UNIVERSIDADE FEDERAL DO PARANÁ

LARISSA VUITIKA

MAPEAMENTO ESTRUTURAL DO SÍTIO ATIVO DE FOSFOLIPASES D PRESENTE NO VENENO DE ARANHA-MARROM (*Loxosceles intermedia*): CARACTERÍSTICAS BIOQUÍMICAS E BIOLÓGICAS

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Tese apresentada como requisito à obtenção do grau de doutor em Biologia Celular e Molecular, no curso de Pós-graduação em Biologia Celular e Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná

Orientador: Prof.º Dr.º Silvio Sanches Veiga
Co-orientador: Prof.ª Dr.ª Olga Meiri Chaim

CURITIBA
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Programa de Pós-graduação Biologia Celular e Molecular

Departamento de Biologia Celular
Setor de Ciências Biológicas
Universidade Federal do Paraná

DECLARAÇÃO

Declaramos para os devidos fins que ***Larissa Vultika***, no dia 25 de Fevereiro de 2016, no Programa de Pós-Graduação em Biologia Celular e Molecular (UFPR) defendeu sua Tese de Doutorado em Biologia Celular e Molecular, intitulada: "Mapeamento estrutural do sítio ativo de fosfolipases-D presente no veneno de aranha-marrom (Loxosceles intermedia): caracteristicas bioquímicas e biológicas", com a banca examinadora composta pelos Profs. Drs. Raghuvir Krishnaswamy Ami (Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP) Lia Sumie Nakao (Universidade Federal do Paraná - UFPR), Rafael Bertoni da Silveira (Universidade Estadual de Ponta Grossa - UEPG), Edvaldo da Silva Trindade (Universidade Federal do Paraná - UFPR), e Silvio Sanches Veiga (Orientador e presidente da banca examinadora da Universidade Federal do Paraná), e foi Aprovada.

A solicitação de emissão do Diploma de Doutor em Biologia Celular e Molecular ficará condicionada à implementação das correções sugeridas pelos membros da banca examinadora, bem como ao cumprimento integral das exigências estabelecidas no Regimento interno deste Programa de Pós-Graduação.

Esta declaração tem validade de 60 (sessenta) dias.

Curitiba, 25 de Fevereiro de 2016

Prof. Dr.Ciro Alberto de Oliveira Ribeiro
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Dedico essa tese primeiramente à minha ***Vó Josefina Vuitika*** *(Dona Iuja) que desde sempre me incentivou a estudar.*

Dedico também aos meus pais ***Vilmar Vuitika*** *e* ***Cristina Vuitika****, pelo imenso apoio em todos os momentos, pelo incentivo de nunca desistir dos sonhos e pelo exemplo de humildade e honestidade.*

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Tinha eu 14 anos de idade
Quando meu pai me chamou
Perguntou me se eu queria
Estudar filosofia
Medicina ou engenharia
Tinha eu que ser doutor

Mas a minha aspiração
Era ter um violão
Para me tornar sambista
Ele então me aconselhou
Sambista não tem valor
Nesta terra de doutor
E seu doutor
O meu pai tinha razão

Vejo um samba ser vendido
E o sambista esquecido,
O seu verdadeiro autor
Eu estou necessitado
Mas meu samba encabulado
Eu não vendo não senhor

(14 anos- Paulinho da Viola e Élton Medeiros)

RESUMO

Os membros da família das fosfolipases D, também referidas como toxinas dermonecróticas, estão entre as moléculas mais bem estudadas presentes no veneno de aranhas-marrons do gênero *Loxosceles.* Estas toxinas são biologicamente ativas, uma vez que induzem dermonecrose, resposta inflamatória exacerbada, agregação plaquetária, hemólise e insuficiência renal aguda. Apesar da sua importância biomédica, o papel dos resíduos de aminoácidos abrangendo a interface catalítica na atividade das fosfolipases D e seus efeitos biológicos ainda não estão totalmente esclarecidos. Para abordar esta questão, foi realizada uma investigação mutacional e funcional utilizando a isoforma LiRecDT1 (primeira isoforma de fosfolipase D identificada de *Loxosceles intermedia*) como modelo. As formas mutadas H12A, H12A-H47A e E32A-D34A (sítio catalítico), C53A-C201A (ponte dissulfeto adicional), K93A, Y228A e W230A (ligação ao substrato lipídico) e G96A (controle) foram produzidos em modelos heterólogos e purificados. Todas as variantes mutantes foram reconhecidas pelos anticorpos policlonais anti-veneno total e anti-LiRecDT1, mostrando que mantiveram os epítopos imunogênicos conservados. Além disso, observamos que a conformação das variantes mutantes permaneceu semelhante a isoforma original, mostrada pelo dicroismo circular (CD) e calorimetria diferencial de varredura (DSC). As variantes H12A, H12A-H47A e E32A-D34A apresentaram atividade fosfolipásica, hemolítica, alteração da permeabilidade vascular e dermonecrótica diminuídas para valores residuais, reforçando a participação desses resíduos na atividade catalítica. O maior destaque foi para a mutação no resíduo Y228, que se mostrou igualmente importante para as atividades bioquímicas e biológicas, indicando um papel essencial no reconhecimento e ligação ao substrato. Por outro lado, a variante mutante C53A-C201A (mutação que suprime a ponte dissulfeto adicional nas fosfolipases D de classe II) não bloqueou a atividade fosfolipásica, hemolítica, permeabilidade vascular e dermonecrótica em comparação com a LiRecDT1. Estes resultados confirmam os determinantes estruturais envolvidos com as atividades biológicas das fosfolipases D do veneno de aranhas-marrons, abrindo portas para pesquisas de estrutura/função por método de cristalografia, estudos de vias sinalização celular e mediadores pró-inflamatórios, e também na prospecção de inibidores moleculares específicos para estas enzimas.

Palavras-chave: aranha-marrom, fosfolipase D, mutações sitio-dirigidas, atividade fosfolipásica.

ABSTRACT

Brown spider phospholipases D are among the most widely studied toxins in *Loxosceles* venom. These toxins are very active, as they induce dermonecrosis, exacerbated inflammatory responses, increased vascular permeability, hemolysis, and acute renal failure. Despite their biomedical importance, the role of residues at the catalytic interface of phospholipase D activity and other biological effects have not yet been determined. In the present study, a comprehensive mutational investigation was conducted using the *Loxosceles intermedia* phospholipase D (LiRecDT1) as model. All variants were identified using whole venom serum antibodies and a specific antibody to wild-type LiRecDT1 and showed native-like conformation according to circular dichroism (CD) and differential scanning calorimetry (DSC) analyses. The phospholipase activity of the variants H12A, H12A-H47A, and E32A-D34A, such as vascular permeability, dermonecrosis, and hemolytic effects were inhibited, suggesting the involvement of these residues in catalysis. The mutant Y228A was equally detrimental to biochemical and biological effects of phospholipase D, suggesting an essential role of this residue in substrate recognition and binding. On the other hand, the mutant C53A-C201A (which suppresses the hallmark disulfide bond of class II phospholipases D) did not block phospholipase activity, but compared to the wild-type LiRecDT1, the enzyme capacity to hydrolyze phospholipids and to promote dermonecrosis, hemolytic, and vascular effects was diminished. The results confirm the structural features of the biological activity of phospholipases D previously proposed by X-ray crystallographic studies, and leave open the possibility for the design of synthetic and specific inhibitors to Brown spider venom phospholipases D.

Keywords: brown spider, venom, phospholipase D, site-directed mutagenesis, activity modulation.

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LISTA DE ABREVEATURAS E SÍMBOLOS

A: Alanina

Ala: Alanina

Å: Ângstroms

Asn: Asparagina

Asp: Asparagina

APS: Persulfato de amônio

BOD: do Inglês, *Biological Oxygen Demand*

C: Cisteina

Cys: Cisteína

CD: do ingês, *Circular Dichroism*

$CaCl_2$: Cloreto de Cálcio

°C: Grau Celsius

C1P: Ceramida 1-Fosfato

D: Ácido Aspártico

D.O.: Densidade óptica

DIC: do inglês, *Differential Interface Contrast*

DNA: Ácido desoxirribonucléico

DSC: do inglês, *Differential Scanning Calorimetry*

E: Ácido Glutâmico

E. coli: *Escherichia coli*

Egg Lyso PC: Lisofosfotidilcolina de ovo

Egg SM: Esfingomielin de ovo

g: Grama

xg: Giro

h: Hora

H: Histidina

HPTLC: do inglês, *High-Performance Thin-Layer Chromatography*

Hys: Histidina

ICK: do inglês, *Inhibitor Cystine Knot*

IgG: Imunoglobulina do tipo G

IPTG : Isopropil-β-D-tiogalactopiranosídeo

IRA: Insuficiência renal aguda.

K: Lisina

KCl: Cloreto de potássio

kDa: quilodaltons (unidade de massa molecular equivalente a mil daltons)

kg: Kilogramas

L: Litro

L: Leucina

LALP: do inglês, ***L**oxosceles* **A**stacin-**l**ike **P**roteases

LB: Luria-Bertani

LoxTox: do inglês, ***Lox**osceles **Tox**ins*

LiRecDT: do inglês, ***L**oxosceles **i**ntermedia **Rec**ombinat **D**ermonecrotic **T**oxin*

LPA: Ácido Lisofosfatídico

Lys: Lisina

M: Mol por litro

mg: Miligrama

$MgCl_2$: Cloreto de magnésio

$MgSO_4$: Sulfato de Magnésio

Mg^{+2}: forma iônica do Magnésio

mL: Mililitro

mM: Milimol por litro

mRNA: RNA mensageiro

NaCl: Cloreto de sódio

N: Asparagina

nm: Nanômetros

PBS: do inglês, *Phosphate Buffered Saline*

PCR: Reação em cadeia da polimerase

PLD: Fosfolipase D

pH: Potencial de hidrogeniônico

pI: Ponto isoelétrico

RNA: Ácido ribonucléico

rpm: Rotações por minuto

rP (1, 2, 3): do inglês, Recombinant Phospholipase D

SDS: do Inglês, Dodecilsulfato de Sódio

SDS-PAGE: do Inglês, *Sodium dodecyl sulfate polyacrylamide gel*

Electrophoresis

SMase: Esfingomielinase

SicTox: do inglês, ***Sic****arridae* ***Tox****ins*

TCTP: do inglês, *Translationally Controlled Tumor Protein*

TEMED: N,N,N'N'-dimetilaminoetano

TM: temperatura de desnaturação

Trp: Triptofano

Tyr: Tirosina

UFPR: Universidade Federal do Paraná

UV: Ultravioleta

V: Volts

v/v: proporção volume/volume

W: Triptofano

Y: Tirosina

µg: Micrograma

µL: Microlitro

µM: Micromol por litro

α: Alfa

β: Beta

SUMÁRIO

1 INTRODUÇÃO

Fosfolipases D (PLD) (fosfatidilcolina fosfatidohidrolases) pertencem a uma superfamília de enzimas que catalisam a hidrólise de ligações fosfodiéster de fosfolipídeos, gerando como produto colina e ceramida 1-fosfato (C1P) (quando o substrato hidrolisado é esfingomielina); colina e ácido lisofosfatídico (LPA) (quando o substrato hidrolisado é fosfatidilcolina) (SELVY, *et al.,* 2011; DAMNJANOVIC e IWASAKI, 2013; GOMEZ-CARBRONERO, 2014). Estas enzimas foram primeiramente descritas em plantas e posteriormente muitas outras foram descobertas em vírus, procariotos e organismos eucaróticos (SELVY, *et al.,* 2011; ABDLKAFI e ABOUSALHAM, 2011; DAMNJANOVIC e IWASAKI, 2013).

As fosfolipases D identificas e descritas nos venenos de aranhas do gênero *Loxosceles* são proteínas de massa molecular que varia entre 30-35 kDa (KALAPOTHAKIS *et al.,* 2007) e que exibem grande representatividade dentro do veneno. O estudo do transcriptoma da glândula produtora de veneno de *Loxosceles intermedia* revelou que 20,2% dos transcritos codificadores de toxinas estavam relacionados às fosfolipases-D (GREMSKI *et al.,* 2010).

A notável proporção das fosfolipases D dentro do veneno total impulsionou os estudos para a obtenção destas toxinas na sua forma recombinante. Como resultado, muitas isoformas foram identificadas e caracterizadas bioquimicamente e biologicamente, o que resultou no agrupamento dessas moléculas em uma família de toxinas. Dentre as atividades bioquímicas e biológicas já descritas, podemos destacar a hidrólise de vários tipos de fosfolipídeos (LEE e LYNCH, 2006; CHAIM *et al.,* 2011a), lesão dermonecrótica, alterações na permeabilidade vascular, indução de intensa resposta inflamatória, agregação plaquetária, citotoxicidade, nefrotoxicidade, hemólise e letalidade em modelo animal (da SILVA *et al.,* 2004; APPEL *et al.,* 2005; CHAIM *et al.*, 2011b; GREMSKI *et al.;* 2014).

Estruturalmente, as fosfolipases D de *Loxosceles* possuem a forma distorcida de um barril $(\alpha/\beta)_8$, formada por folhas β e α-hélices conectadas por 3 alças (*loops*), denominados de *loop* flexível, *loop* hidrofóbico e *loop* catalítico

(MURAKAMI *et al.,* 2006; GIUSSEPE *et al.,* 2011). O *loop* catalítico contém os resíduos His12, Glu32, Asp34, Asp91, His47, Asp52, Trp230, Asp233 e Asn252, os quais são conservados entre as fosfolipases D de aranhas-marrons e, juntamente com a coordenação do íon Mg^{+2}, formam o sítio catalítico capaz de hidrolisar o substrato lipídico (MURAKAMI *et al.* 2005 e 2006; GUISEPPE *et al.,* 2011; DIAS-LOPES *et al.,* 2013). Além dos resíduos que participam da atividade catalítica da enzima e da coordenação do íon Mg^{+2}, é proposto na literatura que outros resíduos podem estar relacionados com o reconhecimento e ligação do substrato lipídico, uma vez que foi evidenciado que as fosfolipases D são toxinas capazes de se ligarem na membrana citoplasmática de células em cultivo (KUSMA *et al.*, 2008; CHAVES-MOREIRA, *et al.,* 2009; PALUDO *et al.*, 2009; CHAIM *et a.l*, 2011a; WILLE *et al.*, 2013). Além disso, as fosfolipases D de aranhas-marrons podem ser agrupadas em duas classes: Classe I e Classe II, baseado na sequência aminoacídica, na estrutura e atividade catalítica. Estudos apontam que a sequência aminoacídica que compõem o sítio catalítico das fosfolipases D é conservada. No entanto, é proposto que qualquer substituição de resíduos-chave poderia modificar a sua capacidade catalítica e/ou atividade biológica, além de alterar sua especificidade a diferentes substratos (LEE e LYNCH, 2005; MURAKAMI *et al.,* 2006, GIUSEPPE *et al.,* 2011; DIAS-LOPES *et al.,* 2013).

A partir de dados estruturais de cristalografia das fosfolipases D de *Loxosceles*, o presente estudo teve como objetivo identificar e mapear os resíduos de aminoácidos que estão envolvidos na atividade catalítica, na coordenação do íon metal Mg^{+2} e no reconhecimento do substrato lipídico. A técnica de mutação sítio-dirigida foi utilizada para produzir isoformas mutadas, tomando como molde a isoforma recombinante LiRecDT1 de *L. intermedia*. Os resíduos de aminoácidos alvos do presente estudo foram: His12 e His47 (sítio catalítico), Glu32 e Asp34 (coordenação do íon Mg^{+2}), Lys93, Tyr228 e Trp230 (ligação ao substrato), Cys53 e Cys201 (ponte dissulfeto adicional) e Glu96 (mutação controle). A caracterização bioquímica e biológica destas isoformas mutadas foi realizada com o propósito de aprofundar o conhecimento sobre a relação estrutura/função destas toxinas.

2 REVISÃO DE LITERATURA

2.1 Veneno loxoscélico

O veneno loxoscélico é um líquido cristalino e incolor, de natureza essencialmente proteica, produzido por um par de glândulas situadas no cefalotórax, ligadas a um par de quelíceras (Figura 1A) (dos SANTOS *et al.*, 2000). Os venenos de *Loxosceles laeta*, *Loxosceles intermedia*, *Loxosceles gaucho* e *Loxosceles reclusa* possuem perfil eletroforético similar, revelando a predominância de moléculas com massas variando de 5 a 10 kDa e 30 a 35 kDa (Figura 1C) (MOTA e BARBARO, 1995; BARBARO *et al*., 1996). Aranhas adultas desse gênero inoculam um volume de apenas alguns microlitros na vítima, contendo cerca de 20 a 200 µg de proteínas (BINFORD e WELLS, 2003; da SILVA *et al.,* 2004; SENFF-RIBEIRO *et al.,* 2008). Os venenos loxoscélicos são compostos essencialmente por moléculas de natureza protéica, com ação tóxica e/ou enzimática (CHAIM *et al.,* 2011b; GREMSKI *et al*., 2014)

A quantidade e o conteúdo de veneno produzido dependem de diversos fatores tais como a espécie de *Loxosceles*, o tamanho, o sexo, a idade e estado nutricional do animal. Por meio de análises de SDS-PAGE foi possível visualizar diferenças no conteúdo de veneno entre *L. intermedia* e *L. laeta* (de OLIVEIRA *et al*. 2005). Estas variações inter-específicas no conteúdo proteico podem modular a atividade biológica do veneno. Foi observado experimentalmente que a atividade dermonecrótica do veneno de *L. laeta* é mais exacerbada que o veneno de *L. intermedia*. Além disso, o veneno das fêmeas de aranhas-marrons provoca lesão dermonecrótica mais acentuada quando comparado com as lesões provocadas pelos venenos dos machos (de OLIVEIRA *et al.,* 2005).

Muitos estudos focam na identificação dos constituintes dos venenos loxoscélicos e algumas toxinas já foram descritas (da SILVA *et al.*, 2004; APPEL *et al.,* 2005; CHAIM *et al.,* 2011b; GREMSKI *et al.,* 2014). Aproximadamente 16,4% dos transcritos da glândula produtora de veneno de

L. laeta correspondem a sequências codificantes para toxinas já conhecidas como metaloproteases, serino-proteases, fosfolipases D, lectinas do tipo C e hialuronidases (FERNANDES-PEDROSA *et al.,* 2008). Gremski e colaboradores (2010) estudaram o perfil de transcritos da glândula produtora de veneno de *L. intermedia,* no qual aproximadamente 43,5% dos transcritos correspondem a toxinas (Figura 1B). Dentre eles, podemos observar a presença de diversas isoformas de fosfolipases D, peptídeos potencialmente inseticidas, neurotoxinas, serinoproteases, TCTP (do inglês, *Translationally Controlled Tumor Protein*), alérgenos, hialuronidases, inibidor de serinoproteases e metaloproteases.

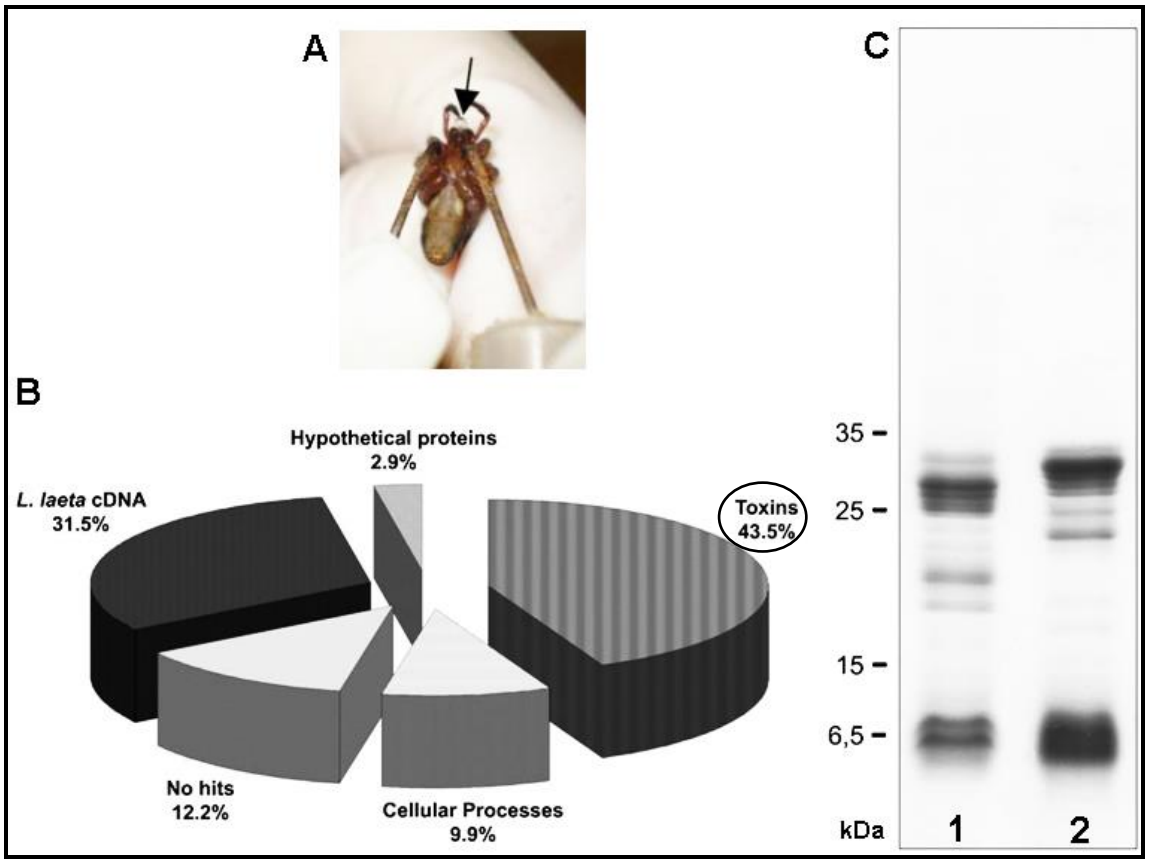

FIGURA 1. PERFIL PROTEICO DO VENENO DE ARANHA-MARROM. **A-** Extração do veneno de um espécime de *Loxosceles* (a seta indica o líquido incolor correspondente ao veneno). **B-** Perfil dos transcritos da glândula produtora de veneno de *L. intermedia*. **C-** Perfil eletroforético do veneno de *L. intermedia* em condições não redutoras (1) e redutoras (2). Adaptado de CHAIM *et al*., 2011b; GREMSKI *et al*., 2014).

Até o momento, foram identificadas várias toxinas nos venenos de aranhas-marrons, além de enzimas como fosfatase alcalina, 5’ ribonucleotídeo

fosfohidrolases (FUTRELL, 1992), peptídeos com provável atividade inseticida (de CASTRO *et al.,* 2004; GREMSKI *et al.,* 2010; MATSUBARA *et al.,* 2013), hialuronidases (BARBARO *et al.,* 2005; da SILVEIRA *et al.,* 2007a; dos SANTOS *et al.,* 2009; YOUNG e PINCUS, 2001; FERRER *et al.,* 2013), fosfolipases D (de ANDRADE *et al.,* 2005; CHAIM *et al.,* 2006; da SILVEIRA *et al.,* 2006a, 2006b; KALAPOTHAKIS *et al.,* 2007; APPEL *et al.,* 2008; BINFORD *et al.,* 2009; MAGALHÃES *et al.,* 2013; VUITIKA *et al.,* 2013), serinoproteases (VEIGA *et al.,* 2000), alérgenos (FERNANDES-PEDROSA *et al.,* 2008; dos SANTOS *et al.,* 2009) e metaloproteases (FEITOSA *et al*., 1998; da SILVEIRA *et al.,* 2007b; TREVISAN-SILVA *et al.,* 2010). Muitos autores sugerem que a toxicidade do veneno de aranha-marrom é decorrente de efeitos danosos sinérgicos e/ou somatórios de todos os seus componentes (GEREN *et al.,* 1976; da SILVA *et al.,* 2004; APPEL *et al.,* 2005; KALAPOTHAKIS *et al.,* 2007). A Tabela 1 mostra resumidamente os principais efeitos biológicos descritos para as principais toxinas presentes nos venenos de *Loxosceles.*

TABELA 1- VISÃO GERAL DAS CARACTERÍSTICAS BIOLÓGICAS DESCRITAS DAS PRINCIPAIS TOXINAS PRESENTES NOS VENENOS DE *Loxosceles.*

TOXINA	PESO MOLECULAR (kDa)	CARACTERÍSTICAS BIOLÓGICAS DESCRITAS
Fosfolipases D	30-35 kDa	Várias isoformas com diferentes atividades: - Dermonecrose. - Hidrólise de fosfolípideos. - Hemólise. - Agregação plaquetária. - Atividade inflamatória. - Edema. - Distúrbio Renal. - Letalidade. - Citotoxicidade *"in vitro".*
Peptídeos inseticidas	5-8 kDa	- Peptídeos relacionados à Magi 3. - LiTx: Letal para *S. frugiperda* (paralisia flácida). - LiTx3: atua sobre canais de Na^+.
Metaloproteases	28-35 kDa	- Metaloproteases do tipo Astacinas (LALPs). - Presente no veneno de diferentes espécies de *Loxosceles*. - Atividade hidrolítica sobre gelatina, fibronectina, fibrinogênio e entactina.
Hialuronidases	41-43 kDa	- Classificadas como hidrolases do tipo endo-β-Nacetyl-d hexosaminidases. - Atividade degradadora sobre ácido hialurônico e condroitin-sulfato. - Presente no veneno de diferentes espécies de *Loxosceles*. - Fator de espalhamento gravitacional.
Serinoproteses	85-95 kDa	- Atividade gelatinolítica. - Ativada *in vitro* pela tripsina. - Presente no veneno de *L. intermedia* e *L. laeta.*
Inibidor de Serinoproteases	N.D.	- Pertencente à família das Serpinas. - Identificada em transcriptomas e proteomas de *Loxosceles* sp. - Relacionada aos processos de coagulação, fibrinólise e inflamação.

Adaptado de: Chaim *et al.* (2011b).

2.2 Fosfolipases D

A ação do veneno loxoscélico pode ser melhor compreendida quando observada a ação da fosfolipase D, pois esta toxina obtida isoladamente foi capaz de reproduzir experimentalmente grande maioria dos efeitos observados em pacientes acometidos pela picada por aranha-marrom (Loxoscelismo). Algumas atividades biológicas relacionadas às fosfolipases D presentes nos venenos de aranhas-marrons já foram elucidadas, a exemplo de dermonecrose, hemólise, agregação plaquetária, distúrbios renais, citotoxicidade, nefrotoxicidade, alterações na permeabilidade vascular, coagulação intravascular disseminada, edema e massiva resposta inflamatória (FUTRELL, 1992; da SILVA *et al.,* 2004; APPEL *et al.,* 2005; SWANSON e VETTER, 2006; TAMBOURGI *et al,* 2010; CHAIM *et al.,* 2011b, GREMSKI *et al.,* 2014).

Inicialmente, as fosfolipases D encontradas no veneno de aranhas do gênero *Loxosceles* foram denominadas esfingomielinases D (SMase D) devido sua alta capacidade de hidrolisar esfingomielina. Atualmente, sabe-se que tais enzimas são capazes de atuar sobre um amplo espectro de fosfolipídeos, tornando o termo esfingomielinase limitado para descrevê-las (LEE e LYNCH, 2005; CHAIM *et al.,* 2011a; LAJOIE *et al.*, 2015a, 2015b). Outra denominação encontrada na literatura para fosfolipase D de aranhas-marrons é de fator/proteína dermonecrótica, devido sua principal atividade biológica de induzir dermonecrose (APPEL *et al.*, 2005). A partir de estudos filogenéticos, Binford e colaboradores (2009) propuseram uma nomenclatura para esta família de genes denominando-as de *SicTox* (*Sic*ariidae *Tox*in). Estas proteínas também foram agrupadas em seis grupos distintos, compondo uma família de toxinas denominada Loxtox *(Lox*osceles tox*in*) (KALAPOTHAKIS*, et al.,* 2007).

Vários trabalhos foram realizados para identificar e caracterizar bioquimicamente e biologicamente as isoformas de fosfolipases D de *Loxosceles.* Para *L. reclusa,* foram descritas 4 isoformas ativas, com aproximadamente 32 kDa, as quais reproduzem experimentalmente a lesão dermonecrótica, hemólise e agregação plaquetária (FUTRELL, 1992; da SILVA *et al.,* 2004; VETTER, 2011a, 2011b). Também foram descritas duas isoformas

de fosfolipases D no veneno de *L. laeta*, denominadas SMase I (32 kDa) e SMase II (35 kDa), que experimentalmente, possuem atividade esfingomielinásica, dermonecrótica e hemolítica (TAMBOURGI *et al.,* 2000; FERRARA *et al.,* 2009; MURAKAMI *et al.,* 2005). Catalán e colaboradores (2011) descreveram duas novas isoformas de fosfolipases D de *L. laeta*, as quais foram chamadas de rLIPLD1 e rLIPLD2, e demonstraram que a primeira possui atividade esfingomielinásica e hemolítica, enquanto a segunda parece ser inativa. Além disso, uma análise proteômica do veneno de *L. gaucho,* identificou 11 isoformas da família das fosfolipases D, as quais foram denominadas de Loxonecroginas (MACHADO *et al.,* 2005). Recentemente, Magalhães e colaboradores (2013) produziram uma isoforma recombinante de *L. gaucho* (denominada LgRecDT1) com atividade esfingomielinásica, dermonecrótica, hemolítica e agregação plaquetária.

Foram identificadas e caracterizadas bioquimicamente sete isoformas de fosfolipases D de *L. intermedia.* Estas enzimas foram clonadas a partir de uma biblioteca de cDNA da glândula produtora de veneno e foram denominadas de *Loxosceles intermedia Recombinante Dermonecrotic Toxins:* LiRecDT1 (CHAIM *et al.,* 2006), LiRecDT2 e LiRecDT3 (da SILVEIRA *et al.,* 2006a), LiRecDT4 e LiRecDT5 (da SILVEIRA *et al.,* 2006b), LiRecDT6 (APPEL *et al.,* 2008) e LiRecDT7 (VUITIKA *et al.,* 2013). Além disso, Gremski e colaboradores (2010) demosntraram que além das isoformas caracterizadas, foram identificados no transcriptoma de *L. intermedia* mais 4 novos *clusters* similares a fosofolipases D, considerados como prováveis isoformas dessa enzima. Recentemente, uma análise proteômica do veneno total de *L. intermedia* identificou mais 25 "*spots"* que foram imunologicamente reconhecidos como isoformas de fosfolipases D (WILLE *et al.,* 2013). Os estudos utilizando a biologia molecular e proteômica como ferramentas possibilitaram a identificação e a caracterização de várias isoformas de fosfolipases D presentes nas espécies de *Loxosceles,* sugerindo que estas isoformas poderiam ser agrupadas em uma família de toxinas e que a atuação sinérgica destas toxinas entre si e com outras moléculas está associado com o efeito tóxico do veneno total (KALAPOTHAKIS *et al.,* 2007).

Quanto à atividade enzimática, as fosfolipases D de aranhas-marrons realizam a hidrólise da esfingomielina, com a formação da ceramida 1-fosfato

(C1P) e colina, subprodutos envolvidos em importantes fenômenos biológicos (MARCHESINI e HANNUN, 2004). Lee e Lynch (2005) demonstram que uma fosfolipase D de *L. reclusa* foi capaz de hidrolisar não somente esfingomielina, mas também outros fosfolipídeos como lisofosfolipídeos, tais como lisofosfatidilinositol e lisofosfatidilcolina, gerando ácido lisofosfatídico (LPA) e ácido fosfatídico cíclico. Chaim e colaboradores (2011a), observaram a atividade fosfolipásica da isoforma recombinate LiRecDT1, mostrando que esta possui especificidade por diferentes substratos como esfingomielina e lisofosfatidilcolina (maior atividade), fosfotidilcolina, lisofosfatidilinositol, lisofosfatidiletanolamina, lisofosfatidilserina e 1-Ohexadecyl- 2-hydroxy-sn-glycero-3-phosphocholine (lisoPAF) (menor intensidade). Além disso, foi evidenciado a formação de produtos de fosfatos cíclicos a partir da hidrólise de esfingomielina e lisofosfolipídeos, resultado de uma atividade intramolecular de transfosfatidilação de fosfolipase D de *L. arizonica* (LAJOIE *et al.,* 2013 e 2015a). Recentemente, Lajoie e colaboradores (2015b) propuseram que fosfolipases D de aranhas-marrons possuem preferência por substratos lipídicos carregados positivamente (colina), grupamento presente nos principais substratos desta enzima (esfingomielina e lisofosfatidilcolina). No entanto, uma isoforma de fosfolipase D de *Sicarius terrosus* (grupo irmão do gênero *Loxosceles*) possui maior afinidade por fosfatidiletanolamina (também carregado positivamente). O amplo espectro de substratos lipídicos hidrolisados pelas fosfolipases D de *Loxosceles* pode explicar os diferentes níveis de toxicidade dessas enzimas, devido às diferenças nas proporções de fosfolipídeos que compõem as diversas membrana celulares, bem como elucidar a diversidade de presas que essas aranhas possuem.

