *L. laeta*) (B, D, F), and incubated with different dilutions of rabbit anti-rP1 (A, B), rP2 (C and D) or rSmase I (E and F) sera followed by GAR-HRPO. The absorbance of the samples were determined at 490 nm. The data presented correspond to mean OD₄₉₂ value $\pm$ S.D. of experiments carried out in duplicate.

shown in Fig. 4, antisera were most effective in detecting the antigen they were raised against. Cross-reactivity was observed and reactivity was highest within the same species. Reactivity of anti-*L. intermedia* recombinant proteins P1 and P2 was lowest against *L. laeta* Smase I or whole

venom and vice versa. Western blot analysis of whole venoms and recombinant proteins probed with antisera raised against the recombinant proteins again showed that reactivity was stronger against the homologous species antigens (Fig. 5).

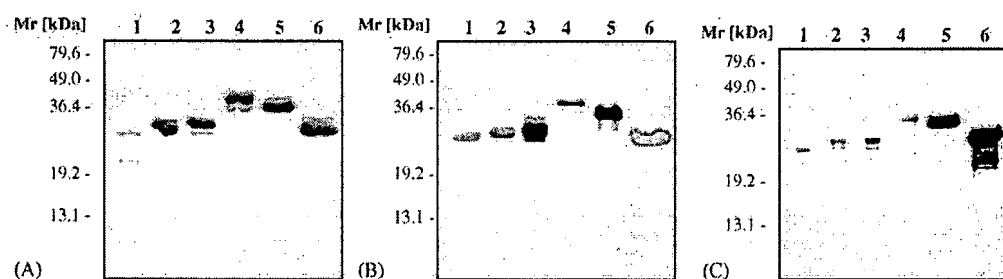


Fig. 5. Cross-reactivity of anti-recombinant toxins as analyzed by western blotting. Venom (10 μ g) from *L. laeta* (1), *L. gaucha* (2) and *L. intermedia* (3) and the recombinant proteins (2 μ g) P1 (4), P2 (5) and Smase I (6) were run on 12% SDS-PAGE gel under non-reducing conditions and western blotted using anti-sera raised against rP1 (A), rP2 (B) and rSmase I (C).

4. Discussion

We have cloned and expressed the two isoforms P1 and P2 of the sphingomyelinase toxins of *L. intermedia* (Fig. 1; Genbank accession numbers: AY304471 and AY304472, respectively). The two isoforms showed a high degree of homology with each other (85%). Comparative DNA and protein sequence analysis with a sphingomyelinase cloned from *Loxosceles arizonica* (AF512953; unpublished; activity not established) also revealed a high degree of homology (72%). A lower level of homology was observed with the sphingomyelinase we recently cloned and expressed from *L. laeta* (57% identity with H17; AY093599; Fernandes Pedrosa et al., 2002) and the sphingomyelinase like proteins we recently cloned, but have not yet expressed, from *L. laeta* (57% identity with H13; and 42% identity with H10; Genbank accession numbers: AY093600 and AY093601; Fernandes Pedrosa et al., 2002). Specific regions of the sphingomyelinase proteins are highly conserved (Fig. 1A), suggesting that these residues may be important for the enzymatic activity and binding to the substrate.

Based on the spider morphology and geographic distribution, these three spider species can be classified in different groups. *L. arizonica* belongs to the “*reclusa* group” of Gertsch (Gertsch, 1958), which includes 50 species distributed in North America, Central America and West Indies. *L. laeta* belongs to “*laeta* group” (Gertsch, 1967), which includes 24 species distributed in South and Central America; *L. intermedia*, which has been identified in Brazil and Argentina, belongs to “*spadicea* group” (Gertsch, 1967), which includes two other species, *L. spadicea*, found in Bolivia and *L. hisurta*, found in Brazil, Paraguay and Argentina.

Comparison of the cDNA and amino acid sequences suggests that *L. intermedia* (*spadicea* group) is more closely related to *L. arizonica* (*reclusa* group) than to *L. laeta* (*laeta* group) (Fig. 1A and B). It is interesting to observe that even though *L. laeta* and *L. intermedia* are spider species endemic to South America, their Smases genes and amino acid sequences are less homologous with one and other than with the one from *L. arizonica*, an endemic North America spider species. The low level of homology between *L. intermedia* and *L. laeta* sphingomyelinases however, may predict a lower level of cross-reactivity of anti-sera raised against the individual toxins.

The *L. intermedia* spider venom P1 and P2 recombinant proteins were assessed for their functional activities and we show here that they are both endowed with all biological properties ascribed for the whole venom and for the native toxins, e.g., dermonecrotic and complement-dependent hemolytic activities and ability of hydrolyzing sphingomyelin (Fig. 3). These results clearly demonstrate that the sphingomyelinases are the major or unique toxins responsible for the main symptoms of both systemic and cutaneous forms of loxoscelism. The cloning and expression of the toxins also provides useful tools to study the mechanism of pathology induced by the *Loxosceles* venom and these data

will help facilitate the generation of an effective neutralizing antiserum. Recently, the cloning of a partial sequence of a dermonecrotic protein (LiD1) from *L. intermedia* was reported (Kalapothakis et al., 2002; Genbank accession number: AY340702). This protein exhibited a high degree of homology with P1, differing by a few residues; however, these authors failed to obtain a functionally active recombinant molecule.

The comparison of the amino acid sequences of the toxins of the different *Loxosceles* species and of the different isoforms within *L. intermedia*, predicted a relatively low level of cross-reactivity of antisera between the species, but a relatively high level of cross-reactivity between the isoforms. Indeed results presented here show that although anti-rP1 and rP2 sera were highly cross-reactive against specific antigens (rP1, rP2 and *L. intermedia* venom), they exhibit a low level of recognition to Smase I of *L. laeta* and to the related components present in *L. laeta* and *L. gaucho* venoms. Similar results were also obtained with anti-Smase I sera which exhibited a low level of cross-reactivity against non-*L. laeta* related species antigens (Figs. 4 and 5). These data suggest that in order to obtain a suitable neutralizing antiserum using recombinant toxin as immunogen, a mixture of the recombinant toxins from the different species is a necessary requirement.

In conclusion, we have cloned, expressed and characterized the toxins from *L. intermedia* venom and shown that they display all the functional characteristics of whole venom. These reagents allow us to further investigate the mechanism of pathology induced by the *L. intermedia* venom. The cloning and functional expression of P1 and P2 *L. intermedia* venom toxins will permit further characterization of the functionally important domains of these proteins. The identification of the active site(s) would aid in the design and testing of suitable anti-sphingomyelinase compounds in the development of novel therapies to treat Loxoscelism.

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Molecular cloning and expression of a functional dermonecrotic and haemolytic factor from *Loxosceles laeta* venom[☆]

Matheus de F. Fernandes Pedrosa,^a Inácio de L.M. Junqueira de Azevedo,^{b,c}
Rute M. Gonçalves-de-Andrade,^a Carmen W. van den Berg,^d Celso R.R. Ramos,^{b,e}
Paulo Lee Ho,^{b,c,e} and Denise V. Tambourgi^{a,*}

^a Laboratório de Imunoquímica, Instituto Butantan, Av. Prof. Vital Brazil, 1500, CEP 05503-900, São Paulo, SP, Brazil

^b Centro de Biotecnologia, Instituto Butantan, Av. Prof. Vital Brazil, 1500, CEP 05503-900, São Paulo, SP, Brazil

^c Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil

^d University of Wales, College of Medicine, Cardiff, UK

^e Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil

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Abstract

The bite of spiders of the genus *Loxosceles* can induce a variety of biological effects, including dermonecrosis and complement-dependent haemolysis. The aim of this study was to generate recombinant proteins from the *Loxosceles* spider gland to facilitate structural and functional studies in the mechanisms of loxoscelism. Using “Expressed Sequencing Tag” strategy of aleatory clones from, *L. laeta* venom gland cDNA library we have identified clones containing inserts coding for proteins with significant similarity with previously obtained N-terminus of sphingomyelinases from *Loxosceles intermedia* venom [1]. Clone H17 was expressed as a fusion protein containing a 6× His-tag at its N-terminus and yielded a 33 kDa protein. The recombinant protein was endowed with all biological properties ascribed to the whole *L. laeta* venom and sphingomyelinases from *L. intermedia*, including dermonecrotic and complement-dependent haemolytic activities. Antiserum raised against the recombinant protein recognised a 32-kDa protein in crude *L. laeta* venom and was able to block the dermonecrotic reaction caused by whole *L. laeta* venom. This study demonstrates conclusively that the sphingomyelinase activity in the whole venom is responsible for the major pathological effects of *Loxosceles* spider envenomation.

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Loxoscelism is the clinical condition produced by the venom of spiders belonging to the genus *Loxosceles*. Human cases of loxoscelism have been reported in diverse countries of different continents in temperate and tropical regions. There are numerous *Loxosceles* species, including more than 17 in Africa, two in Europe, and more than 50 in North and South America [2]. *Loxosceles laeta*, possibly the most toxic and dangerous of all the species, is endemic primarily in South and Central

America, although it has been found as far as the north of Canada [3,4]. Loxoscelism can be observed as two well-defined clinical variants: cutaneous loxoscelism (CL) and systemic or viscerocutaneous loxoscelism (VCL) which occur in around 83.3% and 16.7% of the cases, respectively [5]. Pain, oedema, and livedoid plaque, which develops later into a necrotic scar, are the predominant local manifestations in CL. In VCL, haematuria and haemoglobinuria are always observed, while jaundice, fever, and sensorial involvement occur in most of the cases. Although systemic loxoscelism is less common than the cutaneous form, it is the main cause of death associated with *Loxosceles* envenomation. Most of the deaths occur in children and are related to the South American species, *L. laeta* [6]. Due to a limited

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* Corresponding author. Fax: +55-11-3726-1505.

E-mail address: dvtambourgi@butantan.gov.br (D.V. Tambourgi).

understanding of the venom's mechanism of action, effective treatment is still not available. Sphingomyelinase D, the primary constituent of the venom, has been implicated in both local tissue destruction as well as haemolysis [7].

We have recently identified, purified, and characterised the toxins from *Loxosceles intermedia* venom that are responsible for all the local and systemic effects induced by whole venom [1,8]. Two highly homologous proteins with M_r 35 kDa were purified to homogeneity and shown to be endowed with sphingomyelinase activity. These proteins, termed P1 and P2, were both able to induce dermonecrosis in experimental animals and rendered human erythrocytes (E) susceptible to lysis by Complement (C) in vitro. In a mouse model of *Loxosceles* envenomation, we showed that the toxins also induced intravascular haemolysis and provoked a cytokine response, which resembles that seen in endotoxic shock [9]. The spider toxins P1 and P2 have probably originated by gene duplication and show a high level of amino acid sequence identity with two other molecules in the spider venom. The latter molecules named P3 and P4, despite the high degree of identity in N-terminal amino acid sequence with P1 and P2, were ineffective in all activity assays [1].

The haemolysis induced by *Loxosceles* venom is dependent on activation of complement via the alternative and classical pathway [8,10,11]. The toxins P1 and P2, unlike some other sphingomyelinases, do not directly induce lysis of erythrocytes, but render the cells susceptible to complement lysis by two different mechanisms. P1 and P2 induced cleavage of glycoporphins from the erythrocyte surface, making the cell surface susceptible to complement activation via the alternative pathway resulting in haemolysis [10]. The cleavage was not the result of a direct action of P1 and P2 on glycoporphins and was prevented by metalloproteinase inhibitors, suggesting that the sphingomyelinase activity of the toxins induces activation of an endogenous membrane bound metalloproteinase, which then cleaves glycoporphins. More recently, it was observed that the *L. intermedia* toxin P1 can induce activation of the classical pathway of complement, possibly by inducing loss of membrane asymmetry [11]. The exact mechanisms of these events remain to be elucidated.

One of the limiting factors to study the mechanisms involved in loxoscelism is the difficulty in obtaining large amounts of venom and purified venom components. In addition, although the toxins from *L. intermedia* have been purified to apparent homogeneity, it cannot be excluded that small contaminants in these venom-derived preparations contribute to the biological effects. The recombinant expression of the sphingomyelinase is thus necessary for further functional and structural characterisation of the toxin and is a pre-requisite for

site-directed mutagenesis studies that may be informative in further dissecting the mechanism of sphingomyelinase action on human blood cells and tissues. This represents the first report of a cDNA cloning and expression of a functional dermonecrotic and complement-dependent haemolytic factor obtained from a cDNA library of *Loxosceles* spider venom glands.

Materials and methods

Antibodies. Monoclonal antibody against Glycophorin C (Bric 4: extracellular epitope, aa 16–23) was from the International Blood Group Reference Laboratory (IBGRL, Bristol, UK) [12]. Goat antibodies against human complement components C4, C3, and fB were from Quidell. Rabbit anti-mouse IgG-FITC, goat anti-rabbit IgG-FITC (GAR/IgG-FITC), and rabbit anti-goat IgG-FITC (RAG/IgG-FITC) were from Amersham Pharmacia Biotech. Goat anti-rabbit IgG-alkaline phosphatase (GAR/IgG-AP) was from Promega.