Como mencionado anteriormente, a maioria dos produtos da hidrólise de fosfolipídeos são moléculas bioativas relacionadas com importantes funções biológicas como sobrevivência, resposta inflamatória, divisão celular, apoptose, proliferação celular, agregação plaquetária e hemólise (CHALFANT e SPIEGEL, 2005; OHANIAN e OHANIAN, 2001; YANG *et al*., 2000). Ceramida 1-fosfato (C1P) e ácido lisofosfatídico (LPA) são metabólitos lipídicos originados pela hidrólise de esfingomielina e lisofosfatidilcolina, respectivamente, relacionados principalmente a processos inflamatórios (CHALFANT e SPIEGEL, 2005; GOMEZ-MUÑOZ *et al.*, 2015). Estudos

envolvendo veneno total e fosfolipases D recombinantes de *Loxosceles* propõem que estes metabólitos lipídicos (C1P e LPA) agem como mensageiros intracelulares capazes de estimular a produção e secreção de citocinas e quimiocinas próinflamatórias, como: IL-6, IL-8, IL-1b, CCL5, CXCL1, CXCL2, TNF-α, MPC1, KC (DRAGULEV *et al.,* 2007; BARBARO, *et al.,* 2010; HORTA *et al.,* 2013; RIVERA *et al.,* 2015). Foi proposto que IL-6, IL-8 e TNF-α estão envolvidos na histopatologia do Loxoscelismo por estimular a migração massiva de neutrófilos, necrose, edema e proliferação de fibloblasto no local de inoculação (DRAGULEV et al., 2007). Além disso, estes mediadores lipídicos podem estar relacionados na ativação da degranulação de mastócitos, migração de macrófagos e produção de mediadores inflamatórios eucosanóides como prataglandinas (PGE_2) e leucotrienos (CHALFANT e SPIEGEL, 2005; PALUDO *et al.,* 2009; RIVERA *et al*., 2015). As fosfolipases D de *Loxosceles* mostram-se como potenciais bioferramentas para estudos de metabolismo de membrana e formação de mediadores lipídicos envolvidos em processos inflamatórios, contribuindo para a discussão da participação destas moléculas na histopatologia do Loxoscelismo, bem como na elucidação de novos alvos na intervenção terapêutica (DRAGULEV *et al.,* 2007; BARBARO, *et al.,* 2010; HORTA *et al.,* 2013).

2.3 Estrutura das fosfolipases D de *Loxosceles sp*

A sequência polipeptídica primária das fosfolipases D de *Loxosceles* é composta em média por 284 ou 285 resíduos de aminoácidos que são bastante conservados (55-99%) (KALAPOTHAKIS *et al.,* 2007, FERRARA *et al.,* 2009). A cadeia polipeptídica é submetida a processos de dobramento (*"folding"*) para formar uma estrutura em forma de barril distorcido, onde a face interna do barril é composta por oito β-folhas dispostas paralelamente (A-H), que são interligadas por oito alças ("*loops"*) flexíveis em α-hélice (1-8) que formam a superfície da estrutura $(\alpha/\beta)_8$, também denominada de TIM-barril (Figuras 2 e 3A). Os *loops* de ligação são de caráter hidrofílico e hidrofóbico, na porção superior e inferior, respectivamente. Observa-se a presença de uma pequena β-folha (B`) entre a cadeia B e a hélice 2, e também duas pequenas hélices (3'e

4') entre a hélice 3 e cadeia D, e hélice 4 e cadeia E. Devido à presença de α-hélices, β-folhas e *loops* que variam de tamanho e de característica, a estrutura em forma de barril é distorcida e possui uma área de 11,254 $Å^2$ (MURAKAMI *et al.,* 2005). O interior do barril é composto por resíduos de aminoácidos hidrofóbicos e por porções curtas das regiões N- e C- terminal. A porção C-terminal é caracterizada por possuir uma pequena hélice (8`) e uma β-folha (H`). As porções dos *loops* que estão mais próximos do barril possuem caráter mais hidrofóbico, e uma cavidade estreita promove o acesso ao sítio catalítico, que é caracterizado por um anel de resíduos de aminoácidos negativamente carregados (MURAKAMI *et al.,* 2005 e 2006; GUISEPPE *et al.,* 2011; DIAS-LOPES *et al.,* 2013; GREMSKI *et al*., 2014).

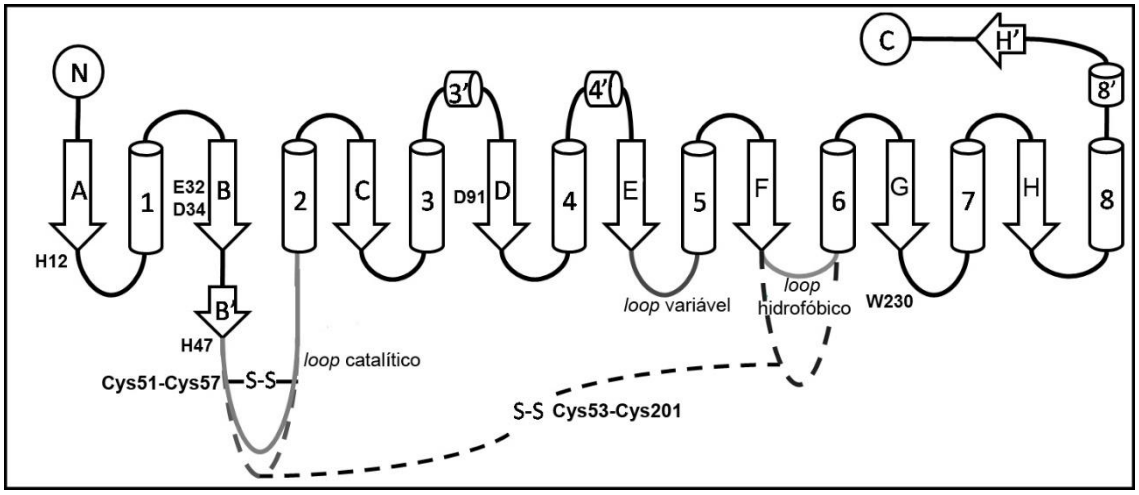

FIGURA 2. ESQUEMA DA TOPOLOGIA DE FOSFOLIPASES D DE *L. laeta* (CLASSE I) e *L. intermedia* (CLASSE II). B-folhas (flexas A-H), α-helices (cilindros *1-8*) que formam a estrutura em forma de barril $(\alpha/\beta)_8$. *Loop* catalítico, *loop* variável, *loop* hidrofóbico, ponte dissulfeto (S-S entre Cys51-Cys57) e ponte dissulfeto adicional presente na enzima da classe II (S-S Cys53-Cys201). Letras C e N se referem às porções carboxi e amino-terminal das proteínas, respectivamente. (Adaptado de GREMSKI *et al.,* 2014).

Estruturalmente, as fosfolipases D dos venenos de aranhas do gênero *Loxosceles* podem ser agrupadas em duas classes, baseado na sua sequência aminoacídica, na sua estrutura e atividade catalítica. As fosfolipases D da classe I, que são representadas pela isoforma recombinate PLD I de *L. laeta*, são caracterizadas pela presença de uma única ponte dissulfeto entre os resíduos Cys51 e Cys57 e um extenso *loop* flexível. As fosfolipases D da classe II, representadas pelas PLDs do veneno *L. intermedia*, contêm duas

pontes dissulfeto, entre os resíduos Cys51 e Cys57 e Cys53 e Cys201, que liga o *loop* flexível e o *loop* catalítico (Figura 3A). Dependendo da sua capacidade de hidrolisar esfingomielina, são divididas em subclasses IIa (contém as isoformas mais ativas) e subclasse IIb (contém as isoformas menos ativas ou inativas). É postulado que as diferenças eletrostáticas no sítio catalítico entre as duas classes de fosfolipases D estão associadas às diferentes maneiras de orientar o substrato na fenda catalítica, resultando em diferentes afinidades pelo substrato (Figura 3B e C) (MURAKAMI *et al.,* 2006; GIUSEPPE *et al.,* 2011).

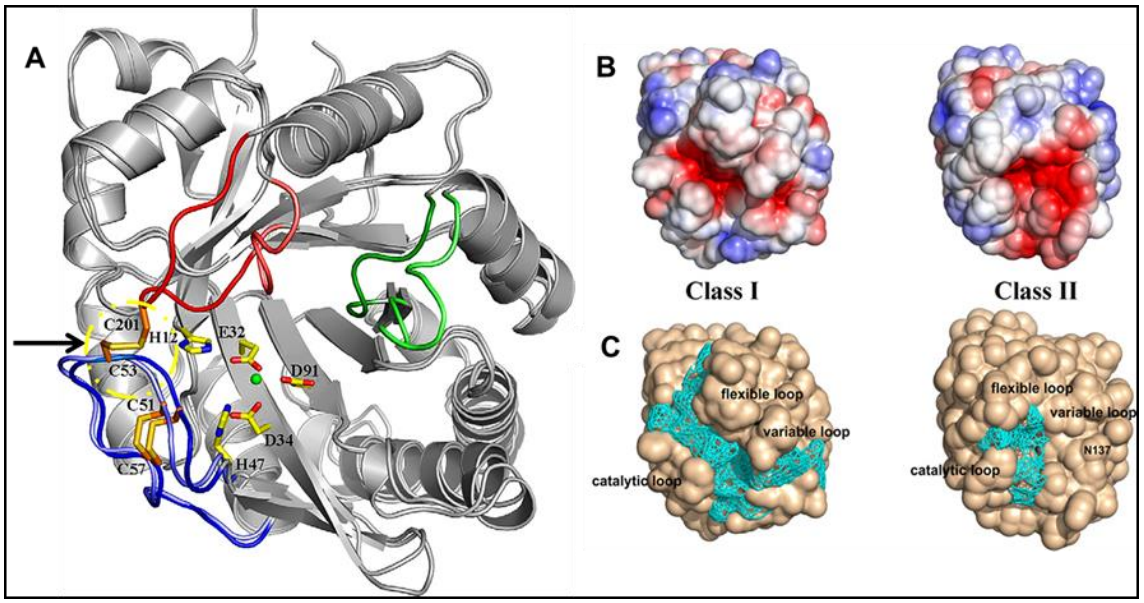

FIGURA 3. ALINHAMENTO ESTRUTURAL DA ISOFORMA LiRecDT1 DO VENENO DE *L. intermedia* (CLASSE II) E PLD DE *L. laeta* (CLASSE I). **A-** Sobreposição de LiRecDT1 e PLD de *L. laeta* mostrando os resíduos de aminoácidos envolvidos na ligação do íon Mg^{+2} (esfera verde). Os *loops* catalítico, flexível e variável estão representados em azul, vermelho e verde, respectivamente. Os resíduos envolvidos na formação das pontes dissulfeto estão representados na cor laranja. A ponte dissulfeto adicional presente nas fosfolipases D da classe II (Cys53 e Cys201) está indicado na seta. **B-** Diferenças eletrostáticas entre as fosfolipaases D de classe I e de classe II (-2kv em vermelho e +2kv em azul). **C-** Representação do tamanho da cavidade catalítica entre as duas classes de fosfolipases D de aranhas-marrons. Adapatado de: GIUSEPPE *et al.* (2011).

O *loop* catalítico, *loop* variável e *loop* hidrofóbico estão localizados na mesma face do barril (Figura 2). O *loop* catalítico (resíduos 46-60) contém os resíduos de His12, Glu32, Asp34, Asp91, His47, Asp52, Trp230, Asp233 e Asn252, os quais são conservados entre fosfolipases D de várias espécies, e juntamente com a coordenação do íon Mg^{+2} formam o sítio catalítico capaz de hidrolisar esfingomielina (MURAKAMI *et al.* 2005 e 2006; GUISEPPE *et al.,*

2011; DIAS-LOPES *et al.,* 2013). O íon Mg^{+2} é essencial para a catálise e tem coordenação octaédrica (com uma média de distância Mg^{2+}-O de 1,98 Å) pelos átomos de oxigênios dos ácidos carboxílicos das cadeias laterais dos resíduos Glu32 e Asp34. Também existe a participação de duas moléculas de água que estão fortemente ligadas ao átomo de oxigênio do ácido carboxílico do resíduo Asp91, e por outra molécula de água ligada por uma ponte de hidrogênio ao átomo do resíduo Glu32. Neste mesmo estudo, os autores sugerem um mecanismo catalítico do tipo ácido-base para esta enzima (Figura 4). Neste mecanismo proposto, a His47 inicia o processo da hidrólise, comportando-se como um nucleófilo que ataca a ligação fosfodiéster do substrato, gerando uma estrutura penta-coordenada. Já a His12 doa um próton (H^+) para esta estrutura penta-coordenada gerando a colina. A His12 retira um átomo de H^+ da molécula de água, iniciando um segundo ataque nucleófilo sobre o intermediário da reação, que dessa vez, resulta na formação e liberação da ceramida 1-fosfato (C1P). O íon Mg^{+2} é importante para o reconhecimento, ligação e estabilização do estado intermediário do mecanismo catalítico.

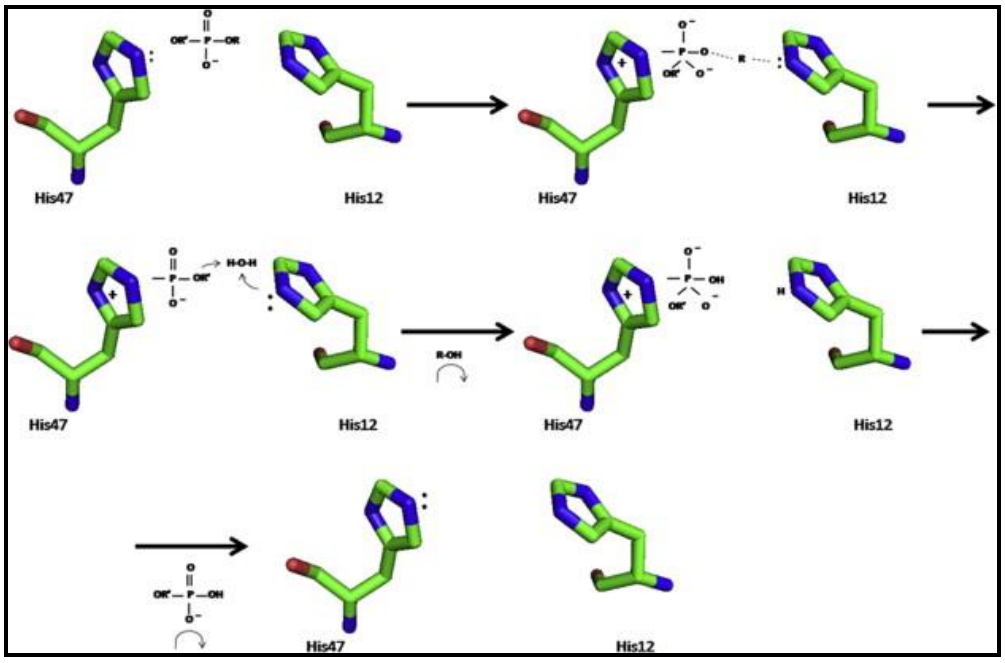

FIGURA 4. MECANISMO CATALÍTICO DAS FOSFOLIPASES D DOS VENENOS DE ARANHAS DO GÊNERO *Loxosceles.* A atividade fosfolipásica do tipo D é uma hidrólise dependente de ataques nucleófilos das histidinas sobre a ligação fosfodiéster do substrato. Adaptado: GREMSKI *et al*. (2014).

Até o momento, a literatura aponta que os resíduos de aminoácidos que compõem o sítio catalítico das fosfolipases D dos venenos de aranhas do gênero *Loxosceles* são conservados. No entanto, é proposto que qualquer substituição destes resíduos poderia modificar a sua capacidade catalítica e/ou atividade biológica, além de alterar sua especificidade a diferentes substratos (LEE e LYNCH, 2005; MURAKAMI *et al.,* 2006, GIUSEPPE *et al.,* 2011; DIAS-LOPES *et al.,* 2013).

Andrade *et al.* (2006) caracterizaram duas isoformas recombinates de fosfolipase D de veneno de *L. intermedia*, denominadas de rP1 e rP2. Neste estudo, foi observado que a isoforma rP1 hidrolisa menos esfingomielina que rP2, tal fato que pode ser atribuído a substituição P203L e de outros resíduos que compõem o canal hidrofóbico. Lee e Lynch (2005) produziram várias isoformas recombinates de SMase D do veneno de *L. reclusa* com mutações sítio-dirigidas e verificaram que isoformas com a mutação nos resíduos H37N e His73N perderam a capacidade de hidrolisar esfingomielina e lisofosfatidilcolina, além de não causar hemólise. Estudos realizados com uma isoforma mutada de fosfolipase D do veneno de *L. intermedia* (LiRecDT1 H12A) demonstraram que esta molécula estimula resposta inflamatória, porém possui uma diminuição significativa na atividade catalítica, dermonecrótica, hemolítica e citotóxica (KUSMA *et al.,* 2008, CHAVES-MOREIRA *et al,* 2009; CHAVES-MOREIRA *et al.,* 2011; CHAIM *et al.,* 2011a; WILLE *et al,* 2013). Embora a isoforma LiRecDT1 H12A não foi capaz de induzir o mesmo nível de atividade catalítica e biológica que a isoforma original, foi observada que esta foi capaz de se ligar na superfície celular ou a lipídeos mobilizados (KUSMA *et al.*, 2008; PALUDO *et al.*, 2009; CHAIM *et al.*, 2011a; WILLE *et al.*, 2013). A partir disso é proposto que outros domínios da proteína, além dos resíduos do sítio catalítico, podem ser responsáveis pela interação da toxina com a membrana celular ou com os substratos lipídicos.

Alguns resíduos de aminoácidos presentes nas estruturas de fosfolipases D são propostos como sítios de reconhecimento e de ligação ao substrato lipídico. Estudos por cristalografia de fosoflipases D de *Loxosceles* sugerem que os resíduos de aminoácidos Lys93 e Trp228 estão relacionados com a ligação e estabilização do substrato na fenda catalítica (MURAKAMI et al., 2005; MURAKAMI et al. 2006; GUISEPPE et al. 2011). Recentemente,

Catalán *et al*. e colaboradores (2014) sugeriram por meio de mutagênese que os resíduos Trp256 e Asp259 são importantes para a atividade de uma isoforma recombinante de *L. laeta* e que esses resíduos podem estar relacionados à ligação do substrato. Em glicerofosfodiéster fosfodiesterase (GDPD) de *Thermoanaerobacter tengcongensis* foi proposto que o resíduo Lys121 é importante para a ligação do substrato lipídico (SHI *et al*., 2008).

Estes estudos reforçam a ideia de que os resíduos de aminoácidos que compõem o sítio ativo são importantes para a atividade catalítica das fosfolipases D, uma vez que substituições desses resíduos poderiam alterar a atividade catalítica, a acessibilidade e reconhecimento da enzima pelo seu substrato. Além disso, outros domínios e outros resíduos podem estar assumindo um papel estrutural e de reconhecimento e ligação ao substrato lipídico que os tornam tão importantes quanto os resíduos relacionados com a catálise. A partir destas evidências, houve grande interesse em mapear e estudar mais profundamente a importância de alguns resíduos de aminoácidos na atividade catalítica e avaliar biologicamente as propriedades destas moléculas. Por isso, a produção e caracterização de proteínas mutantes é uma essencial ferramenta para a caracterização estrutural, bem como para buscar o esclarecimento funcional das proteínas.

3 OBJETIVOS

3.1 Objetivo Geral

O objetivo principal do presente trabalho é o mapeamento estrutural do sítio ativo das fosfolipases D de *Loxosceles*, a fim de contribuir para o melhor entendimento sobre a relação entre a estrutura e a função destas toxinas.

3.2 Objetivos Específicos

- Obter isoformas recombinantes de fosfolipase D (LiRecDT1) com as seguintes mutações sítio-dirigidas: G96A, H12A, H12A-H47A, E32A-D34A, K93A, Y228A, W230A e C53A-C201A.
- A partir de experimentos de cunho bioquímico e biológico, determinar e descrever a importância dos resíduos de aminoácidos no papel catalítico das fosfolipases D, na coordenação do íon Mg^{+2} e na ligação do substrato lipídico.
- Analisar o impacto dos resíduos de aminoácidos mutados na interface estrutura/atividade das fosfolipases D por modelagem molecular utilizando ferramentas de bioinformática.
- Utilizar as isoformas de fosfolipases D mutadas como bioferramentas para futuros estudos estruturais (cristalografia por difração de raios-X), bem como o estudo de inibidores para estas moléculas.

4 RESULTADOS

O presente trabalho de Tese descreve os resultados relativos à caracterização funcional de toxinas derivadas da isoforma 1 (LiRecDT1) de fosfolipase D de *L. intermedia* com mutações sítio-dirigidas. As alterações na sequência de aminoácidos permitiram o mapeamento dos resíduos importantes que compoêm o sítio ativo desta enzima.

Item 1: manuscrito submetido em dezembro de 2015 (Vuitika *et al.*). O manuscrito está na formatação exigida pela revista (inserido nos resultados).

Outros artigos publicados, produzidos durante o doutorado estão anexados (Item 8 – Anexos).

ANEXO 1: Artigo publicado na revista Journal of Cellular Biochemistry em primeira autoria (Vuitika et al. 2013), **Identification, cloning and functional characterization of a novel phospholipase D (dermonecrotic toxin) from brown spider (*Loxosceles intermedia*) venom containing a conservative mutation (D233E) in the catalytic site.**

ANEXO 2: Artigo como co-autora: Gremski *et al.*, 2014, **Recent advances in the understanding of brown spider venoms: From the biology of spiders to the molecular mechanisms of toxins**, Toxicon.

ANEXO 3: Artigo como co-autora: Coronado *et al.,* 2015, **Structural Insights into Substrate Binding of Brown Spider Venom Class II Phospholipases D,** Current Protein e Pepitide Science.

Os artigos foram incluídos nesta primeira versão de Tese na sua forma original, com intuito de facilitar a apreciação dos membros da banca.

Resultados - Item 1: Active site mapping of *Loxosceles* phospholipases D: biochemical and biological features.

Vuitika[a], L.; Chaves-Moreira[a], D.; Caruso[b], I.; Lima[c], M. A.; Matsubara[a], F. H.; Murakami[d], M. T.; Takahashi[c], H. K.; Toledo[c], M. S.; Coronado[b], M. A.; Nader[c], H. B.; Senff-Ribeiro, A[a].; Chaim[a], O. M; Arni[b], R. K.; Veiga[a*], S. S.

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Highlights

- The key aminoacids residues for the catalytic activity of PLD from *Loxosceles sp.*
- Tyrosine 228 residue is essential for enzymatic activity and substrate binding.
- Biochemical and biological activities highlighted features of Brown spider PLD.

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Active site mapping of *Loxosceles* phospholipases D: Biochemical and biological features

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ABSTRACT

Brown spider phospholipases D from *Loxosceles* venoms are among the most widely studied toxins since they induce dermonecrosis, triggering inflammatory responses, increase vascular permeability, cause hemolysis, and renal failure. The catalytic (H12 and H47) and metal-ion binding (E32 and D34) residues in *Loxosceles intermedia* phospholipase D (LiRecDT1) were mutated to understand their roles in the observed activities. All mutants were identified using whole venom serum antibodies and a specific antibody to wild-type LiRecDT1, they were also analyzed by circular dichroism (CD) and differential scanning calorimetry (DSC). The phospholipase D activities of H12A, H47A, H12A-H47A, E32, D34 and E32A-D34A, such as vascular permeability, dermonecrosis, and hemolytic effects were inhibited. The mutant Y228A was equally detrimental to biochemical and biological effects of phospholipase D, suggesting an essential role of this residue in substrate recognition and binding. On the other hand, the mutant C53A-C201A reduced the enzyme's ability to hydrolyze phospholipids and promote dermonecrosis, hemolytic, and vascular effects. These results provide the basis understanding the importance of specific residues in the observed activities and contribute to the design of synthetic and specific inhibitors for Brown spider venom phospholipases D.

1. Introduction

Accidental bites from the genus *Loxosceles* (brown spiders, also known as violin spiders) have been reported to induce several clinical signs, such as necrotic skin lesions with gravitational spreading (the hallmark of *Loxosceles* envenomation), hematological disturbances (thrombocytopenia and hemolytic anemia), and renal failure [1–4]. Brown spiders have a cosmopolitan distribution and the variation of species is reflected by their geographical prevalence. Accidents involving brown spiders have been reported in America, Europe, Asia, Africa, and Oceania [1–4].

Molecular biological technologies such as, the construction of a cDNA library from venom glands, cloning procedures, transcriptome analysis, heterologous recombinant toxin expression, and proteomics studies have revealed the complex composition of brown spider venoms. They contain at least three major classes of molecules: (1) phospholipases D (also known as *Loxosceles intermedia* Recombinant Dermonecrotic Toxins [LiRecDTs], based on their involvement in dermonecrosis following accidents); (2) metalloproteinases (which are members of the *Loxosceles* Astacin-like Proteinase [LALP] family) [5,6]; and (3) low molecular mass molecules, putatively classified as insecticidal toxins (Cystine Knot peptides [ICK]) [7,8]. The venom of Brown spiders also contains toxins with low levels of expression, such as hyaluronidases, translationally controlled tumor protein (TCTP), serine proteinases, serine proteinase inhibitors, and venom allergens [9–12].

Previous reports have proposed the existence of a family of phospholipase D toxins in *Loxosceles* spider venoms. Kalapothakis and colleagues classified the phospholipases D from *Loxosceles* spider venoms as the LoxTox family, which was further extended to include the phospholipase D homologs identified in Sicariid spiders, which are referred to as SicTox [13,14]. Wood et al. compiled the ArachnoServer database of toxins from spider venoms, which includes several toxins characterized as homologs of phospholipases D derived from *Loxosceles* species [15]. The existence of an intra-species family of antigenically and structurally related members has also been supported by experimental approaches, including the immunodetection of multiple homologs of phospholipase D in the venom of *Loxosceles* species [15,16].

Among the venoms of Brown spiders, the phospholipase D family and its homologs are the most widely studied. Members of this family of

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toxins hydrolyze sphingomyelin to ceramide 1-phosphate and choline, and also hydrolyze lysoglycerophospholipids, such as lysophosphatidylcholine, lysophosphatidylinositol, lysophosphatidylethanolamine, lysophosphatidylserine, and lyso platelet activating factor (LPAF), to produce the lipid mediator lysophosphatidic acid (LPA) [17–19]. With the aid of ^{31}P NMR spectroscopy and mass spectrometry, the formation of cyclic phosphate products from intramolecular transphosphatidylation, catalyzed by a recombinant phospholipase D from the venom of *Loxosceles arizonica* has been reported recently and the authors postulated that Brown spider phospholipases D catalyze transphosphatidylation rather than hydrolysis, to form cyclic phosphate products from substrates, such as sphingomyelin and lysophosphatidylcholine [20]. It is presently accepted that members of this family of enzymes possess both sphingomyelinase D and lysophospholipase D activity [17].

Brown spider venom recombinant phospholipases D have been produced and purified from various *Loxosceles* species, such as *L. laeta* [21,22]; *L. reclusa* and *L. boneti* [18,23]; *L. intermedia* [24–28]; *L. gaucho* [29]; and *L. arizonica* [30,31]. Under laboratory conditions, purified recombinant brown spider venom phospholipase D isoforms from *L. intermedia* can induce the major effects elicited by whole venom, such as dermonecrosis, deregulated inflammatory responses, hemolysis, platelet aggregation, increased vascular permeability, and acute renal failure [31–39]. With the aid of X-ray crystallography and other *in silico* structural analyses studies on recombinant phospholipases D from *L. laeta* [40,41] and *L. intermedia* [42,43] venoms have demonstrated that these toxins consist of an $(\alpha/\beta)_8$-barrel catalytic domain with conserved residues involved in catalysis (H12 and H47) and metal-ion coordination (E32 and D34). In summary, these studies have confirmed the existence of an intra- and inter-specific family of phospholipases D and their biological importance in the life cycle of Brown spiders [40–43].

The phospholipase D from *L. intermedia* venom (LiRecDT1) was used as a model [17,25,33,34,36] to perform a systematic mutational and functional investigation of the residues that have been proposed to be involved in catalysis (H12A, H47A and H12A-H47A), metal-ion binding (E32A, D34A and E32A-D34A), substrate recognition (K93A, Y228A, and W230A), and stabilization of the flexible loop (C53A-C201A). In addition, the mutant G96A was used as a control of the experimental procedures which recombinants toxins followed after site-directed mutagenesis.

2. Material and methods

2.1. Reagents

Whole venom from *L. intermedia* was extracted from spiders captured in the wild with the authorization of the Brazilian Governmental Agency "Instituto Chico Mendes de Conservação da Biodiversidade" number 29801-1, in accordance with the methods outlined by Feitosa and colleagues [44]. Polyclonal antibodies against *L. intermedia* venom toxins and phospholipase D ("dermonecrotic toxin" - LiRecDT1) were produced in rabbits as previously described [25]. Evans Blue dye was purchased from Vetec (São Paulo, Brazil). NaCl, KCl, $CaCl_2$, Na_2HPO_4, KH_2PO_4, Na_2PO_4, NaH_2PO_4, imidazole, and agar were purchased from Merck (Darmstadt, Germany). Tryptone and yeast extract were obtained from Himedia (Mumbai, India) and chloramphenicol and ampicillin were purchased from USB Corporation (Cleveland, USA). Coomassie Blue, tris and sucrose were purchased from Bio-Rad (Hercules, USA) and Sigma–Aldrich (St. Louis, USA), respectively. Xylazine and ketamine were purchased from Rhobifarma (São Paulo, Brazil).

2.2. Site-directed mutagenesis

The QuikChange® Site-Directed Mutagenesis Kit from Stratagene (Santa Clara, USA) was selected for *in vitro* site-directed mutation. The wild-type toxin LiRecDT1 cloned in the pET-14b vector was used as a template to construct and code mutated homologs. LiRecDT1 toxin is the most abundant in *L. intermedia* venom and following the site-directed mutations; it generated eleven mutants (H12A, H47A, H12A-H47A, E32A, D34A, E32A-D34A, K93A, Y228A, W230A, C53A-C201A, and G96A). Sense oligonucleotide and antisense mutagenic primers were designed using the PrimerX tool (Table 1) and PCR was performed using the *Pfu-Turbo* polymerase High Fidelity kit as described by Scott and colleague [45]. PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and exposed to ultraviolet (UV) light. The corresponding bands were cut and purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). Digestion of the non-mutated, methylated template strand was performed and the methylated fragment was identified by the endonuclease *DpnI* enzyme kit (target sequence 5′-3′-Gm6ATC). XL1-Blue supercompetent cells from a strain of *Escherichia coli* were transformed with the mutated constructions. Positive clones were identified by a sequencing reaction (BigDye Terminator v 3.1 Cycle Sequencing Kit, Applied Biosystems) using the DNA 3500 Genetic Analyzer automatic sequencer (Applied Biosystems, Warrington, UK).

2.3. Wild-type LiRecDT1 and mutants expression

Constructs in pET-14b vector were expressed as fusion proteins with a 6xHis-Tag at the N-terminus and transformed into One Shot BL21(DE3)pLysS *E. coli* competent cells (Invitrogen, Waltham, USA) and plated on LB agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. A single colony was inoculated into 50 mL of LB broth (100 μg/mL ampicillin and 34 μg/mL chloramphenicol) and left overnight at 37 °C. From this overnight culture, 10 mL was inoculated into 1 L of LB broth/ampicillin/chloramphenicol at 37 °C, until the optical density (O.D.) at 550 nm reached 0.5. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.05 mM and the culture was incubated for an additional 3.5 h at 30 °C (with vigorous shaking). Cells were harvested by centrifugation (4000 × *g*, 7 min) and the resultant pellet was frozen at −20 °C overnight [25].

Table 1
Sense and antisense mutagenic oligonucleotides were designed using the PrimerX tool (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi).

Mutation	Oligonucleotide sequence (5′–3′)	Orientation
G96A	GTGTTCGACTTAAAGACA**GCC**AGCCTCTACGATAATCAAG	Sense
G96A	CTTGATTATCGTAGAGGCT**GGC**TGTCTTTAAGTCGAACAC	Antisense
H12A	ATTTACCAT**GGC**CCCCATGATC	Sense
H12A	GATCATGGGG**GCC**ATGGTAAAT	Antisense
H47A	CAATCCTGAGTATACTTAT**GCC**GGCATTCCATGTGATGCCG	Sense
H47A	CGGCATCACATGGAATGCC**GGC**ATAAGTATACTCAGGATTG	Antisense
H12A-H47A	CCAATCCTGAGTATACTTAT**GCC**GGCATTCCATGTGATTGTGG	Sense
H12A-H47A	CCACAATCACATGGAATGCC**GGC**ATAAGTATACTCAGGATTGG	Antisense
E32A	CTTGGAGCAAACTCCATC**GCC**ACAGACGTGTCTTTCGATG	Sense
E32A	CATCGAAAGACACGTCTGT**GGC**GATGGAGTTTGCTCCAAG	Antisense
D34A	GCAAACTCCATCGAAACA**GCC**GTGTCTTTCGATGACAATG	Sense
D34A	CATTGTCATCGAAAGACAC**GGC**TGTTTCGATGGAGTTTGC	Antisense
E23A-D34A	CTTGGAGCAAACTCCATC**GCC**ACA**GCC**GTGTCTTTCGATTG	Sense
E23A-D34A	CAATCGAAAGACAC**GGC**TGT**GGC**GATGGAGTTTGCTCCAAG	Antisense
K93A	CTGGTCTTAGTCGTGTTCGACTTA**GCC**ACAGGTAGCCTCTACG	Sense
K93A	CGTAGAGGCTACCTGT**GGC**TAAGTCGAACACGACTAAGA CCAG	Antisense
Y228A	CGGATTCATTAACAAAGTG**GCC**TACTGGACAGTGGACAAGC	Sense
Y288A	GCTTGTCCACTGTCCAGTA**GGC**CACTTTGTTAATGAATCCG	Antisense
W230A	CAAAGTGTACTAC**GCC**ACAGTGGACAAGCGCTCAACGACC AGAG	Sense
W230A	CTCTGGTCGTTGAGCGCTTGTCCACTGT**GGC**GTAGTACACT TTG	Antisense
C53A	CTTATCACGGCATTCCATGTGAT**GCC**GGAAGGAATTGCAA GAAATATG	Sense
C53A	CATATTTCTTGCAATTCCTTCC**GGC**ATCACATGGAATGCCG TGATAAG	Antisense
C201A	GAGCGATGGTATCACCAAC**GCC**TTACCACGTGGCCTTAGTC	Sense
C201A	GACTAAGGCCACGTGGTAA**GGC**GTTGGTGATACCATCGCTC	Antisense

2.4. Recombinant protein purification

The cells were thawed and disrupted by mechanical lysis and centrifuged (9000 ×*g*, for 30 min, at 4 °C), and the supernatants were incubated with 1 mL of Ni^2-NTA agarose beads for 1 h at 4 °C. The binding suspensions were loaded onto a column and the packed gel was washed with a wash buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 8.0, 500 mM NaCl and 20 mM Imidazol). The pure proteins were obtained with elution buffer (50 mM NaH_2PO_4/Na_2HPO_4 pH 8.0, 500 mM NaCl and 250 mM Imidazole). The fractions were analyzed by SDS–PAGE under reducing conditions using β-mercaptoethanol. The fractions were pooled and dialyzed against phosphate-buffered saline (PBS) [17,25].

2.5. Immunological cross-reactivity of LiRecDT1 and mutant toxins

Protein quantification was performed by the Coomassie Blue method according to the procedure outlined by Bradford [46]. Protein profiles of recombinant toxins (2.5 μg) were analyzed by 12.5% SDS–PAGE under reduced conditions. The toxins were transferred onto nitrocellulose membranes for immunoblotting and were immunostained with polyclonal antibodies raised against either phospholipase-D isoform 1 (LiRecDT1) (1:10,000) or *L. intermedia* whole venom (1:10,000) [26,32,39]. This was followed by detection using secondary alkaline phosphatase-coupled *anti*-IgG (1:8000) (Sigma-Aldrich) and visualization of immunoreactions through the BCIP/NBT substrate reaction (Promega). Control of primary antibody specificity was performed using pre-immune serum (collected before immunization) for immunodetection.

2.6. Circular dichroism spectroscopy (CD)

Recombinant wild-type LiRecDT1 and mutated toxins were dialyzed at 4 °C against a phosphate buffer (20 mM NaH_2PO_4/Na_2HPO_4 pH 7.4 and 150 mM NaCl) to a final concentration of 0.5 mg/mL. The spectra were recorded in a Jasco J-815 spectropolarimeter (Jasco Corporation) using a 2 mm cuvette. The spectra of 0.5 nm intervals were the average of eight measurements. Each test was performed at a rate of 50 nm/min using a response time of 8 s and a band width of 1 nm. The temperature was maintained constant at 20 °C [24–26]. Measurements were performed in triplicate. The secondary structures of toxins were estimated from the spectra using the K2D3 web Server [47].

2.7. Differential scanning calorimetry (DSC)

The experiments were performed using *N*-DSC III (TA Instruments) in the range of 20–80 °C at a heating and cooling scan rate of 1 °C/min [48]. Samples were diluted in phosphate buffer (20 mM NaH_2PO_4/Na_2HPO_4 pH 7.4 and 150 mM NaCl) to a final concentration of 1.5 mg/mL. Both calorimeter cells were loaded with buffer solution, equilibrated at 20 °C for 10 min and scanned repeatedly. The sample cell was subsequently loaded with the recombinant toxin (LiRecDT1) and/or the mutated form and scanned. The baseline was obtained by subtracting the buffer scan from the corresponding protein scan. Measurements were performed in triplicate.

2.8. Phospholipase activity

Phospholipase activity was measured using the Amplex Red Assay Kit (Thermo Fisher Scientific, Waltham, USA). In this assay, phospholipase D activity was monitored using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2 [24]. Recombinant phospholipase D first hydrolyzed the lipid substrate to yield ceramide 1-phosphate and choline. Choline was then oxidized by choline oxidase to betaine and H_2O_2. In the presence of horseradish peroxidase, H_2O_2 reacted with the Amplex Red reagent in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin. LiRecDT1 (positive control) and mutated toxins (10 μg each, in five trials) were added to the Amplex Red reagent mixture in reaction buffer (Tris-HCl 100 mM pH 7,4 containing 10 mM $MgCl_2$). The negative control was obtained by incubation of the Amplex Red reagent mixture in the absence of toxins. The reaction tubes were incubated at 37 °C for 1 h, and the fluorescence was measured in a microplate fluorimeter (Tecan Infinite M200, Männedorf, Switzerland) using excitation and emission detection wavelengths of 540 nm and 570 nm, respectively. These procedures were used to test the ability of phospholipase D wild-type and mutants to hydrolyze solubilized Egg SM (Sphingomyelin Egg, Chicken) and/or solubilized Egg LPC (L-α-lysophosphatidylcholine Egg, Chicken) in reaction buffer containing Triton X-100 all below CMC (Avanti Polar Lipids, Inc. Alabaster, USA).

2.9. High-performance thin-layer chromatography

To analyze the hydrolytic process, 50 μg of LiRecDT1 or each mutant toxin was incubated for 2 h with 1 mg/mL of Sphingomyelin Egg, Chicken (SM) and lysophosphatidylcholine Egg, Chicken (LPC). All samples were recovered directly by partition with 2 mL of water-saturated 1-butanol and the butanol fraction was dried, resuspended in chloroform, and analyzed by HPTLC. Analytical HPTLC was performed on silica gel 60 plates (Merck) using 40% chloroform-methanol-methylamine (65:35:10 v/v/v) as the mobile phase. Lipid samples were dissolved in chloroform and 20 μL of each resultant solution was applied to respective plates using a micropipette. The samples were then visualized under UV light after being sprayed with 0.01% primulin in 90% aqueous acetone [17]. Differences in lipid degradation following enzyme treatments were quantified by densitometry of the digital images of HPTLC plates, acquired with the GeneSnap software for G: Box Chemi XL (Syngene, Cambridge, UK) and quantified by the Quantity One software for Chemic Doc XRS (BioRad). For comparison, the percentage of hydrolysis by LiRecDT1 was considered as 100% (The extent to which LiRecDT1 hydrolyzes SM and LPC under experimental conditions is 85% and 53%, respectively).

2.10. Determination of hemolytic activity and morphological alterations of human erythrocytes

The hemolysis assay was performed as previously described [33,34]. Washed human red blood cells (1×10^8 cells) were added to microtubes containing 25 μg/mL of LiRecDT1 or mutant toxin in Tris buffer sucrose (TBS: 250 mM sucrose; 10 mM Tris/HCl, pH 7.4) containing 1 mM $CaCl_2$. Experiments were performed in pentaplicate at different intervals (0, 3, 6, 12, and 24 h). Red blood cells in TBS with 1 mM $CaCl_2$ served as the negative control, and for the positive control, red blood cells were incubated in 0.1% v/v Triton X-100. Each incubation was performed with gentle agitation, after which the controls and samples were centrifuged, using a refrigerated microfuge (Centrifuge 5804 R, Eppendorf, Hamburg, Germany), for 3 min at 350 ×*g*. Absorbance of the supernatants was immediately measured at 550 nm (Meridian ELx 800, BioTek Instruments, Winooski, USA). Absorbance was converted to percentage hemolysis using the absorbance of the positive control (Triton X-100) as 100% hemolysis.

Human erythrocytes were washed with Ringer's Solution (125 mM NaCl; 5 mM KCl; 1 mM $MgSO_4$; 32 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]; 5 mM glucose; and 1 mM $CaCl_2$, pH 7.4) and incubated with 5 μg/mL of LiRecDT1 or mutated toxin for morphological observation. Cells were analyzed at various intervals (0, 3, 6, 12, and 24 h) using the Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany) at a magnification of 400× for differential interface contrast (DIC). Captured images were representative of each treatment. The negative control comprised red blood cells in the presence of Ringer buffer with 1 mM $CaCl_2$. Experiments were performed in triplicate. The AxioVision

LE software was used for snapshot processing in the Zeiss image format (ZVI).

2.11. Animals

Adult Swiss mice (25–30 g) from the Central Animal House of the Federal University of Paraná and adult rabbits (~3 kg) from the Production and Research Center of Immunobiology (CPPI) were randomly selected for *in vivo* experiments. All procedures involving animals were performed in accordance with Brazilian Federal Laws, in accordance with the Ethical Subcommittee on Research Animal Care Agreement number 743 of the Federal University of Paraná.

2.12. Vascular permeability

Detection of changes in capillary permeability was based on the leakage of plasma protein-bound dye into the extravascular compartment of the skin [28,37]. Evans Blue Dye diluted in PBS was administered to mice intravenously (30 mg/kg) 5 min prior to injections of toxin samples. Recombinant LiRecDT1 and mutant homologs (10 μg) were administered intradermally to the dorsum of mice (n = 5 per treatment). For the negative control, animals received PBS injection only. After 1 h, the animals were anesthetized using ketamine and xylazine, sacrificed, and the dorsal skin was resected for the visualization of dye extravasation. Mice were selected because this animal model does not develop dermonecrosis, which would prevent local hemorrhage following toxin exposure, and potentially confound the interpretation of vascular permeability.

2.13. Dermonecrosis in vivo

To evaluate the dermonecrotic effect, 10 μg of each toxin was injected intradermally into a shaven area of rabbit dorsum skin. Animals were observed over the course of the evolution of the dermonecrotic lesion. Macroscopic images were acquired after 0, 3, 6, and 24 h of the respective toxin applications using a Sony DSC-W55 camera (Tokyo, Japan). Experiments were performed in triplicate [24,26–28,37].

2.14. Statistical analysis

The data were analyzed by analysis of variance and Tukey's test for average comparisons in GraphPad InStat 3.0. Mean and SD values were used to build the figures in GraphPad Prism 6.0. Results of $p \leq 0.001$ were considered significant.

3. Results

3.1. Expression, purification, immunological cross-reactivity, and biophysical characterization of LiRecDT1 toxin and mutants

Eleven mutants of LiRecDT1 were obtained by site-directed mutagenesis. Mutations were in the histidine residues at positions 12 and 47 (LiRecDT1 H12A, LiRecDT1 H47A and LiRecDT1 H12A-H47A); glutamic acid, 32 and aspartic acid 34 residues (LiRecDT1 E32A, LiRecDT1 D34A and LiRecDT1 E32A-D34A); cysteine residues 53 and 201 (LiRecDT1 C53A-C201A); lysine residue 93 (LiRecDT1 K93A) tyrosine residue 228 (LiRecDT1 Y228A); tryptophan residue 230 (LiRecDT1 W230A); and glycine residue 96 (LiRecDT1 G96A) (Fig. 1A). LiRecDT1 enzyme and its mutants, cloned into the pET-14b system, were expressed in *E. coli* cells and purified by affinity chromatography (Ni^{2+}-NTA) (Fig. 1B). Recombinant toxins were identified by both phospholipase D specific [25] and whole venom antibodies [49] and these mutants contained sequence/epitope identity and antigenic cross-reactivity with LiRecDT1 and the native venom phospholipase D (Fig. 1C). In addition, to verify structural similarity and the precise folding of the mutants, circular dichroism (CD) measurements were recorded. CD spectra revealed that all mutants were in the soluble form and adopted a native-like conformation, containing alpha-helices and beta-sheets (Fig. 2A). Based on the thermograms obtained by DSC, we observed that the stability of most mutations remained unaltered, except for the double substitution of the C53 and C201 residues that eliminates a disulfide bridge in the catalytic interface, thereby connecting the catalytic loop to the flexible loop (Fig. 2B).

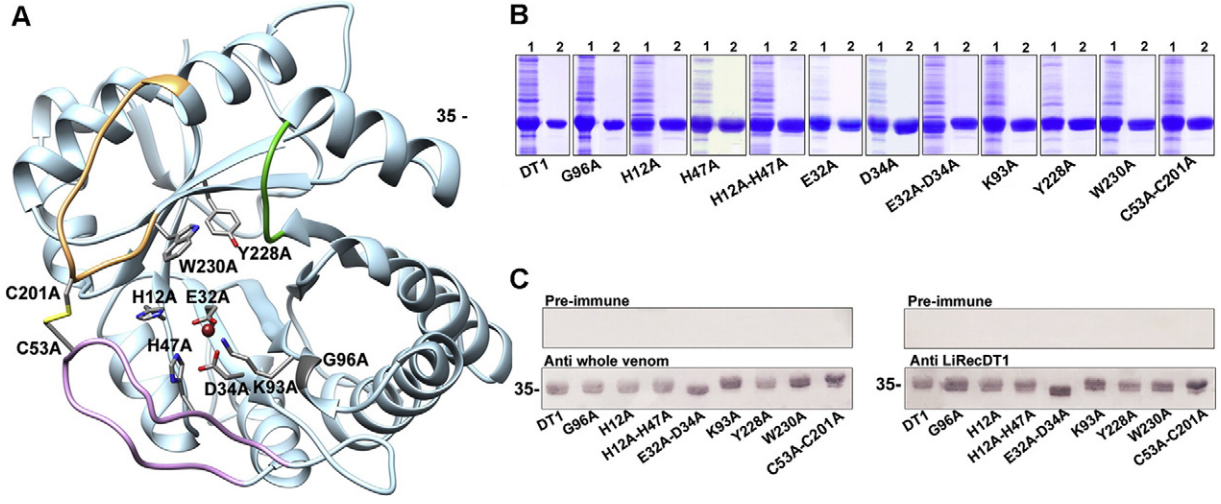
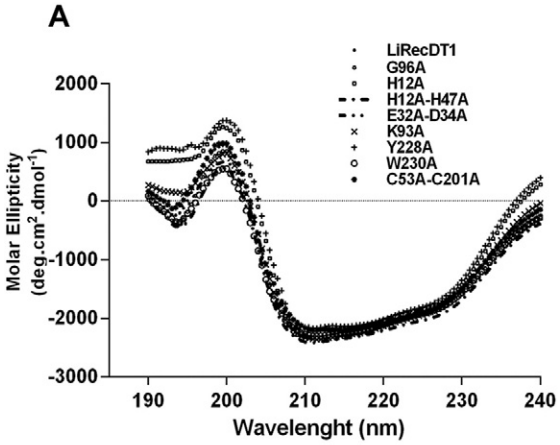
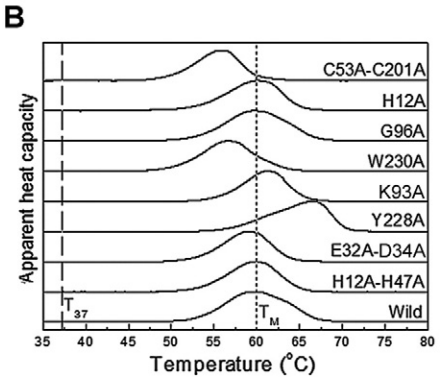


Fig. 1. Molecular cloning, expression, purification, and immunological cross-reactivity of Brown spider venom phospholipase D (LiRecDT1) and its recombinant site-direct mutagenesis derivatives. (A) Cartoon representation of the LiRecDT1 structure (blue) highlighting the mutated residues H12, H47, E32, D34, C53, C201, K93, Y228, W230, and G96. These residues are shown as sticks with carbon atoms in grey and the magnesium ion represented by a red sphere. In pink is represented the catalytic loop, in orange the flexible loop and in green the variable loop. (B) SDS-PAGE analysis of protein expression levels and purification of LiRecDT1 (DT1) and its mutants (12.5% gel under reducing conditions. Proteins were stained by Coomassie blue dye. Lane 1 shows *E. coli* BL21(DE3)pLysS cells collected by centrifugation after induction for 3.5 h with 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG); lane 2 depicts purified toxins by Ni-NTA agarose bead affinity chromatography; supernatants of cell lysates were obtained by freeze-thawing and mechanical lysis in extraction buffer. (C) Purified toxins (2.5 μg) were separated SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes that were exposed to antibodies against whole venom of *L. intermedia* (1: 10,000) or antibodies against LiRecDT1 (1: 10,000). Detection of immunoreactions used secondary alkaline phosphatase-coupled anti-IgG (1:8000) and the BCIP/NBT substrate reaction. The nitrocellulose membrane strips indicate no reactions in the presence of pre-immune serum (control for antibody specificity) (1:10,000).



	CD DECONVOLUTION (%)		
PROTEIN	α helix	β strand	Others
LiRecDT1	24.4	24.1	51.5
G96A	21.6	25.1	53.3
H12A	23.6	24.1	52.3
H12A-H47A	23.4	24.2	52.4
E32A-D34A	23.1	23.9	53
K93A	23.1	24.0	52.9
Y228A	23.6	24.1	52.3
W230A	23.1	23.9	53
C53A-C201A	23.4	24.1	52.5



PROTEIN	MELTING TEMPERATURE (°C)
C53A-C201A	56 ± 1.0
H12A	60 ± 1.0
G96A	60 ± 1.0
W230A	57 ± 1.0
K93A	61 ± 1.0
Y228A	67 ± 1.0
E32A-D34A	59 ± 1.0
H12A-H47A	60 ± 1.0
WILD	60 ± 1.0

Fig. 2. Circular dichroism (CD) and differential scanning calorimetry (DSC) analyses of LiRecDT1 and its mutants. (A) Circular dichroism (CD) spectra of the purified LiRecDT1 (wild-type) and mutated toxins. Spectra were obtained using proteins in Phosphate buffer, pH 7.4, at 20 °C and deconvoluted. (B) Thermogram obtained in the range of 20–80 °C at a scan rate of 1 °C/min in Phosphate buffer, pH 7.4, at 20 °C. Measurements were recorded in triplicate (SD ± 1).

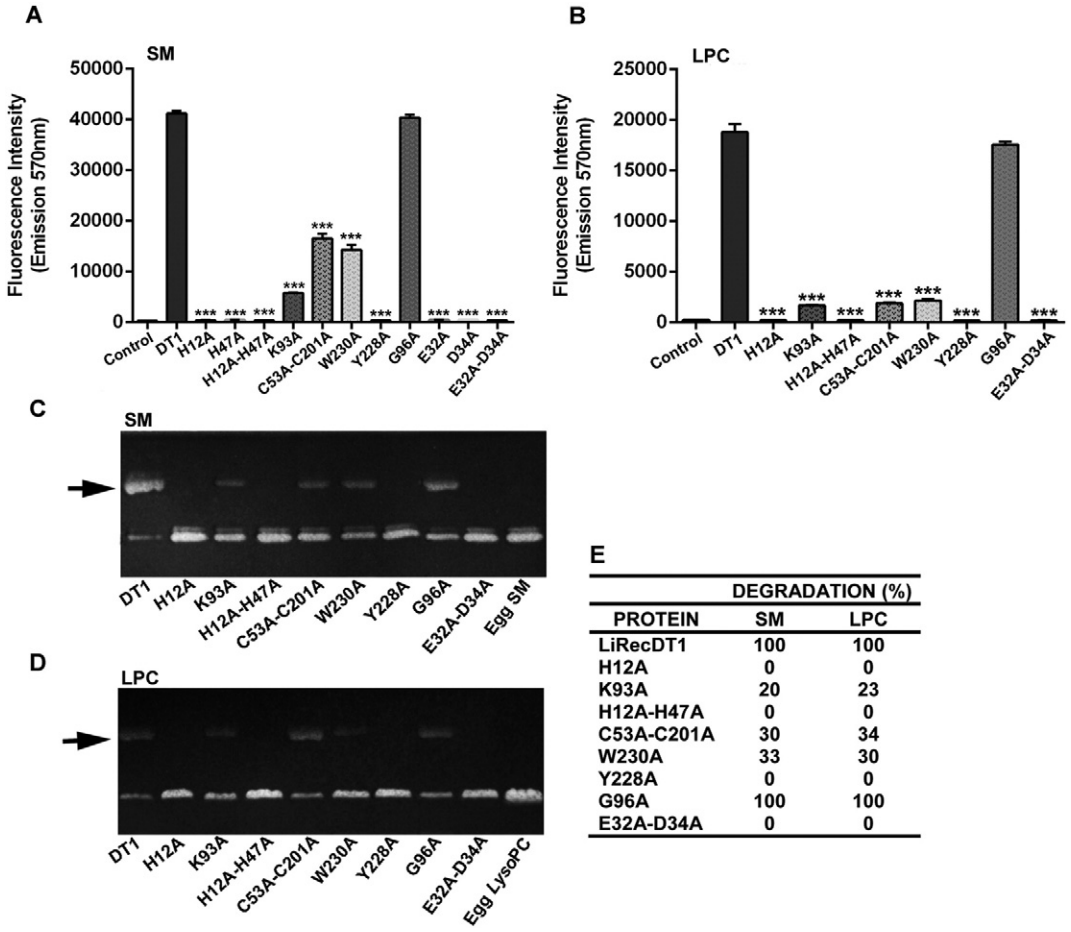
3.2. Phospholipase activity of LiRecDT1 toxin and mutants

Phospholipases D (PLDs), and specifically LiRecDT1, reportedly exhibits sphingomyelinase D and lysophospholipase D hydrolytic activity [17]. Purified LiRecDT1 and H12A, H47A, H12A-H47A, E32A, D34A, E32A-D34A, C53A-C201A, K93A, Y228A, W230A, and G96A mutants were tested for their activities on sphingomyelin and lysophosphatidylcholine. A fluorometric method based on choline release from phospholipid substrates was employed for enzymatic assays, using sphingomyelin (Fig. 3A) or lysophosphatidylcholine (Fig. 3B). Purified LiRecDT1 was used as a positive control and the mutant G96A was used as a control for the influence of site-direct mutagenesis and experimental manipulation on the recombinant toxins. Phospholipase activity was almost abolished in H12A, H47A, H12A-H47A, E32A, D34A and E32A-D34A on both phospholipid substrates. Phospholipase D activity was also significantly reduced to residual values by the mutant Y228A. The mutants C53A-C201A, W230A, and K93A displayed reduced enzymatic activity than LiRecDT1 did; however, phospholipid hydrolysis was not completely eliminated.

In addition, to support the fluorometric assays, the hydrolysis of sphingomyelin (SM) (Fig. 3C) and lysophosphatidylcholine (LPC) (Fig. 3D) by the respective toxins was also determined using HPTLC. After incubation with LiRecDT1 (positive control), the intensity of primulin-positive bands corresponding to lipid substrates was reduced and the amount of metabolites generated after degradation increased. The enzymatic activity of the mutants H12A, H12A-H47A, and E32A-D34A were abolished. Similarly, the catalytic activity of Y228A was suppressed. Phospholipids were degraded by C53A-C201A, W230A, and K93A, suggesting partial retention of their catalytic activity. Degradation of SM was 20% for K93A, 33% for W230A, and 30% for C53A-C201A when compared to LiRecDT1 and G96A (100%). H12A, H12A-H47A, Y228A, and E32A-D34A mutants showed no hydrolytic/catalytic activity against LPC (Fig. 3E). In comparison to LiRecDT1 and/or G96A (100%), other mutants degraded LPC to a lesser extent as follows: K93A, 23%; C53A-C201A, 34%; and W230A, 30%.

3.3. Hemolytic effects of LiRecDT1 toxin and mutants

Previous reports have described the direct hemolytic activity and morphological alterations of human erythrocytes caused by brown spider venom phospholipases D [33–35]. To evaluate the alterations in the biological effects of the enzyme, all eight mutants were tested for their hemolytic activity upon human erythrocytes while incubated in serum-free medium. As previously described, wild-type LiRecDT1 and the G96A mutant were used as controls. As illustrated in Fig. 4(A–B), different levels of hemolysis and morphological changes could be observed following treatment of the erythrocytes with the respective mutants. The hemolytic and morphological activities of H12A, H12A-H47A, and E32A-D34A mutants were significantly reduced. Hemolytic effect of the Y228A mutant was strongly inhibited and no morphological alterations were observed. On the other hand, treatment of human erythrocytes with purified K93A, C53A-C201A, or W230A induced hemolysis and morphological alterations, such as poikilocytosis and anisocytosis. Similar effects were observed on treatment with LiRecDT1 (wild-type) and G96A; however, the hemolytic effects of K93A, C53A-C201A, and W230A were to a comparatively lesser extent (50%, 38%, and 50%, respectively). Fig. 4B presents representative images of morphological alterations observed in all treated samples with controls and mutants. The present results strongly support the theory that these mutants mildly alter the biochemical activity of phospholipase D or the hemolytic effects of LiRecDT1.



E

	DEGRADATION (%)	
PROTEIN	SM	LPC
LiRecDT1	100	100
H12A	0	0
K93A	20	23
H12A-H47A	0	0
C53A-C201A	30	34
W230A	33	30
Y228A	0	0
G96A	100	100
E32A-D34A	0	0

Fig. 3. Phospholipase activity of LiRecDT1 and its mutants. (A) Sphingomyelinase D activity on 10 μg of LiRecDT1 (DT1), G96A, H12A, H47A, H12A-H47A, E32A, D34A, E32A-D34A, K93A, Y228A, W230A, and C53A-C201A variants. (B) Lysophospholipase D activity on 10 μg of LiRecDT1 (DT1), G96 A, H12A, H12A-H47A, E32A-D34A, K93A, Y228A, W230A, and C53A-C201A variants were evaluated with the Amplex Red Assay Kit at 37 °C for 1 h. The product of the reaction was fluorimetrically measured at wavelengths of 540 nm (excitation) and 570 nm (emission) after substrates solubilization in reaction buffer containing Triton X-100. Negative control was obtained by incubation of Amplex Red reagent mixture in the absence of toxins. The average ± standard errors are shown, with significance levels $^{***}P \leq 0.001$ comparing activities of LiRecDT1 and its mutants. Alternatively, high performance thin layer chromatographic (HPTLC) analysis of hydrolysis of purified sphingomyelin (egg SM) (C) and purified lysophosphatidylcholine (egg LPC) (D) produced by these toxins were performed. Purified phospholipids were incubated with 50 μg of either LiRecDT1 (DT1) or its mutant homologs for 2 h (non-treated samples were used as the negative control). Recovered lipids post-butanol extractions were visualized by primuline reagent in HPTLC plates. Arrows show degradation products after toxin treatments. (E) Band intensities were estimated densitometrically and were plotted as a percentage of the extent of degradation relative to LiRecDT1 samples (considered 100%).

3.4. Mouse vascular permeability and rabbit dermonecrosis of LiRecDT1 toxin and mutants

Previous studies have reported that native and recombinant wild-type brown spider venom phospholipases D (LiRecDTs) are able to increase vascular permeability and trigger dermonecrotic lesions *in vivo* [24,26–28,37]. For this reason, we evaluated the effects of mutated toxins (10 μg) on vascular permeability (Miles assay) in mice. As depicted in Fig. 5A, LiRecDT1 and the G96A mutant caused diffuse leakage of dye from the blood vessels, whereas the mutants H12A, H12A-H47A, E32A-D34A, and Y228A induced no changes in vascular permeability. The mutants C53A-C201A, K93A, and W230A stimulated a slight increase in vessel permeability.

Necrotic skin lesions induced by the LiRecDT1 toxin and respective mutants were evaluated in rabbits (Fig. 5B). The macroscopic lesions at the site of toxin (10 μg) inoculation were evaluated at 0, 3, 6, and 24 h. Animals that received recombinant LiRecDT1 and the G96A mutant responded similarly in a time-dependent manner. Lesions showed an ischemic halo and local hemorrhage around the site of inoculation. In addition, lesions were characterized by erythema, edema, and gravitational spreading of necrosis. Intradermal injections of H12A, H12A-H47A, E32A-D34A, and Y228A mutants induced no dermonecrotic features. Signs of intermediate skin necrosis (edema, erythema, hemorrhage, ischemia, and necrosis) were observed following inoculation with C53A-C201A, K93A, and W230A mutants, corroborating the aforementioned results of the present study.

4. Discussion

Previous studies have reported that enzymatic hydrolysis of membrane phospholipids is necessary for the biological activities triggered by these PLD toxins. Phospholipase D homologs devoid of catalytic activity are unable to induce dermonecrotic lesions in rabbits [17,24–28,37]. Moreover, toxins with low or residual enzymatic activity on synthetic sphingomyelin exhibit reduced or residual hemolytic activity on human erythrocytes and weaker or residual effects on platelet aggregation and vascular permeability [17,24–28,37].

The crystal structures of purified recombinant phospholipases D from *L. laeta* and *L. intermedia*, were recently determined and divided into two classes [40–42,50]. The PLD class I enzymes, represented by the *L. laeta* toxin, SMase I, possess a single disulfide bridge and an extended hydrophobic loop [40,41]. Phospholipases D from *L. intermedia* belong to class II, which contains an additional intra-chain disulfide bridge that links the shortened flexible loop to the catalytic loop, hereby altering the accessibility and conformation of the catalytic interface [42,50]. Based on the crystal structures and multiple alignment analysis

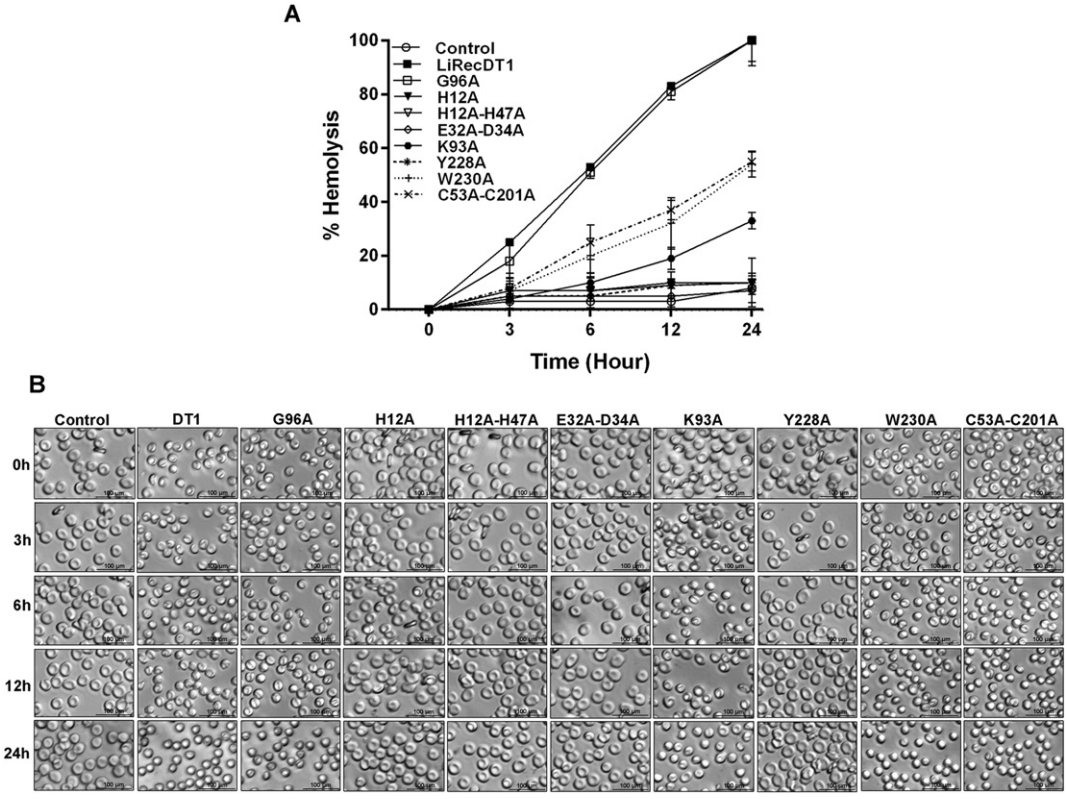


Fig. 4. Effect of LiRecDT1 and its mutants upon erythrocytes: hemolysis and morphology. (A) Human erythrocytes were exposed to 10 μg of various purified toxins (LiRecDT1, G96A, H12A, H12A-H47A, E32A-D34A, K93A, Y228A, W230A, and C53A-C201A) for 0.5, 1, 3, 6, 12, and 24 h at 37 °C in Sucrose buffer. Evaluation of hemolysis was measured spectrophotometrically at 550 nm. As a negative control, erythrocytes were incubated in the absence of toxins, under similar laboratory conditions; positive control consisted of erythrocytes in the presence of Triton X-100. The results show an average of five experiments ± SEM. $^{***}P < 0.001$. (B) Human red blood cells suspended in Ringer's Solution were incubated with 2 μg of each toxin or without (control) for different time (3, 6 and 12 h). Morphological alterations of cells were observed with an inverted microscope in 400× magnification.

of the cDNA deduced amino acid sequences for Brown spider phospholipases D homologs, the catalytic site of these toxins was determined and contains the residues His12, Glu32, Asp34, His47, Asp52, Asp91, Trp230, Asp233, and Asn252, which are highly conserved throughout the genus [30,40–42,50]. Moreover, it has been proposed that the catalytic mechanism of Brown spider venom phospholipases D involves an acid-base reaction and an Mg^{2+} metal-binding site. In the proposed catalytic mechanism both histidines (H12 and H47) can act as nucleophile. The interaction between the enzyme and the phospholipid is stabilized by the interaction of the Mg^{2+} ion with the phosphate group of the substrate. Mg^{2+} ions are coordinated by the residues Glu32, Asp34, and Asp91 and water molecules. According to this model, the catalytic process involves the key residues His12 and His47, which are supported by a network of hydrogen bonds between the residues Asp34, Asp52, Trp230, Asp233, and Asn252 [42,50].