***Loxosceles laeta* spider venom.** *Loxosceles laeta* spiders were provided by "Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil." The venom was obtained by electro-stimulation as previously described [8,13].

cDNA library construction. One hundred *L. laeta* female spider specimens were milked to stimulate the production of mRNAs in the venom glands. After 5 days, the spider venom glands were collected and kept in liquid nitrogen until use. For total RNA extraction, Trizol reagent (Gibco-BRL Life Technologies) was used according to manufacturer's protocol. An oligo(dT) cellulose column (Amersham Pharmacia Biotech) was used for mRNA purification. The cDNAs were synthesised from 5 µg mRNA using the Superscript Plasmid System for cDNA Synthesis and Cloning (Gibco-BRL Life Technologies), linked to *EcoRI* adapters (Amersham Pharmacia Biotech), and cloned in pGEM11Zf⁺ plasmid (Promega) at *EcoRI/NotI* sites [14]. *Escherichia coli* DH5α cells were transformed with the cDNA library plasmids and then plated on 2YT (Gibco-BRL Life Technologies) agarose plates containing 100 mg/mL ampicillin [15].

Sequence identification. Random colonies were inoculated in 2YT culture medium, grown overnight, and the DNA was purified and sequenced on an ABI 377 automatic sequencer using BigDye2 dideoxymutators (Applied Biosystems). The primers used to sequence were M13 forward and M13 reverse. Complete cDNA sequence was obtained from three clones by sequencing and deposited at GenBank under Accession Nos.: AY093599, AY093600, and AY093601. Sequence analysis was performed by using Vector NTI Suite software (Informax). DNA sequences were compared to GenBank nucleotide and protein databases using Blastn and Blastx programs at NCBI.

Recombinant protein expression. The cDNA encoding the mature *L. laeta* protein was amplified by PCR using clone H17 as template. The *L. laeta* protein primer (5'-CTC GAG GGA TCC GCT GAT AAC CGT CGT CCA AT-3') was designed to contain a *Bam*HI restriction site (underlined) and the nucleotide sequence coding the first seven amino acids of the putative mature protein (excluding the signal sequence) (Fig. 1). The reverse primer used was (5'-ATT TAG GTG ACA CTA TAG-3') which anneals to the SP6 promoter located in the pGEM11Zf⁺ vector. The PCR product was cloned into pGEM-T plasmid (Promega). pGEM-T plasmid DNA containing the mature *L. laeta* protein coding sequence was digested with *Bam*HI and *Hind*III enzymes. The excised insert was subcloned in a T7-based promoter plasmid (pAE) digested with the same enzymes [14], resulting in the pAE-*L. laeta* H17 cDNA construct. The correct cloning was confirmed by sequencing. The recombinant mature *L. laeta* protein was expressed as a fusion protein, including a 6× His-tag at the N-terminus and a four amino acid linker (N-terminal amino acid sequence before the coding sequence of the mature protein: MHHHHHHLEGS).

Fig. 1. Complete cDNA sequence of *L. laeta* Smase I (clone H17) and its deduced protein sequence. (A) The putative signal peptide is underlined by dots and the polyadenylation signal is between parentheses. The arrow corresponds to N-terminal primer H17. The asterisk denotes the stop codon. (B) Alignment of the complete deduced amino acid sequences of *L. laeta* Smase I (clone H17) and clones H13 and H10. The N-terminus amino acid position of the mature toxins is located at position +1.

For the production of the *L. laeta* recombinant protein, pAE-*L. laeta* H17 cDNA transformed *E. coli* BL21 (DE3) cells were inoculated in 50 mL of 2YT/amp and grown overnight at 37 °C. The culture was diluted with 2YT/amp medium to 500 mL and grown until the OD at 600 nm reached 0.6. IPTG was added to a final concentration of 1 mM and the induced culture was incubated for additional 2 h at 37 °C. The cells were then collected by centrifugation and the bacterial pellet was resuspended in extraction buffer (100 mM Tris-HCl, pH 8.0; 300 mM NaCl) and disrupted by French-pressure. The supernatant was loaded onto a Ni(II)-Chelating Sepharose Fast Flow column (Pharmacia, Sweden, 1.0 × 6.4 cm). The column was washed with 10 volumes of extraction buffer, followed by 10 volumes of washing buffer (100 mM Tris-HCl, pH 8.0; 300 mM NaCl; 60 mM imidazole) and the recombinant protein was eluted with the elution buffer (100 mM Tris-HCl, pH 8.0; 300 mM NaCl; 0.8 M imidazole). Fractions of 1 mL were collected and analysed by SDS-PAGE. Samples were dialysed against

PBS and the protein was determined by BCA Protein Assay Kit (Pierce).

Sphingomyelinase activity. Sphingomyelinase activity of *L. laeta* venom and of the recombinant protein was assayed by measuring the enzyme-catalysed hydrolysis of TNPAL-sphingomyelin (Sigma) as described [16].

Antisera. Adult rabbits were injected intradermally with 3 µg recombinant *L. laeta* protein adsorbed with Al(OH)₃. The injections were repeated four times at weekly intervals. Blood samples were collected 1 week after the last injection and the sera were stored at -20 °C.

Electrophoresis and Western blotting. Samples of gland extract, recombinant protein or venom from *L. laeta* were solubilised in reducing sample buffer and run on 12% SDS-PAGE [17]. Gels were blotted onto nitrocellulose. After transfer, the membrane was blocked with PBS containing 5% BSA and incubated with rabbit serum anti-

recombinant protein (diluted 1:1000) for 1 h at room temperature. Membrane was washed three times with PBS/0.05% Tween 20 for 10 min each wash and incubated with GAR/IgG-AP (1/3000) in PBS/1% BSA for 1 h at room temperature. After washing three times with PBS/0.05% Tween 20 for 10 min each wash, blot was developed using NBT/BCIP according to manufacturer's instructions (Promega).

Normal human serum and erythrocytes. Human blood was obtained from healthy donors. Blood samples drawn to obtain sera were collected without anticoagulant, allowed to clot for 2 h at room temperature, and the normal human serum (NHS) was stored at -80°C . C9-depleted human serum (C9d-HS) was obtained by passage of NHS over a mAb anti-C9 Sepharose 4B column. Blood samples drawn to obtain erythrocytes (E) for subsequent use as target cells were collected in anticoagulant (Alsever's old solution: 114 mM citrate, 27 mM glucose, and 72 mM NaCl, pH 6.1).

Treatment of erythrocytes with *L. laeta* recombinant protein/venom. E were washed and resuspended at 2% in VBS²⁺ (Veronal Buffered Saline: 10 mM Na barbitone, 0.15 mM CaCl_2 , and 0.5 mM MgCl_2 , pH 7.4), and incubated with different concentrations of the recombinant protein or venom for 1 h at 37°C . Control samples were incubated with VBS²⁺. The cells were washed five times, resuspended to the original volume in VBS²⁺, and analysed in a haemolysis assay, following the procedures described for Tambourgi et al. [8], or prepared for flow cytometry.

Deposition of serum complement components on E. Erythrocytes were incubated with recombinant *L. laeta* protein, venom, or buffer and subsequently incubated with C9d-HS for 45 min using conditions as described for the haemolysis assay. C9d-HS did not cause any haemolysis. Cells were washed twice in VBS²⁺ and subsequently analysed by flow cytometry.

Flow cytometry. Analysis of cell surface antigens and deposition of complement components on erythrocytes was assessed by flow cytometry as previously described [8,10].

Dermonecrotic activity. Samples of 1, 2.5, and 5 μg *L. laeta* venom or recombinant protein in PBS were injected intradermally in the shaved back of adult rabbits. Control sites were injected with equal volume of PBS. The size of the lesions was measured after 24 h.

Serum neutralisation. Neutralisation of *L. laeta* venom toxicity was determined by measuring the dermonecrotic activity remaining in samples of 1, 2.5, and 5 μg venom, preincubated for 60 min at 37°C with normal or anti-recombinant protein rabbit sera (diluted 1:2). The size of the lesions was measured after 72 h.

Results

Identification and characterisation of *L. laeta* spider venom toxin by EST sequencing

A cDNA library was constructed from the venom glands of *L. laeta* in order to characterise toxins with C-dependent haemolytic and dermonecrotic activities from this specialised tissue. DNA sequences of the random clones obtained were searched with Blastn and Blastx programs [18] for similar sequences in DNA and protein databases. From the first 40 clones analysed, 3 revealed (clone H10, H13, and H17) significant similarity at the amino acid level with partial characterised N-terminus of Smases P1 (EC 3.1.4.12; Swiss Prot no. P83045) and P2 (EC 3.1.4.12; Swiss Prot no. P83046) from *L. intermedia* spider venom [1], mammalian toxin from *L. reclusa* venom (ac: A38107) [19], and dermonecrotic factors from *L. laeta* and *L. gaucho* venom (ac:

AAB50796.1; ac: AAB50794.1) [20]. The longest cDNA clone (H17) revealed an open reading frame of 311 amino acids with an ATG start codon at position 12 and the TAG stop codon at position 944 (Fig. 1A). The deduced protein has a predicted signal peptide composed of the first 26 amino acids, according to Nilsen's algorithm [21]. A molecular mass of 32 kDa was predicted for the putative mature protein. Similar procedure was developed to determine the complete nucleotide sequences of clones H10 and H13 (GenBank Accession Nos.: AY093601 and AY093600, respectively). The deduced complete amino acid sequences of the clones H13 and H17 showed a high degree of identity with each other (81%) while the identity of these clones with clone H10 was still significantly but much lower (40%) (Fig. 1B).

Production of recombinant mature *L. laeta* protein

The amino acid sequence deduced for clone H17 showed the highest degree of identity with the known *Loxosceles* toxins N-terminus and was selected for expression in a bacterial system. The cDNA fragment encoding the mature *L. laeta* protein from clone H17 was amplified by PCR, cloned in pGEM-T, and subcloned in the T7-based promoter plasmid pAE [14]. The final construct, pAE-*L. laeta*, encodes a methionine, 6 $\times$ His-tag, and four amino acid linker (LEGS) followed by the mature *Loxosceles* protein. When *E. coli* BL21(DE3) cells were transformed with pAE-*L. laeta*, a 33 kDa recombinant protein was clearly visible by SDS-PAGE in the bacterial cell extracts and the intensity of this band was amplified by further incubation with IPTG. No band was observed in cells transformed with the vector only (data not shown). The recombinant *L. laeta* protein was purified from the soluble fraction of cell lysates by Ni^{2+} -chelating chromatography and eluted from the resin in extraction buffer containing 0.8 M imidazole at greater than 95% purity, resulting in 17.4 mg of per litre of culture (Fig. 2A). Monospecific rabbit polyclonal anti-serum raised against the recombinant protein recognised the purified *L. laeta* recombinant protein as well as a band of approximately 32 kDa in venom and gland extract of *L. laeta* by Western blotting (Fig. 2B). The slightly higher M_r of the recombinant protein is due to the extra N-terminal histidine-tag and linker amino acids, which increase the M_r by approximately 1.0 kDa.

Sphingomyelinase activity

As described above, the deduced protein sequence of the cloned *L. laeta* molecule presented a significant level of identity with the N-terminal amino acid sequences of the active sphingomyelinases (Smase) P1 and P2 of *L. intermedia* venom. The ability of the purified recombinant *L. laeta* protein to degrade sphingomyelin

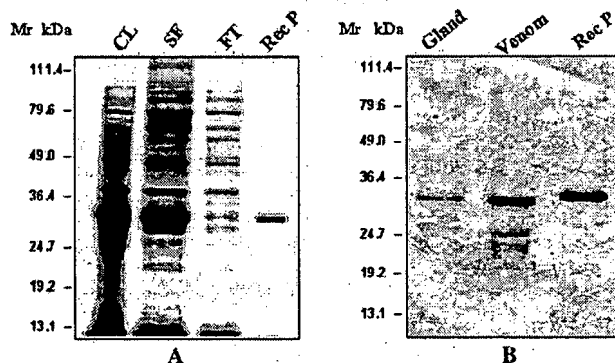


Fig. 2. SDS-PAGE and Western blotting analysis of recombinant mature *L. laeta* Smase I. (A) Extracts of *E. coli* BL21(DE3) transformed with the plasmid pAE-*L. laeta* H17 clone were induced with IPTG for 2 h. The cells were collected by centrifugation (CL), resuspended in extraction buffer, and disrupted by French-pressure. The supernatant (SF) was loaded onto a Ni(II)-Chelating Sepharose Fast Flow column, the flowthrough was collected (FT), the column was washed, and the recombinant protein (Rec protein) was eluted with the elution buffer. Samples were separated by SDS-PAGE (12% gel) under reducing conditions and stained with Coomassie blue. (B) Western blot analysis of purified recombinant protein (Rec Protein), *L. laeta* venom (Venom), and gland extract (Gland) was run on 12% SDS-PAGE gel under non-reducing conditions and Western blotted using anti-serum raised against recombinant Smase I protein.

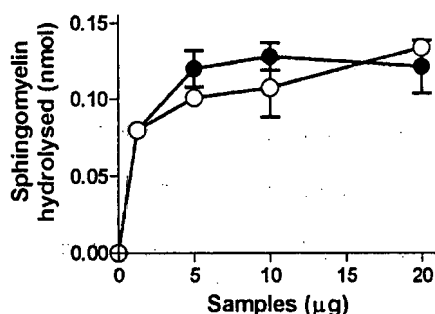


Fig. 3. Sphingomyelinase activity of *L. laeta* recombinant protein. TNPAL-sphingomyelin was incubated with different amounts of buffer, *L. laeta* H17 rec protein (●) or crude venom (○). After 2 h of incubation at 37 °C, the reaction was stopped, the absorbance was determined, and the sphingomyelinase activity was expressed as nmol TNPAL-sphingomyelin hydrolysed. Results are representative for three separate experiments expressed as means of duplicates $\pm$ SD.

was investigated by measuring hydrolysis of TNPAL-sphingomyelin, a synthetic coloured derivative of sphingomyelin. Crude *L. laeta* venom as well as the recombinant protein contained significant sphingomyelinase activity as shown by the breakdown of TNPAL-sphingomyelin (Fig. 3). Considering these data we named the recombinant protein *L. laeta* Smase I.

Loxosceles laeta Smase I induces complement susceptibility

Sphingomyelinases isolated from *L. intermedia* spider venom have been shown to be able to transform human

erythrocytes (E) in activators of the complement system [1,8,10,11]. In order to assess whether the *L. laeta* Smase I could also induce C-dependent haemolysis, E were incubated with increasing amounts of *L. laeta* Smase I or crude venom. As shown in Fig. 4, *L. laeta* Smase I as well as *L. laeta* venom were both able to render E susceptible to lysis by autologous C in a dose-dependent way. *L. laeta* Smase I-, venom-, and buffer-treated erythrocytes were incubated with C9d-HS and deposition of C-components was measured by flow cytometry. Deposition of C3, C4, and fB was found on both *L. laeta* Smase I- and venom-treated E but not on control cells (Fig. 4). These results show that both alternative and classical pathways are initiated after incubation of E with the *L. laeta* Smase I.

Loxosceles laeta Smase I induces release of GPs

We have previously shown that removal of glycoporphins (GPs) is responsible for the increased C-susceptibility of treated erythrocytes with P1 toxin purified from *L. intermedia* venom [10]. To investigate if *L. laeta* Smase I induced a similar response, E were incubated with the *L. laeta* Smase I, venom, or buffer and analysed for the expression of glycoporphin C (GPC) by flow cytometry. A significant reduction in binding of anti-GPC antibodies, recognising extracellular epitopes close to the membrane, was observed after treatment of E with *L. laeta* Smase I or crude venom (Fig. 5A). The disappearance of GPC epitopes was associated with the incorporation of the Smase into the E cell surface as detected by anti-*L. laeta* Smase I anti-serum (Fig. 5B).

Dermonecrotic action of *L. laeta* Smase I and serum neutralisation

The ability of *L. laeta* Smase I to induce dermonecrotic lesions was tested by injecting rabbits with 1, 2.5, and 5 μ g *L. laeta* Smase I. The animals received buffer or *L. laeta* venom (1, 2.5, and 5 μ g) as negative and positive controls, respectively. A typical loxoscelic lesion, as revealed by the presence of oedema, erythema, and mild tenderness, developed in the skin area injected with *L. laeta* Smase I or venom within a few hours of injection and approximately 24 h post-injection, necrosis with gravitational spread and scar were observed at the inoculation site (Fig. 6A).

To determine whether the dermonecrotic effect of *L. laeta* venom could be inhibited by antibodies against the recombinant *L. laeta* Smase I, in vitro-in vivo neutralisation experiments were performed using 1, 2.5, and 5 μ g of *L. laeta* venom and hyper-immune serum against recombinant *L. laeta* Smase I. Fig. 6B shows that anti-recombinant Smase I nearly completely blocked the dermonecrotic inducing-activity of *L. laeta* venom. In

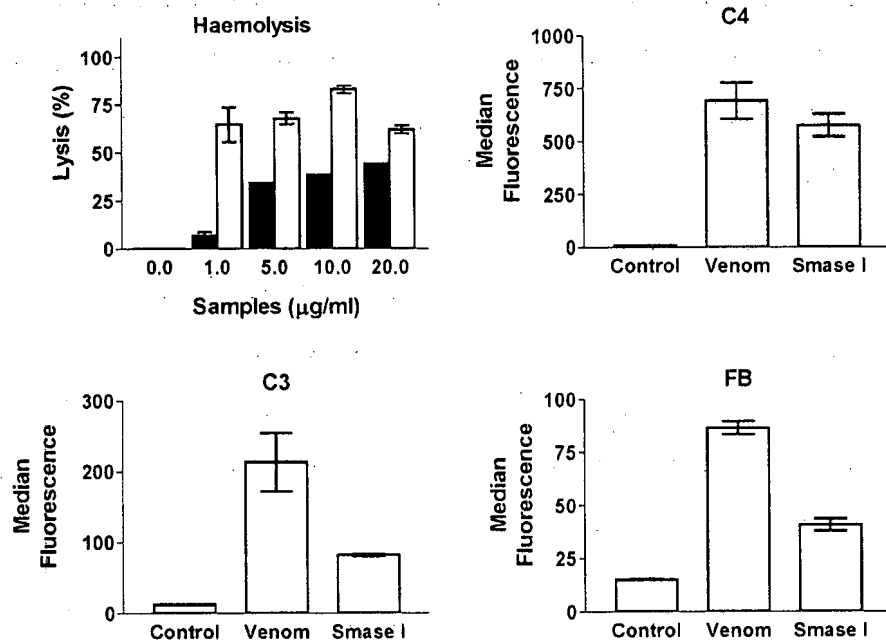


Fig. 4. Induction of complement susceptibility by *L. laeta* Smase I. E pre-treated with different amounts of purified *L. laeta* Smase I (■), venom (□) or VBS²⁺ were incubated with NHS. After incubation for 1 h at 37°C, unlysed cells were spun down; the absorbance of the supernatant was measured at 414 nm and expressed as percentage of lysis (Haemolysis). E incubated with buffer (Control), *L. laeta* Smase I or spider venom (5 µg/mL) were exposed to C9d-HS and deposition of C components C3, C4, or fB was assessed by flow cytometry analysis. Results are representative of three different experiments carried out in duplicate and represented as means ± SD.

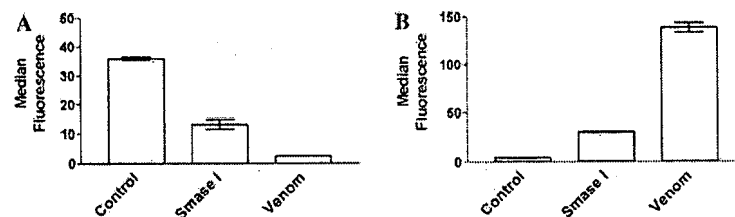


Fig. 5. *Loxosceles laeta* Smase I incorporates into erythrocytes and causes loss of expression of glycophorins. (A) E were treated with buffer, *L. laeta* Smase I or spider venom and analysed for the expression of GPC by flow cytometry using the MoAb Bric 4. (B) The ability of the *L. laeta* Smase I as well as the Smases present in *L. laeta* venom to insert into the E surface was analysed using monospecific polyclonal rabbit serum against the *L. laeta* Smase I. Results are representative for three different experiments expressed as means of duplicates ± SD.

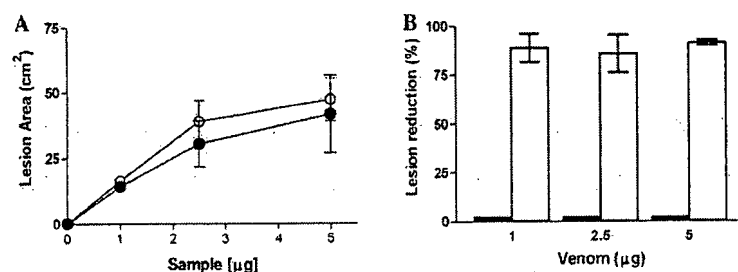


Fig. 6. Dermonecrotic activity of *L. laeta* Smase I and serum neutralization. (A) Adult rabbits were injected intradermally with different amounts (1, 2.5, and 5 µg) *L. laeta* Smase I (●) or crude venom (○). The animals received buffer for negative control reactions. The areas of the dermonecrotic lesions were determined 24 h after injection. (B) Neutralisation of *L. laeta* venom toxicity was determined by measuring the dermonecrotic activity remaining in samples of 1, 2.5, and 5 µg venom, preincubated for 60 min at 37°C with normal rabbit serum (solid bars) or anti-*L. laeta* Smase I (open bars) rabbit serum (diluted 1:2). The size of the lesions was measured after 72 h. Results are expressed as percentage reduction of the size of the dermonecrotic lesion of three separate experiments ± SD.

the same experimental conditions normal rabbit serum had no effect on the development of the lesion induced by venom.

Discussion

The aim of this study was to generate recombinant proteins from the *Loxosceles* spider gland to facilitate further structural and functional studies in the mechanisms of loxoscelism. Using a strategy of sequencing aleatory clones from a *L. laeta* venom gland cDNA library we have been able to generate ESTs (expressed sequence tags) representing the abundant transcripts from this specialised tissue. Sequencing of only few clones allowed us to identify cDNA clones coding for putative toxins after searching with Blastn and Blastx programs [18], for similar sequences in DNA and protein databases. Of 40 clones, three (H10, H13, and H17) contained inserts coding for proteins with significant similarity at the amino acid level with the sphingomyelinases P1 and P2 from *L. intermedia* venom. The longest cDNA insert from clone H17, which shared a high percentage of identity with these toxins, was further subcloned and expressed in a bacterial system. The *L. laeta* spider venom recombinant protein was endowed with all biological properties ascribed for the whole venom and for the toxins P1 and P2 from *L. intermedia* venom, e.g., dermonecrotic and complement-dependent haemolytic activities and ability of hydrolysing sphingomyelin. Considering its lipase activity, the recombinant *L. laeta* toxin expressed from clone H17 was named *L. laeta* Smase I. The antiserum raised against *L. laeta* Smase I recognised a 32-kDa protein in *L. laeta* venom and gland extract (Fig. 2B) as well the purified P1 and P2 sphingomyelinases from *L. intermedia* venom (data not shown).

We show here that the recombinant toxin, like the native sphingomyelinases from the venom of *L. intermedia* [8,10,11], was able to bind to erythrocyte surface, transforming the cell into an activator of the autologous complement system, as demonstrated by the increase of lysis susceptibility in the presence of complement and deposition of C-components from both classical and alternative pathways on E surface. However, the recombinant protein was less active than the crude venom and, as shown in Fig. 5B, the binding of *L. laeta* Smase I to the erythrocyte surface was less efficient than the toxins present in *L. laeta* crude venom. This is most likely due to some inappropriate folding of the recombinant protein resulting in the expression of only partial active protein. Sphingomyelinase generally have two important domains: a calcium-dependent lipid binding domain and an enzymatic lipase domain [22]. Inappropriate folding of either of these domains will have an effect on the final specific

activity. However, the venom and recombinant toxin Smase I had similar activities in the sphingomyelinase assay. It is not known what the sphingomyelinase toxins bind on to erythrocytes but in the sphingomyelinase assay the substrate TNPAL-sphingomyelin dispersed in detergent and inappropriate folding of the binding domain will not have an effect on the enzymatic lipase domain. The venom and recombinant Smase I also had similar activities in the dermonecrosis assay. The mechanism of dermonecrosis is still completely unresolved and our data suggest that the putative lipid binding domain is not of importance in this process.

We have previously shown [10] that purified P1 and P2 toxins induced removal of the rich sialic portions of GPA, GPB, and GPC and that the cleavage of glycoporphins could not be reproduced using purified toxins and GPA. The data suggested that the venom proteins, through their sphingomyelinases activities, altered the membrane environment and fluidity, causing the activation of an as yet unknown endogenous metalloproteinase, which then cleaved glycoporphins. The recombinant *L. laeta* Smase I, devoid of any proteolytic activity, was also able to induce the cleavage of glycoporphins. This unequivocally demonstrated that our previously report about cleavage of the glycoporphins was not due to a metalloproteinase in the venom and further supports our hypothesis that the sphingomyelinase activity of *Loxosceles* spider venom on the erythrocyte membrane results in the activation of an endogenous metalloproteinase responsible for the glycoporphin cleavage.

It has been suggested that metalloproteases, serineproteases, and oligosaccharide moieties play an important role in the development of the dermonecrotic reaction by *Loxosceles* spiders envenoming [23–25]. The data we are presenting here show that our recombinant *L. laeta* Smase I expressed in a bacterial system, in which glycosylation does not take place, was fully able to induce all the pathological effects of the *L. laeta* venom. Furthermore, the antiserum raised against this toxin was able to block the dermonecrotic reaction induced by the whole spider venom (Fig. 6). Our results clearly demonstrate that the sphingomyelinases, but not metalloproteases or serineproteases, are the major or unique toxins responsible for the main symptoms of both systemic and cutaneous forms of loxoscelism.

One of the limiting factors in *Loxosceles* research is the difficulty in obtaining large amounts of venom and purified venom components since, using electro-stimulation procedures for venom extraction, it is possible to collect no more than 30 µg venom protein per spider. The cloning and expression of the *L. laeta* toxin described here allowed us to obtain 17.4 mg of pure toxin per litre of culture. Thus, enough

recombinant toxin is now available not just to be used as antigen for the production of neutralizing antiserum for human therapy but also as an important tool to further investigate the mechanism of action of these spider toxins.