PLD mutants studied herein were constructed based on the aforementioned structural and catalytic characteristics of Brown spider venom phospholipases D [30,40–42,50] (Fig. 1A). The catalytic residues (H12 and H47) and the metal-binding residues (E32 and D34) were mutated to alanine to confirm their respective roles in the toxin's activities. In addition, the importance of the additional disulfide bridge C53-C201 was analyzed, as these cysteine residues connect the flexible loop to the catalytic loop in Class II PLD toxins. Residues in the substrate binding site were also mutated to yield K93A, Y228A, and W230A toxins. The positive controls were LiRecDT1 and the mutant G96A. LiRecDT1 G96A was included in all assays to discard possible interferences due to experimental procedures of site-directed mutagenesis and recombinant toxin activity. We investigated the relationship among these mutants, their enzymatic activity, and biological effects. All mutants were successfully produced and purified in a native-like conformation as evidenced by CD, DSC, and immunological studies (Figs. 1 and 2). CD spectra confirm that all mutant toxins showed non-denatured and non-aggregate forms, similar to the LiRecDT1 spectrum. DSC analyses showed the stability of the mutants under experimental conditions (37 °C and below, temperatures in which biochemical and biological experiments were performed). Changes in thermostability were observed only in much higher temperatures. C53A-C201A mutant melting temperature was altered, probably because of the loss of an intra-chain disulfide bridge, which led to increased molecular flexibility. The Y228A mutant exhibited the opposite effect, probably because of a protein compaction effect [51,52]. Additional DSC experiments showed that the thermal transitions of the wild-type *L. intermedia* PLD and its mutants are irreversible.

To verify the effectiveness of the proposed site-directed mutations on the biochemical properties of phospholipases D, the activity of the mutants against synthetic sphingomyelin and lysophosphatidylcholine was evaluated. Two methods (fluorimetric quantification of choline and HPTLC detection of substrates) were performed for a more comprehensive analysis. As depicted in Fig. 3, the absence of phospholipase D activity in the H12A, H12A-H47A, and E32A-D34A mutants suggests the critical role of these residues in modulating phospholipid hydrolysis and supports previous reports [30,40–42,50]. Phospholipase D members of class I and II present a disulfide bond between C51 and C57 residues; however, only class II PLDs have a second disulfide bridge at the C53A-C201A residues [30,40–42,50]. Analysis of the C53A-C201A variant, which abolishes the characteristic class II disulfide bond between those residues, revealed that this mutation decreases catalytic activity of the toxin on phospholipid substrates by 30% when compared to LiRecDT1 (Fig. 3). On the other hand, class II PLDs hydrolyze sphingolipids more effectively. However, compared to LiRecDT1, the C53A-C201A mutant showed no change in its preference for SM. Based on the reduced hydrolytic activity and SM preference displayed

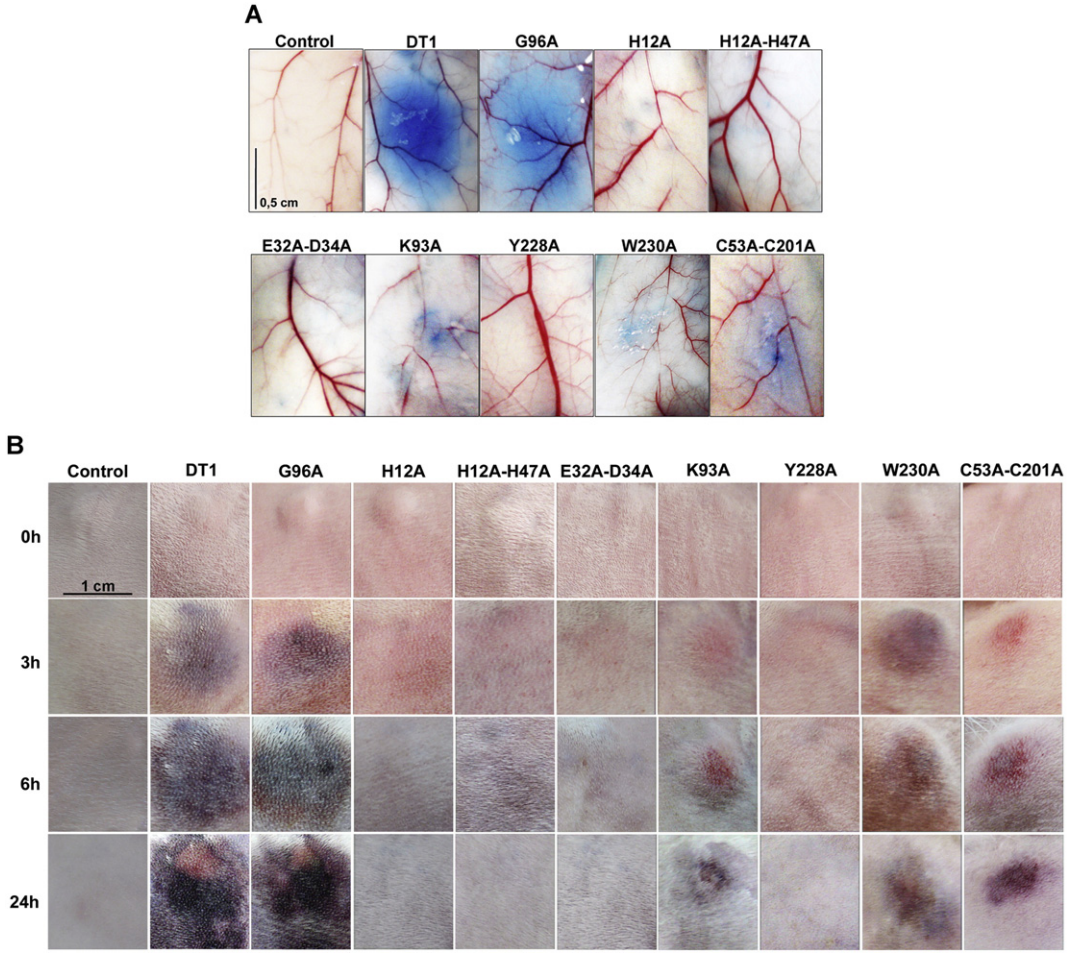


Fig. 5. Effect of LiRecDT1 and its mutants on *in vivo* vascular permeability and dermonecrotic responses. (A) Vascular permeability of cutaneous blood vessels (Miles assay). Mice were administered intradermal injections of LiRecDT1 (10 μg) or each mutated toxin; 5 min after Evans Blue Dye diluted in PBS was administered intravenously (30 mg/kg). After 1 h, the dorsal skin was removed for visualization of dye extravasation. PBS was used as a control for baseline permeability levels. The photographs show the site of toxin injection. Positive controls (LiRecDT1 and G96 A) show increased dye leakage. (B) Dermonecrosis in rabbits. Macroscopic analysis of skin lesions following intradermal injection of 10 μg of purified recombinant homologs of phospholipases D (LiRecDT1, G96A, H12A, H12A-H47A, E32A-D34A, K93A, Y228A, W230A, and C53A-C201A). Animal skin samples were observed and photographed 0, 3, 6, and 24 h after injection of toxins.

by the mutant, we can infer that structural changes in the molecule could disrupt its activity and removal of the disulfide bond only is insufficient to mimic the catalytic interface and the substrate preference of a class I member. It is possible that other amino acid substitutions and/or insertions in the loops forming the catalytic interface also contribute to substrate binding and catalysis of class II enzymes. The 100% activity for LiRecDT1 hydrolysis of substrate in the assays is for conditions where most of the substrate was hydrolyzed. Thus, mutants with reduced activities are likely to be comparatively more inactive than presented in this Fig. 3.

In relation to the substrate-binding site, the mutants K93A and W230A were effective in reducing hydrolytic activity by 20–30%, as compared to LiRecDT1. These findings could be attributed to the loss of hydrophobic and cation-π interaction of W230 with choline in the substrate and loss of the hydrogen bond of K93 with the tail of the substrate. The substitution of a unique tyrosine residue in the substrate-binding site (Y228A) completely abolished enzymatic activity, indicating the participation of this residue in the catalytic cycle. According to structural analysis followed by molecular dynamics simulations, the Y228 residue may interact with the polar head of the substrate *via* hydrogen bond and cation-π interaction.

The biological activities of LiRecDT1 and mutants were tested *in vitro* (hemolysis) and *in vivo* (vascular permeability and dermonecrosis). These biological activities are well established and have been reported for phospholipase D homologs and *Loxosceles* venoms [17,24–28,37]. H12A, H12A-H47A, and E32A-D34A mutant toxins did not trigger any cytotoxic activity against red blood cells (hemolytic assay, Fig. 4), thus corroborating their predicted involvement in catalysis and metal ion coordination seen in phospholipid hydrolysis experiments [40–42,50]. These results are in agreement with previous reports of the importance of catalysis in hemolysis [33,34]. On the other hand, the C53A-C201A mutant induced hemolysis and morphological changes in red blood cells, albeit in a moderate manner (50%) compared to LiRecDT1 and G96A (100%) (Fig. 4). This finding is consistent with those of phospholipid hydrolysis and confirms that removal of the C53-C201 disulfide bridge from a PLD class II member does not block its cytotoxic effects. Likewise, the mutations K93A and W230A could reduce the hemolytic activity of LiRecDT1 more efficiently than the loss of a disulfide bridge. The Y228A mutant toxin did not cause erythrocyte hemolysis or morphological changes, supporting reports of the involvement of this residue in phospholipid hydrolysis. Erythrocytes morphology following treatment with toxins revealed a time-dependent effect. Poikilocytosis, anisocytosis, and the appearance of spherocytes were observed in LiRecDT1 and G96A treatments. When erythrocytes were exposed to K93A, W230A, and C53A-C201A mutants, mild changes were observed mostly with long-term exposures. H12A, H12A-H47A, E32A-D34A, and Y228A mutants triggered no

morphological changes in erythrocytes, which had a similar appearance to the normocytic negative control cells (biconcave disks flattened and depressed in the center).

The *in vivo* effects of these mutants were evaluated using vascular permeability in mice and dermonecrosis in rabbits. As depicted in Fig. 5, the mutations related to acid-base pairs and metal binding (H12A, H12A-H47A, and E32A-D34A) induced no changes to vascular permeability in mice nor signs of skin injury in rabbits. These results reinforce the importance of enzymatic activity in eliciting toxicity. *In vivo* data proved the importance of H12, H47, E32, and D34 residues in PLD activity. When compared to the effects of LiRecDT1, the mutation in the residues C53-C201, K93 and W230, resulted in reduced vascular permeability and dermonecrosis activity. Complete suppression of these *in vivo* effects was observed with the Y228A mutation. In some instances, the Y228A mutation completely blocked the biochemical, cellular, and *in vivo* activities induced by LiRecDT1. To our knowledge, this is the first time that the importance of the Y228 residue has been experimentally described for PLD activities. Several wild-type phospholipases D from Brown spider *L. intermedia* (LiRecDT1 [25], LiRecDT6 [24], and LiRecDT7 [28]); *L. laeta* (SMase I [40,41]); and *L. gaucho* (LgRec1 [29]) have two sequential tyrosine residues at positions 228 and 229, present high phospholipid hydrolytic activity, and induce hemolysis and dermonecrosis. We should highlight that other wild-type recombinant Brown spider phospholipases D (LiRecDT3 [26] and LiRecDT5 [27]) that carry a mutation at position 229 with the substitution Y229T exhibit just residual or low phospholipid hydrolytic activity and do not induce hemolysis or dermonecrosis. The substitution Y229T is conservative, as an uncharged polar amino acid usually occupies that position. This is probably the reason for the maintenance of residual activity. We can infer that these two tyrosine residues (228 and 229) of wild-type toxins are necessary hydrophobic motifs for substrate binding, and thereby stabilize the native-like conformation of the active-site pocket. Our results reinforce the importance of the role of these tyrosine residues, particularly the indispensable participation of residue 228 in interactions with phospholipid substrates.

5. Conclusion

In conclusion, our mutational and functional approach to determine the importance of specific residues in the biochemical and biological activities of phospholipases D from Brown spiders expands the possibilities to design and develop drugs that directly interact with the active sites and thereby inhibit the toxins. These inhibitors could be tested *in vitro* and/or *in vivo* and could be optimized to develop a specific treatment for Loxoscelism.

Transparency document

The Transparency Document associated with this article can be found, in online version.

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5 DISCUSSÃO

Os papéis funcionais e estruturais de resíduos de aminoácidos específicos de uma proteína de interesse podem ser estudados por comparação entre uma proteína mutante e sua forma selvagem/original, por meio da realização de alteração/troca dos resíduos de aminoácidos na proteína original (LING e ROBINSON, 1997). A mutação sítio-dirigida é uma importante técnica usada para estudar a relação entre a sequência do gene e a sua função, mostrando-se uma das principais técnicas de elucidação da relação estrutura-função de uma proteína (AN *et al.,* 2005). As mutações sítio-dirigidas realizadas neste presente estudo mostraram ser bastante eficientes. Ling e Robinson (1997), a partir de um estudo geral sobre as várias estratégias de mutagênese, constataram que a substituição dos resíduos de aminoácidos geralmente ocorre com uma eficiência de 50-100%, corroborando com os nossos resultados.

A ausência de resíduos de aminoácidos portadores de cargas em regiões importantes da proteína podem comprometer suas funções catalíticas e biológicas. No presente estudo, os resíduos de interesse foram substituídos pelo resíduo do aminoácido alanina, o qual possui característica apolar com uma estrutura bastante simples, permitindo avaliar a importância dos resíduos originais para a atividade da fosfolipase D de aranha-marrom. Vale ressaltar que em geral, a substituição por alanina não interfere no dobramento correto das proteínas preservando a estrutural tridimensional (SAMBROOK e RUSSEL, 2001).

A escolha do modelo de expressão heteróloga, bem como da cepa de expressão é muito importante para a produção de proteínas recombinantes solúveis e ativas, e algumas questões devem ser levadas em consideração. Cepas de expressão devem ser deficientes em proteases e manter a expressão do plasmídeo estável (SORENSEN e MORTENSEN, 2005). As cepas bacterianas são geralmente escolhidas para a expressão de proteínas recombinantes devido a sua facilidade de manipulação, por possuírem crescimento rápido em meios de cultura simples, por geralmente expressarem grandes quantidades de proteína recombinante e por ser um método

relativamente barato. Devido ao fato das várias isoformas de fosfolipases D de *L. intermedia* terem sido expressas de forma bem-sucedida na cepa *E. coli* BL21(DE3)pLysS (LiRecDT1 - CHAIM *et al.,* 2006; LiRecDT2 e LiRecDT3 - da SILVEIRA *et al.,* 2006a; LiRecDT4 e LiRecDT5 - da SILVEIRA *et al.,* 2006b; LiRecDT6 - APPEL *et al.,* 2008; LiRecDT1 H12A - KUSMA *et al.,* 2008), optamos por utilizar essa mesma cepa para obtenção das isoformas com mutação sítio-dirigida. Esta cepa possui um sistema bastante simples e também possui o plasmídeo pLysS, o qual reprime a expressão basal antes da indução da expressão por IPTG. Para todas as isoformas citadas acima, esta cepa demonstrou ser bastante vantajosa por expressar grandes quantidades das proteínas em condições solúveis e ativas bioquimica e biologicamente.

Normalmente, a purificação de proteínas é facilitada por adição de *"tags"* de fusão, que permitem a rápida e eficiente purificação por cromatografia de afinidade. Como já mencionado, usamos a isoforma LiRecDT1 e LiRecDT1 H12A (CHAIM, *et al*., 2006; KUSMA *et al.,* 2008) como molde para a produção das isoformas com mutação sítio-dirigidas, as quais foram subclonadas em vetor de expressão pET-14b, a fim de inserir na porção N-terminal a etiqueta *6xHis-tag,* possibilitando assim a purificação por cromatografia de afinidade com resina Ni^{+2} -NTA agarose e .a imunodetecção da proteína expressa por *Western blot* utilizando anticorpos anti-*His tag*.

A presença de contaminantes em preparados de proteínas recombinantes não é incomum. Usualmente é necessário estabelecer quais contaminantes não podem estar presentes, pois irão afetar os experimentos, e qual deve ser proporção da proteína em relação aos contaminantes. O processo de purificação utilizando IMAC (*Immobilized Metal-Ion Affinity Chromatography*), como a cromatografia em resina Ni^{2+}-NTA agarose, é uma metodologia simples e rápida, que com frequência, resulta em produtos com pureza que varia de 70 a 90%. A pureza da proteína recombinante produzida depende de diversos fatores como o tipo de célula em que a proteína foi expressa, condições cromatográficas como pH, concentração de sal e tensoativos, presença de proteínas que interajam com a matriz ou com o metal, ou mesmo uma baixa proporção da proteína recombinante na solução do lisado bacteriano (CROWE *et. al.,* 1995; GUPTA *et. al.,* 2003).

A produção de grandes quantidades de proteína, da ordem de miligramas, para estudos estruturais e funcionais, requer uma purificação eficiente (DONNELLY *et al.,* 2006). Algumas estratégias foram usadas para tentar aumentar a pureza das proteínas recombinantes mutadas neste trabalho. Primeiramente, foi padronizada a relação entre a quantidade de resina e de proteína recombinante. Um excesso de resina resulta em sítios livres para uma interação inespecífica com outras proteínas bacterianas. A melhor relação observada, resultando em uma proteína de maior pureza e menor perda da proteína no *void*, foi de 1 mL de resina Ni^{2+}-NTA para cada litro de cultura, o qual foi ressuspenso em 20 mL de tampão de ligação à resina. O pH dos tampões da cromatografia pode ser um ponto chave na remoção de contaminantes, uma vez que pHs alcalinos (acima de 8,0) facilitam a interação de proteínas com o metal. Em pHs mais baixos, apenas proteínas que interagem fortemente com o metal permanecem ligadas. Sabendo que os pI das proteínas do presente estudo variam entre 7.72 – 8.5, todos os tampões utilizados no procedimento de purificação para cada isoforma sempre foram ajustados uma unidade acima do pI da proteína, com o objetivo de obter alta pureza das amostras e também para evitar a precipitação das proteínas recombinantes (SAMBROOK e RUSSEL, 2001; CUTLER, 2004). Por exemplo, para a isoforma LiRecDT1 H12A-H47A, que possui pI 7.72, os pHs dos tampões foram ajustados para 8.72. Ao final do processo de purificação, obtivemos um rendimento bastante razoável para cada isoforma com mutação sítio-dirigida, quantificado entre 15-30 mg por litro de cultura. Para a LiRecDT1, a qual utilizamos como a proteína molde para as mutações sítio-dirigidas, obteve-se após a purificação até 24 mg/L de cultura (CHAIM *et al.*, 2006).

Após expressar e purificar todas as isoformas recombinantes com mutações sítio-dirigidas, a primeira etapa foi determinar a estrutura secundária e verificar se as mutações alteraram o dobramento e a solubilidade dessas isoformas. Sabemos que uma mutação em um único nucleotídeo pode acarretar em mudanças na estrutura da proteína e, consequentemente, interferir em sua função proteica. A partir da estrutura primária específica de uma proteína, ocorrem interações entre os monômeros vizinhos e com as moléculas de solvente circundantes. Estas interações entre monômeros acabam por dar origem a padrões repetitivos de organização espacial,

denominados de estrutura secundária. A importância do conhecimento da estrutura secundária de biomoléculas reside, principalmente, no fato de que estes elementos se organizam de forma tridimensional no espeaço, dando origem ao que chamamos de estrutura terciária. Em outras palavras, a estrutura terciária de uma dada biomolécula corresponde à montagem dos seus elementos de estrutura secundária. Por outro lado, é a estrutura terciária (ou a quaternária em alguns casos) que irá exercer a função biológica da biomolécula em questão. Os diversos elementos de estrutura secundária de uma dada molécula se organizam em uma estrutura terciária através de um fenômeno denominado enovelamento (também chamado em português de dobramento, do termo em inglês *folding*). Neste processo, uma combinação de forças converge para que a biomolécula adote uma conformação mais estável no meio em que ela está imersa (VERLI, 2014).

A técnica espectroscópica denominada de dicroísmo circular (CD) é um importante método na biologia estrutural que permite a determinação da estrutura secundária de proteínas, detecção do dobramento *folding*/*unfolding*, estabilidade proteica, formação de complexos macromoleculares e caracterização de interações entre proteína-ligante (GREENFIELD, 2006; RANJBAR e GILL, 2009, GADER *et al.,* 2011).

O perfil de espectros gerados pelo CD sugere que todas as toxinas com mutações sítio-dirigidas estão na forma solúvel e na sua conformação secundária correta. Podemos observar uma predominância de α-hélices e estrutura em β-folhas em todas as toxinas, tendo em vista que as estruturas secundárias mais frequentemente observadas incluem três grupos de elementos principais: as alças, as α-hélices e as folhas-β (VERLI, 2014). Até o momento não foi possível obter os dados de deconvolução espectral de todas as isoformas (dados em processamento com a colaboração com pesquisadores da Escola Paulista de Medicina-UNIFESP). No entanto, dados de deconvolução mostrados em trabalhos anteriores com a isoforma recombinante LiRecDT1 mostraram que esta proteína possui 22% α-hélice, 22% β-folha, 13% β-turn e 40% outros (RIBEIRO*, et al.,* 2007). Andrade e colaboradores (2005) também obtiveram dados de deconvolução para as isoforma de fosfolipases-D de *L. intermedia* (rP1, rP2, rP3) e *L. laeta* (SMase I), nos quais também foram observadas a predominância de α-hélices e β-folhas.

Além disso, não foram observadas alterações importantes na estrutura, embora espectros acima de 200 nm não foram acessíveis. Provavelmente isso se deve à composição do tampão em que as toxinas estão solúveis (tampão fosfato-PBS) e, assim, análises com outros tampões poderão ser testadas.

A técnica de calorimetria diferencial por varredura (DSC) é uma ferramenta utilizada para estudar a termoestabilidade de uma amostra submetida à diminuição ou aumento de temperatura. Em estudos bioquímicos de biomoléculas, é comum utilizar essa técnica para avaliar a transição conformacional e a estabilidade térmica de proteínas (GILL *et al,* 2010; SPINK, 2015). Até o momento não encontramos na literatura estudos envolvendo a termoestabilidade das fosfolipases D de veneno de aranhas-marrons. Os nossos resultados por DSC apontam que todas as toxinas estudadas, incluindo as isoformas controles e as variantes mutadas, possuem alta estabilidade térmica, pois podemos observar que a perda de conformação ocorre a partir de 60ºC. Podemos observar no termograma que as mutações realizadas na isoforma original (LiRecDT1) não alteraram significantemente a termoestabilidade das variantes mutantes na temperatura de 37ºC (temperatura utilizada em nossos experimentos). Os resultados obtidos pelo DSC complementam os resultados obtidos pelo CD, reforçando a ideia de que as mutações realizadas não alteraram a conformação tridimensional das variantes mutantes, e que todos os resultados obtidos pelos experimentos de cunho bioquímico e biológico são decorrentes da participação dos resíduos de aminoácidos que atuam na atividade catalítica dessa toxina.

Após verificar a estrutura secundária, o dobramento correto, a solubilidade das toxinas e a termoestabilidade das toxinas com mutações sítio-dirigidas, o próximo passo foi avaliar a atividade catalítica utilizando como substrato lipídico esfingomielina (SM) e lisofosfatidilcolina (lisoPC). Avaliamos a atividade catalítica por dois métodos distintos: Amplex Red Kit e Cromatografia em Camada Delgada de Alta Performance (HPTLC). De modo geral, quando comparamos todas as isofomas mutadas com a isoforma original LiRecDT1 e com a isoforma controle mutada LiRecDT1 G96A, podemos observar que as formas mutantes diminuíram significantemente a sua capacidade de hidrolisar SM e lisoPC. Outro resultado muito importante é observado na comparação da atividade da isoforma LiRecDT1 sobre SM e lisoPC. Observamos que a

atividade frente a lisoPC diminui aproximadamente pela metade do valor em relação à atividade esfingomielinásica. Já é de conhecimento que as fosfolipases D de *L. intermedia* possuem preferência hidrolítica por SM, mas já foi evidenciado que esta enzima também é capaz de hidrolisar um amplo espectro de fosfolipídeos (CHAVES-MOREIRA *at al.,* 2011; CHAIM *et al.,* 2011a; WILLE *et al.,* 2013; LAJOIE *et al.,* 2015b). Como mencionado anteriormente, foi demonstrado que a isoforma LiRecDT1 foi capaz de hidrolisar fosfatildilglicerídeos, em especial a lisofosfatidilcolina e também lipídeos obtidos da membrana plasmática de eritrócitos humanos e células em cultivo celular (CHAIM *et al.,* 2011a; CHAVES-MOREIRA *et al.,* 2011, WILLE *et al.,* 2013).

Primeiramente, podemos observar que as isoformas variantes H12A, H12A-H47A e E32A-D34A apresentaram uma redução bastante significativa na sua atividade fosfolipásica. Estudos anteriores mostraram que a isoforma LiRecDT1 H12A possui uma drástica diminuição na atividade fosfolipásica, corroborando com nossos resultados (KUSMA *et al.*, 2008; CHAVES-MOREIRA *et al.*, 2009; CHAVES-MOREIRA, *et al.*, 2011; CHAIM *et al.*, 2011b, WILLE *et al.*, 2013). Como já foi mencionado, encontram-se no *loop* catalítico das fosfolipases D de *Loxosceles* os resíduos His12 e His47 e o íon Mg^{+2} (cofator) que é hexacoordenado pelos resíduos Glu32, Asp34 e Asp91. Todos esses resíduos de aminoácidos são altamente conservados entre as espécies de *Loxosceles* (MURAKAMI *et al.*, 2005 e 2006; GUISEPPE *et al.*, 2011; DIAS-LOPES *et al.*, 2013). Lee e Lynch (2005) também demonstraram que uma fosfolipase D de *L. reclusa* com mutação sítio-dirigida nos seus resíduos de histidina (H37N e H73N) não possuía atividade fosfolipásica sobre diversos substratos lipídicos.

Além dos resíduos que estão envolvidos diretamente na hidrólise do substrato, também é proposto que outros resíduos presentes na fenda catalítica estão envolvidos no reconhecimento, estabilização e direcionamento do substrato lipídico. Entre as isoformas estudadas com essas propriedades, a qual mostrou menor atividade fosfolipásica foi a isoforma LiRecDT1 Y228A. As outras isoformas LiRecDT1 K93A e LiRecDT1 W230A possuem uma significante diminuição da atividade catalítica, mas não foram capazes de abolir completamente a sua atividade. A partir desses resultados, podemos propor

que, provavelmente, esses resíduos de aminoácidos possuem importância no reconhecimento, estabilização e direcionamento do substrato lipídico na fenda catalítica das fosfolipases-D de *L. intermedia*. Foi proposto em trabalhos anteriores que o resíduo Trp230, localizado muito próximo ao *loop* flexível, está altamente conservado entre as SMasesD sugerindo que este também poderia orientar o substrato na fenda catalítica permitindo o primeiro ataque nucleófilo da His47, ou também, poderia estar participando estabilização do estado intermediário da reação. Já o resíduo Lys93, localizado no bolsão catalítico da enzima, também é altamente conservado e pode estar desempenhando um papel crucial em equilibrar as cargas durante a catálise ou na orientação e/ou acoplamento do substrato (MURAKAMI *et al.*, 2005 e 2006, GUISEPPE *et al.*, 2011). Nestes mesmos estudos, também é proposto a participação do resíduo Tyr228 no reconhecimento e direcionamento do substrato, mas a maneira pela qual esse processo ocorre durante a hidrólise do substrato não foi elucidado (GUISEPPE *et al.*, 2011). Sabe-se que o resíduo de tirosina (Y) é um aminoácido hidrofóbico que raramente participa no processo de catálise nas enzimas, mas pode reconhecer e se ligar a substratos que também possuem caráter hidrofóbico, tais como os lipídeos (BETTS e RUSSELL, 2003). Além dos resíduos de aminoácidos discutidos acima, também é proposto que o íon Mg^{+2} está posicionado entre duas moléculas de água e que ele poderia estar participando na orientação do grupo fosfato do substrato a entrar na fenda catalítica, ou também, agindo como estabilizador do composto intermediário formado durante a hidrólise (MURAKAMI *et al.*, 2005 e 2006, GUISEPPE *et al.*, 2011).

Também avaliamos o perfil da atividade catalítica da isoforma LiRecDT1 C53A-C201A que teve uma diminuição significativa na sua atividade fosfolipásica comparado com a LiRecDT1. A isoforma LiRecDT1 está classificada como PLD da classe IIa (ativas), que possuem na sua estrutura duas pontes dissulfetos entre os resíduos Cys51-Cys57 e Cys53-Cys201. Esta ponte dissulfeto adicional (Cys53-Cys201) favorece o deslocamento do *loop* flexível em direção ao *loop* catalítico, ocluindo a fenda eletrostática e diminuindo a cavidade do sítio catalítico (ANDRADE *et al.* 2006; GUISEPPE *et al.*, 2011). Por isso, propomos que a mutação nestes dois resíduos de cisteínas (Cys53→Ala e Cys201→Ala) não possibilitou a formação da ponte dissulfeto

adicional durante a expressão heteróloga dessa proteína. Consequentemente, não ocorreu o deslocamento do *loop* flexível em direção ao *loop* catalítico, deixando a fenda eletrostática um pouco mais aberta, aumentando a cavidade do sítio catalítico. Esta provável alteração proporcionada pelo deslocamento entre os *loops* não foi evidenciada nos espectros de dicroísmo circular. Por fim, podemos propor que a presença da ponte dissulfeto adicional entre os resíduos Cys53 e Cys201 na isoforma LiRecDT1 é importante para a organização estrutural e para o potencial eletrostático da fenda catalítica, e estas propriedades são fundamentais para a atividade hidrolítica desta enzima.