Acknowledgments

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Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of *Loxosceles intermedia* spider venom

Evanguedes Kalapothakis^{a,*}, Simone Costa Araujo^a, Cibele Soares de Castro^a,
Thais Melo Mendes^a, Marcus Vinícius Gomez^a, Oldemir C. Mangili^b,
Ida C. Gubert^b, Carlos Chávez-Olórtégui^c

^aDepartamento de Farmacologia, Divisão de Biologia Celular, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, Belo Horizonte, MG, Cep 31270901, Brazil

^bDepartamento de Patologia Básica, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, Cep 81531970, Brazil

^cDepartamento de Bioquímica-Imunologia, ICB, UFMG, Belo Horizonte, MG, 31270901, Brazil

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Abstract

The present report describes the identification and molecular characterization of LiD1, a protein expressed in the venom gland of the brown spider *Loxosceles intermedia*. LiD1 belongs to a family of proteins with dermonecrotic activity and members of this family have been found in spiders from the genus *Loxosceles*. The necrotic lesions caused by this group of proteins may lead to serious socio-economic problems such as surgical tissue reconstitution and even patient death. LiD1 was cloned using a cDNA library constructed from the venom gland of *L. intermedia* and antibodies against proteins with dermonecrotic activity isolated from the crude venom of this spider. The amino acid sequence deduced from the cDNA revealed a mature protein of approximately 31 kDa, with a pI of 7.37. The cDNA also revealed the existence of a signal peptide, a propeptide and also an untranslated 3' region with 218 nucleotides. LiD1 was expressed as a protein fused with β -galactoside protein using the vector pBK-CMV, resulting in the recombinant protein recLiD1 with important immunological properties. recLiD1 was strongly recognised by anti-dermonecrotic antibodies and was also able to generate reactive antibodies against native dermonecrotic proteins isolated from the venom of *L. intermedia*.

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Keywords: *Loxosceles intermedia*; Dermonecrotic toxin; cDNA; Expression; LiD1

1. Introduction

Loxosceles intermedia is a medium-sized spider very well adapted to urban regions where it can capture different types of insects in its web. However, this spider frequently leaves the web during the night looking for food or searching for a site to build a new web. This behavior leads to an inconvenient contact with humans that normally occurs when the victims press the small brown spider by moving their body during sleep or when dressing. Despite the small size (up to 1 cm in length and 2–3 cm in leg span for an adult specimen) and sometimes painless bites of this spider, its venom may cause skin damage. It is not

Abbreviations: bp, base pair(s); BSA, Bovine serum albumin; Da, daltons; EDTA, ethylenediamine tetra-acetic acid; Ig, immunoglobulin; IPTG, isopropyl- β -D-thiogalactoside; Kb, kilobase(s); PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TAE, Tris–acetate–EDTA buffer; TBS, Tris–buffered saline; Lx, *Loxosceles intermedia* spider; LxD1, *Loxosceles intermedia* Dermonecrotic protein; Xgal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

* Corresponding author. Tel.: +55-31-3499-2173; fax: +55-31-3499-2695.

E-mail address: ekalapo@icb.ufmg.br (E. Kalapothakis).

uncommon to observe the astonishing evolution of a necrotic skin lesion of several square centimeters (normally, 1–10 cm²) at the site of the bite, requiring surgical treatment with skin grafts. The necrotic lesion is caused by a group of dermonecrotic proteins with intense activity against the cellular matrix. The mechanism by which the venom acts on the basement membrane seems to be closely similar for this group of spiders including *Loxosceles reclusa* found in North America, *Loxosceles gaucho* and *intermedia* native to Brazil, and *Loxosceles laeta* native to Chile (Mota and Barbaro, 1995). Strong sphingomyelinase activity has been reported as the major biological activity responsible for the ulceration of the skin and also hemolytic action that may cause anemia and acute renal insufficiency. Kurpiewski et al. (1981) reported the purification and the sphingomyelinase D activity of four toxins from the venom gland of *L. reclusa*. Tambourgi et al. (1998) described the dermonecrosis activity and also complement-dependent hemolysis of toxins isolated from the venom of *L. intermedia*. Fibronectinolytic and fibrinogenolytic activities were also reported as harmful effects of the venom of *L. intermedia* (Feitosa et al., 1998). A recent review of the mechanism of action of *Loxosceles* venom on extracellular matrix molecules was published by Veiga et al. (2001).

The medical treatment of *Loxosceles* bites is controversial. Non-specific therapy involves the administration of corticosteroids and, depending on the evolution of the patient, antibiotic administration, surgical excision of necrotic lesions, blood transfusion and hemodialysis (Ribeiro et al., 1993). Recently, Monteiro et al. (2002) showed the presence of *Clostridium perfringens* in the venom of *L. intermedia*. The authors also demonstrated the role of this anaerobic bacterium as an element that favors the exacerbation of the dermonecrotic process, suggesting the need to associate antibiotic therapy in the treatment of *Loxoscelism*. Specific treatment by serotherapy with polyvalent anti-arachnid horse anti-venom is often adopted. The results of this therapy are also controversial, because the anti-arachnid serum is not very efficient in neutralizing the lethal and dermonecrotic activities of *L. intermedia* venom (Braz et al., 1999). Clearly, there is an urgent need to develop specific anti-sera against *L. intermedia* which might prove much more efficient than the polyvalent anti-sera. It has been demonstrated that the 32–35 kDa antigens are the most immunogenic components of the venom, showing intense cross-reactivity when species-specific anti-sera were used (Barbaro et al., 1994). The ability of polyclonal antibodies against the dermonecrotic protein to neutralize the dermonecrotic activity and lethal effects of whole *Loxosceles* venom (Rees et al., 1981; Guilherme et al., 2001) indicates that this protein is a good candidate for the production of an *L. intermedia*-specific anti-venom to be used in the treatment of *Loxosceles* spider bites. However, some problems still need to be solved. The immunization of animals for the production of this anti-venom with such a very toxic native protein from the whole

venom is obviously a problem, and the use of a fused non-toxic recombinant protein could be an alternative strategy.

Considering these issues, we decided to isolate the cDNA coding for a dermonecrotic toxin. Our group has been involved in the isolation and characterization of cDNAs encoding toxins to be used in the production of recombinant proteins and specific anti-serum (Guatimosim et al., 2000). The present investigation describes the isolation and characterization of a cDNA encoding a new protein expressed in the venom gland of the spider *L. intermedia*, called here LiD1 (*L. intermedia* dermonecrotic protein 1). In addition, the cDNA was used to express the LiD1 and the recombinant protein characterized. Basically, this approach is justified by the difficulties encountered in obtaining the venom of this small spider and the possibility to analyze and correlate structure and function using the deduced amino acid sequence (Chavéz-Olórtegui et al., 2002). Furthermore, several authors have demonstrated the importance of improving *loxoscelism* immunotherapy (Barbaro et al., 1994; Braz et al., 1999; Guilherme et al., 2001).

2. Material and methods

2.1. Venom extraction

L. intermedia spiders were provided by the Interdisciplinary Laboratory for Research on Venomous Animals, Federal University of Paraná, Brazil. The venom was obtained by electric stimulation of adult specimens and was pooled, centrifuged, filtered, lyophilized and stored at –20 °C before use.

2.2. Fractionation of *L. intermedia* venom

The dermonecrotic fraction (DF) of crude *L. intermedia* venom was separated by gel filtration chromatography on Sephadex G-100 according to the general purification procedure described by Barbaro et al. (1994). Dermonecrotic protein was obtained from DF by cation exchange chromatography on a carboxy methyl cellulose (CM52) column. The column (1.6 × 35.5 cm²) was eluted with 20 mM ammonium acetate, pH 4.7, using a linear 0–700 mM sodium chloride gradient at a flow rate of 30 ml h^{–1}. The fractions separated were assayed for dermonecrotic activity by intradermal injection in rabbits.

2.3. Preparation of anti-*L. intermedia* venom antibodies

Hyperimmune horse anti-*L. intermedia* venom plasma was obtained according to the standard immunization schedules of the Immunobiological Production Unit of the Center of Production and Immunobiological Research, CPPI, Piraquara, PR, Brazil. Briefly, after collection of pre-immune serum from four adult horses, each animal

received an initial s.c. injection of 5 mg of soluble *L. intermedia* venom in complete Freund's adjuvant. Four additional injections of 5 mg of the venom in incomplete Freund's adjuvant (two) and aluminium hydroxide (two) were performed at 1-week intervals and the horses were bled 1 week after the last injection to obtain hyperimmune anti-*L. intermedia* plasma. Specific antibodies to *L. intermedia* were purified in a single step by immunoaffinity chromatography of the immobilized *L. intermedia* venom on a CNBr–Sephacryl column prepared according to manufacturer instructions (Pharmacia). Anti-*L. intermedia* venom IgG F(ab')₂ fragments were obtained by ammonium sulfate precipitation and partial digestion of hyperimmune plasma with pepsin and purified from IgG F(ab')₂ fragments and undigested IgG by filtration through a Protein A–Sephacryl 4B column as described by Ey et al. (1978). Anti-*L. intermedia* IgG F(ab')₂ fragments were purified by affinity chromatography on an *L. intermedia*–Sephacryl column as described by Chávez-Olortegui et al. (1994). Anti-*L. intermedia* IgGs F(ab')₂ were also conjugated to horseradish peroxidase (HRP type IV; Sigma) according to the method of Nakane and Kawoi (1974). The conjugate was stored at –20 °C and titrated as described by Chávez-Olortegui et al. (1994).

For rabbits, after collection of pre-immune serum, each animal received an initial injection of 20 µg of *L. intermedia* whole venom, dermonecrotic native protein or LiDI recombinant protein (recLiDI) intradermally in complete Freund's adjuvant. Two subcutaneous booster injections of 20 µg protein in incomplete Freund's adjuvant were given at 2 week intervals. A control group received Freund's adjuvant alone under the same conditions. Blood samples were withdrawn 1 week after the last injection.

2.4. Indirect ELISA for the determination of anti-*Loxosceles* antibodies

Sera (1:1000 to 1:32,000) from rabbits inoculated with *L. intermedia* whole venom, DF or recLiDI protein were tested by enzyme-linked immunosorbent assay (ELISA). Microtitration plates (Falcon 3912, Becton Dickinson, Oxnard, CA 93030) were coated overnight at 5 °C with 100 µl of a 5 µg ml^{–1} solution of *L. intermedia* venom in 0.02 M sodium bicarbonate buffer, pH 9.6, and the assay was conducted as previously described (Chávez-Olortegui et al., 1991). Absorbance was measured at 492 nm with a Titertek Multiscan spectrophotometer. All measurements were performed in duplicate. Pre-immune sera used under the same conditions were added to the plate as controls.

2.5. General molecular biology

Standard recombinant DNA techniques (plasmid purification, phenol extraction, ethanol precipitation, electrophoresis, etc.) were carried out as described by Sambrook et al. (1989).

2.6. Construction and analysis of a venom gland cDNA library

Venom glands from 200 *L. intermedia* spiders were removed 2 days after venom extraction, pulverized with a mortar and pestle in dry ice and lysed in the presence of guanidinium thiocyanate according to published procedures (Chirgwin et al., 1979). Total RNA was passed through an oligo(dT)–cellulose column using the Poly(A) Quick mRNA isolation kit from Stratagene (La Jolla, CA) and eluted as poly(A⁺) RNA. Complementary DNA synthesis was performed using the ZAP–cDNA Synthesis kit from Stratagene. The cDNA molecules were prepared from 5 µg of *L. intermedia* venom gland mRNA and inserted directionally into a Uni-Zap™ XR vector at the XhoI and EcoRI positions according to the manufacturer specifications. The vector was subsequently packaged into viable lambda ZAP phage particles using the Gigapack II Packaging Extracts kit (Stratagene, La Jolla, CA). The cDNA library was titrated and tested for color selection with IPTG–X–gal according to Sambrook et al. (1989). The insert sizes were analyzed by PCR (Saiki et al., 1988) using the primers M13R (5'-GGAAACAGCTATGACCATG-3') and M13U (5'-GTAAACGACGCGCCAGT-3').

2.7. cDNA library screening

Molecular screening was carried out using Sandwich ELISA basically as described by Kalapothakis et al. (2001). In vivo excision of the pBK–CMV phagemid from the Uni-Zap XR vector was carried out using the ExAssist helper phage according to manufacturer specifications (Zap–cDNA synthesis manual, Stratagene). After excision, bacteria (XLORL strain) containing the pBK–CMV vector were plated onto agar L broth containing 100 µg ml^{–1} ampicillin, 200 µg ml^{–1} X–Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 250 µg ml^{–1} IPTG (isopropylthiogalactoside) and incubated at 37 °C for 10 h. Individual white colonies were picked off with sterile 200 µl tips and transferred to 2 ml of L broth containing 100 µg ml^{–1} ampicillin and 0.5 mM IPTG in a sterile 15 ml tube. The collected material was allowed to grow at 37 °C for 8–10 h and harvested by centrifugation (3000g for 5 min) at 4 °C. The pellet was resuspended in 0.5 ml of carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6 and 0.5 mM PMSF) and cells were disrupted by sonication (2 pulses of 15 s at a power setting of 3.5). Sandwich ELISA was performed using anti-*L. intermedia* IgGs F(ab')₂. Microtitration plates (Falcon 3912, Becton Dickinson, Oxnard, CA 93030) were coated for 8–12 h with 100 µl well^{–1} of a 10 µg ml^{–1} solution of immunoglobulins in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). After blocking and washing, 100 µl of induced bacterial supernatant sonicated in incubation buffer was added and incubated for 1 h at 37 °C. The plates were washed and incubated with anti-*L. intermedia* IgGs F(ab')₂ conjugated with horseradish

peroxidase (diluted 1:1000 in incubation buffer) for 1 h at 37 °C. The wells were washed six times and the assay was completed with citrate buffer (50 mM Na_2HPO_4 , 24 mM citric acid, pH 5.0), 2 mg ml^{-1} of OPD (*o*-phenylenediamine) and 0.2 $\mu\text{l ml}^{-1}$ of H_2O_2 . After incubation at 25 °C for 20 min the reaction was stopped by the addition of 20 $\mu\text{l well}^{-1}$ of a 1:20 dilution of sulfuric acid. Absorbance values were determined at 492 nm with a Titertek Multiscan plate spectrophotometer. A control group (a clone prepared as described earlier but not induced by IPTG) was always used.

2.8. DNA sequencing and computer analysis

Large scale plasmid isolations from *E. coli* were carried out according to the alkaline lysis SDS method and purified on CsCl–ethidium bromide gradients as described by Sambrook et al. (1989). DNA sequencing reactions were performed on both strands using the chain termination method of Sanger et al. (1977). All reactions were carried out using the Automated ABI PRISM, 310 Genetic Analyzer and BigDye Terminator Cycle Sequencing kit (ABI PRISM). Nucleic acid sequences were analyzed using the MacVector™ 5.0 program (Oxford Molecular Group PLC). After editing, the sequences were compared to sequences in the GenBank databases (Altschul et al., 1997).

2.9. Expression and purification of recombinant protein

The isolated clone with the LiD1 cDNA (*L. intermedia* dermonecrotic protein 1) was grown overnight at 37 °C in 2 ml of L broth medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin and then used to inoculate 1000 ml of L broth medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. When a density of $A_{600} = 0.5$ was reached the culture was induced by the addition of IPTG to a final concentration of 0.5 mM. The cells were then grown for an additional 8–12 h at 37 °C to allow expression of the fusion protein. Next, the cells were harvested by centrifugation and sonicated in PBS with short pulses of 15 s for 2 min and subjected to centrifugation at 9000g for 30 min. The supernatant was applied to a Sephacryl S-200 column and the fractions were analyzed by ELISA. Positive fractions for anti-*Loxosceles*-venom antibodies were pooled and the recombinant protein affinity-purified using anti-*L. intermedia* Sepharose-IgGs F(ab')_2 . The yield of purified recombinant fusion protein was approximately 3 mg l^{-1} . The pBK-CMV vector was directly used resulting in a protein fused with β -galactosidase protein.

2.10. Immunoblot analysis

The expressed protein recLiD1 (3 μg), *L. intermedia* whole venom (10 μg) and the DF (2 μg) were resolved by SDS-PAGE on 10% acrylamide gel by the method of Laemmli (1970). Samples were treated with loading buffer

containing 75 mM Tris-HCl, pH 6.8, 2% SDS, 0.55 M β -mercaptoethanol, 0.003% bromophenol blue, and 10% glycerol. After electrophoresis, gels were stained with Coomassie Blue G-250 for identification of protein bands or transferred to a 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) as described previously for antibody recognition (Kalapothakis et al., 2001). Briefly, non-specific binding sites were blocked for 1 h in PBS containing 0.3% (v/v) Tween 20. The blot was incubated with rabbit (a) anti-*L. intermedia* whole venom, (b) anti-DF, (c) anti-recLiD1 antibodies diluted 1 $\mu\text{g ml}^{-1}$ in PBST (PBS containing 0.05% (v/v) Tween 20) for 1 h at room temperature. The nitrocellulose membrane was washed four times in PBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibodies (Sigma Chemical Co., St Louis, MO, USA), diluted 1:1000 in PBST. After four washes the reaction was developed by the addition of the peroxidase substrate (diaminobenzidine plus chloronaphthol).

3. Results and discussion

To gain more insight into the dermonecrotic process induced by the bite of *L. intermedia*, we decided to clone a gene coding for a protein belonging to the dermonecrotic toxin family. A cDNA library was constructed using mRNA isolated from the venom gland of this small spider, resulting in a library with 1.8×10^6 independent recombinants. The isolation of cDNAs was based on a screening using ELISA and, to this end, anti-*Loxosceles* venom antibodies were developed and purified to be used in the process. Using this methodology we identified several positive clones. In an initial screening of 500 colonies we found approximately 10% positive clones that showed different levels of immunoreactivity. In contrast, 90% of the clones did not show higher reactivity than the control group.

Clone Lx38 showed the strongest signal and was selected for further characterization. The DNA sequencing of this clone revealed a 1127 pb cDNA coding for a mature protein with a calculated molecular weight of 31,252.55 (Fig. 1), and this toxin was named LiD1 for *L. intermedia* dermonecrotic protein 1. The cDNA of LiD1 showed a small propeptide composed of 10 residues, and an incomplete signal peptide of 12 residues with a consensus cleavage point for a signal peptidase (Von Heijne, 1983). The Met initiation codon for LiD1 was not found, probably due to incomplete elongation during the cDNA synthesis by reverse transcriptase; however, the toxin was cloned in frame with the β -galactosidase protein. The sequencing analysis of several spider toxins has shown the same overall organization (Kalapothakis et al., 1998a). They contain a short 5'-untranslated region and an open reading frame encoding peptides that are longer than the mature toxin.

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      10      20      30      40      50      60
-22 GCA CGA GTA GTA TTG GGG TGT TGG AGC GTC TTG TCC CAG GCT GCT CAA ACA GAT GAT GAA GAA CGC -1
    A R V V L G C W S V L S Q A A C T D D E E R
      70      80      90     100     110     120     130
+1  GCA GGT AAT CGT CGG CCT ATA TGG ATC ATG GGG CAC ATG GTA AAT GCC ATC GGT CAG ATA GAC GAG
    A G N R R R P L W I M G H W V N A I G Q I D E 22
      140     150     160     170     180     190
    TTC GTG AAC CTT GGA GCA AAC TCC ATC GAA ACA GAC GTG TCT TTC GAT GAC AAT GCC AAT CCT GAG
    F V N L L G L A N S I E T D V S F D D N A N P E 44
      200     210     220     230     240     250     260
    TAT ACT TAT CAC GGC ATT CCA TGT GAT TGT GGA AGG AAT TGC AAG AAA TAT GAG AAT TTT AAC GAT
    Y T V H G I P C D C G R N C K K Y E N F N D 66
      270     280     290     300     310     320     330
    TTT CTG AAA GGT CTC CGA AGC GCC ACA CCT GGT AAT TCA AAG TAT CAG GAA AAA CTG GTC TTA
    F L K G L R S A T T P G N S K Y Q E K L V L 88
      340     350     360     370     380     390
    GTC GTG TTC GAC TTA AAG ACA GGT AGC CTC TAC GAT AAT CAA GCC AAC GAC GCC GGA AAG AAA TTG
    V V F D L K T G S L Y D N Q A N D A G K K L 110
      400     410     420     430     440     450     460
    GCG AAG AAT CTC TTA CAA CAT TAC TGG AAC AAT GGC AAT AAT GGT GGA AGA GCA TAC ATA GTG TTA
    A K N L L Q H Y W N N G N W G G R A V I V L 132
      470     480     490     500     510     520
    TCG ATC CCA GAC CTT AAT CAT TAT CCA CTG ATT AAA GGA TTC AAA GAC CAG CTT ACA AAG GAC GGA
    S I P D L N H Y P L I K G F K D Q L T K D 154
      530     540     550     560     570     580     590
    CAC CCA GAG TTG ATG GAC AAA GTT GGA CAC GAC TTC TCC GGA AAC GAC GAC ATC AGC GAC GTT GGA
    H P E L M D K V G H D E S G N D D I S D V G 176
      600     610     620     630     640     650     660
    AAG GCT TAC AAG AAA GCA GGA ATA ACT GGT CAT ATT TGG CAG AGC GAT GGT ATC ACC AAC TGT TTA
    K A V K K A C A G I T A G H I M Q S D G I T N C L 198
      670     680     690     700     710     720
    CCA CGT GGC CTT AGT CGT GTG AAC GCA GCT GTG GCA AAC AGA GAT TCC GCA AAC GGA TTC ATT AAC
    F R G L S R V N A A V A N R D S A N G I L N 220
      730     740     750     760     770     780     790
    AAA GTG TAC TAC TGG ACA GTG GAC AAG CGC TCA ACG ACC AGA GAT GCA CTT GAT GCT GGA GTT GAC
    K V Y Y W T V D K R S T T R D A L D A G V D 242
      800     810     820     830     840     850
    GGC ATA ATG ACC AAC TAC CCG GAT GTT ATC ACT GAT GTT CTC AAC GAA GCC GCA TAC AAG AAG AAA
    G I M T N Y P D V I T D V L N E A A Y K K R 264
      860     870     880     890     900     910     920
    TTC CGA GTT GCC ACA TAC GAC GAT AAT CCA TGG GTG ACA TTC AAG AAA TAA att ctg cag gtt gat
    F R V A T Y D D N P W V T F R K 280
      930     940     950     960     970     980     990
    tgt gaa aaa aca cat ggc aat ctg gat ttc acg att ttc att gaa ctt tgt tga aaa acc aat ttg
      1000     1010     1020     1030     1040     1050
    atg cga aaa att aaa aac tat gca cta tgg aag ctt tgt tca aaa tat tgt ttg tta tta ttg taa
      1060     1070     1080     1090     1100     1110     1120
    aca tgt ttg aaa aag tat ttt tca gaa ata aat ttt cat cca tgt aaa aaa aaa aaa aaa aaa
    aaa aa

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Fig. 1. Nucleotide-sequence of DNA encoding LiD1. The coding region is shown in capital letters with the 3' untranslated region in lower case; the 5' region was not present in the cDNA. The segment that corresponds to the mature toxin is given in gray box. The incomplete signal peptide (in bold) and the propeptide (underlined) are also indicated.

Immature toxins composed by a typical hydrophobic signal peptide followed by a hydrophilic intervening propeptide and extra C-terminal residues (C-terminal peptide) are frequently found in immature toxins (Kalapothakis et al.,

1998a). Our cDNA sequencing data, together with supporting biochemical evidence (Barbaro et al., 1996a,b; Tambourgi et al., 1998), indicate the exact N-terminal point for proteolytic excision of the mature toxin; however,

1	5	10	15	20	25	30	35	40	
AGNRRPIWIMGHMVNAIGQIDFVNLGA-NSIE-TDVSFDDN	LiD1 <i>L.intermedia</i>								
AGNRRPIWIMGHMVNAIGQIDFVNLGA-NSIEITDVFFD	P1 <i>L.intemedia</i>								
ADKRRPIWIMGHMVNALAQIDFVNLGA-N	P2 <i>L.intemedia</i>								
ADKRRPIWIMGHMVNAIEWIDFVNLGA-NNIEX-DVAF	P3 <i>L.intemedia</i>								
AGNKRPIWIMGAMVNAIKDIXDFVNLGA-NNIXK	35 Kda <i>L.intermedia</i>								
ADNKRPIWVMGGMVNSLAQIKETVGLGLDMS-EK-DNKWYQ	35 Kda <i>L.gaucho</i>								
ADNRRPIWNLGHMVNALKQIPTFLKDGA-NA	32 Kda <i>L.laeta</i>								
A-NKRPFVIMGHMVNAVYQIDFVNLGA-NSID-TDVS	<i>L.reclusa</i>								

Fig. 2. LiD1 was aligned with the partial amino acid sequences of previously characterized toxins with dermonecrotic activity. The peptides P1, P2 and P3 described by Tambourgi et al. (1998) were purified from the venom of *L. intermedia*. Peptides of 35 kDa (Genebank accession 1911682), 35 kDa (Genebank accession 1911681) and 32 kDa (Genebank accession 1911683) from the venom of *L. intermedia*, *L. gaucho* and *L. laeta*, respectively, were described by Barbaro et al. (1996a,b). The last toxin described by Cisar et al. (1989, Genebank accession 102825) is a deduced peptide from a genomic clone (*L. reclusa*). Similarities are indicated by closed boxes and conserved residues are underlined.