Também avaliamos a atividade catalítica das isoformas com mutação sítio-dirigidas frente a lisoPC. Podemos observar que todas as isoformas com mutações sítio-dirigidas diminuíram significativamente a sua capacidade de hidrolisar lisoPC em relação a isoforma controle LiRecDT1. Com base nestes resultados podemos propor que as mutações sítio-dirigidas realizadas em resíduos de aminoácidos associados ao sítio ativo da enzima, coordenação do íon metal Mg^{+2}, reconhecimento do substrato lipídico e envolvidos na parte estrutural da proteína são muito importantes para a atividade enzimática desta toxina. Além disso, e com base na literatura, também podemos propor que as fosfolipases-D de classe IIa (ativas) possuem maior afinidade pela SM em relação a lisoPC, sendo que o percentual de hidrólise desta última corresponde a aproximadamente metade do que é observado para SM (CHAIM *et al.,* 2011b; CHAVES-MOREIRA *et al.,* 2011, WILLE *et al.,* 2013). Embora a maior afinidade catalítica demonstrada seja para SM, também foi evidenciada atividade fosfolipásica frente a lisoPC e também lipídeos obtidos da membrana plasmática de eritrócitos humanos e células em cultivo celular (CHAIM *et al.,* 2011b; CHAVES-MOREIRA *et al.,* 2011, WILLE *et al.,* 2013). Isoformas de fosfolipase D de outras espécies de *Loxosceles*, principalmente as fosfolipses D de classe I, possuem maior afinidade por lisoPC. Uma isoforma de fosfolipase D de *L. reclusa* foi capaz de hidrolisar lisofosfolipídios, lisofosfatidilinositol, lisofosfatidilglicerol, ácido lisofosfatídico e ácido fosfatídico cíclico (LEE e LYNCH, 2005).

Nesse mesmo estudo, uma isoforma com mutação sítio-dirigida nos resíduos H37N e H73N não apresentou atividade sobre substratos lipídicos, mostrando a importância desses resíduos na atividade catalítica. Uma SMase

D de *L. laeta* apresentou atividade sobre lisoPC, gerando ácido lisofosfatídico (LPA) (van MEETEREN *et al.,* 2004). Sabemos que as fosfolipases D de *Loxosceles* da classe I possuem apenas uma ponte dissulfeto na sua estrutura, a qual é estabelecida entre os resíduos Cys51 e Cys57 (ANDRADE *et al.* 2006; MURAKAMI *et al.,* 2005 e 2006; GUISEPPE *et al.*, 2011) e que essas enzimas possuem maior afinidade pelo substrato lipídico lisoPC do que pela esfingomielina (LEE e LYNCH, 2005; van MEERETEN *et al*., 2004). No entanto, há discussões propondo que essas diferenças observadas na topologia e no potencial eletrostático entre as fosfolipases D das classes I e II sugerem que estas enzimas possuem diferentes maneiras de orientar o substrato na fenda catalítica (ANDRADE *et al.* 2006; GUISEPPE *et al.*, 2011). Nesses mesmos estudos foram propostos um mecanismo catalítico do tipo ácido-base para estas enzimas, envolvendo principalmente os dois resíduos de histidina (His12 e His47) combinado com a estabilização do íon metal (Mg^{+2}) pelos resíduos de ácido aspártico e ácido glutâmico (Glu32 e Asp34). Baseado nesse mecanismo de catálise já proposto para outras enzimas análogas de outros organismos, a exemplo da ribonuclease I (JONES *et al.*, 1996), PLD e SMase C de *Bacilus cereus* (STUCKEY *et al.*, 1999; OBAMA *et al.*, 2003; LEIROS *et al.*, 2004), propõem-se então que as fosfolipases D de *L. laeta* e *L. intermedia* atuam por meio do mesmo mecanismo de catálise ácido-base (LEE e LYNCH, 2005; ANDRADE *et al.* 2006, MURAKAMI *et al.* 2005 e 2006, GUISEPPE *et al.*, 2011). Embora o mecanismo catalítico do tipo ácido-base, a participação dos resíduos na hidrólise, a coordenação do íon metal, o reconhecimento, orientação e estabilização do substrato tenham sido bastante discutidos, nenhum trabalho mostrou como esse mecanismo realmente ocorre. Alguns estudos com proteínas análogas às fosfolipases D de *Loxosceles* sugerem a participação de resíduos de aminoácidos específicos no mecanismo catalítico dessas enzimas. Um dos métodos mais descritos na literatura que é bastante utilizado para este propósito é a mutação sítio-dirigida. Estudos com fosfolipases D de *Bacillus cereus* mostram que as mutações sítio-dirigidas nos resíduos H151A, H296A e E195A, que são altamente conservados entre PLDs de bactérias e mamíferos, aboliram completamente a atividade esfingomielinásica e hemolítica dessas enzimas. Com base nos resultados de atividade catalítica e analogia com enzimas da mesma família, foi proposto que

o mecanismo catalítico para esta enzima é do tipo ácido-base, com a participação dos resíduos His195 e His296 no ataque nucleofílico ao substrato, e o resíduo Asp195 coordenando o íon metal Mg^{+2} (OBAMA *et al.,* 2004). Para uma enzima GDPD (glicerofosfodiéster fosfodiesterase) de *Thermoanaerobacter tengcongensis*, foi utilizada a mutação sítio-dirigida para esclarecer quais resíduos formam o sítio-catalítico (His17 e His19), quais resíduos estabilizam e coordenam o íon metal Ca^{+2} (Glu44, Asp46 e Glu119) e esse estudo também identificou e sugeriu um possível resíduo que reconhece e se liga ao substrato (Lys121). Assim, um mecanismo catalítico ácido-base também foi proposto para esta enzima (SHI *et al.,* 2008). Podemos observar que os resíduos que foram propostos nos trabalhos discutidos acima são os mesmos resíduos que são mencionados em estudos com as fosfolipases D de *Loxosceles* (LEE e LYNCH, 2005; ANDRADE *et al.* 2006; MURAKAMI *et al.* 2005 e 2006, GUISEPPE *et al.*, 2011; DIAS-LOPES *et al.*, 2013), sugerindo uma alta similaridade estrutural e catalítica entre diversas enzimas de diferentes organismos.

Após avaliar a atividade fosfolipásica utilizando como substrato a SM e a lisoPC, o próximo passo foi avaliar a atividade hemolítica tempo-dependente e as alterações morfológicas dos eritrócitos tratados com as isoformas com mutações sítio-dirigidas. Escolhemos o eritrócito para os testes por ser um excelente modelo experimental devido à facilidade de obtenção e preservação celular. Além disso, é de conhecimento que a hemólise causada pelo veneno de *L. intermedia* é um efeito direto da atividade catalítica das fosfolipases D (CHAVES-MOREIRA *et al.,* 2009 e 2011; VUTIKA *et al.,* 2013). Outros estudos também mostraram que a hemólise pode ser indireta, ou seja, por um mecanismo dependente da ativação do sistema complemento, tanto pela via clássica, quanto pela via alternativa (TAMBOURGUI *et al.,* 2010; TAMBOURGI *et al.,* 2007; TAMBOURGI *et al.,* 2004). Analisando os nossos resultados, podemos observar que as variantes H12A-H47A e E32A-D34A apresentaram baixa atividade hemolítica. Estudos envolvendo a isoforma LiRecDT1 H12A mostraram que esta não desencadeia hemólise, corroborando com os resultados obtidos para a isoforma com dupla mutação LiRecDT1 H12A-H47A (CHAVES-MOREIRA *et al.*, 2009 e 2011). Os resultados sugerem que os resíduos de His^{12} e His^{47} são fundamentais para a atividade catalítica e,

consequentemente, também estão envolvidos na atividade hemolítica dessas enzimas. Também foi constatada uma baixa no percentual de hemólise referente a uma isoforma de fosfolipase D de *L. reclusa* com mutação sítio-dirigida nos resíduos H37N e H73N (LEE e LYNCH 2005). A baixa atividade hemolítica da variante E32A-D34A pode ser atribuída devido a estes resíduos mutados, uma vez que os resíduos originais participam na coordenação do íon metal Mg^{+2}, muito importante para a atividade catalítica das fosfolipases D (MURAKAMI *et al.*, 2005 e 2006; GUISEPPE *et al.*, 2011; DIAS-LOPES *et al.,* 2013). Podemos propor que a ausência dos resíduos originais comprometeria a coordenação do íon Mg^{+2} e, consequentemente, resultaria na diminuição da atividade hemolítica dessa toxina. Estudos de Chaves-Moreira e colaboradores (2009) mostraram uma diminuição significante da atividade hemolítica da isoforma LiRecDT1 quando tratada com quelantes de metais divalentes como EDTA, EGTA e 1-10, fenantrolina. A partir desses resultados, podemos reforçar que a presença dos resíduos de aminoácidos que coordenam o íon metal Mg^{+2} é fundamental para a atividade catalítica destas enzimas.

Podemos observar uma atividade hemolítica diminuída, embora não completamente abolida, para as células tratadas com as isoformas mutadas LiRecDT1 K93A, LiRecDT1 W230A e LiRecDT1 C53A-C201A. Estes resultados corroboram com a atividade fosfolipásica estudada, inferindo que estes resíduos estão envolvidos no reconhecimento de substratos lipídicos específicos, os quais também são encontrados na membrana do eritrócito. Quando analisamos a atividade hemolítica da isoforma LiRecDT1 C53A-C201A observamos um percentual de hemólise consideravelmente alto, semelhante ao resultado observado para a LiRecDT1. No entanto, observamos anteriormente que esta isoforma possui moderada atividade fosfolipásica. Para explicar esses resultados, podemos propor que a alta atividade hemolítica observada para a variante C53A-C201A se deve ao metabolismo de fosfolipídeos presentes nas membranas celulares e suas respostas biológicas. É de conhecimento que a atividade das fosfolipases D sobre SM gera colina e ceramida 1-fosfato (C1P) e quando hidrolisa lisoPC gera colina e ácido lisofosfatídico (LPA) (FUTRELL, 1992). C1P e LPA são metabólitos lipídicos que exercem funções biológicas essenciais na célula exemplo de: transdução de sinais, alteração no ciclo celular, apoptose, viabilidade da membrana plasmática, diferenciação celular,

resposta inflamatória, agregação plaquetária, hemólise e outros (YANG *et al.,* 2000; OHANIAN e OHANIAN, 2001; ANLIKER e CHUN, 2004; CHALFANTAND e SPIEGEL, 2005; MITSUTAKE e IGARASHI, 2007). Estudos mostraram que quando eritrócitos humanos são incubados com os produtos de degradação de SM e lisoPC gerados pela atividade catalítica da LiRecDT1, ou seja, C1P e LPA, ocorre hemólise (CHAVES-MOREIRA *et al.,* 2011). A partir disso, podemos inferir que a toxina LiRecDT1 C53A-C201A também estimula a produção de C1P e LPA a partir da hidrólise de SM e de lisoPC, e que estes metabólitos bioativos podem desencadear hemólise em eritrócitos humanos decorrente de eventos de sinalização intracelular. Além disso, foi relatado que a atividade de LiRecDT1 acarreta um influxo de cálcio em eritrócitos humanos através de um canal de cálcio do tipo L (CHAVES-MOREIRA *et al.,* 2011). Essas observações sugerem que o influxo de cálcio, os mediadores lipídicos C1P e LPA podem ativar várias cascatas de sinalização intracelular que pode também contribuir para a hemólise (CHAVES-MOREIRA *et al.,* 2011; CHAIM *et al.,* 2011b).

Além disso, pudemos observar que a isoforma mutada LiRecDT1 Y228A não apresentou atividade hemolítica, corroborando com os resultados obtidos na avaliação da atividade fosfolipásica. Estudos de imunomarcação específica para a isoforma original LiRecDT1 mostraram que esta toxina se liga na membrana dos eritrócitos e altera a morfologia e estrutura dessas células (CHAVES-MOREIRA, *et al.,* 2009). Nesses mesmos estudos foi mostrado, por meio de microscopia confocal, que a isoforma LiRecDT1 fusionada com a proteína verde fluorescente (GFP) também se liga na membrana eritrocitária. Também foi evidenciado por imunomarcação que a toxina mutada LiRecDT1 H12A se liga na membrana do eritrócito e não altera a morfologia da célula devido a mutação no resíduo que faz parte do sítio catalítico (CHAVES-MOREIRA, *et al.,* 2009). Essas referências encontradas na literatura corroboram com a nossa proposta de que existem sítios de ligação e reconhecimento de fosfolipídeos sintéticos e fosfolipídeos presentes na membrana eritrocitária. Além disso, podemos propor que o resíduo Lys228 é o que melhor reconhece e direciona os substratos lipídicos na fenda catalítica das fosfolipases D, e que os resíduos Tyr93 e o Trp230 também possuem

participação na ligação de substratos lipídicos, como discutido anteriormente nos resultados de cinética enzimática.

Para reforçar os resultados da citotoxicidade sobre eritrócitos, avaliamos também as alterações morfológicas dessas células tratadas com as toxinas mutadas. Nesse ensaio verificamos alterações morfológicas nas células tratadas com a toxina original e com a toxina utilizada como controle (LiRecDT1 e LiRecDT1 G96A, respectivamente), e também para as células tratadas com as toxinas mutadas LiRecDT1 K93A, LiRecDT1 W230A e LiRecDT1 C53A-C201A. Chaves-Moreira e colaboradores (2009) mostraram que a isoforma LiRecDT1 altera a morfologia dos eritrócitos tratados, resultando em formas de esferócitos, estomatócitos e knizócitos acompanhados pela diminuição do volume celular no decorrer do tempo de exposição. Os eritrócitos tratados com as toxinas mutadas LiRecDT1 H12A, LiRecDT1 H12A-H47A, LiRecDT1 E32A-D34A e LiRecDT1 Y228A não apresentaram alterações na morfologia das células, corroborando com os nossos resultados de hemólise tempo-dependente. Esse resultado já era esperado, uma vez que essas variantes mutadas não apresentam atividade catalítica sobre fosfolipídeos sintéticos e fosfolipídeos de membrana eritrocitária devido à ausência dos resíduos responsáveis pela hidrólise, coordenação do íon metal e ligação ao substrato lipídico.

Já é bem descrito que as fosfolipases D de *Loxosceles* são capazes de causar isoladamente dermonecrose e intensa resposta inflamatória em pele de coelho (da SILVA *et al.*, 2004; APPEL *et al.*, 2005, CHAIM *et al.*, 2011b). Porém, havia a necessidade de esclarecer se este evento é dependente da sua atividade catalítica e/ou apenas pelo fato das fosfolipases D se ligarem ao substrato lipídico. O ensaio de permeabilidade vascular em camundongos também foi realizado, correlacionando esses efeitos com a atividade catalítica dessas enzimas, pois já foi mostrado que isoformas recombinantes e ativas de fosfolipases D de *L. intermedia* podem desencadear essas respostas (APPEL *et al.* 2008; VUITIKA *et al.*, 2013).

A permeabilidade vascular é um processo essencial nos tecidos normais e está envolvido no transporte de moléculas como gases, água e pequenas moléculas que podem atravessar a barreira de células endoteliais em capilares sanguíneos. Alterações na permeabilidade vascular são ocasionadas a partir

de processos inflamatórios agudos e patologias associadas com a angiogênese e tumores (NAGY *et al.*, 2008; NIDAVANI *et al.*, 2014). O ensaio de Milers é geralmente usado experimentalmente para avaliar o extravasamento vascular de proteínas plasmáticas. Este método se baseia no uso do corante Azul de Evans (EDB) injetado por via intravenosa e que se liga de forma não covalente à albumina, formando o complexo albumina-corante que se acumula na pele do animal (NAGY *et al.*, 2008; NIDAVANI *et al.*, 2014). Foram utilizados camundongos porque este modelo animal não desenvolve dermonecrose e hemorragia local após a exposição ao veneno total da aranha-marrom ou fosfolipase D recombinante, um evento que poderia mascarar a interpretação da permeabilidade vascular. Estudos anteriores mostraram que a permeabilidade vascular aumentada é um evento biológico causado *in vivo* pelo veneno total da aranha-marrom e toxina fosfolipase D nativa ou recombinante (da SILVEIRA *et al.,* 2006; APPEL *et al*., 2008; PALUDO *et al*., 2009; VUITIKA *et al.,* 2013). No presente estudo, observamos que as isoformas mutadas LiRecDT1 H12A, LiRecDT1 H12A-H47A, LiRecDT1 E32A-D34A e LiRecDT1 Y228A não acarretaram aumento na permeabilidade vascular. Como discutido anteriormente, essas toxinas possuem mutações que aboliram sua atividade catalítica e, consequentemente, essas enzimas não apresentam funcionalidade biológica. Para as outras toxinas mutadas LiRecDT1 K93A, LiRecDT1 W230A e LiRecDT1 C53A-C201A e controles LiRecDT1 e LiRecDT1 G96A, verificamos um aumento significativo na permeabilidade vascular. Assim, nossos resultados reforçam a ideia de que o aumento da permeabilidade capilar causada pelo veneno total e fosfolipases D recombinantes de *Loxosceles* depende da atividade catalítica destas enzimas (da SILVEIRA *et al.*, 2006; APPEL *et al.*, 2008; VUITIKA *et al.*, 2013). Para a isoforma mutada LiRecDT1 H12A verificamos uma atividade biológica residual, verificada por uma discreta alteração da permeabilidade vascular, corroborando com os resultados de Paludo e colaboradores (2009). Os subprodutos originados a partir da hidrólise de fosfolipídios de membrana de células endoteliais, como a ceramida 1-fosfato (C1P) e ácido lisofisfatídico (LPA) possuem papel importante em processos inflamatórios. Além disso, os efeitos citotóxicos diretos dessas toxinas em células endoteliais podem ser responsáveis pelo desencadeamento intenso de infiltração de células

inflamatórias. Paludo e colaboradores (2009) propuseram que a capacidade das fosfolipases D de evocar eventos inflamatórios, a exemplo do aumento da permeabilidade vascular, é dependente da ativação de mastócitos e, posteriormente, da liberação de histamina. De acordo com a literatura, podemos sugerir uma classificação para o aumento da permeabilidade vascular causada por fosfolipases D de *L. intermedia* como hiperpermeabilidade vascular aguda (AVH). A AVH ocorre quando a microvasculatura é exposta de forma aguda a qualquer tipo de fatores vasculares, ou seja, agentes de hiperpermeabilidade que provocam degranulação de mastócitos, como por exemplo VEGF-A, serotonina, histamina, PAF, alergia, picadas de insetos, etc. O fluido que extravasa em casos de AVH é muito rico em proteínas do plasma, tais como albumina, fibrinogênio (resultando em edema) e a coagulação do sangue em cascata (NAGY *et al.,* 2008).

Na literatura as fosfolipases D de aranha-marrom são também conhecidas como "toxinas dermonecróticas" devido a sua capacidade biológica, em condições experimentais, de causar dermonecrose *in vivo* (APPEL *et al.,* 2005; CHAIM *et al.,* 2011; Gremski *et al.*, 2014). Por este motivo, foi avaliada a capacidade das toxinas com mutações sítio-dirigidas em causar dermonecrose *in vivo.* Os resultados mostraram que a toxina original LiRecDT1 e toxina controle mutada LiRecDT1 G96A possuem alta capacidade de causar dermonecrose. Para essas toxinas foi observada uma área de necrose no local de aplicação das toxinas, eritema profundo, um edema difuso ao redor da lesão e uma hemorragia notável. Estudos histopatológicos com o veneno total e fosfolipases D recombinantes de espécies de *Loxosceles* mostraram atividade dermonecrótica e massiva diapedese de células inflamatórias, áreas hemorrágicas, degeneração dos vasos sanguíneos e formação de trombo, desorganização das fibras de colágeno e deposição de fibrina (OSPEDAL *et al.,* 2002; CHAIM *et al.,* 2006; da SILVEIRA *et al.,* 2006; 2007; APPEL *et al.,* 2008; PEREIRA *et al.,* 2012; VUITIKA *et al.,* 2013). Nossos resultados também mostraram que as toxinas com mutações LiRecDT1 K93A, LiRecDT1 W230A e LiRecDT1 C53A-C201A foram capazes de causar dermonecrose em um nível “intermediário” ao observado para os controles. Também podemos observar que a isoforma LiRecDT1 Y228A não reproduziu nenhum efeito dermonecrótico. Todos esses resultados observados na avaliação da atividade

dermonecrótica estão em consonância com os ensaios que demonstraram a dependência de atividade catalítica das enzimas, sugerindo que a dermonecrose além de estar relacionada com a atividade catalítica das fosfolipases D, também está associada com o reconhecimento, ligação e estabilização do substrato dentro do bolsão catalítico da enzima.

Alguns estudos propõem mecanismos para tentar explicar o desencadeamento da lesão dermonecrótica e a grande resposta inflamatória. Podemos destacar que a atividade catalítica das fosfolipases D provoca efeito nocivo na pele de coelho, pois foi relatado em muitos trabalhos que essas enzimas apresentam ação citotóxica *in vitro* em diferentes tipos celulares como por exemplo eritrócitos (CHAVES-MOREIRA *et al.,* 2009; 2011; VUTIKA *et al.,* 2013), célula endotelial de rim canino (linhagem MDCK) (CHAIM *et al.,* 2006; KUSMA *et al.,* 2008), células endoteliais de aorta de coelho (linhagem RAEC) (CHAIM et al., 2011b), melanoma murino (linhagem B16-F10) (WILLE *et al.,* 2013), fibroblasto humano (linhagem CRL-1635) (DRAGULEV *et al.*, 2007) e queratinócitos humano (linhagem HaCAT) (PAIXÃO-CAVALCANTE *et al.,* 2006). Podemos destacar outro mecanismo que envolve a expressão de citocinas e quimiocinas pró-inflamatórias estimuladas pelos produtos de hidrólise de fosfolipídeos presente na membrana plasmática de fibroblastos (DRAGULEV *et al.*, 2007). Estudos mais recentes (HORTA *et al.,* 2013) identificaram que o ácido lisofosfatídico (LPA) foi capaz de estimular a produção e liberação de interleucinas (IL-6, IL-8, CXCL1 e CXCL2) via receptor de LPA em linhagem de fibroblasto humano (Linhagem HFF-1).

Por último, foi proposto um modelo preliminar das isoformas mutantes, a fim de ilustrar os possíveis resultados das substituições dos resíduos de aminoácidos na interação com a esfingomileina (Figura 6 do item 4-Resultados). Exceto a mutação G96A, que foi estrategicamente realizada em um local distante do sítio ativo da enzima, todas as demais substituições alteraram a interação da toxina com o substrato. De acordo com os resultados, podemos propor que na ausência dos resíduos H12 e H47, resíduos importantes para o ataque nucleofílico e para a estabilização do produto intermediário, ocorreu uma maior distância entre a enzima e o substrato, que por sua vez, pode ter alterado a configuração do sítio catalítico. Na isoforma mutante E32A-D34A, a ausência dos resíduos E32 e D34 promoveu a perda de coordenação do íon magnésio

(co-factor), o que pode ter provocado a falta de estabilidade do substrato durante a catálise (o resíduo de alanina é incapaz de coordenar o Mg^{+2}). Além disso, o mutante Y228A exibiu uma perda da interação entre o grupo amina carregado positivamente da colina (esfingomielina) e o oxigênio carregado negativamente do resíduo Y228, que podem ter alterado a configuração do local de ligação ao substrato. A isoforma mutante W230A também pode ter perdido a interação hidrofóbica com a cabeça polar da esfingomielina. Além disso, o mutante K93A pareceu perder a interação com a cauda do substrato. O mutante C53A-C201A perdeu uma ligação dissulfueto, o qual pode ter facilitado a expansão da fenda catalítica e o deslocamento do resíduo catalítico H47, não tormando-o acessível para o ataque nucleófilo.

6 CONCLUSÃO

A produção de isoformas mutantes mostrou ser uma ferramenta muito importante para o estudo da relação estrutura/função das fosfolipases D de *L. intermedia*. A partir dos resultados bioquímicos, biológicos e bioinformáticos, podemos concluir que o conjunto de resíduos de aminoácidos que realizam o ataque nucleofílico (His12 e His47), que estabilizam o íon metal Mg^{+2} (Asp32 e Glu34) e que se ligam ao substrato lipídico (Lys93, Tyr228 e Trp230) são muito importantes para o desempenho da atividade catalítica e função biológica dessas enzimas. Além disso, os resíduos Cys53 e Cys201 mostraram ser bastante importantes para a mesma relação estrutura/função da toxina, pois a desestabilização da alça catalítica em relação à alça flexível pode alterar a fenda eletrostática do sítio-catalítico.

Além disso, os resultados obtidos neste trabalho são muito relevantes no sentido de abrir caminhos para novas pesquisas na área da biologia estrutural das fosfolipases D por método de cristalografia, estudos de vias de sinalização celular e mediadores pró-inflamatórios, e também na prospecção de inibidores moleculares para fosfolipases D.

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8 ANEXOS

Demais trabalhos publicados durante o peíodo do doutorado:

ANEXO 1:

Identification, cloning and functional characterization of a novel phospholipase D (dermonecrotic toxin) from brown spider (Loxosceles intermedia) venom containing a conservative mutation (D233E) in the catalytic site

Autores: Larissa Vuitika, Luiza Helena Gremski, Matheus Regis Belisário, Daniele Chaves-Moreira**,** Valéria Pereira Ferrer, Andrea Senff-Ribeiro, Olga Meiri Chaim, Silvio Sanches Veiga.

Revista: Journal of Cellular Biochemistry, 2013 (I.F.= 3.263).

ANEXO 2:

Recent advances in the understanding of brown spider venoms: From the biology of spiders to the molecular mechanisms of toxins.

Autores: Luiza Helena Gremski, Dilza Trevisan-Silva, Valéria Pereira Ferrer, Fernando Hitomi Matsubara, Gabriel Otto Meissner, Ana Carolina Martins Wille, Larissa Vuitika, Camila Dias-Lopes, Anwar Ullah, Fábio Rogério de Moraes, Carlos Chávez-Olórtegui, Katia Cristina Barbaro, Mario Tyago Murakami, Raghuvir Krishnaswamy Arni, Andrea Senff-Ribeiro, Olga Meiri Chaim, Silvio Sanches Veiga.

Revista: Toxicon, 2014 (I.F.=2.492).

ANEXO 3: **Structural Insights into Substrate Binding of Brown Spider Venom Class II Phospholipases D.**

Autores: Monica Coronado, Anwar Ullah, Luciane Sussuchi Silva, Daniele Chaves Moreira, Larissa Vuitika, Olga Meiri Chaim, Silvio Sanches Veiga, Jorge Chahine, Mario Tyago Murakamy, Raghuvir Krishnaswamy Arni

Revista: Current Protein e Pepitide Science, 2015 (I.F.=3.154).

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Brown Spider Phospholipase-D Containing a Conservative Mutation (D233E) in the Catalytic Site: Identification and Functional Characterization

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ABSTRACT

Brown spider (*Loxosceles* genus) bites have been reported worldwide. The venom contains a complex composition of several toxins, including phospholipases-D. Native or recombinant phospholipase-D toxins induce cutaneous and systemic loxoscelism, particularly necrotic lesions, inflammatory response, renal failure, and hematological disturbances. Herein, we describe the cloning, heterologous expression and purification of a novel phospholipase-D toxin, LiRecDT7 in reference to six other previously described in phospholipase-D toxin family. The complete cDNA sequence of this novel brown spider phospholipase-D isoform was obtained and the calculated molecular mass of the predicted mature protein is 34.4 kDa. Similarity analyses revealed that LiRecDT7 is homologous to the other dermonecrotic toxin family members particularly to LiRecDT6, sharing 71% sequence identity. LiRecDT7 possesses the conserved amino acid residues involved in catalysis except for a conservative mutation (D233E) in the catalytic site. Purified LiRecDT7 was detected as a soluble 36 kDa protein using anti-whole venom and anti-LiRecDT1 sera, indicating immunological cross-reactivity and evidencing sequence-epitopes identities similar to those of other phospholipase-D family members. Also, LiRecDT7 exhibits sphingomyelinase activity in a concentration dependent-manner and induces experimental skin lesions with swelling, erythema and dermonecrosis. In addition, LiRecDT7 induced a massive inflammatory response in rabbit skin dermis, which is a hallmark of brown spider venom phospholipase-D toxins. Moreover, LiRecDT7 induced in vitro hemolysis in human erythrocytes and increased blood vessel permeability. These features suggest that this novel member of the brown spider venom phospholipase-D family, which naturally contains a mutation (D233E) in the catalytic site, could be useful for future structural and functional studies concerning loxoscelism and lipid biochemistry. Highlights: 1- Novel brown spider phospholipase-D recombinant toxin contains a conservative mutation (D233E) on the catalytic site. 2-LiRecDT7 shares high identity level with isoforms of *Loxosceles* genus. 3-LiRecDT7 is a recombinant protein immunodetected by specific antibodies to native and recombinant phospholipase-D toxins. 4-LiRecDT7 shows sphingomyelinase-D activity in a concentration-dependent manner, but less intense than other isoforms. 5-LiRecDT7 induces dermonecrosis and inflammatory response in rabbit skin. 6-LiRecDT7 increases vascular permeability in mice. 7-LiRecDT7 triggers direct complement-independent hemolysis in erythrocytes. J. Cell. Biochem. 114: 2479–2492, 2013.

KEY WORDS: BROWN SPIDER; VENOM; DERMONECROTIC TOXIN; PHOSPHOLIPASE-D; CLONING; RECOMBINANT PROTEIN

The authors declare no conflict of interest.

GenBank data deposition information for *L. intermedia* cDNA clone: LiRecDT7, GenBank accession no. KC237286.

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Spiders of the *Loxosceles* genus are commonly known as brown spiders based on their body colors that range from fawn to dark brown. At present, 103 species of spiders from the *Loxosceles* genus have been described and are distributed worldwide [Platnick, 2012]. There are 12 species of *Loxosceles* genus spiders in Brazil [Gonçalves-de-Andrade et al., 2012]. Loxoscelism is the term used to describe the lesions caused from bites by *Loxosceles* genus spiders [da Silva et al., 2004; Appel et al., 2005; Chaim et al., 2011a]. Traditionally, loxoscelism is divided in two clinical conditions: cutaneous and systemic loxoscelism. Cutaneous loxoscelism refers to the typical skin lesion with dermonecrosis, edema, erythema and gravitational spreading that appears after most accidents. Systemic loxoscelism is less common than the cutaneous form, but is responsible for complications following accidents, which might include hematological disturbances, such as thrombocytopenia, disseminated intravascular coagulation, hemolytic anemia, and renal failure [Futrell, 1992; da Silva et al., 2004; Isbister and Fan, 2011].

The volume of venom inoculated by *Loxosceles* genus spiders is typically minute (some microliters) and enriched in proteins of low molecular mass (20–45 kDa). To date, a great number of brown spider venom toxins have been identified in different *Loxosceles* species, including hyaluronidases [Young and Pincus, 2001; Barbaro et al., 2005; da Silveira et al., 2007a; dos Santos et al., 2009], metalloproteases from the astacin family [da Silveira et al., 2007b; Trevisan-Silva et al., 2010], serine proteases [Veiga et al., 2000; Gremski et al., 2010], translationally controlled tumor protein (TCTP) [Sade et al., 2012], insecticidal peptides of the ICK family [Gremski et al., 2010], serine protease inhibitors [Gremski et al., 2010], and members of the phospholipase-D family, also known as dermonecrotic toxins, based on their biological activity [Kalapothakis et al., 2007; Appel et al., 2008; Binford et al., 2009].

Previous studies using native dermonecrotic toxins purified from crude venom have biochemically characterized this toxin as a sphingomyelinase based on its ability to hydrolyze sphingomyelin into choline and ceramide-1-phosphate [Futrell, 1992]. Using recombinant isoforms of dermonecrotic toxins from different *Loxosceles* species, it has recently been postulated that these molecules are phospholipase-D enzymes based on their abilities to hydrolyze other synthetic phospholipids, such as lysophosphatidylcholine, lysophosphatidylinositol, lysobisphosphatidic acid, cyclic phosphatidic acid, and lysoplatelet-activating factor [Lee and Lynch, 2005; Chaim et al., 2011b]. The final release of lipid metabolites such as ceramide-1-phosphate (C1P) from sphingomyelin or lysophosphatidic acid from a lysophospholipid was already described for *Loxosceles* phospholipases-D [van Meeteren et al., 2004; Chalfant and Spiegel, 2005; Lee and Lynch, 2005]. These metabolites are bioactive mediators that play a major role in complex signaling pathways that control several cellular processes and also in various pathophysiologic processes [Hannun, 1994; Anliker and Chun, 2004; Moolenaar et al., 2004]. Thus, these lipid mediators could induce toxicity by activating signaling pathways involved with a variety of pathophysiologic changes [Chaim et al., 2011b].

The phospholipase-D toxins in *Loxosceles* genus spider venoms represent the most biologically and biochemically characterized brown spider venom components and various isoforms of these molecules have previously been reported for different species [Chaim et al., 2011a]. Four biochemically related isoforms of phospholipase-D in a native purified dermonecrotic toxin have been reported in *L. reclusa* venom [Futrell, 1992]. The results from N-terminal sequence studies of *L. boneti* venom have revealed three different isoforms of phospholipase-D in this species [Ramos-Cerrillo et al., 2004]. Similarly, based on proteomic analyses such as two-dimensional electrophoresis, N-terminal amino acid sequencing and mass spectrometry, the presence of at least eleven isoforms of phospholipase-D have been reported for *L. gaucho* venom [Machado et al., 2005]. Six isoforms of phospholipase-D have been identified in *L. intermedia* venom using molecular biology techniques such as cloning, heterologous expression, amino acid alignment and phylogenetic studies [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Appel et al., 2008]. Additional studies have also described multiple members of the phospholipase-D family in other *Loxosceles* venoms, and these isoforms differ in various features, such as their biological activity and substrate specificity [Lee and Lynch, 2005; Kalapothakis et al., 2007; Catalán et al., 2011; Chaim et al., 2011b]. The transcriptome analysis of the venom gland of *L. intermedia* revealed that phospholipases-D represent 20.2% of the total toxin-encoding transcripts present in the gland [Gremski et al., 2010]. An inter-species family of similar phospholipase-D toxins has been postulated, and the noxious effects of *Loxosceles* spp. whole venom are due to the family synergism of these related toxins [Kalapothakis et al., 2007]. Many studies indicate that the gene family of venom phospholipase-D toxins has undergone frequent multiple duplications and occasional functional evolution [Binford et al., 2009].

Here, we describe the cloning, heterologous expression, purification, and functional evaluation of a novel phospholipase-D isoform from a cDNA library of *L. intermedia* venom gland. This novel isoform has conservative the amino acid residues involved in the catalysis and metal ion coordination that are important for phospholipase activity, as previously reported for the other members of the family [Murakami et al., 2006; de Giuseppe et al., 2011], except for a conservative mutation (D233E) in the catalytic site. Taken together, these results strengthen the concept of a gene family encoding different phospholipase-D toxins in the venom of *L. intermedia*. Here, we present evidence of a novel isoform and illustrate the features of a dermonecrotic toxin with a natural mutation in an amino acid residue in the catalytic site.

METHODS

REAGENTS

Whole venom from *L. intermedia* was extracted from spiders captured in the wild with the authorization of the Brazilian Governmental Agency "Instituto Chico Mendes de Conservação da Biodiversidade" number 29801-1, according to [Feitosa et al., 1998]. Polyclonal antibodies to *L. intermedia* crude venom toxins and phospholipase-D "dermonecrotic toxin" (LiRecDT1) were produced in rabbits as previously described [Chaim et al., 2006]. Evans Blue dye was purchased from Vetec (São Paulo, Brazil). NaCl, KCl, $CaCl_2$, Na_2HPO_4, KH_2PO_4, Na_2PO_4, NaH_2PO_4, Imidazole, Lysozyme, and Ágar XLT4 were purchased from Merck (Darmstadt, Germany). Tryptone Type I, Yeast Extract (Himedia, Mumbai, India). Chloramphenicol and

Ampicillin Trihydrate were purchased from USB Corporation (Cleveland, OH). Tris and Sucrose were purchased from Bio-Rad (Hercules, CA) and Sigma–Aldrich (St. Louis, MO), respectively. Xylasine and Ketamine were purchased from Rhobifarma (Hortolândia, São Paulo, Brazil).

cDNA CLONING

The partial cDNA sequence for the dermonecrotic toxin isoform 7 described herein was isolated from a previously constructed venom gland cDNA library [Gremski et al., 2010]. Briefly, venom gland mRNAs were purified from total RNA using a magnetic separation kit, PolyATtract mRNA Isolation System III (Promega Corporation, Madison, WI). A directional *L. intermedia* venom gland cDNA library was constructed using a Creator SMART cDNA Library Construction Kit (BD Biosciences Clontech, Mountain View, CA). The first strand of cDNA was synthesized from purified mRNA, and the second strand was obtained through long distance PCR (LD-PCR), according to the manufacturer's instructions. The cDNA was size fractionated via chromatography to avoid contaminating the library with short length sequences. Competent *Escherichia coli* DH5α cells were transformed with the cDNA library plasmids to amplify the cDNA. The transformants were selected on LB (Luria-Bertani) agar plates containing 34 μg/ml chloramphenicol, and more than 20,000 colonies were obtained.

AMPLIFICATION OF THE 5′ END OF THE cDNA

To obtain the complete 5′ end of phospholipase-D isoform 7 cDNA, a 5′RACE (Rapid Amplification of cDNA Ends) protocol was performed according to Sambrook and Russell [2001], with some modifications. Approximately 1 μg of total RNA was obtained from *L. intermedia* venom glands. The first-strand cDNA was synthesized using the gene-specific reverse primer R1 (5′-CGAACACAACTAGGGT-CAGTTCTG-3′) and Improm-II Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. The cDNA was recovered through ethanol precipitation in the presence of ammonium acetate and was subsequently poly (A)-tailed with terminal deoxynucleotidyl transferase (Fermentas, Hanover, MD) according to the manufacturer's instructions. The modified cDNA was PCR amplified with the (dT) 17-adaptor primer (5′-CGGTACCATG-GATCCTCGAGTTTTTTTTTTTTTTTT-3′) and the nested gene-specific reverse primer R2 (5′-CTTGTGACACCACCTTCTGCAATC-3′) using Pfu DNA polymerase (Fermentas). The PCR product was gel-purified using a PerfectPrep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions and was sequenced on both strands using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on a DNA 3500 Genetic Analyzer automatic sequencer (Applied Biosystems, Warrington, UK). The putative protein product from the sequenced cDNA was used to search the GenBank protein databases at NCBI.

RECOMBINANT PROTEIN EXPRESSION

The cDNAs encoding putative mature phospholipase-D isoform 7 (LiRecDT7) were PCR amplified using primers designed to contain *Nde*I (Fermentas) restriction sites at the 5′ ends (forward primers) and *Bam*HI (Fermentas) sites at the 3′ ends (reverse primers). The PCR products were digested with *Nde*I and *Bam*HI restriction enzymes and subcloned into the pET-14b plasmid (Novagen, Madison, USA) digested with the same enzymes. The recombinant construct was expressed as a fusion protein, with a 6× His-Tag at the N-terminus and a 13 amino acid linker, including a thrombin site between the 6× His-Tag and the mature protein. The expression construct was transformed into *E. coli* SHuffle T7 Express *lysY* cells (New England Biolabs, Ipswich, MA, UK) and plated on LB plates containing 100 μg/ml ampicillin. Single colonies of LiRecDT7 construct were inoculated into LB broth (100 μg/ml ampicillin) and grown overnight at 30°C. This culture was diluted 1:50 into 2 L fresh LB broth/ampicillin and incubated at 30°C until the $OD_{600\,nm} = 0.5$. Recombinant expression was induced with the addition of 0.05 mM IPTG (isopropyl β-D-thiogalactoside; Fermentas) and cells were incubated for 4 h at 30°C. The cells were harvested through centrifugation (4,000*g*, 7 min, 4°C), resuspended in 40 ml of extraction buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole, and 1 mg/ml lysozyme) and frozen overnight at −20°C.

PROTEIN PURIFICATION

The cells were thawed and disrupted through mechanical lysis. The lysed materials were centrifuged (9,000*g*, 30 min, 4°C), and the supernatants were incubated with 500 μl of Ni^{2+}-NTA beads for 1 h at 4°C. The suspensions were loaded onto a column and the packed gel was washed with a buffer containing 50 mM sodium phosphate, pH 8.0, 500 mM NaCl and 30 mM imidazole. The recombinant protein was eluted with 2 ml of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 250 mM imidazole), and the fractions were collected and analyzed using 12.5% SDS–PAGE under β-mercaptoethanol reducing conditions. The fractions were pooled and dialyzed against phosphate-buffered saline (PBS).

MOLECULAR MODELING OF THE LiRecDT7 AND STRUCTURAL ANALYSIS OF PROTEINS USING BIOINFORMATICS TOOLS

We used the SWISS-MODEL [Kiefer et al., 2009] software (http://swissmodel.expasy.org/) to construct a prediction of the three-dimensional structure of LiRecDT1 and LiRecDT7. The protein used as a model for analysis of the predictions was LiRecDT1 (PDB code: 3RLH), as this toxin has been crystallized and its 3D structure has been solved [de Giuseppe et al., 2011]. The production of the figure presented in the results was produced with the software Open Astex Viewer 3.0 (http://openastexviewer.net/web/), a program for molecular visualization.

SPHINGOMYELINASE-D ACTIVITY ASSAY

The Sphingomyelinase-D activity of purified LiRecDT7 was measured using an Amplex Red Assay Kit (Molecular Probes, Eugene, USA) through the analysis of the sphingomyelinase-D activity of recombinant toxin. In this assay, sphingomyelinase-D activity was monitored using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2 [Chaim et al., 2011b]. First, sphingomyelinase-D hydrolyzes sphingomyelin to yield C1P and choline. Choline is then oxidized to betaine and H_2O_2 through coline oxidase. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with the Amplex reagent at 1:1 stoichiometry to generate the highly fluorescence product, resorufin. LiRecDT1 (positive control) or recombinant LiRecDT7 (5, 10, and 20 μg, in

five trials) were added to the Amplex Red reagent mixture. The reaction tubes were incubated at 37°C for 30 min, and the fluorescence was measured in a microplate fluorimeter (Tecan Infinite® M200, Männedorf, Switzerland) using excitation at 540 nm with emission detection at 570 nm.

DETERMINATION OF HEMOLYTIC ACTIVITY

The hemolysis assay was performed as previously described [Chaim et al., 2011b; Chaves-Moreira et al., 2011]. Washed red blood cells (10^8 cells) were added to each Eppendorf tube containing the appropriate concentrations of LiRecDT7 (0.025, 0.25, 2.5, and 25 μg/ml) in Tris buffer sucrose (TBS; 250 mM sucrose, 10 mM Tris/HCl, pH 7.4 and 280 mOsm\kg H_2O) containing 1 mM $CaCl_2$. For this assay, the experiments were performed in pentaplicate along with negative (in presence of the appropriate amount of TBS with 1 mM $CaCl_2$) and positive (red blood cells in 0.1% v/v Triton X-100) controls. After 24 h of incubation with gentle agitation, the controls and samples were centrifuged (refrigerated Eppendorf microfuge) for 3 min at 1,600 rpm, and the absorbance values for the supernatants were immediately measured at 550 nm (Meridian ELx 800, Auto Reader Diagnostics, Inc., USA). The absorbance values were converted to percent hemolysis using the absorbance values of the positive control as 100% lysis. Blood collection from voluntary students was authorized through agreement of the ethical committee of the Federal University of Paraná.

ANIMALS

Adult Swiss mice (25–30 g) and adult rabbits weighting approximately 3 kg from the Central Animal House of the Federal University of Parana were used for the in vivo experiments with whole venom and recombinant toxins. All procedures involving animals were performed in accordance with "Brazilian Federal Laws," following Ethical Subcommittee on Research Animal Care Agreement number 565 of the Federal University of Parana.

IN VIVO STUDIES ON RABBITS

For the evaluation of the dermonecrotic effect, 10 μg of purified recombinant LiRecDT7 diluted in PBS was injected intradermally into a shaved area of rabbit skin. Dermonecrosis was assessed at 3, 6, and 24 h after injection. For a negative control to assure that bacterium constituent contamination during purification did not influence the results, we used purified recombinant green fluorescent protein (GFP) obtained under the same conditions for LiRecDT7. Rabbits were used in experiments for dermonecrosis because this animal model reproduces skin lesions consistent with those observed in accidents with humans [da Silva et al., 2004]. The experiments were repeated twice for each sample.

HISTOLOGICAL METHODS FOR LIGHT MICROSCOPY

Rabbit skin was collected from animals anesthetized with ketamine and xylazine, and subsequently fixed in "ALFAC" (ethanol absolute 85%, formaldehyde 10%, and glacial acetic acid 5%) for 16 h at room temperature. After fixation, the samples were dehydrated in a graded series of ethanol before paraffin embedding (for 2 h at 58°C). Thin sections (4 μm) were processed for histology. The tissue sections were stained with hematoxylin and eosin (H&E) as previously described [Chaim et al., 2006; da Silveira et al., 2006]. The images were obtained using an Axio Imager Z2 microscope (Carl Zeiss, Jena, DE) equipped with a motorized scanning module VSlider (MetaSystems, Altlussheim, DE).

MEASUREMENT OF VASCULAR PERMEABILITY

The changes in capillary permeability were based on the leakage of plasma protein-bound dye into the extravascular compartment of the skin [Appel et al., 2008]. Evans Blue dye diluted in saline was administered intravenously (30 mg/kg of mice) 5 min prior to sample injections. Whole venom and the recombinant toxins LiRecDT1 and LiRecDT7 (10 μg) were injected intradermally into the dorsal skin of mice ($n = 5$ per treatment). After 30 min, the animals were anesthetized using ketamine and acepromazine, sacrificed and the dorsal skin was removed for the visualization of dye extravasation. For the negative control, animals received only a saline injection without venom toxins. Mice were used because this animal model does not develop dermonecrosis and local hemorrhage following brown spider venom exposure, an event that could mask the interpretation of vascular permeability.

GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Protein content was determined using the Coomassie Blue method (BioRad, Hercules, CA). For protein analysis, 12.5% SDS–PAGE under reduced conditions. For immunoblotting, the proteins were transferred to nitrocellulose filters and immunostained polyclonal with antibodies raised against phospholipase-D isoform 1 (LiRecDT1) or against whole venom toxins. The molecular mass markers were acquired from Sigma.

STATISTICAL ANALYSIS

The statistical analyses were performed using analysis of variance (ANOVA) with a post hoc Tukey test using GraphPad InStat program version 5.00 for Windows XP. Statistical significance was considered when $P \leq 0.05$.

RESULTS

CLONING OF A NOVEL PHOSPHOLIPASE-D ISOFORM FROM *L. intermedia* VENOM GLAND

A partial cDNA encoding for a novel phospholipase-D isoform was obtained through screening clones of a cDNA library of *L. intermedia* venom gland [Gremski et al., 2010]. The complete cDNA sequence was obtained using RT-PCR in a 5′ RACE protocol. The putative protein product from this cDNA was referred to as LiRecDT7 (from *L. intermedia* recombinant dermonecrotic toxin). The other isoforms previously described were the following: LiRecDT1 [Chaim et al., 2006], LiRecDT2 and LiRecDT3 [da Silveira et al., 2006], LiRecDT4 and LiRecDT5 [da Silveira et al., 2007c], and LiRecDT6 [Appel et al., 2008]. The complete cDNA sequence of LiRecDT7 comprises 1,200 bp with a single ORF coding for 300 amino acids with a hydrophobic and putative signal peptide of 18 amino acids (Fig. 1). The calculated molecular mass of the predicted mature protein for LiRecDT7 was 34.4 kDa, with a pI of 5.94.

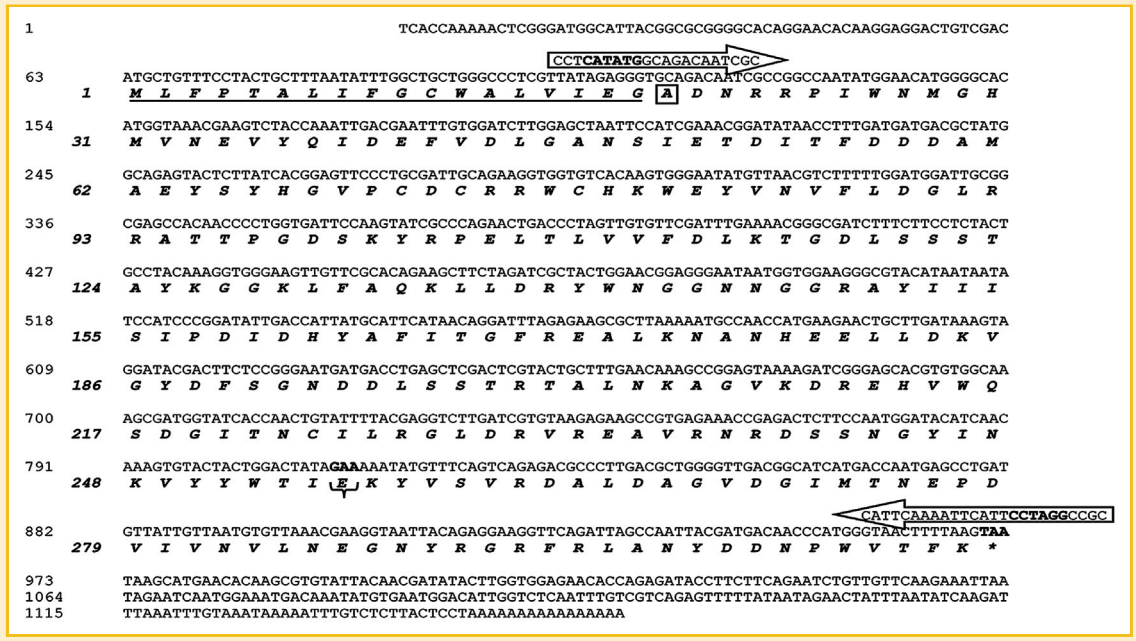

Fig. 1. Molecular cloning of a novel phospholipase-D toxin from *L. intermedia* venom gland cDNA library. Nucleotide and deduced amino acid sequences of cloned phospholipase-D. In the protein sequence, the predicted signal peptide is underlined. The arrows show the annealing positions for the primers used for subcloning into the expression vector, and the restriction sites are highlighted in bold. The alanine in the box indicates the first amino acid of the mature protein with 282 amino acids. The nucleotide and amino acid residue for the conservative substitution for LiRecDT7 (D233E) is highlighted in the bracket. The asterisk corresponds to the stop codon TAA.

MULTIPLE ALIGNMENT ANALYSIS OF THE cDNA-DEDUCED AMINO ACID SEQUENCE AND THE SIMILARITY OF LiRecDT7 WITH OTHER PHOSPHOLIPASE-D TOXIN FAMILY MEMBERS

The BLAST GenBank database search revealed that LiRecDT7 has structural similarity to other LiRecDT family members. The overall identity of LiRecDT7 is approximately 63% with LiRecDT1. From all *L. intermedia* phospholipases-D described, LiRecDT7 is more similar to LiRecDT6, sharing 71% sequence identity. When compared with the other *L. intermedia* dermonecrotic toxin isoforms, LiRecDT7 shows 64% sequence identity to LiRecDT2, 45% identity to LiRecDT3, 60% identity to LiRecDT4, and 46% identity to LiRecDT5 (Fig. 2A). LiRecDT7 also shares 58% identity with LoxTox i5 (EF535254), whose sequence has been previously described [Kalapothakis et al., 2007]. A similarity analysis (Fig. 2B) of these seven phospholipase-D isoform toxins and the cloned cDNAs of other brown spider dermonecrotic toxins revealed that LiRecDT7 was most identical to the *L. hirsuta* toxin (GenBank accession number ACN48948; 91%). These data are consistent with the idea of an intra and inter-species family of brown spider venom phospholipases-D, now containing a novel member.

EXPRESSION, PURIFICATION, AND IMMUNOLOGICAL RELATIONSHIP OF LiRecDT7 TO OTHER BROWN SPIDER MEMBERS

LiRecDT7 was expressed using the N-terminal tag of six histidine residues. Expression experiments were performed in *E. coli* SHuffle T7 Express *lysY* cells. The expression of recombinant protein was optimal when induced for 4 h with 0.05 mM of IPTG. Recombinant phospholipase-D was purified using the soluble fraction of cell lysates under native conditions using Ni^{2+} NTA agarose-chelating chromatography to obtain a 200 μg/L sample of the purified recombinant protein. The SDS–PAGE mobility of the purified recombinant protein, reduced through β-mercaptoethanol treatment, was 36 kDa (Fig. 3A). Differences between the deduced molecular mass and SDS-electrophoretic mobility of LiRecDT7 resulted from the 6× His-tag fusion peptide. Immunoblot analysis using a LiRecDT1 specific antibody and antibodies for whole venom toxins established an immunological relationship between purified recombinant LiRecDT7 and native venom phospholipases-D (dermonecrotic toxins) and LiRecDT1 (Fig. 3B). Both antibodies reacted with purified LiRecDT7, demonstrating that whole venom contains proteins similar to this recombinant toxin, and LiRecDT7 contains similar sequence/epitopes and antigenic cross-reactivity with LiRecDT1, the prototype of this family of toxins.

MOLECULAR MODELING OF LiRecDT7

To model the 3D structure of LiRecDT7, the isoform LiRecDT1 was chosen as a template because the crystallized protein in Protein Data Bank (PDB) shows more identity with the novel isoform [de Giuseppe et al., 2011]. In addition, LiRecDT1 was used as a basis for comparison in all assays performed in the present work. As the images displayed in Figure 4 demonstrate, the overall structure of the novel isoform closely resembles the LiRecDT1 general outline, despite the differences observed in the primary structure. The presence and location of the disulfide bridges (Cys51–Cys57 and Cys53–Cys201), the Mg^{2+} binding site and the two catalytic histidine residues (His12 and His47) are conserved in LiRecDT7 model, which are components of the active-site pocket of spider venom PLDs [de Giuseppe et al., 2011].

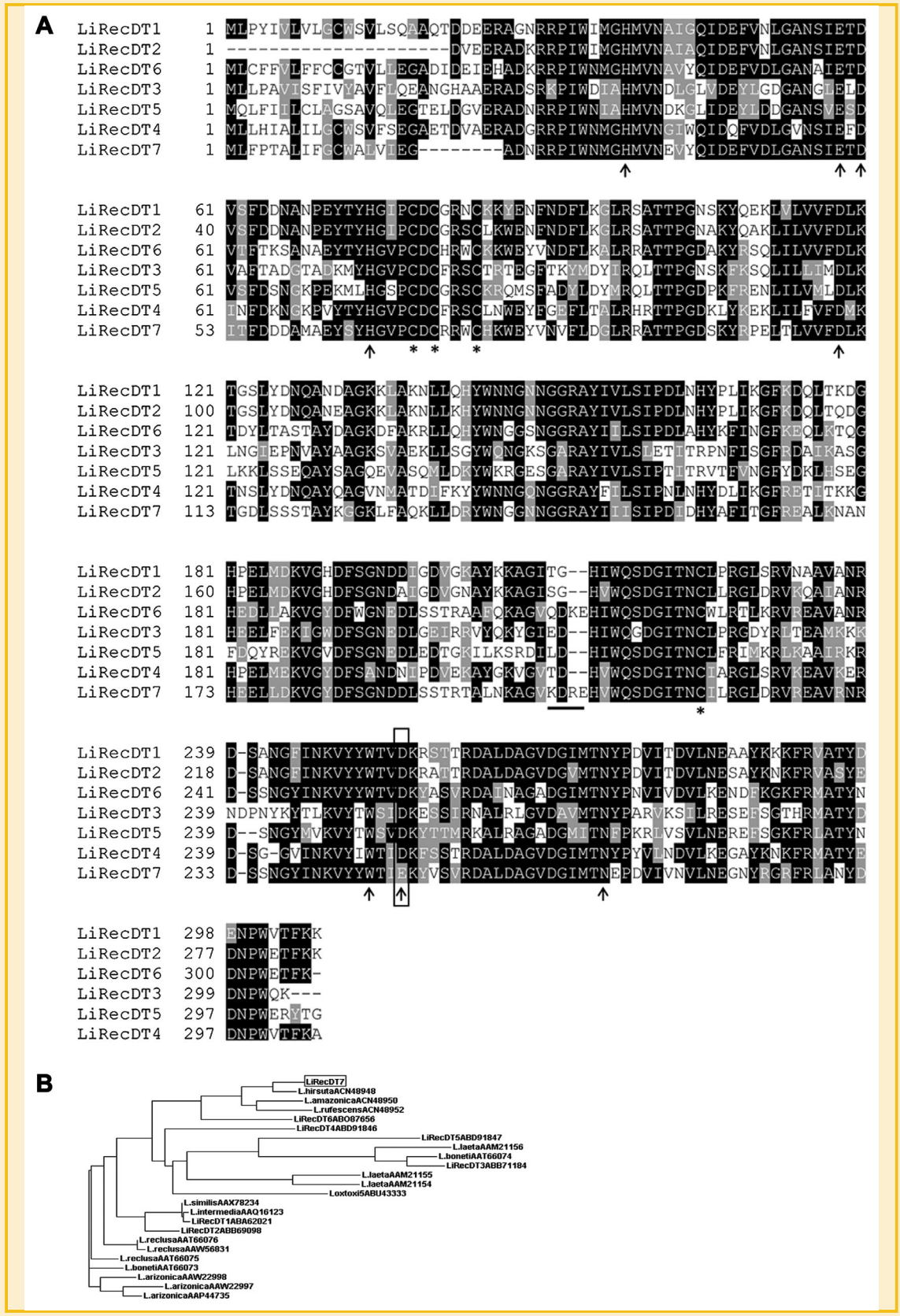

Fig. 2. Multiple alignment analysis of the cDNA-deduced amino acid sequence and the similarity relationship of described phospholipase-D with other phospholipase-D toxin family members from brown spider venoms. The sequences were aligned using the CLUSTAL W program (www.ebi.ac.uk/CLUSTAL). A: The black shaded regions show amino acid identity, the gray shaded regions show conservative substitutions, and the arrows point to amino acid residues of catalytic site of sphingomyelinases-D. The asterisks show cysteine residues. The conservative D223E substitution of LiRecDT7 is featured in the box. The line indicates the amino acids residues of the prominent loop. B: Similarity cladogram of the cloned phospholipase-D toxin members based on sequence alignment from GenBank data. The tree was constructed using the CLUSTAL program as described above. LiRecDT7 is highlighted in the box.

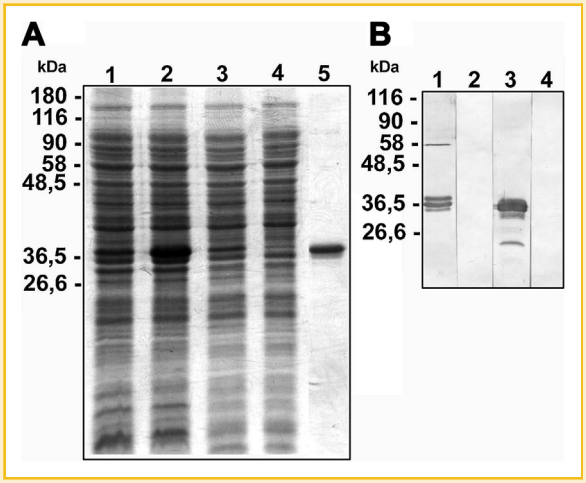

Fig. 3. Expression, purification, and immunological relationship of recombinant dermonecrotic toxin LiRecDT7 with other brown spider phospholipase-D members. A: The expression and purification of recombinant toxin was analyzed using 12.5% SDS–PAGE under reducing conditions and Coomassie blue dye staining. Lane 1 depicts *E. coli* SHuffle T7 Express *lysY* cells before 4 h of induction with 0.05 mM IPTG. Lane 2 shows the proteins of cells after induction with 0.05 mM IPTG. Lanes 3 and 4 depict the supernatant obtained through freeze, thawing and mechanical lysis in extraction buffer before and after affinity chromatography using a Ni^{2+}-NTA column, respectively. Lane 5 shows purified recombinant protein LiRecDT7. The molecular protein mass standards are shown on the left. B: Crude venom (lanes 1 and 2) and purified recombinant toxin LiRecDT7 (lanes 3 and 4; 2.5 μg) were separated using 12.5% SDS–PAGE under reducing conditions, transferred onto nitrocellulose membranes that were incubated with polyclonal antibodies against LiRecDT1 (lane 1) or polyclonal antibodies against whole venom toxins (lane 3). Lanes 2 and 4 indicate reactions in the presence of pre-immune serum (control for antibody specificity). The molecular mass markers are shown on the left.

However, it is possible to observe a significant variation in the contour of the loop located just behind the variable loop of both models. In the LiRecDT7 model, this loop is more prominent (directed toward the surface of the protein) compared with that in LiRecDT1, where this loop, although still prominent, is more positioned in the interior of the protein. As presented in Figure 4, this condition can be easily detected in LiRecDT7, as this loop follows the beginning of the near helix, while in LiRecDT1 this loop follows a different path, away from the near helix and toward the interior of the molecule.

In addition, in both models residue 233 is highlighted, illustrating the difference in the structures of aspartic acid (Asp) and glutamic acid (Glu); it is clear that Glu (LiRecDT7) has a bulkier side chain compared with to Asp (LiRecDT1), which protrudes towards the flexible loop.

SPHINGOMYELINASE AND HEMOLYTIC ACTIVITIES FOR LiRecDT7

To further assess the recombinant molecule functionality, LiRecDT7 was tested for its activity as a sphingomyelinase-D because the native brown spider dermonecrotic toxin and other LiRecDT's family members have sphingomyelinase-D activity. The sphingomyelinase activity was measured using an Amplex-R-Red Sphingomyelinase Assay Kit (as described in material and methods). LiRecDT1 (10 μg) was used as a positive control, and PBS was used as a negative control. Samples of 5, 10, and 20 μg of LiRecDT7 were tested in three subjects. As shown in Figure 5A, the recombinant LiRecDT7 toxin exhibited sphingomyelinase-D activity in a concentration-dependent manner. Nevertheless, as the graph depicts, LiRecDT7 exhibited lower sphingomyelinase-D activity than LiRecDT1.

In addition, we reported the hemolytic activity for *L. intermedia* recombinant phospholipases-D [Chaves-Moreira et al., 2009, 2011]. This hemolytic activity is attributed to the hydrolytic activity of toxins on erythrocyte membrane phospholipids, as toxins induce different degrees of hemolysis, which is proportional to sphingomyelinase-D activity. LiRecDT1H12A, a molecule without sphingomyelinase-D activity, also exhibits residual hemolytic activity [Chaves-Moreira et al., 2011]. Herein, we provide additional data suggesting the direct hemolysis activity induced though brown spider venom phospholipase-D. Figure 5B shows the hemolysis in human red blood cells incubated in serum-free medium containing different amounts of LiRecDT7 at 37°C. As observed, hemolysis occurred in a dose-dependent manner, indicating the direct hemolytic activity of LiRecDT7 in human erythrocytes.

SKIN LESIONS AND INFLAMMATORY RESPONSE EVOKED BY RECOMBINANT LiRecDT7

Because brown spider venom phospholipases-D possess dermonecrotic and inflammatory activities [da Silva et al., 2004; Appel et al., 2005; Chaim et al., 2011b], we evaluated the functionality of recombinant LiRecDT7. For this purpose, LiRecDT7 (10 μg) was injected intradermally into shaved rabbit skin. As a negative control, we used recombinant GFP (devoid of dermonecrotic activity), which was expressed and purified under the same conditions as those used for LiRecDT7. The macroscopic lesions were assessed 3, 6, and 24 h after injection, and the tissue samples were collected and histologically analyzed using a light microscope at 24 h after toxin exposure (Fig. 6). The animals that received recombinant toxin LiRecDT7 showed lesions with a deep erythema, a diffuse edema surrounding the lesion and a remarkable hemorrhage after 6 h. After 24 h, these signs were exacerbated, and an area of necrosis was observed surrounding the injection site. Gravitational spreading was not observed after the inoculation of LiRecDT7 compared with the clear spreading triggered by whole venom and LiRecDT1. The histopathological findings of skin biopsies after recombinant toxin exposure (LiRecDT7) showed a massive inflammatory response (Fig. 7C), with inflammatory cells (neutrophil leukocytes; Fig. 7D) diffusely spread within the dermis. Figure 7A,B shows panoramic views of sections of negative control and LiRecDT7, respectively, and clearly illustrate the edema triggered using the recombinant toxin, as evidenced by the length of the skin structures. As observed, the panoramic view of the tissue section after LiRecDT7 exposure is wider compared with that of the control, indicating an event induced through tissue edema. In addition, in Figure 7E it is possible to observe the disorganization of collagen fibers in the dense connective tissue inside the dermis, which also indicates edema. The images also depict areas of necrosis, including the degeneration of blood vessel walls (Fig. 7F,G). We also detected thrombus formation into dermal blood vessels after LiRecDT7 inoculation (Fig. 7H). Thus, the recombinant toxin LiRecDT7 is functional and a member of the family of dermonecrotic toxins.