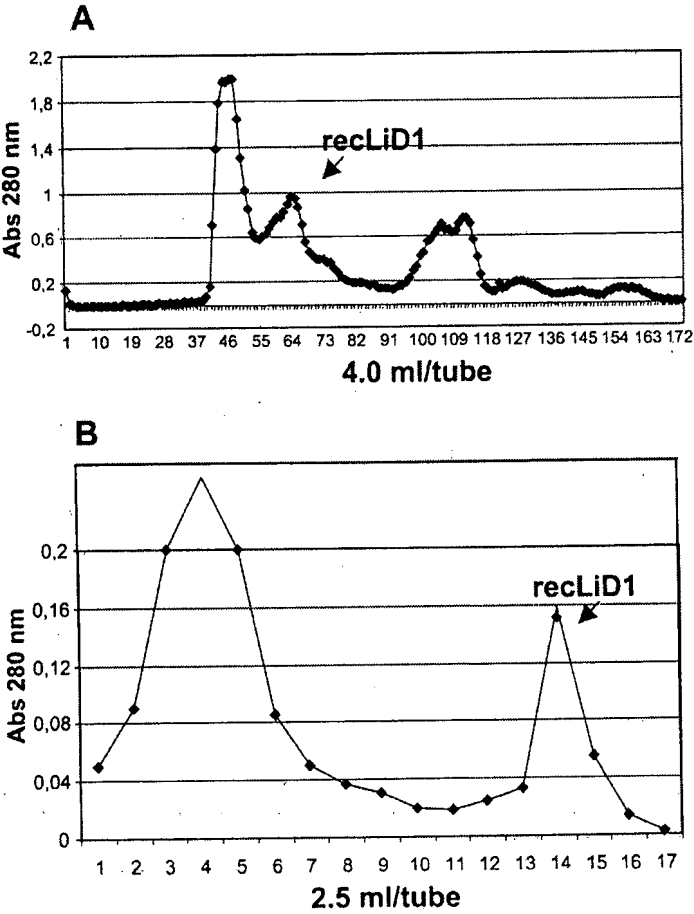


Fig. 3. Purification of recLiD1. The bacterial extract was applied to a Sephacryl S-200 column and the fractions were analyzed by ELISA. Positive fractions for anti-*Loxosceles* venom antibodies were pooled and the recombinant protein affinity-purified using Sepharose-IgGF(ab')₂ anti-*L. intermedia* whole venom. The yield of purified recombinant fusion protein was approximately 3 mg l⁻¹. The pBK-CMV vector was directly used, resulting in a protein fused with β-galactosidase protein.

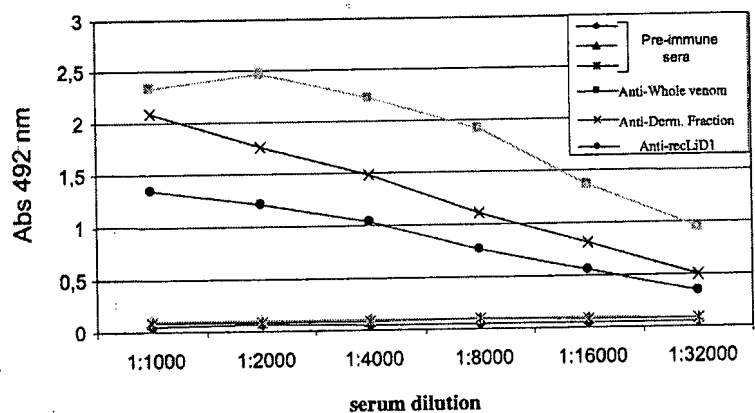


Fig. 4. Reactivity of *L. intermedia* venom with serum from immunized rabbits. Antibody levels were measured by indirect ELISA in sera from rabbits immunized with *L. intermedia* venom (■), *L. intermedia* DF (×) and *recLiD1* protein (♦). Pre-immune sera were used as control. Plates were coated with *L. intermedia* venom ($5 \mu\text{g ml}^{-1}$) and sera were diluted 1:1000 to 1:32,000. Values are means of duplicates.

it is not clear whether or not any C-terminal residue is processed. The number of residues in the C-terminal peptide is variable and the longest described for a spider toxin is composed of 11 residues and was found in the Tx3-2 toxin cloned from the venom gland of *Phoneutria nigriventer* (Kalapothakis et al., 1998b). The dermonecrotic protein family seems to be composed of toxins with a high level of identity and similarity in the N-terminal region (Fig. 2).

However, the sequence comparison was carried out on the N-terminal sequences due to the absence of data in the literature describing the complete sequences of proteins belonging to this family. Considering the whole molecule expressed by the isolated clone, we expected a molecular weight of 37,395.04 Da, including a fragment of the fusion protein (β -galactosidase), 12 residues of the incomplete signal peptide, 10 residues of the propeptide, and 280

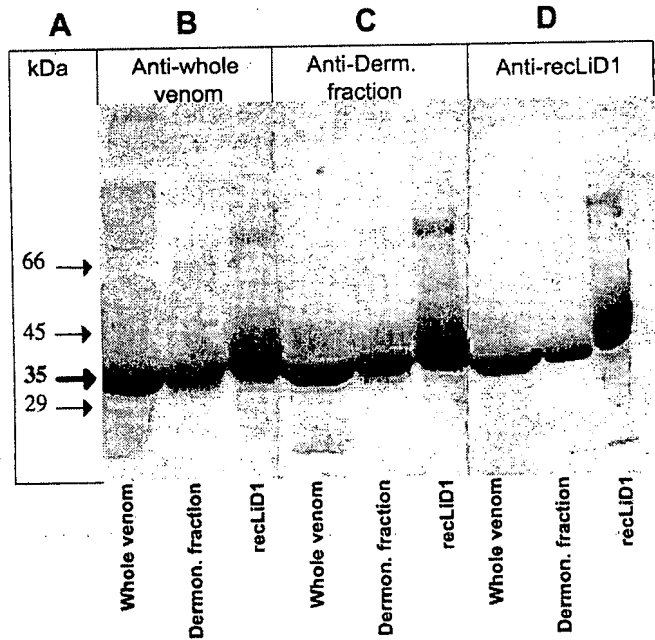


Fig. 5. Immunochemical identification of *L. intermedia* dermonecrotic proteins. Samples were applied to 10% polyacrylamide SDS gel and proteins were transferred to a nitrocellulose membrane. *L. intermedia* whole venom, purified DF and the purified recombinant protein LiD1 were probed against anti-*L. intermedia* whole venom (B), anti-*L. intermedia* DF (C) and anti-recLiD1 antibodies (D). The molecular weight standards are indicated on the left (A).

residues of the mature toxin (Fig. 1). As indicated earlier, the calculated molecular weight for mature LiD1 is approximately 31 kDa, an expected value since literature data describe molecular weights ranging from 32 to 35 kDa for dermonecrotic toxins isolated from different *Loxosceles* species (Barbaro et al., 1994, 1996a,b; Tambourgi et al., 1998). LiD1 protein could also be an isoform expressed at low level in the venom gland and therefore not detected by Western blotting analysis. Considering the ability of venomous animals to paralyse and kill so many different preys, we may expect the presence of isoforms of toxins in the venom composition and in fact they have been identified (Rash and Hodgson, 2002). This fact and post-translational modifications may contribute to small differences in molecular weight and also explain differences in the pharmacological activity of toxins from the same family.

Since the molecular cloning of LiD1 was done by ELISA using the pBK-CMV vector, we did not attempt to subclone the cDNA in another expression vector. The expression and purification of recLiD1 was carried out in few steps. After induction, the bacterial pellet was disrupted and the soluble proteins applied to a Sephacryl S-200 column. Once more, we used ELISA to identify the fractions containing recLiD1 and positive fractions were pooled and affinity-purified (Fig. 3). The yield of purified recombinant fusion protein was approximately 3 mg l^{-1} . The amount of purified protein was sufficient for the production of anti-recLiD1 antibodies that showed a strong reactivity against *L. intermedia* whole venom (Fig. 4). However, the results indicated that the immunization protocol could be improved when compared to the results obtained with antibodies generated against the DF and whole venom. The improvement could be related to the presence of different isoforms of the dermonecrotic family in the whole venom and in the purified fraction, that are absent in the antibody production with recLiD1. The possible misfolding of the recombinant protein due to problems in disulfide bond formation and the presence of β -galactosidase in the recombinant fusion protein may also contribute to its lower immunoreactivity. However, recLiD1 was able to generate reactive antibodies against native dermonecrotic proteins. Fig. 5 also illustrates the reactivity of the antibodies generated in the present investigation and supports the results obtained by ELISA (Fig. 4). It is important to emphasize the slightly higher molecular weight shown by the polyacrylamide gel for recLiD1 since the recombinant protein is a fused protein, as described earlier. Preliminary results showed that recLiD1 is devoid of dermonecrotic activity, although further investigations are needed to clarify this point. The molecular cloning and expression of recLiD1 opens up the opportunity to use the recombinant protein to correlate structure and function and may also supply sufficient amounts of protein for the development of different immunological assays leading to the production of specific neutralizing antibodies for clinical use, and will certainly contribute to identifying the real role played by this family of toxins in the process of skin lesion.

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Genetic and enzymatic characterization of sphingomyelinase D isoforms from the North American fiddleback spiders *Loxosceles boneti* and *Loxosceles reclusa*

Blanca Ramos-Cerrillo^a, Alejandro Olvera^a, George V. Odell^a, Fernando Zamudio^a,
Jorge Paniagua-Solís^b, Alejandro Alagón^a, Roberto P. Stock^{a,*}

^aDepartamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México,
Av. Universidad 2001, Cuernavaca, Morelos 62210, Mexico

^bDirección de Investigación. Laboratorios Silanes SA de CV, Mexico DF 83100, Mexico

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Abstract

In this study we report the isolation and characterization of several sphingomyelinase D isoforms from the venoms of the North American spiders *Loxosceles boneti* and *Loxosceles reclusa*, from Mexico and the United States, respectively. We have measured their enzymatic activity, their capacity to induce necrotic lesions in rabbits, cloned the cDNAs coding for the mature forms of two of the isoforms from *L. boneti* and two of *L. reclusa* based on N-terminal sequence information of the purified proteins, and performed a comprehensive comparison of the sequence data generated by us with that reported for other sphingomyelinase genes to date.

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Keywords: *Loxosceles boneti*; *Loxosceles reclusa*; Sphingomyelinase activity; Cloning

1. Introduction

Loxosceles spiders are cosmopolitan, and are the cause of medical conditions which can range from moderately morbid to life-threatening and fatal. Numerous species of *Loxosceles* have been described, mostly in the Americas and Africa, although European species have been found. The effects of poisoning by *Loxosceles* may range from localized necrotic conditions of varying severity, called cutaneous loxoscelism (CL), to systemic envenomation (VCL, viscero-cutaneous loxoscelism) which may be not infrequently fatal, particularly in children (Sezerino et al., 1998).

Loxosceles spiders have venoms with a toxic component for mammals with a rare enzymatic activity, termed sphingomyelinase D (SMD). SMD is a relatively abundant protein component of pure *Loxosceles* venom, comprising a significant portion of venom protein along with components of lower molecular weight (by SDS-PAGE analysis, Binford and Wells, 2003). Sphingomyelinases have a molecular mass of approximately 33 kDa and catalyze the cleavage of sphingomyelin, releasing 1-phosphoceramide and choline (Kurpiewski et al., 1981). Sphingomyelins, in turn, are integral components of many cell membranes (such as erythrocytes and cells of the vascular epithelium), and although the natural targets of SMD action are not known, there is convincing evidence which points to the role of the enzymatic activity in the initiation of events which may lead to extensive necrosis and, on occasion, severe systemic

* Corresponding author. Tel: +52-777-3-291-602; fax: +52-777-3-172-388

E-mail address: rstock@ibt.unam.mx (R.P. Stock).

poisoning. It has been suggested that bacterial cells injected with the venom may enhance, or even be responsible for, the necrotizing effects of the *Loxosceles* bite (Monteiro et al., 2002). While some bacteria may contribute to the necrotic process, there is very strong experimental evidence that SMD activity is sufficient cause for the observed lesions. Besides the fact that SMDs render erythrocytes susceptible to complement-mediated lysis in vitro (Tambourgi et al., 1995), and therefore could conceivably induce damage in other cell types, the most compelling evidence to date for the requirement of SMD in the development of the necrotic wounds characteristic of loxoscelism comes from a study by Fernandes Pedrosa et al. (2002), who reported the cloning and expression of an active sphingomyelinase D from the South American species *Loxosceles laeta*. In their study, they show that heterologously expressed and purified recombinant SMD retains enzymatic activity and is sufficient for induction of necrosis in experimental animals. This necrosis, in turn, was abolished by incubation of the venom or recombinant SMD with antiserum generated against the recombinant toxin. Kalapothakis et al. (2002) have also reported the cloning and characterization of a SMD from another South American species, *L. intermedia*.

The aim of the present study was to characterize SMD from two common North American species of *Loxosceles*, *L. boneti* (abundant in central and southwestern Mexico) and *L. reclusa* from central and southeastern United States. We report the purification of three isoforms of SMD from *L. boneti* and a characterization of their enzymatic activity, as well as of two from *L. reclusa*. From amino-terminal sequencing information of the purified enzymes from *L. boneti* we also cloned and characterized two of the three genes coding for the isoforms isolated from *L. boneti*, as well as two isoforms from *L. reclusa*. A comprehensive comparison of the primary sequence data for all SMD genes available to date is also presented and discussed.

2. Materials and methods

2.1. Spiders

Specimens of *L. boneti* were collected in Tuxpan, Guerrero, Mexico. *Loxosceles reclusa* specimens were collected in Stillwater, Oklahoma, USA. Identification of the species was done by Dr Norman I. Platnick of the American Museum of Natural History, New York.