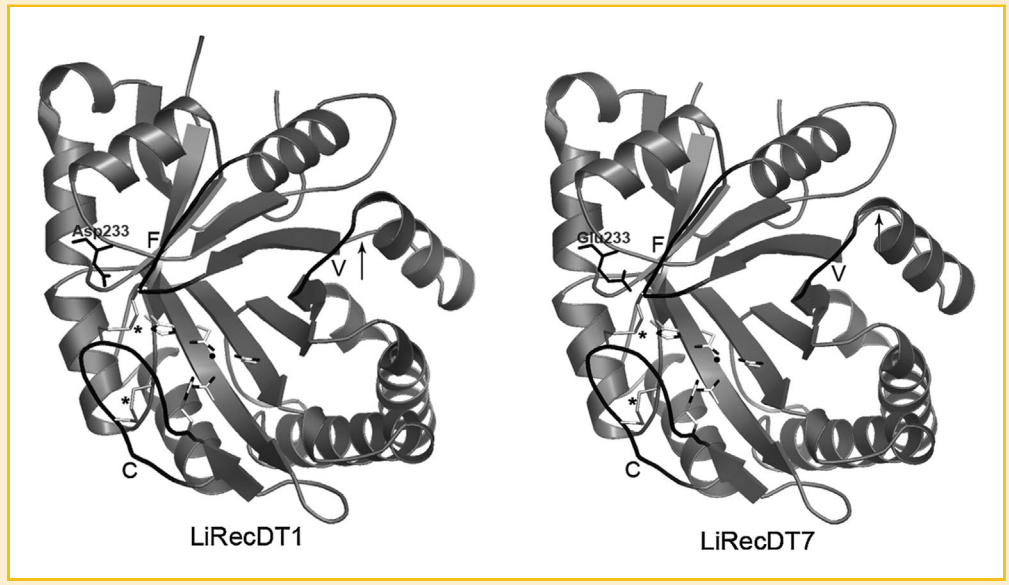

Fig. 4. Molecular modeling of LiRecDT1 and LiRecDT7. We used beta SWISS-MODEL to build a prediction of the three-dimensional structure of LiRecDT1 and LiRecDT7. Asp 233 in LiRecDT1 and Glu233 in LiRecDT7 are indicated. The black arrow indicates the significant variation of the loops in LiRecDT7 and LiRecDT1. The catalytic (C), variable (V), and flexible (F) loops are indicated. The asterisks indicate the disulfide bridges. The main amino acid residues involved in the coordination of the Mg^{2+} (represented by a sphere) in the catalytic site are highlighted (His12, Asp 32, Glu34, and His47).

RECOMBINANT TOXIN LiRecDT7 INCREASED VASCULAR PERMEABILITY

The data obtained from previous literature reported that brown spider phospholipase-D toxins increase vessel permeability [da Silveira et al., 2006, 2007c]. To examine whether LiRecDT7 could change vessel integrity and permeability in vivo, purified recombinant LiRecDT7, along with the appropriate negative control (PBS) and a positive control (LiRecDT1), was injected into the skin of mice that had been previously blue dye-perfused (Miles assay). The LiRecDT7 injection induced increased Evans blue extravasation compared with the negative control. Nevertheless, LiRecDT7 showed a lower activity compared with that of LiRecDT1 (Fig. 8).

DISCUSSION

Based on its ability to hydrolyze the phospholipid sphingomyelin into choline and C1P, previous studies have characterized brown spider venom phospholipase, also called "dermonecrotic toxin," as a sphingomyelinase-D molecule [da Silva et al., 2004]. However, the recent results obtained from lipid biochemical research have suggested that this toxin is a phospholipase-D enzyme because it degrades not only sphingophospholipids but also glycerophospholipids, generating C1P or lysophosphatidic acid (LPA) [Lee and Lynch, 2005; Chaim et al., 2011b; Chaves-Moreira et al., 2011]. It has been postulated that by degrading phospholipids and generating important lipid mediators, such as C1P or LPA, brown spider venom phospholipase-D toxin activates signaling pathways in different cells causing pathophysiological changes, such as inflammatory response, platelet aggregation, increased blood vessel permeability, hemolysis, and nephrotoxicity [Chaim et al., 2011b; Chaves-Moreira et al., 2011; Wille et al., 2013].

Currently, the idea of a family of similar molecules of phospholipase-D toxins in brown spider venom species is evident. This hypothesis was first suggested based on a biochemical characterization of *L. reclusa* venom, which contains four phospholipase-D isoforms [Futrell, 1992]. Additional studies revealed antigenic cross-reactivity for phospholipases-D from different brown spider venoms, including *L. gaucho*, *L. laeta*, and *L. intermedia* [Barbaro et al., 1996]. The biochemical and immunological analyses of *L. deserta* and *L. reclusa* venoms showed antigenic cross-reactivity and biochemical homologies (amino acid composition) for phospholipase-D toxins [Gomes et al., 2011]. Two phospholipase-D-like toxins were described in *L. gaucho* [Cunha et al., 2003] and four toxins were described in *L. boneti* venom [Ramos-Cerrillo et al., 2004]. Through proteomic analysis, several phospholipase-D isoforms were identified in *L. gaucho* venom [Machado et al., 2005], thereby confirming the idea that these molecules belong to a family of related toxins. Through molecular biology studies, the concept of an intra- and inter-species family of brown spider venom phospholipase-D was further confirmed by the cloning and expression of phospholipase-D toxins from a variety of *Loxosceles* spiders. Binford et al. [2005] reported three cDNA sequences for phospholipase-D toxins identified in *L. arizonica*. Chaim et al. [2006], da Silveira et al. [2006, 2007c], and Appel et al. [2008] cloned, expressed, and identified differential functionality for six related phospholipase-D molecules using a cDNA library obtained from the venom gland of *L. intermedia*. The transcriptional profile of the *L. intermedia* venom gland obtained through the construction of a wide cDNA library showed that members of phospholipase-D family represent 20.2% of the total

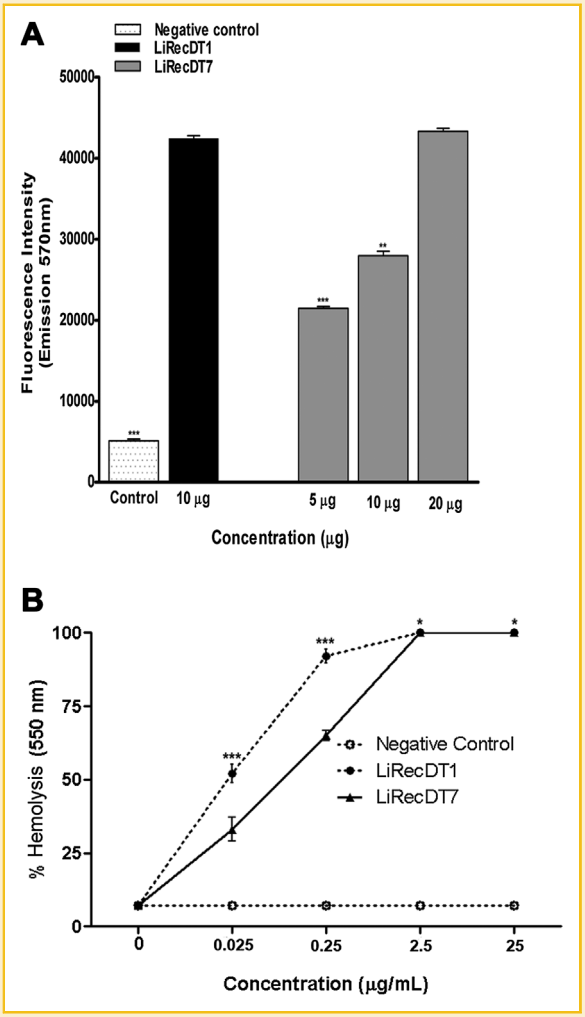

Fig. 5. Sphingomyelinase-D and hemolytic activities of LiRecDT7. A: Sphingomyelinase-D activities of recombinant dermonecrotic toxins LiRecDT1 and LiRecDT7 were evaluated using an Amplex Red Assay Kit at 37°C for 1 h, and the product of the reaction was determined at 550 nm. PBS was used as a negative control. Reactions used 5, 10, and 20 μg of LiRecDT7 and 10 μg of LiRecDT1 ($n = 5$). The means ± standard errors are shown, with significance levels $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ comparing activities of LiRecDT1 and LiRecDT7. B: Human erythrocytes suspended in TBS were incubated with different concentrations of LiRecDT7, or in the absence of toxin (negative control), for 24 h at 37°C. The absorbance values of the supernatants were measured at 550 nm, and the percentage of hemolysis was determined using the absorbance values induced with 0.1% (v/v) Triton X-100 as 100% hemolysis (positive control). The results represent the means of five experiments ± SEM. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

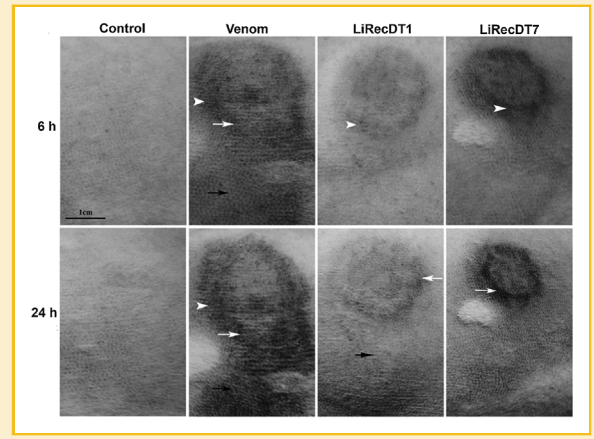

Fig. 6. Macroscopic and histological changes in rabbit skin exposed to whole venom and the recombinant toxins LiRecDT1, LiRecDT7 and negative control recombinant—Green Fluorescent Protein (GFP). Macroscopic visualization of dermonecrosis in rabbits intradermally injected with 10 μg of whole venom and the purified toxins LiRecDT1 and LiRecDT7. Lesions were observed at 6 and 24 h after the injections. The arrowhead indicates a hemorrhagic area surrounding the lesion. The white arrow indicates a necrotic area. The black arrows indicate gravitational spreading of lesions.

toxin-encoding transcripts [Gremski et al., 2010]. Brown spider venom toxins that have phospholipase-D activity are currently grouped into a family. It was postulated that the noxious effects induced by *Loxosceles* species crude venom might reflect the synergism among these related phospholipase-D toxins [Kalapothakis et al., 2007] and other components of venom, as the venom is a complex mixture containing additional constituents, such as insecticidal peptides, astacin-like metalloproteases, neurotoxins, serine proteases, venom allergen, translationally controlled tumor protein, hyaluronidases, serine protease inhibitors, and other components [Gremski et al., 2010].

Herein, we described the cloning, heterologous expression, affinity purification, and the functionality of a novel phospholipase-D "dermonecrotic toxin" family member from brown spider venom, strengthening the proteomic, immunological, and molecular biology data previously reported. The recombinant toxin identified was designated LiRecDT7 (GenBank Accession no. KC237286). The name is a reference to other previously identified brown spider (*L. intermedia*) venom phospholipase-D isoforms (LiRecDT1, LiRecDT2, LiRecDT3, LiRecDT4, LiRecDT5, and LiRecDT6) [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Appel et al., 2008].

Initially, our experimental strategy for cloning this novel member of brown spider venom phospholipase-D toxin cDNA was based on the random sequence analysis obtained from a cDNA library of *L. intermedia* venom gland [Gremski et al., 2010] using a BLAST search for similarities with previous cloned phospholipase-D toxins and 5′ RACE amplification to obtain the complete cDNA sequence.

The complete sequence of this novel brown spider phospholipase-D includes a signal peptide and a mature protein with high similarity to other previously reported brown spider venom phospholipase-D molecules [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Kalapothakis et al., 2007; Appel et al., 2008]. The deduced LiRecDT7 protein displayed an amino acid sequence identity of 63% compared with LiRecDT1. The similarities between LiRecDT7 and the other isoforms of *L. intermedia* varied showing the highest similarity with LiRecDT6 (71%) and the lowest similarity with LiRecDT3 (45%). Compared with phospholipase-D toxins from other *Loxosceles* species, the highest sequence similarities to LiRecDT7 were observed for sphingomyelinase-D alphaIV2 of *L. hirsuta* (ACN48948—91% amino acid identity). Binford et al. [2009] showed that recent gene duplications are apparent in groups of closely related species, which is

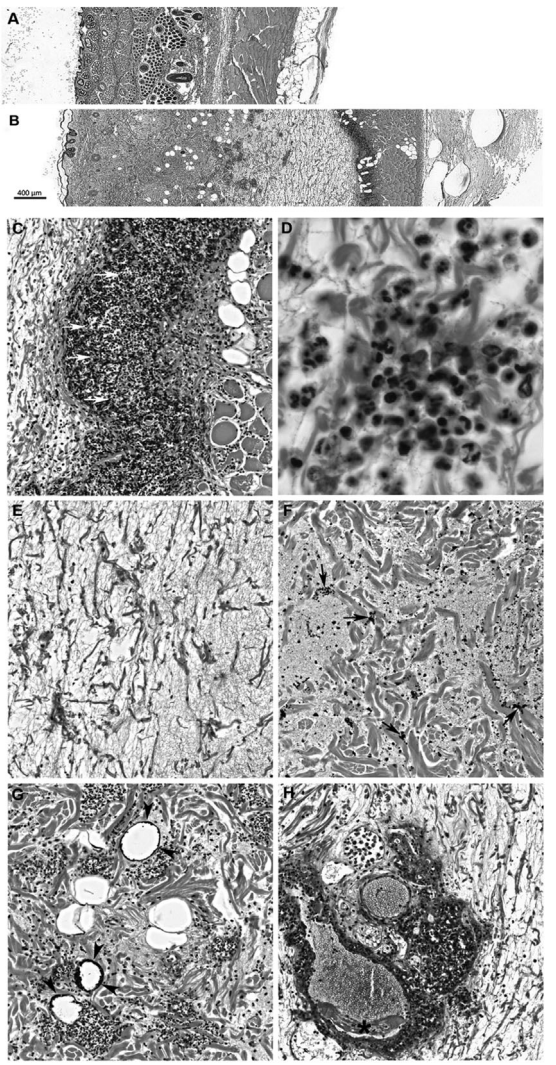

Fig. 7. Histopathology features of rabbits' skin 24 h following LiRecDT7 exposure. Light microscopy analysis of sections of dermonecrotic lesions stained with H&E. A,B: Panoramic views of sections of negative control recombinant—green fluorescent protein (GFP)—and LiRecDT7, respectively, clearly demonstrating the edema triggered by the recombinant toxin. At this stage, there was a massive inflammatory response (white arrows) (C), with the presence of neutrophils in the dermis (D), the disorganization of collagen fibers (E), areas of necrosis (black arrows; F), including the degeneration of blood vessel walls (black arrowheads; G), and thrombus formation into dermal blood vessels (asterisk; H). Magnification of panoramic views: 15×; (C,E–H) 100×; (D) 630×.

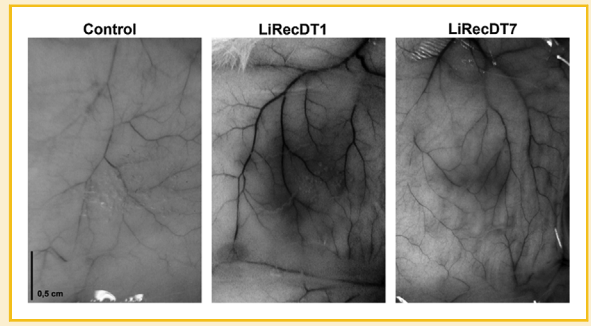

Fig. 8. Effect of LiRecDT7 on vascular permeability of skin vessels. The mice were administered with intradermal injections (10 μg) of LiRecDT7, LiRecDT1, and PBS (control for baseline permeability level; n = 5 per treatment). The photographs show increased dye leakage after recombinant toxin LiRecDT1 and LiRecDT7 exposure compared with minimal vessel permeability due to the negative control PBS.

the case for *L. intermedia* and *L. hirsuta*, members of the *spadicea* group. Thus, the high similarity observed between LiRecDT7 and sphingomyelinase-D alphaVI2 certainly reflects the closeness among the species. Some authors argue that to mantain effectiveness against preys and predators, the genes encoding venom peptides and proteins have undergone multiple duplication events. The duplicated genes, in turn, acquire related or even novel functions through adaptive evolution [Ma et al., 2012].

Moreover, LiRecDT7 contains the conserved amino acid residues involved in catalysis or metal ion coordination, which are important for phospholipase-D activity [Murakami et al., 2006], except for a punctual and conservative substitution at position 233 in the catalytic site. While all *L. intermedia* phospholipase-D isoforms contain a conserved an Asp residue in this position, LiRecDT7 has a Glu (D233E).

Structural analyses of LiRecDT1 show that the catalytic site of this enzyme is formed by two histidines at positions 12 and 47 and an Mg^{2+} ion. This catalytic site is hexacoordinated by Glu32, Asp34, and Asp91, a water molecule and two PEG4 oxygens [de Giuseppe et al., 2011]. This ion coordination is stabilized through hydrogen bonds formed by Asp34, Asp52, Trp230, Asp233, Asn252, and Gly480 [Murakami et al., 2005, 2006; de Andrade et al., 2006; de Giuseppe et al., 2011].

Asp is frequently involved in the formation of hydrogen bonds that assist in stabilizing protein structures. This residue might also be involved in active or binding sites for interactions with other proteins. Glu is also a charged amino acid. However, Asp has a less bulky side chain and is less flexible when located in the interior of a protein. These properties confer a slight preference for the inclusion of Asp in protein active sites, as is observed with the Asp233 residue in *Loxosceles* phospholipases-D, which is highly conserved. This phenomenon has also been observed in the classic example of the active site of serine proteases, whose mechanism is based in the amino acid triad Asp-His-Ser [Betts and Russell, 2003]. In this context, the substitution of Asp for Glu (Asp → Glu) is quite rare, although it is possible that Glu in the position 233 might play a similar role stabilizing the ion coordination structure of LiRecDT7, consistent with the observed biochemical and biological properties of this toxin.

Several other structural and biochemical characteristics provide confirmation of LiRecDT7 as a member of the brown spider phospholipase-D venom toxin family. First, the molecular mass of LiRecDT7 (36 kDa) and the calculated isoelectric point from the deduced amino acid sequence of the mature protein (pI 5.94) are similar to those described for other phospholipase-D members (ranging from 31 to 33 kDa and pI 5.3 to 8.7) [Kalapothakis et al., 2007]. Using immunological approaches, LiRecDT7 cross-reacted with antibodies against LiRecDT1, confirming antigenic homology

with LiRecDT1. Moreover, antibodies for crude venom toxins also cross-reacted with recombinant LiRecDT7, demonstrating that the structures of crude venom proteins are similar to LiRecDT7 and that LiRecDT7 shows sequence/epitope similarities with native venom toxins. Taken together, these results demonstrate that the phospholipase-D epitopes are strong antigenic determinants.

Full length LiRecDT7 was heterogeneously expressed in *E. coli* SHuffle T7 Express *lysY* cells and purified using single step affinity chromatography. LiRecDT7 was eluted in a pure form as visualized through SDS–PAGE. Using this approach, it was possible to obtain purified material for biochemical and functional analyses.

The native brown spider venom phospholipase-D toxins and previously described LiRecDT1 to 6 isoforms [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Appel et al., 2008] exhibit sphingomyelinase-D activity. As expected, the amino acid alignment and similarity analysis demonstrated the homology of LiRecDT7 with other brown spider phospholipase-D isoforms, and LiRecDT7 demonstrated sphingomyelinase-D activity through the generation of choline in a concentration-dependent manner. The substitution of the Asp residue with Glu at position 233 in the catalytic site did not abolish the sphingomyelinase-D activity of isoform LiRecDT7, although a reduction in the enzymatic activity was observed compared with LiRecDT1. Nevertheless, these results confirm the biochemical nature of LiRecDT7 as a sphingomyelinase-D and strongly suggest functionality for this LiRecDT isoform. However, with our results, it is not possible to infer whether this novel isoform is able to hydrolyze other phospholipids as observed for other members of this family [Chaim et al., 2011b]. Further studies specifically focusing on these activities of LiRecDT7 are needed to identify such aspects. As previously reported, all pathophysiological events triggered by brown spider venom recombinant isoforms, such as nephrotoxicity, dermonecrosis, inflammatory response, mice lethality and hemolysis are dependent on phospholipase-D catalysis [Kusma et al., 2008; Paludo et al., 2009; Chaim et al., 2011a; Chaves-Moreira et al., 2011].

The data obtained from the literature have described brown spider venom phospholipases-D as remarkable inducers of necrotic skin lesions and inflammatory response. Due to these effects, brown spider venom phospholipase-D toxins are also referred to as "dermonecrotic toxins." Indeed, the hallmark of accidents following brown spider bites is dermonecrosis with gravitational spreading surrounding the bite site; thus, loxoscelism is also referred to as necrotic arachnidism or gangrenous arachnidism [da Silva et al., 2004; Senff-Ribeiro et al., 2008]. Skin lesions triggered by brown spider phospholipase-D toxins are the consequence of a massive inflammatory response observed in the epidermis and dermis. This event is histopathologically referred to as "aseptic coagulative necrosis" and is a consequence of dysregulated endothelial cell-dependent neutrophil activation [Ospedal et al., 2002]. As previously described for other LiRecDT isoforms, LiRecDT7 demonstrated necrotic and inflammatory activities, providing further evidence of the activity of this toxin. LiRecDT7 induced noxious responses upon injection in rabbit skin (a macroscopic lesion), with swelling, erythema, hemorrhage, ischemia, and necrosis. Histological studies of samples collected from macroscopic lesions at 24 h after toxin exposure provided additional evidence for LiRecDT7 as an inducer of the inflammatory response. The observed histopathological changes included diffuse dermal edema, proteinaceous exudation and a massive and diffuse aggregation of leukocytes into the dermis. In addition, LiRecDT7 induced tissue necrosis, including degeneration of blood vessel walls. The novel phospholipase-D isoform also triggered thrombus formation into dermal blood vessels. These events are consistent with the literature data and resemble the observations reported after crude venom exposure [Veiga et al., 2001; Ospedal et al., 2002; da Silva et al., 2004].

Furthermore, the functionality of LiRecDT7 was also supported through the observed increase in capillary permeability in mice after treatment with purified recombinant toxin. This event has been described in *L. intermedia* whole venom, native brown spider venom phospholipase-D toxins and other LiRecDT isoforms [da Silva et al., 2004; da Silveira et al., 2007c; Ribeiro et al., 2007; Appel et al., 2008]. Notably, the increase in blood vessel permeability triggered by LiRecDT1 was more prominent than that induced by LiRecDT7. As described for other LiRecDTs, this ability is associated with the level of enzymatic activity of the isoform, as observed for LiRecDT3 and LiRecDT5, which show lower sphingomyelinase-D activity than LiRecDT1 and also trigger a less prominent increase in blood vessel permeability [da Silveira et al., 2006, 2007c]. Apparently, through the induction of inflammatory response and leukocyte infiltration into the dermis of mice [Sunderkotter et al., 2001], phospholipase-D toxins increase vessel permeability. In addition, a direct phospholipase-D effect upon endothelia, inducing endothelial cell activation and cytotoxicity, as previously reported for brown spider crude venom [Veiga et al., 2001; Zanetti et al., 2002; Paludo et al., 2009] increases vessel permeability through a disruption of the blood vessel wall.

LiRecDT7 also induced the direct lysis of human erythrocytes. The hemolytic effect of brown spider venom has been demonstrated through the clinical and laboratory features observed in accident victims. These features include hematuria, hemoglobinuria, elevated creatine kinase levels, proteinuria and shock [Kusma et al., 2008]. We have previously described the direct hemolytic effect of LiRecDT1 upon erythrocytes of different animal sources. This activity is not dependent on the ABO or Rhesus systems, but rather is dependent on the animal species, as human, sheep, and rabbit erythrocytes were lysed, but erythrocytes from horses were less severely damaged after toxin treatment, and these results depend upon the membrane composition of the cells. This event is also dependent on the catalytic activity of the phospholipase-D, as a site-directed mutation in the catalytic site (H12A) completely abolished the hemolytic effect of the mutated isoform LiRecDT1H12A [Chaves-Moreira et al., 2009]. The results described herein using LiRecDT7, demonstrate the direct hemolytic effect of this recombinant phospholipase-D on human erythrocytes. Toxin-dependent hemolysis occurs in a concentration-dependent manner, supporting the specificity of this effect.

Based on the crystallography results obtained using recombinant phospholipase-D toxin isoforms from *L. laeta* and *L. intermedia* venom and the structural and sequence alignment comparison data [Murakami et al., 2006; de Giuseppe et al., 2011], a classification system was proposed, which considers the phospholipase-D activity of these toxins. According to the authors, brown spider venom dermonecrotic toxins can be divided into two categories: class I molecules, containing a single intrachain disulfide bond and one

extended hydrophobic loop (*L. laeta* isoform) and class II molecules, containing an additional disulfide bond linking the catalytic loop to a second flexible loop. The class II phospholipases can be subdivided into classes IIa and IIb according to their ability, or lack thereof, to hydrolyze sphingomyelin, respectively. Based on this classification, LiRecDT7 belongs to class IIa, as this isoform possesses two putative disulfide bonds (Cys51–Cys57 and Cys53–Cys201) and is able to hydrolyze sphingomyelin.

The molecular modeling data also support the classification of LiRecDT7 as a class II sphingomyelinase-D, as it revealed the presence of two disulfide bridges in the putative structure of this novel isoform, as observed in LiRecDT1 model. The 3D modeling of LiRecDT7 also demonstrated that the structural basis for Mg^{2+} ion coordination and the two catalytic histidine residues (His12 and His47), which play key roles in the active-site pocket of spider venom phospholipases-D, is maintained compared with LiRecDT1.

In LiRecDT1, these two catalytic histidine residues (His12 and His47), are supported through a network of hydrogen bonds between Asp34, Asp52, Trp230, Asp233, and Asn252. Because LiRecDT7 possesses a substitution of Asp233 to Glu233, this residue is highlighted in the 3D modeling of both isoforms (LiRecDT1 and LiRecDT7) to illustrate this natural mutation. It is clear that the side chain of Glu in LiRecDT7 is bulkier than the side chain of Asp in LiRecDT1. There is also an obvious variation in the positioning of the side chain of these residues, as Glu in LiRecDT7 protrudes toward the flexible loop. It is not possible to infer whether these differences affect the network of hydrogen bonds between this residue and the two catalytic histidine residues. Indeed, this natural mutation did not abolish the enzymatic and biological actions of this novel isoform. The molecular modeling of LiRecDT7 also revealed that one of the loops, located near the variable loop, is more prominent (directed toward the surface of the molecule) compared with the same loop in LiRecDT1. When comparing the primary structure of this specific region, we observed the presence of lysine (207), Asp (208), arginine (209) and Glu (210) residues in LiRecDT7. These amino acids generally prefer to reside on the surface of the protein. Lysine and arginine are also frequently involved in *salt-bridges* where they pair with a negatively charged Asp or Glu to stabilize hydrogen bonds that are important for protein stability [Betts and Russell, 2003]. These features might explain the prominent configuration of this loop in LiRecDT7. In addition, as shown in Figure 2, LiRecDT1 has only two amino acid residues in this region. Thus, the presence of two additional residues in this region of LiRecDT7 might also explain the presence of a longer loop in this isoform. Moreover, Figure 2 also shows that the only isoform containing the four conserved amino acid residues in this region is LiRecDT6, although it is not possible to infer a direct relation concerning their structures based on this observation.

The results presented herein, demonstrate that LiRecDT7 possesses an intermediate ability for sphingomyelin hydrolysis and biological activities compared with LiRecDT1. A putative explanation for the differences described could be inferred from a mutation in the catalytic site (D233E) that, despite involving amino acids from the same characteristics (negative charged residues), in some way could destabilize and disorganize the catalytic site, affecting enzyme/substrate interactions in the catalytic cleft as the Glu residue contains an additional CH_2 group. However, it is not possible to infer that D233E substitution alone is responsible for the observed differences, as some members of the phospholipase-D family in *L. intermedia* exhibit lower sphingomyelinase-D activity, even with all amino acids of the catalytic site conserved (e.g., LiRecDT3, LiRecDT4 and LiRecDT5) [da Silveira et al., 2006, 2007c]. As observed for these mentioned isoforms, LiRecDT7 also possesses several substitutions in the amino acid residues neighboring the catalytic site. As previously proposed [da Silveira et al., 2006] these residues might be involved in the stabilization and organization of the catalytic site or even in the synergistic domains of these toxins. Thus, in addition to the specific substitution present in LiRecDT7, these differences might also explain the variations in functionality between these isoforms.

Venom toxin molecules have recently been used to investigate molecular and cellular mechanisms, as models for the design of novel drugs or even for diagnostic or therapeutic uses [Senff-Ribeiro et al., 2008; Chaim et al., 2011a; Horta et al., 2013]. The development of a novel recombinant *Loxosceles* phospholipase-D toxin can provide an agonist molecule as an additional tool to study the inflammatory response or to design and identify antagonist molecules using co-crystallization techniques and X-ray diffraction procedures. Additionally, this novel phospholipase-D can be used as a tool in biochemical lipid research protocols or as a recombinant antigen for serum therapy applications.

In summary, we have identified a novel brown spider venom phospholipase-D "dermonecrotic toxin" family member. This molecule, referred to as LiRecDT7, was cloned, heterogeneously expressed and purified. LiRecDT7 degraded sphingomyelin to generate choline in a concentration-dependent manner, induced dermonecrosis in rabbit skin and increased inflammation in the dermis of these animals. LiRecDT7 also increased vascular permeability in mice and induced direct hemolysis in human erythrocytes. Together, these results provide new insights into loxoscelism, contribute to the understanding of venom phospholipase-D and present the possibility of applying venom toxins as biotechnological tools for lipid research.

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Review

Recent advances in the understanding of brown spider venoms: From the biology of spiders to the molecular mechanisms of toxins

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ABSTRACT

The *Loxosceles* genus spiders (the brown spiders) are encountered in all the continents, and the clinical manifestations following spider bites include skin necrosis with gravitational lesion spreading and occasional systemic manifestations, such as intravascular hemolysis, thrombocytopenia and acute renal failure. Brown spider venoms are complex mixtures of toxins especially enriched in three molecular families: the phospholipases D, astacin-like metalloproteases and Inhibitor Cystine Knot (ICK) peptides. Other toxins with low level of expression also present in the venom include the serine proteases, serine protease inhibitors, hyaluronidases, allergen factors and translationally controlled tumor protein (TCTP). The mechanisms by which the *Loxosceles* venoms act and exert their noxious effects are not fully understood. Except for the brown spider venom phospholipase D, which causes dermonecrosis, hemolysis, thrombocytopenia and renal failure, the pathological activities of the other venom toxins remain unclear. The objective of the present review is to provide insights into the brown spider venoms and loxoscelism based on recent results. These insights include the biology of brown spiders, the clinical features of loxoscelism and the diagnosis and therapy of brown spider bites. Regarding the brown spider venom, this review includes a description of the novel toxins **revealed by molecular biology and proteomics techniques**, the data regarding three-dimensional toxin structures, and the mechanism of action of these molecules. Finally, the biotechnological applications of the

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venom components, especially for those toxins reported as recombinant molecules, and the challenges for future study are discussed.

1. Introduction

Spider bites of the genus *Loxosceles* have been associated with clinical manifestations characterized by dermonecrosis with gravitational spreading near the lesion site and, to a lesser extent, with systemic toxicity, such as the hematological disturbances of intravascular hemolysis, thrombocytopenia, disseminated intravascular coagulation and acute renal failure. The spiders of the genus *Loxosceles* are encountered in all continents and different species have been reported in North America, Central America, South America, Europe, Africa, the Middle East, Oceania and Asia. Five species (*Loxosceles rufescens*, *Loxosceles laeta*, *Loxosceles intermedia*, *Loxosceles gaucho* and *Loxosceles reclusa*) are responsible for most cases of human envenomation by the *Loxosceles* genus, and the pathology and clinical features of these spider bites are termed loxoscelism. Nevertheless, sporadic accidents caused by others *Loxosceles* species (*Loxosceles deserta*, *Loxosceles arizonica*, *Loxosceles anomala*, *Loxosceles similis*, for instance) have been described around the world (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006; Bucaretchi et al., 2010; Isbister and Fan, 2011; Chatzaki et al., 2012).

The venom of the brown spider is a colorless and crystalline liquid, formed by a complex mixture of toxins enriched in proteins, glycoproteins and low molecular mass peptides with a predominance of toxins in the range of 5–40 kDa (Sams et al., 2001; da Silveira et al., 2002; da Silva et al., 2004; Machado et al., 2005; Swanson and Vetter, 2006). Previously published data have described highly expressed molecules, such as phospholipases D, astacin-like metalloproteases and low molecular mass insecticidal peptides (characterized as ICK peptides) (da Silva et al., 2004; de Castro et al., 2004; da Silveira et al., 2007a; Gremski et al., 2010; Matsubara et al., 2013). Together, these three toxin classes comprise the majority of the toxin-encoding transcripts in the venom gland of *L. intermedia* (approximately 95%) (Gremski et al., 2010). Other toxins with low level of expression, such as hyaluronidase, serine proteases, serine protease inhibitors, venom allergens and a TCTP family member, have been identified in the venom (de Castro et al., 2004; Barbaro et al., 2005; Gremski et al., 2010; Sade et al., 2012; Ferrer et al., 2013).

Regarding the hemolymph of brown spiders, no current description of its molecular composition, biological activities or even physical properties exists. Nevertheless, the potential of the hemolymph to contain natural inhibitors, antifungals or antibiotics is significant and is based on the environment in which the spiders live and the toxins that the brown spiders produce.