2.2. Purification of SMD

Purification of SMD was performed by a two-step protocol from whole homogenates of venom glands. Venom apparatuses were dissected and homogenized in 1 ml of 20 mM ammonium acetate pH 4.7 per 50 apparatuses by means of a tight-fitting teflon homogenizer on ice. We observed that contaminating hemocyanin could be removed,

since it precipitated after one freeze/thaw cycle, from the extract by centrifugation at 10,000g for 10 min. After clarification by centrifugation, 3.5 ml of the extract were fractionated on a Sephadex G-75 column and fractions collected. The fractions spanning the molecular weight expected for SMD (Fraction 2) were pooled and loaded onto a Mono-S cation exchange column fitted on a FPLC System (Pharmacia) in 20 mM ammonium acetate pH 4.7, and eluted with a linear NaCl gradient (10–500 mM NaCl in the same buffer). The eluted peaks from *L. boneti* were collected individually and processed for N-terminal sequencing. Part of the eluted peaks of *L. boneti* and *L. reclusa* were used for enzymatic and/or dermonecrotic activity assays.

2.3. Protein determination and measurement of SMD activity

Protein quantitation was done using the Bicinchoninic acid reagent (BCA, Pierce) using the protocols and standards supplied with the reagents. SMD activity of gland homogenates and chromatographic fractions was measured by a coupled assay using the substrate AMPLEX (Molecular Probes) and fluorimetric detection as described by the manufacturer. As control and reference standard, a sphingomyelinase C from *Staphylococcus aureus* was included, in which case alkaline phosphatase was included in the reaction, which was not necessary for the SMD reaction (as the assay utilizes choline oxidase which does not recognize choline phosphate as substrate). Since the fluorogenic substrate detects peroxide generated by the coupled reaction, controls were included which did not contain protein and others which did not contain sphingomyelin to avoid errors due to non-specific peroxide generation. One unit is defined as the amount of activity that will hydrolyze 1 μ mol of TNPAL-sphingomyelin per minute at pH 7.4 and 37 °C.

2.4. Dermonecrosis assay

Dermonecrotic activity was evaluated in New Zealand White (NZW, 2.5–3 kg) rabbits by a single subcutaneous injection of 10 μ g (isoforms Lb1 and Lb2) and 30 μ g (isoform Lb3), all in 300 μ l of phosphate buffered saline (PBS) into separate sites. Lesions were evaluated at 72 h post-injection. A traditional method for quantitation of the extent of necrosis is to measure the two longest perpendicular axes of the necrotic halo for approximate estimation of the area (Rees et al., 1981), however, the technique is inherently very variable, as no two injections are identical, and requires a large number of replicate injections, preferably in several animals, for statistical significance. For this reason, our assays were used only for qualitative assessment of necrosis, that is, presence or absence of a necrotic lesion at the site of the injection 72 h post-injection (p.i.).

2.5. N-terminal sequencing of *L. boneti* isoforms

The amino-terminal sequence of the three main peaks from the cation exchange chromatography of the *L. boneti* Sephadex G-75 fraction 2, were determined by standard procedures (Walsh et al., 1981). The N-terminal sequence data were aligned to those amino terminal sequences reported for *L. laeta*, *L. gaucho*, *L. intermedia* and *L. reclusa* (Barbaro et al., 1996) and *L. deserta* (Gomez et al., 2001).

2.6. Primers for cDNA amplification

Based on the amino acid sequences obtained, degenerate primers were designed in order to amplify the mRNAs coding for them (LbD primers). All possible codons were used as codon usage data is scarce for these spiders. Using the Universal Degenerate Code (I stands for Inosine, Y for C or T, R for A or G, M for A or C and H for A, T or C) the sequences of the primers were as follows:

Name	Sequence	Degeneracy	Use
LbD-1	CCIGCITGGATHATGGG	3	3'RACE
LbD-1.1	GATHATGGGI- CAYATGGT	6	3'RACE
LbD-3	MGICCIAARCCIAHTHTGG	12	3' RACE
Lb-1	CCAGCATCGAGTG- CATCT	None	5'RACE

2.7. General molecular biology

The cDNAs coding for SMD up to the N-terminal sequence determined from *L. boneti* and *L. reclusa* were amplified by the 3' RACE technique (Life Sciences). Briefly, total RNA from glands obtained using the Trizol reagent (Gibco) was reverse-transcribed using oligo-d(T) with an adapter sequence and the resulting cDNAs were amplified by PCR using the degenerate oligos (LbD-1 and the nested LbD-1.1) and an adapter primer to obtain double-stranded DNAs which were cloned in pCR TOPO 2.1 sequencing vectors (Invitrogen) by standard procedures. Sequencing was performed with universal primers by means of an automated system at facilities of the Instituto de Biotecnología. Based on the sequence data obtained from the primary clones, a primer (Lb-1) was designed for sequence confirmation as well as extension into the 5' end of the cDNAs (which had been amplified using degenerate primers) by 5' RACE (Life Sciences). Briefly, total RNA obtained as for 3' RACE were reverse-transcribed with the specific primer designed from the sequence data of the primary clones. Subsequently, a 'tail' of dGTP was added to the 3' end of the cDNAs by means of terminal deoxynucleotidyl transferase, and the cDNAs thus processed were amplified by PCR using the original primer used for

reverse-transcription and an oligo-d(C). The products of amplification were again cloned in the sequencing vector and sequenced as above.

2.8. Sequence analysis and alignments

Sequences were compared using Gene Works 2.5.1 (Intelligenetics, Inc.), and visual alignments generated using SeqVu 1.1 (The Garvan Institute of Medical Research). The sequences of *L. intermedia*, *L. laeta* and *L. arizonica* used in the alignment were obtained from the NCBI web server (GenBank Accession numbers AAQ16123, AAM21154, AAP44735, respectively). Identities were calculated by pairwise alignments and pI values were calculated from deduced amino acid sequences using standard pK_a values for the sidechains of individual residues with Gene Works.

3. Results

3.1. Purification of SMD

Venom apparatuses of 500 specimens of *L. boneti* and 500 from *L. reclusa* were homogenized as described above, and fractionated on a Sephadex G-75 column (170 × 2 cm). The elution profiles are shown in Fig. 1, left panel. Absorbance was monitored at 260 and 280 nm and, from left to right, four peaks were apparent. SMD activity was found in the second peak, which was subsequently fractionated on a cation exchange column using a linear NaCl gradient. As shown in the insets, three major peaks were identified for the *L. boneti* fraction, and what are probably three or four incompletely resolved peaks for the *L. reclusa* fraction. For both species, all corresponded to proteins with molecular weights of approximately 30 kDa by SDS-PAGE (Fig. 1, right panel). The second peak, overlapping partially with the first, of the *L. reclusa* chromatogram contained what are probably two mixed isoforms which were not resolved. Henceforth, the three main peaks from the *L. boneti* chromatogram will be referred to as isoforms 1, 2 and 3, respectively. The *L. reclusa* isoforms, in turn, were classified by identity to isoform 1 of *L. boneti*. As we have no N-terminal sequence data (see below) to identify each peak of the cation exchange chromatography to assign to the sequence data obtained from *L. reclusa* mRNA, we have used letters for the 'chromatographic' isoforms (A, BC and D, respectively). The cation exchange chromatograms of the gel filtration fractions of venom from both species also showed minor peaks, which may represent other rarer isoforms, allelic variants, or truncated or otherwise processed forms (see Section 4).

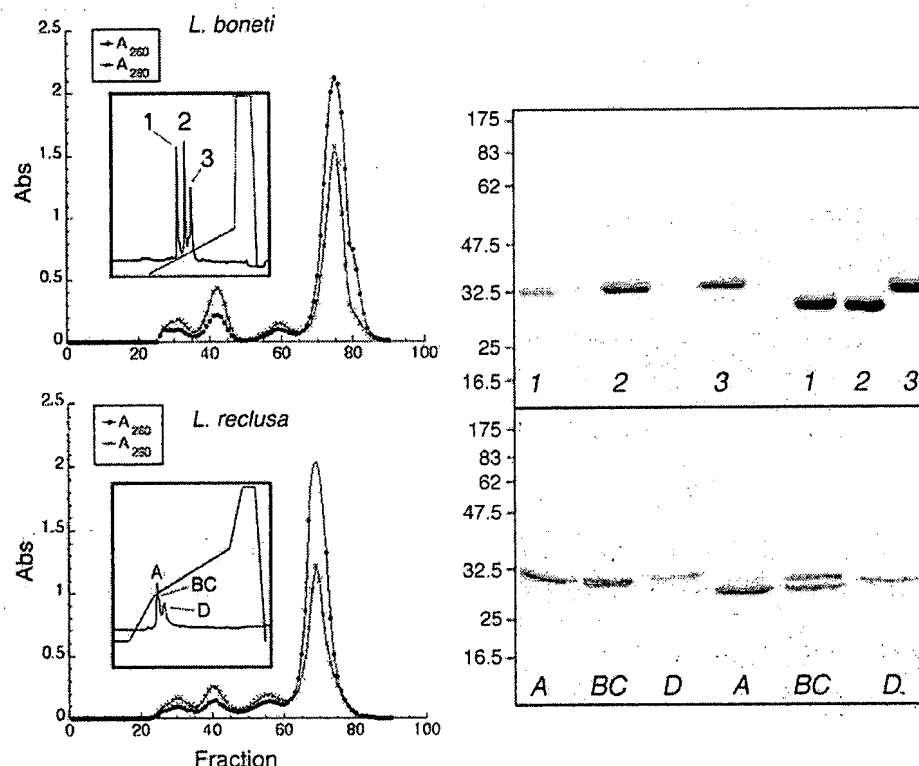


Fig. 1. Purification of SMD from homogenates of *L. boneti* and *L. reclusa* venom glands. Left panel: Top, Gel filtration of *L. boneti* gland extract with cation exchange chromatogram of Fraction 2 (inset); Bottom, gel filtration of *L. reclusa* gland extract with cation exchange chromatogram of Fraction 2 (inset). Ion exchange fractions are indicated. Right panel: SDS-PAGE of cation exchange fractions; Lanes I, II and III are non-reducing, lanes IV, V and VI are reducing. They all correspond to main peaks (indicated below). Molecular weights are in kDa.

3.2. SMD activity of purified *L. boneti* and *L. reclusa* isoforms, and dermonecrotic activity of *L. boneti* isoforms

Sphingomyelinase activity was measured using a coupled assay and fluorescent detection of the products. The assay is exquisitely sensitive, and permits detection of nanograms of enzyme. Of the three *L. boneti* isoforms, isoform 1 showed the highest specific activity, followed by isoform 2 (31% of isoform 1). Interestingly, isoform 3 did not display activity, although its identity to the other isoforms is high enough to warrant its denomination as an isoform (see below). Table 1 lists the specific activities of the three *L. boneti* isoforms, as well as that of the three *L. reclusa* chromatographic fractions.

Both *L. boneti* isoforms 1 and 2 were dermonecrotic when injected subcutaneously in rabbits (Table 1). However, and most interestingly, isoform 3, which is not enzymatically active, was not dermonecrotic when injected at three times the dose of the other isoforms (see Section 4). Similar results were reported for isoforms of *L. intermedia* (Tambourgi et al., 1998).

3.3. N-terminal sequencing of the three main isoforms from *L. boneti*

SDS-PAGE analysis of the individual *L. boneti* fractions showed the proteins to be very pure (Fig. 1, right top panel),

so they were used for N-terminal sequencing. The sequences obtained were congruous with the N-terminal sequence data then reported (Barbaro et al., 1996; Gomez et al., 2001). Isoforms 1 and 2 of *L. boneti* shared an identity of 94% at their N-termini, whereas isoform 3 was the most divergent of the three, with an identity of 36% with respect to isoform 1. In Table 2, the N-terminal sequences of the three *L. boneti* isoforms and percent identities with the then reported N-terminal sequences of several *Loxosceles* species are shown. The amino acids chosen for design of the degenerate primers are underlined and, as those of *L. boneti* isoform 1 perfectly match those of the reported *L. reclusa* sequence, they were also used for amplification of SMD cDNAs of this species.

Table 1
Specific SMD ($n=3$) and dermonecrotic activities of *L. boneti* and *L. reclusa* cation exchange fractions

Name	Specific activity (U/mg)	Necrotic
<i>L. boneti</i> 1	30.5 ± 7.2	+
<i>L. boneti</i> 2	9.5 ± 1.9	+
<i>L. boneti</i> 3	0	–
<i>L. reclusa</i> A	20.3 ± 6.9	NA
<i>L. reclusa</i> BC	11.5 ± 0.2	NA
<i>L. reclusa</i> D	4.59 ± 0	NA

NA, not ascertained.

Table 2

N-terminal sequence data for *L. boneti* isoforms. The percentage of identity is with respect to the sequence of *L. boneti* isoform 1 by pairwise alignment. Amino acids used for degenerate primer design are underlined

Name	Sequence	% Identity	Reference
<i>L. boneti</i> 1	ANKRPVWIMGHMVNAIAQIDEFVNLGANSIETDVS	100	This study
<i>L. boneti</i> 2	ANKRPVWIMAHMVNAVAQIDEFVNLGANSIETD	94	This study
<i>L. boneti</i> 3	AXRPKPIWXVAHVMNDLELVDEYL	36	This study
<i>L. reclusa</i>	ANKRPVWIMGHMVNAVYQIDEFVNLGANSIDTDVS	91	Barbaro et al. (1996)
<i>L. gaucho</i>	ADNKRPIWVMGGMVNSLAQIKEFVGLGLDNSEKDNKWKYQ	58	Barbaro et al. (1996)
<i>L. deserta</i>	ANKRPIWIMGHMVNAIYQIDEFVNLGANSIETDVS	94	Gomez et al. (2001)
<i>L. laeta</i>	ADNRRPIWNLGHMVNALKQIPTFLXDGANA	55	Barbaro et al. (1996)

The X denotes undetermined amino acids.

3.4. Amplification and sequence of SMD cDNA by RT-PCR with degenerate primers and completion of sequence by 5'RACE

With the N-terminal sequence information, degenerate primers were designed to amplify cDNA from isoforms 1 and 2 on one hand; and isoform 3 on the other. The same primers used for amplification of the *L. boneti* cDNAs were also used to amplify cDNA from total RNA of *L. reclusa*. The RT-PCR yielded bands of the expected size which were cloned and sequenced. In the case of *L. boneti*, the deduced amino acid sequences of two distinct clones showed perfect identity to the N-terminal sequence data, corresponding to isoforms 1 and 3. We were not able to detect primary clones which matched the N-terminal sequence of isoform 2. In the case of *L. reclusa*, two distinct sequences were obtained, which we have tentatively called isoforms 1 and 2 based on sequence identity to isoform 1 of *L. boneti*. All these sequences from the primary clones were in turn used to design additional primers for sequence confirmation and to extend the sequences into the 5' region of the cDNA, with the aim of characterizing the complete coding sequences and removing the potential bias introduced by the use of the degenerate primers in the original amplifications. However, it was not possible to extend the sequences beyond those obtained from the mature protein's N-terminal sequences, although unbiased sequences for the portions spanned by the original degenerate primers were obtained.

3.5. Primary sequence of SMD cDNA and alignments

The complete sequences for mature *L. boneti* isoforms 1 and 3, as well as those of *L. reclusa* isoforms 1 and 2 are available from GenBank (accession numbers AY559844, AY559845, AY559846 and AY559847, respectively). In Fig. 2 a complete alignment of the deduced amino acid sequences of *L. boneti* isoforms 1 and 3, *L. reclusa* isoforms 1 and 2, and reported sequences from other *Loxosceles* species (*L. intermedia*, *L. laeta* and *L. arizonica*) starting from the first amino acid of the N-terminal sequence data. To date, the only complete sequences, that is, starting at the start codon are that of *L. laeta* (from a cDNA library,

Fernandes Pedrosa et al., 2002) and that of *L. arizonica*, which was obtained from a genomic library (Genbank AAP44735). Identity between the two *L. boneti* isoforms is 45% and between the two *L. reclusa* isoforms is 90%. Identities are summarized in Table 2. Consistently, the most divergent sequence is that of *L. laeta* (when considering only enzymatically active SMDs). Variability of *L. laeta* SMD includes two amino acid insertions at positions 168 and 201 not found in any of the other proteins, the first of five amino acids and the second a single phenylalanine. SMD from *L. laeta* shares an insertion at position 2 with SMD from *L. intermedia*, although of different amino acids. The number of conserved cysteines is also different for *L. laeta* when compared to all the other sequences, which may have important implications for the structure and/or function of the protein (see Section 4). All in all, the conservation of SMD in species so widely distributed, in geographic and climatic terms, is remarkable, although variability in some residues is not conservative. Theoretical pIs for different isoforms within *L. boneti* and *L. reclusa*, and compared to those of the other species, show considerable variability (Table 3).

4. Discussion

Spiders of the genus *Loxosceles*, which have been recognized as medically relevant, contain venoms with one component, sphingomyelinase D, shown to be responsible for the typical toxicity observed in humans and some animal models. In order to learn more about the toxic activity of *Loxosceles* venom, we purified sphingomyelinase D from venom gland extracts of the North American *L. boneti* and *L. reclusa* by a two-step chromatographic protocol involving a gel filtration step followed by cation exchange. For *L. boneti*, the latter yielded three well defined peaks of high purity which, upon N-terminal sequencing, showed a high identity with the then reported N-terminal sequence for SMD from other *Loxosceles* species. Of the three isoforms, named 1 to 3 by the order of elution, 1 and 2 displayed SMD activity, whereas isoform 3 was inactive in the conditions of our assay. Of the two active isoforms, isoform 1 exhibited

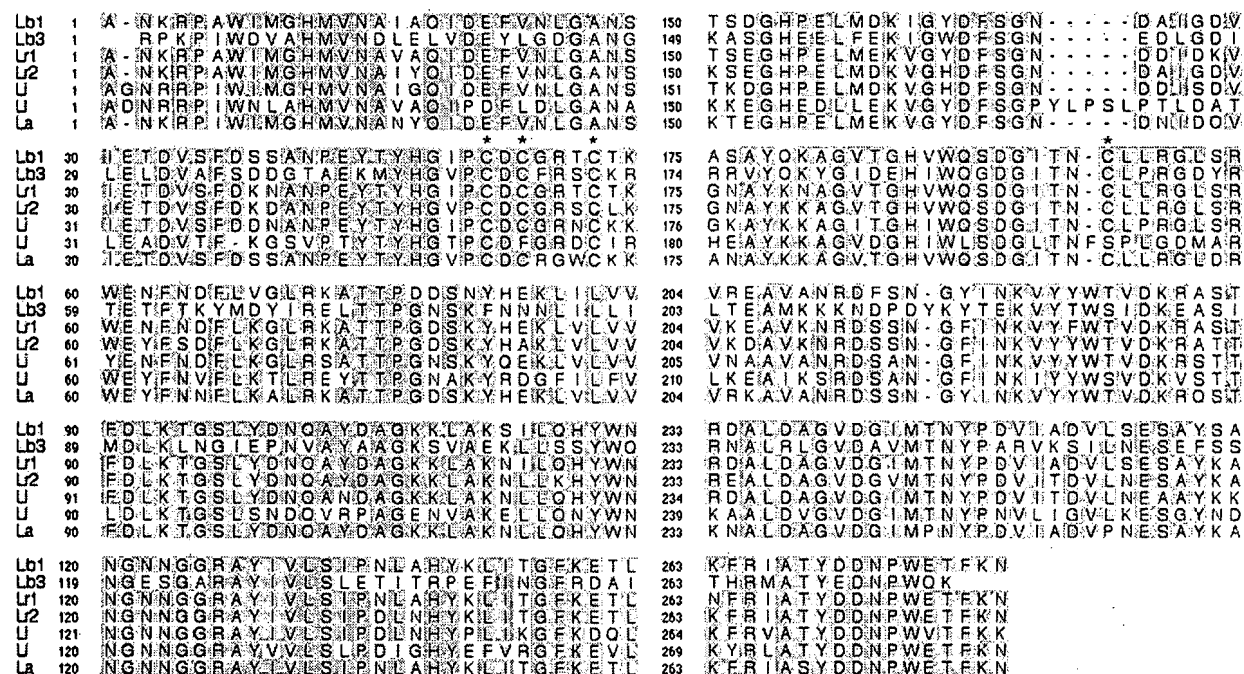


Fig. 2. Deduced amino acid sequences of SMD from *L. boneti* and *L. reclusa* and sequence alignment with all other complete reported sequences for the mature proteins (Lb, *L. boneti*; Lr, *L. reclusa*; La, *L. arizonica*; Li, *L. intermedia*; Ll, *L. laeta*). Cysteines are indicated by asterisks.

the highest specific activity when compared to isoform 2 of the same species. In the case of *L. reclusa*, cation exchange chromatography resulted in two well defined peaks, of which the first seemed to be composed of at least two components which could not be completely resolved. The specific enzymatic activities of the isoforms of *L. reclusa* venom were comparable to those of *L. boneti*. In the case of isoform BC, the activity may correspond to the combination two active isoforms or, alternatively, to one less active and one inactive isoform. Also, the possibility remains that fraction BC corresponds in equal parts to fractions A and D which, if combined in approximately equal amounts (as shown in Fig. 1, bottom SDS-PAGE gel, lanes II and V), would yield a similar average activity.

Interestingly, isoform 3 from *L. boneti*, while clearly a related protein, as determined by sequence alignment, did not exhibit SMD activity while maintaining a high identity with the other isoform of the same species. Consistent with the study of Fernandes Pedrosa et al. (2002), our study supports their assertion that enzymatic activity is necessary and sufficient for, at least, initiation of the necrotic process, as isoform 3 of *L. boneti* venom which is 45% identical to isoform 1, did not exhibit necrotic activity in rabbits. The dermonecrotic assay we used is, at best, semiquantitative, so a fine correlation between enzymatic activity and extent of necrosis can not be, as of yet, unequivocally established.

Although we tried to characterize the complete cDNAs from mRNAs coding for *L. boneti* and *L. reclusa* SMDs, we were not able to obtain cDNAs which contained the amino-terminal sequence absent in the mature protein. A similar

situation is apparent in the case of the SMD cDNA sequence reported for *L. intermedia*. This may be due to secondary structure at the 5' end, an inherent fragility of the specific mRNA or, alternatively, that the mRNA is possibly degraded very soon after, or even during, translation, as we sequenced numerous clones obtained under the most careful conditions of RNA isolation and reverse transcription but could find none that extended to the start codon.

The amino acid sequences of SMDs from different *Loxosceles* species are quite conserved and, interestingly, their theoretical pIs show significant variability among isoforms within a species and between species, ranging from 4.88 in *L. boneti* to 8.23 in *L. arizonica* (Table 3). This variability might be reflected in differences in optimal conditions for SMD activity and/or substrate specificity; characterization of pH and salt optima for activity of pure isoforms will be of use in the understanding of the role of

Table 3
Identities of all reported SMD deduced amino acid sequences

Name (pI)	Lb1						
Lb1 (5.15)	–	Lb3					
Lb3 (4.88)	45	–	Lr1				
Lr1 (6.17)	91	44	–	Lr2			
Lr2 (7.32)	84	46	90	–	La		
La (8.23)	86	43	88	87	–	Li	
Li (7.29)	82	46	84	85	80	–	Ll
Ll (5.42)	57	41	60	60	59	59	–

In parentheses are the theoretical isoelectric points. (Lb, *L. boneti*; Lr, *L. reclusa*; La, *L. arizonica*; Li, *L. intermedia*; Ll, *L. laeta*).

SMD in the natural life circumstances of *Loxosceles* spiders. In this regard, in both *L. boneti* and *L. reclusa* cation exchange chromatograms, minor peaks can be observed and although they may correspond to other minor isoforms, our sequencing of numerous clones which showed slight but persistent variability (data not shown) suggests that significant allelic variation within species exists. As the purification of SMD was from a pool of a large number of specimens, as was the extraction of total RNA for cDNA cloning, it is impossible to establish whether these differences are due to allelic variation or the existence of additional genes for SMD within the species used in this study. Further inquiry into the sequence of SMD genes obtained from single specimens of the same species could be of use in the study of natural populations, for fine-tuning of molecular phylogenies, or even for evolutionary studies.

For example, *L. laeta* is possibly the largest of the genus, and it has been claimed to be one of the most dangerous (Fernandes Pedrosa et al., 2002). SMD from *L. laeta* is also the most divergent of those reported (Table 3), most notably, amongst other things, for the potentially different number of disulphide bonds in the protein, since all other sequences have four conserved cysteines while that of *L. laeta* has only two (and those only five amino acids apart), thus precluding the existence of an intramolecular disulphide bond between C50 (C51 in *L. intermedia*) and C196 (C197 in *L. intermedia*) which could join two distant sites in the molecule, possible in all SMDs sequenced from all other species. It would be most informative to carry out comparisons of sphingomyelinase activity with the smaller species in order to determine whether the higher dermonecrotic potential associated to the *L. laeta* bite is due to higher specific SMD activity, other enzymatic peculiarity (such as stability or cofactor dependence, for example), or, alternatively, due to the larger amounts of venom injected per bite.

Necrotic activities of reptile venoms, mostly related to phospholipases and proteinases, have been under scrutiny for some time. The large quantities of active necrotizing toxins injected by, for example, viperine snakes, have generally been considered sufficient by themselves as cause of the extensive necrosis resulting from envenomation. In the case of SMD as a necrotic toxin, it may be necessary to consider alternative and possibly indirect mechanisms, as the quantities of SMD injected in a *Loxosceles* bite could scarcely exceed micrograms. The factors that affect the development of CL and VCL are not known, however, the fact that VCL is most common in children, and that the severity of CL is very variable between patients, suggests that host mechanisms may be at work as consequence of SMD activity. Sphingosine and ceramide metabolites have been known for some time to be important signaling molecules in a variety of cellular systems, and specific enzymes and pathways exist that control their synthesis and recycling in a variety of cellular processes (Huwiler et al., 2000; Van Meeteren et al., 2004). Studies on the role of 1-phosphoceramide released by SMD action are likely to

contribute to our present meager understanding of the cascade of events that can lead from localized direct damage, possibly at the cell membrane level in the site of the bite, to the sometimes very extensive necrotic processes or systemic syndromes observed in some patients.

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