In recent years, knowledge of brown spider venoms has advanced significantly through the use of molecular biology techniques. The transcriptomes of the *L. laeta* and *L. intermedia* venom glands were analyzed for the first time, and this analysis confirmed the complexity of *Loxosceles* venoms (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). Additionally, by using recombinant DNA technology, heterologous toxins have been expressed and purified. These advances obtained with the recombinant *Loxosceles* venom toxins helped to overcome the obstacles to studying spider toxins, such as the low venom volumes and the difficulty in the purification of native toxins from crude venom. Moreover, these recent advances have enabled researchers to utilize cell biology, biochemistry, immunology, pharmacology and crystallography to clarify the general characteristics of *Loxosceles* toxins.

By using proteomics approaches, such as **two-dimensional gel** electrophoresis, N-terminal amino acid sequencing and mass spectrometry, the venoms of *L. gaucho and L. intermedia* have been investigated (Machado et al., 2005; dos Santos et al., 2009).

Recent advances in protein purification techniques, the application of different models for the synthesis of recombinant toxins, the modeling of domains, the knowledge of the binding or catalytic sites of the toxins of interest and, finally, the availability of the varied cellular and animal models for assessing the products obtained have created possibilities for a broad putative biotechnological use of brown spider venom toxins as important tools (Senff-Ribeiro et al., 2008; Gremski et al., 2010; Chaim et al., 2011a; Wille et al., 2013).

This review focuses on the most recent literature examining brown spider venom and loxoscelism. It discusses the molecular biology techniques used for the characterization of the molecules in brown spider venom, such as transcriptome projects, as well as the production and evaluation of recombinant toxins. Furthermore, it also describes the recent advances in the molecular complexity of venom toxins, and finally, it lists the putative biotechnological applications of several brown spider venom components.

2. Biology of brown spiders

The spiders of the *Loxosceles* genus belong to the *Sicariidae* family, sub-order *Labidognatha*, order *Araneida*, class *Arachnida*, and phylum *Arthropoda* (Platnick, 2013) (Fig. 1). In North America, this genus is popularly referred as recluse spiders, brown recluse spiders and violin spiders (fiddle back), due to a characteristic violin shape on the dorsal surface of the spider's cephalothorax. In South America, they are called brown spiders (da Silva et al., 2004; Vetter, 2008). The name *Loxosceles* means "*slanted legs*" because of the way the spider positions its legs at rest (Vetter, 2008). **Approximately 130 species** of the *Loxosceles* genus have been described and are extensively distributed worldwide (Platnick, 2013). The majority of these spiders are present in the Americas, West Indies and Africa, and some species

Fig. 1. Adult brown spiders. *Loxosceles gaucho* female (A) and male (E); *Loxosceles intermedia* female (B) and male (F); *Loxosceles laeta* female (C) and male (G). An adult brown spider and an ootheca (arrow) (D). The classic violin pattern (arrow) appears on the dorsal surface of the cephalothorax from *Loxosceles gaucho* adult spider (H). Photos are courtesy of Denise Maria Candido from the Instituto Butantan, São Paulo, Brazil. The colored figure refers to the on-line image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have been described in Mediterranean Europe and China (Binford et al., 2008). There is evidence that *Loxosceles* and *Sicarius* originated from a common sicariid ancestor on Western Gondwana, before the separation of the African and South American continents (Binford et al., 2008). Currently, in Brazil, 12 known species of spiders of this genus are present (Bertani et al., 2010; Gonçalves-de-Andrade et al., 2012).

They are small spiders, varying from 1 to 5 cm in length, including the legs. They exhibit sexual dimorphism, with females usually larger than males. The males have palps with modified tarsi with an additional structure specialized for the transfer of sperm, the spermophore (Gilbert and Raunio, 1997). The spiders of the *Loxosceles* genus possess six eyes arranged in non-touching pairs and a U-shaped pattern. This eye positioning has been described as the best means to identify brown spiders (da Silva et al., 2004; Appel et al., 2005; Vetter, 2008; Chaim et al., 2011a).

Brown spiders can survive several months without food or water and can withstand temperatures ranging from 8 to 43 °C (da Silva et al., 2004). The reported longevity for the *L. intermedia* is 1176 ± 478 days for females and 557 ± 87 days for males (Fischer and Vasconcellos-Neto, 2005a). They construct irregular webs that resemble cotton threads (da Silva et al., 2004). Other studies indicated the preference for dead prey (Sandidge, 2004; Fischer and Vasconcellos-Neto, 2005b), although this preference depends on the size and freshness of the live or dead prey (Cramer, 2008; Vetter, 2011a).

The hemolymph of arachnids and insects has many important functions. It participates in homeostatic processes (Ruppert et al., 2004) in the transport of hormones, enzymes and nutrients, as well as metabolic residues for excretion; in the animal's protection; and in the storage of water and lipids (Araújo, 2009).

Although spiders are arachnids and not insects, insecticides are effective in reducing the brown spider population. Many chemicals may not kill the spider but will disrupt the nervous system and other bodily functions (Sandidge and Hopwood, 2005). Lindane and chlordane are insecticides identified as effective, lethal substances to *Loxosceles* spiders, however, these products have carcinogenic effects and, therefore, are no longer used for spider population control (Navarro-Silva et al., 2010). The use of pyrethroids for spider population control has also been evaluated in the field and the laboratory by testing the susceptibility of *L. intermedia* specimens to this class of insecticides. In laboratory tests, microencapsulated lambda-cyhalothrin (ME lambda-cyhalothrin) demonstrated the highest toxicity. A field test confirmed these laboratory results, and the authors concluded that ME lambda-cyhalothrin would be useful in integrated pest management programs for *L. intermedia* (Navarro-Silva et al., 2010). Sandidge (2004) investigated the potential to biologically control *L. reclusa* using the natural arachnid fauna found in most homes. Three common web-building cosmopolitan spiders, *Achaearanea tepidariorum*, *Steatoda triangulosa* and *Pholcus phalangioides*, readily feed on brown recluse spiders and are deemed beneficial in the control of these populations; most importantly, they are relatively harmless to humans. Additionally, vacuum cleaner use in the home has been considered as an effective tool for the integrated management of *L. intermedia* and other spider populations (Ramires et al., 2007). Furthermore, tolerance to the presence of geckos at home, which are considered a natural predator of spiders, has also been considered a promising tool (Ramires and Fraguas, 2004).

3. Clinical features of loxoscelism

The number of loxoscelism cases worldwide is likely underestimated because most cases are not reported (da Silva et al., 2004; Hogan et al., 2004; Dyachenko et al., 2006; Abdulkader et al., 2008; Makris et al., 2009; Pippirs et al., 2009; Pernet et al., 2010; Bajin et al., 2011; Lane et al., 2011; Sanchez-Olivas et al., 2011; Huguet et al., 2012; Ribuffo et al., 2012). Notwithstanding their prevalence as an underreported condition, *Loxosceles* spider bites are considered a public health problem in countries such as Brazil, Chile and Peru because of their frequency and associated morbidity (da Silva et al., 2004; Hogan et al., 2004; Zambrano et al., 2005; Swanson and Vetter, 2006; de Souza et al., 2008; Manríquez and Silva, 2009; Vetter, 2009; Isbister and Fan, 2011; Malaque et al., 2011). As shown in Table 1, since 2001, Brazil has experienced a progressive increase in the number of reported loxoscelism cases. It is currently estimated that approximately 8000 spider bites occur annually, and most of them are reported in the southeastern and southern urban areas of Brazil. The increase in loxoscelism reports in recent years could be a consequence of an ecological imbalance caused by the deforestation and extinction of natural predators, climate change, and pest management practices, which results in the adaptation of spiders to the urban environment (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006; Isbister and Fan, 2011; Saupe et al., 2011; Vetter, 2011a).

Spiders displaced from their natural environment end up inhabiting the breaches, fissures and orifices of human houses. They also seek shelter in storage boxes or in the corners of cupboards and drawers. Accordingly, they can be found inside clothes, towels and bedclothes. As a result,

Table 1
Notifications of *Loxosceles* accidents occurred in Brazil from 2001 to 2012.

Year	Notifications	Deaths	% (deaths/notifications)
2012	7528	2	0.03
2011	8033	6	0.08
2010	7885	2	0.03
2009	8472	2	0.02
2008	7977	3	0.04
2007	9277	12	0.13
2006	7619	5	0.07
2005	7702	3	0.04
2004	8207	1	0.01
2003	7806	3	0.04
2002	6303	3	0.05
2001	5011	5	0.01

http://dtr2004.saude.gov.br/sinanweb/index.php?name=Tnet.
http://dtr2004.saude.gov.br/sinanweb/tabnet/dh?sinan/animaisp/bases/animaisbr.def.
Source: Brazilian Ministry of Health - SINAN/SVS:

most spider bites occur when the victims press the spider against their body; for that reason, victims are most frequently bitten on the trunk, thigh and arm. The fangs of the *Loxosceles* spider are small, and the venom is likely injected by intradermal injection. The spider bites mainly occur during the warmest seasons (spring and summer) (da Silva et al., 2004; Hogan et al., 2004; Isbister and Fan, 2011; Vetter, 2011b; Rader et al., 2012).

Loxosceles bites lead to a mild stinging with clinical signs and symptoms developing only several hours afterward; consequently, the bite is barely noticed, and the spider is rarely captured (~10% of cases) at the time of the bite. Therefore, the diagnosis of loxoscelism is usually presumptive and based on the clinical and epidemiological features presented by the patient at the time of hospital admission, which usually occurs from 12 to 24 h after the bite, when the skin damage is more prominent (da Silva et al., 2004; Hogan et al., 2004; Hubbard and James, 2011; Isbister and Fan, 2011). The classical clinical symptoms caused by *Loxosceles* spider bites are characterized by an intense inflammatory reaction at the bite site followed by local necrosis and can be classified as cutaneous loxoscelism (more than 70% of the cases). The cutaneous loxoscelism is characterized by initial local symptoms, such as burning-stinging, undefined pain, and edema. Most of the time, the patients seek help only several hours after the bite when the signs and symptoms become more severe, such as burning pain, edema, blister formation, erythema, ecchymosis, and paleness (called marble plaques) (Fig. 2 A–C). After several days, a necrotic area forms, which is followed by an ulcer of variable size that scabs over and frequently leaves a sharply defined area surrounded by the raised edges of healthy skin (Fig. 2 D). These necrotic wounds can take several weeks to heal. Other symptoms, such as a scarlatiniform or morbilliform rash, malaise, nausea, vomiting, a low-grade fever or headache, can also occur (da Silva et al., 2004; Hogan et al., 2004; Isbister and Fan, 2011).

Apart from the venom, many other factors associated with the spider (intra- and inter specific variations, the developmental stage and the amount of venom injected) or the patient (the adipose tissue at the bite site, the amount of tissue sphingomyelin, the patient's age and the individual genetic variance) can influence the severity of the bite (da Silva et al., 2004; Hogan et al., 2004; de Oliveira et al., 2005; Tambourgi et al., 2010). For instance, terminal circulation areas or adipose tissue are more sensitive to the venom's action, developing necrosis and severe tissue injury, which may require corrective plastic surgery. Despite the severity of the injury caused by *Loxosceles* venom at the bite site, the development of secondary infections is rare (Hogan et al., 2004; Abdulkader et al., 2008; Isbister and Fan, 2011; Malaque et al., 2011; Huguet et al., 2012; Ribuffo et al., 2012).

Severe cases can be classified as viscerocutaneous or systemic loxoscelism and range from 0.7% to 27% varying geographically or by the *Loxosceles* species responsible for the spider bite (Barbaro and Cardoso, 2003; Hogan et al., 2004; Abdulkader et al., 2008; Isbister and Fan, 2011). For instance, some data have demonstrated that viscerocutaneous loxoscelism has a higher prevalence in several countries, such as Chile (15.7%) and Peru (27.2%), as well as in Santa Catarina state/Brazil (13.1%), where *L. laeta* is found (da Silva et al., 2004; Hogan et al., 2004).

Systemic loxoscelism is characterized by hematuria, hemoglobinuria, jaundice, fever, nausea and disseminated intravascular coagulation (da Silva et al., 2004; Hogan et al., 2004; Isbister and Fan, 2011). Recently, Malaque et al. (2011) found that mild hemolysis is frequent in patients bitten by *L. gaucho* (present in one-third of the cases examined, including those classified as cutaneous loxoscelism). However, acute kidney injury occurred exclusively in patients with extensive hemolysis. Oliguria and dark urine, which can suggest extensive intravascular hemolysis or rhabdomyolysis, can result in acute renal failure, which is the primary cause of death associated with loxoscelism. Nevertheless, the level of mortality (Table 1) caused by *Loxosceles* spider bites is low (França et al., 2002; da Silva et al., 2004; Hogan et al., 2004; Abdulkader et al., 2008; de Souza et al., 2008; Isbister and Fan, 2011; Malaque et al., 2011). Although large case studies report systemic loxoscelism across all age groups, most cases are reported in children (Schenone et al., 2001; Hostetler et al., 2003; da Silva et al., 2004; Hogan et al., 2004; Elbahlawan et al., 2005; Hubbard and James, 2011; Isbister and Fan, 2011; Taskesen et al., 2011; Rosen et al., 2012).

4. Diagnosis and therapy

No consensus treatment for loxoscelism exists, and different therapies are used in different parts of the world. In some countries, the therapy is based on dapsone, antihistamines, analgesics and corticosteroids. However, other treatments, such as the use of steroids, surgical excision, hyperbaric oxygen therapy, and negative pressure wound therapy (vacuum-assisted closure), have been employed in an attempt to remedy the effects of envenomation (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2005; Tutrone et al., 2005; Swanson and Vetter, 2006; Abdulkader et al., 2008; Vetter and Isbister, 2008; Wong et al., 2009; Tambourgi et al., 2010; Andersen et al., 2011; Hubbard and James, 2011; Isbister and Fan, 2011). Another treatment option is the administration of antivenoms against the *Loxosceles* venoms, which are available in Brazil, Argentina and Mexico [horse-derived F(ab′)2 antivenoms] and Peru (whole IgG antivenom) (Isbister et al., 2003; da Silva et al., 2004; Hogan et al., 2004; Pauli et al., 2009; Isbister and Fan, 2011). The antivenom is administered intravenously, and the number of vials used varies according to the clinical severity of the envenomation (cutaneous loxoscelism) present at hospital admission and is administered to all patients with viscerocutaneous loxoscelism (Brasil, 2001).

The delay in seeking medical care by patients (approximately 24 h after the spider bite) can further contribute to the extension of local tissue damage at the bite site because the cutaneous necrosis and systemic clinical symptoms induced by the venom are irreversible and begin a few hours after envenomation (da Silva et al., 2004; Hogan et al., 2004). Accordingly, the type and effectiveness of the treatment are influenced by the amount of time between the spider bite and the diagnosis.

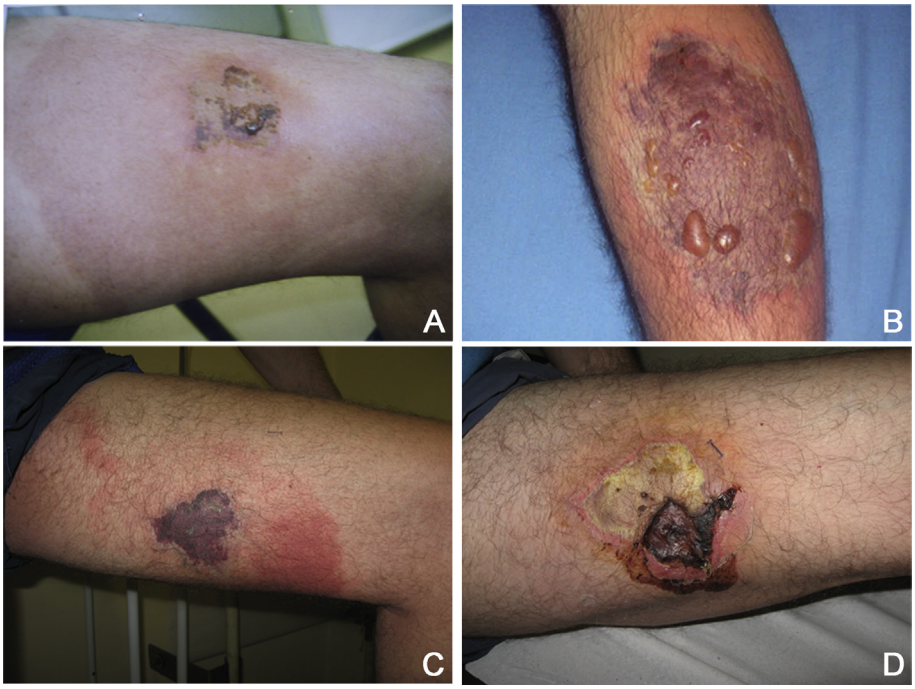

Fig. 2. Cutaneous loxoscelism. A – A female patient bitten on the thigh. The lesion (2 days post-bite) is characterized by edema and erythema, paleness and hemorrhagic areas (marble plaque about 14 cm in diameter), and blistering with hemorrhagic content. B – Male patient bitten on the calf. Two days after spider bite, the injury appeared with extensive bruising, serous blisters that progressed rapidly to hemorrhagic content and burning pain with the additional presence of a cutaneous rash, myalgia and dizziness. Twenty days after bite, the patient presented with desquamation at the injury site without ulcer formation. C and D – Male patient bitten on the inner left thigh while wearing clothes. Four days (C) after the bite, local damage is present and characterized by edema and erythema with ecchymotic, paleness and hemorrhagic areas (marble plaque) and the presence of necrosis 20 days after the bite (D). The presumptive cutaneous loxoscelism because the patient did not bring the spider for formal identification. [Photos are courtesy of Dr. Marlene Entres, Centro de Controle de Envenenamentos de Curitiba, Secretaria de Estado da Saúde, Paraná, Brazil (A, B), and Dr. Ceila M. S. Malaque, Hospital Vital Brazil – Instituto Butantan, São Paulo, Brazil (C, D)]. The colored figure refers to the on-line image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, the effectiveness of the treatments described in the international literature has been widely debated, and a definitive treatment has not yet been established (Isbister et al., 2003; da Silva et al., 2004; Hogan et al., 2004; Pauli et al., 2009; Isbister and Fan, 2011). The bite severity, however, can be estimated by laboratory tests and clinical characteristics, such as evidence of hemolysis (Malaque et al., 2011) and the presence of creatine kinase in the serum, which indicates rhabdomyolysis (França et al., 2002), that can determine the presence of a viscerocutaneous manifestation of envenomation. In these cases of systemic loxoscelism, vigorous hydration and urinary alkalinization should be established early to avoid pigment-induced renal failure (Hogan et al., 2004).

Currently, no commercial test is available to confirm loxoscelism, and most patients never see or capture the offending spider; consequently, the diagnosis is usually based on the clinical features presented by the patient. Moreover, the epidemiological information, such as the circumstances of the bite (sleeping, dressing, etc.), the site of the bite, and the timing of the injury progression (because the clinical signs and symptoms of *Loxosceles* envenomation occur slowly), can aid in the diagnosis of loxoscelism. There are many other medical causes of focal skin necrosis (as described previously by literature data) and laboratory tests can be helpful in determining the presence of these other diseases. In addition, an enzyme-linked immunosorbent assay has been used to detect the venom from patient lesions and sera or the circulating antibodies to the venom, but it is not in widespread clinical use (Gomez et al., 2001, 2002; da Silva et al., 2004; Hogan et al., 2004; Stoecker et al., 2006; Akdeniz et al., 2007; Stoecker et al., 2009).

5. Brown spider venom

Loxosceles spider venom is a colorless and crystalline liquid produced from two bulbous glands situated in the cephalothorax of the spider and flows through an inoculator apparatus composed of a pair of chelicerae (dos Santos et al., 2000; da Silveira et al., 2002; da Silva et al., 2004). Histological findings have revealed that these glands are made up of two adjacent layers of striated muscles fibers, one external and the other internal, in contact with an underlying basement membrane that separates the muscle cells from the secretory epithelium and use a holocrine secretion mechanism (dos Santos et al., 2000).

The volume of venom produced by *Loxosceles* spiders is generally on the order of a few microliters, and it contains approximately 20–200 μg of total protein (Binford and

Wells, 2003; da Silva et al., 2004; Senff-Ribeiro et al., 2008). The amount and the content of the venom produced depend on several factors associated with the spider specimen, including species, size, sex, nutritional state and age. Using SDS-PAGE analysis, de Oliveira et al. (2005) showed that significant variations occurred between the content of the *L. intermedia* and *L. laeta* venoms. These variations in the venom content can be enhanced by other differences in their biological activities, such as the more potent dermonecrotic activity (measured by the lesion size) of *L. laeta* venom compared with *L. intermedia* venom observed in rabbits. In addition, the lesions caused by venom from females were larger in area than those lesions caused by venom from males (de Oliveira et al., 2005). Through 2D electrophoresis (IEF and SDS-PAGE), the venom of *L. intermedia* has been found to be enriched mainly in two groups of proteins at 20–40 kDa and 2–5 kDa (Fig. 3).

In vivo experiments using a rabbit model have shown that *Loxosceles* spp. venoms are associated with the development of a characteristic dermonecrotic lesion with gravitational spreading and ecchymosis. Analyses of rabbit skin exposed to *Loxosceles* venoms shown the following characteristics: an initial edema under the dermis, an increased vascular permeability, an intravascular fibrin network deposition, the thrombosis of dermal blood vessels and the degeneration of the blood vessel walls as well as the infiltration and aggregation of inflammatory cells. At longer exposure times, myonecrosis of the myofibrils and leukocyte infiltration in the skeletal muscle occur. Finally, the destruction of epidermis integrity, massive hemorrhage and the necrosis of surrounding collagen near the epidermis are observed (Ospedal et al., 2002; Tavares et al., 2004; Pretel et al., 2005; Silvestre et al., 2005; Chatzaki et al., 2012). The ability of the venom of *Loxosceles* spiders to be lethal to mice has also been described. Mota and Barbaro (1995) reported this lethality in mice injected with *L. intermedia*, *L. gaucho* and *L. laeta* venoms, and the LD_{50}s determined were 0.48, 0.74 and 1.45 mg/kg, respectively. Appel et al. (2008) found 100% mortality of the mice tested at the concentrations of 50 and 100 μg/kg of *L. intermedia* venom after 16 h post-injection. Silvestre et al. (2005) found an LD_{50} of 0.32 mg/kg for *L. similis* venom, and Pretel et al. (2005) indicated an LD_{50} of 0.696 mg/kg for *Loxosceles adelaida* venom.

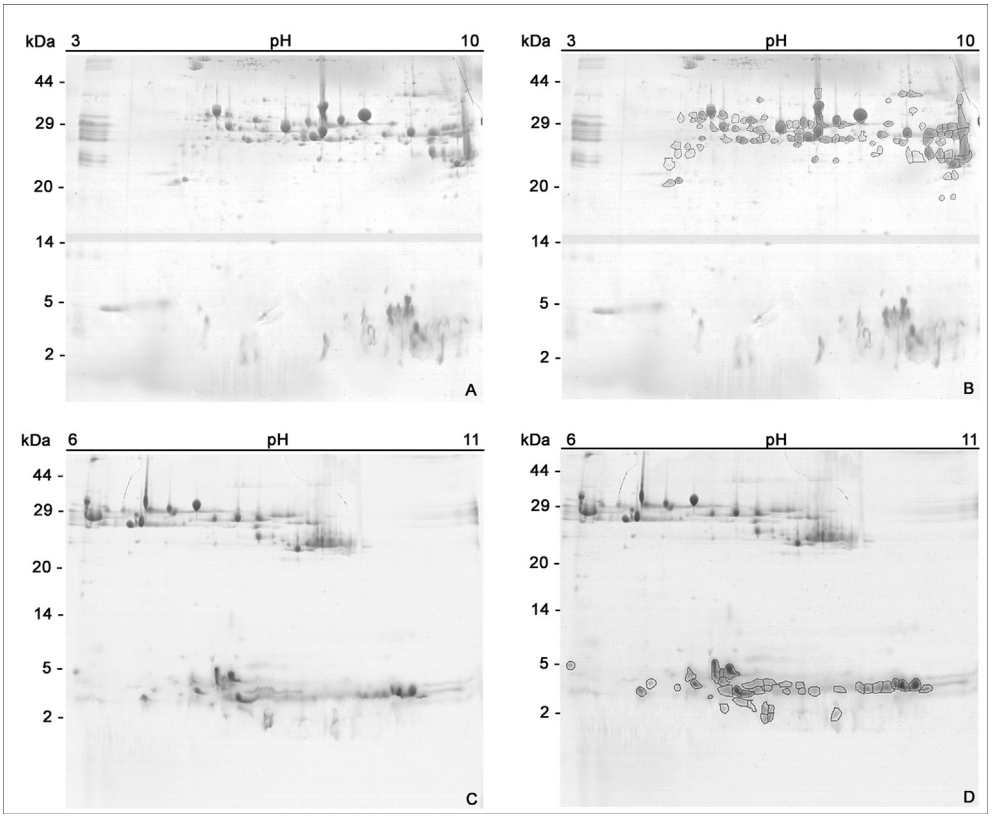

Fig. 3. Two-dimensional (2-DE) protein profile of *Loxosceles intermedia* venom. Samples (150 μg) of *Loxosceles intermedia* crude venom were separated by isoelectric focusing (IEF) on 13-cm immobilized pH gradient (IPG) gel strips with a pH linear range of 3–10 or a pH linear range of 6–11 (GE Healthcare, Piscataway, NJ, EUA) in the first dimension. The second dimension analysis was performed on a 20% polyacrylamide SDS-PAGE gel under reducing conditions. The 2-DE gels were stained with colloidal Coomassie Brilliant Blue. (A) Then, the gels were maintained in a 1% acetic acid solution and scanned using an ImageScanner III LabScan 6.0 (GE Healthcare). The detection of the gel spot and calculation of the isoelectric point (pI) and molecular mass (MM) were obtained using Image Master 2D Platinum software (GE Healthcare). (A) The protein profile of *L. intermedia* venom separated on a pH linear range of 3–10. (B) In total, 97 spots were detected in the 20–40 kDa region, and the spots with pI values from 4.6 to 9.8 are highlighted in circles. (C) The protein profile of *L. intermedia* venom separated on a pH linear range of 6–11. (D) 40 spots were detected in the 2–5 kDa region, and the spots with pI values from 6.1 to 10.4 are highlighted in circles.

The full venom content of the *Loxosceles* spiders is still under investigation; however, many studies have shown that its biochemical composition consists of a complex mixture of biologically active compounds, mainly proteins and peptides with toxic and/or enzymatic action (Veiga et al., 2000a; Gremski et al., 2010). HPLC analysis of whole venom of *L. intermedia* showed the presence of histamine. According results in a sufficient concentration to induce inflammatory responses (Paludo et al., 2009). Additionally, NMR spectroscopy and mass spectrometric analyses of *L. reclusa* crude venom pointed for the presence of sulfated guanosine derivatives as the major small-molecule components of the venom. Nevertheless, results were restricted to chemical analyses and data did not describe for a correlation with biological/pathological activities for these molecules (Schroeder et al., 2008).

In the following sections, the main identified and characterized molecules in the venom of *Loxosceles* spiders will be presented as well as the relevance of these toxins to the understanding of the envenomation process and potential biotechnological applications.

5.1. Proteomic analysis of brown spider venom

Proteomic analyses of brown spider venom are scarce and, in general, are focused on the phospholipase D family members. The first report using mass spectrometry for the identification of the proteins in *Loxosceles* spider venom was in 2003 (Binford and Wells, 2003). The aim of this study was to identify the spider phylogenetic groups with sphingomyelinase and to identify the evolutionary origin of this toxin. Venoms from distinct spiders were analyzed, including *L. laeta* and 9 other *Loxosceles* species from Africa and North America. Sphingomyelinase D (SMD) activity was identified in all surveyed *Loxosceles* species and in two *Sicarius* species (*Loxosceles* sister taxon), and the mass spectrometry analyses found several molecules corresponding to the known SMD size range of 31–35 kDa. The *Loxosceles* venom was first submitted to two-dimensional electrophoresis in 2004 by Luciano and colleagues. They demonstrated that *L. intermedia* venom is enriched in cationic and low molecular mass **proteins** (20–35 kDa). Shortly thereafter, the venoms from *L. adelaida* and *L. gaucho* were subjected to 2D electrophoresis analysis (Pretel et al., 2005). Although the toxins of these venoms displayed a similar distribution with regard to the molecular mass of proteins, only 40% of the components exhibited the same *pI* and molecular mass in the *L. adelaida* and *L. gaucho* venoms. *L. similis* venom was also analyzed by 2D gel electrophoresis and exhibited protein bands ranging from 28 to 112 kDa, and the *pI* values were between 4.0 and 7.0, which matched previous profiles of other *Loxosceles* species (Silvestre et al., 2005).

In 2005, the protein contents of the *L. gaucho*, *L. laeta* and *L. intermedia* venoms were analyzed using 2D electrophoresis. The protein profiles of these three different venoms were similar, possessing the majority of protein bands in the 30–35 kDa range. All *Loxosceles* species presented protein bands of a high molecular mass (45–94 kDa) and exhibited few proteins in the low molecular mass region (14–25 kDa) (Machado et al., 2005).

To identify the dermonecrotic proteins in *L. gaucho* venom, several protein bands present in the 30–35 kDa range after separation by 2D electrophoresis were analyzed using mass spectrometry de novo sequencing combined with N-terminal chemical sequencing. Only eight spots were identified as sphingomyelinase D (Machado et al., 2005). The low abundance of all other protein bands analyzed did not enable their identification. In addition, by LC-MS analysis, 11 distinct proteins were detected in the molecular mass range of the dermonecrotic toxins, suggesting that more isoforms of sphingomyelinase D could be present in *L. gaucho* venom (Machado et al., 2005). The difficulties of protein identification in brown spider venoms using MS approaches are due to the limited amount of *Loxosceles* protein sequences available in online data banks. Thus, sequencing the *Loxosceles* genome is still an ongoing challenge in loxoscelism research that will help guide future studies in this area. More recently, a proteomic analysis was performed using the *L. intermedia* venom by MudPiT (Multidimensional protein identification technology). This approach allowed the identification of 39 proteins; 14 proteins were grouped as toxins generally found in animal venoms and were considered responsible for the tissue damage observed in loxoscelism (dos Santos et al., 2009).

Thus, mass spectrometry and proteomic analysis are underused in the investigation of brown spider toxins. These approaches could be applied in many studies, such as a complete analysis of the protein content of *Loxosceles* venoms to generate a *Loxosceles* protein data bank and the identification of the post-translational modifications of the to toxins. In addition, these techniques could be helpful in analyzing the *in vivo* effects of *Loxosceles* crude venom or a specific recombinant toxin on certain tissues by examining the protein content after treatments.

5.2. Molecular biology approaches for studying brown spider venom

The advent of molecular biology has allowed the development of numerous novel techniques and applications that have enriched the knowledge in many scientific fields. Specifically, molecular biology has introduced new approaches for studying venoms and insights into their mechanisms of action.

One of the most successful tools applied in the study of the *Loxosceles* venoms is undoubtedly the cloning and heterologous expression of recombinant toxins, which circumvent the difficulties presented by the low amounts of venom that can be collected from these spiders (Senff-Ribeiro et al., 2008; Catalan et al., 2011). Fernandes-Pedrosa et al. (2002) published the first report of the cDNA cloning and expression of a *Loxosceles* toxin, in which a functional phospholipase D (SMase I) obtained from a cDNA library of *L. laeta* venom glands was expressed. Thereafter, several recombinant phospholipases D of other *Loxosceles* species were produced and allowed for the complex biological and biochemical characterization of these toxins (Kalapothakis et al., 2002; Araújo et al., 2003; Lee and Lynch, 2005; Tambourgi et al., 2005; Chaim et al., 2006; da Silveira et al., 2006, 2007b; Olvera et al., 2006;

Ribeiro et al., 2007; Appel et al., 2008; Christoff et al., 2008; de Almeida et al., 2008; Kusma et al., 2008; Chaves-Moreira et al., 2009; de Santi Ferrara et al., 2009; Paludo et al., 2009; Catalan et al., 2011; Chaim et al., 2011b; Chaves-Moreira et al., 2011; Zobel-Thropp et al., 2012; Vuitika et al., 2013; Wille et al., 2013). Recently, two recombinant phospholipases D (recLiD1 and LiRecDT1) were developed as tools to assay the sphingomyelinase D activity in crude venoms or recombinant enzymes (Gomes et al., 2011). Concurrently, these techniques also revealed that phospholipases D comprise a family of toxins in *L. intermedia* venom, as several isoforms were described (Chaim et al., 2006; da Silveira et al., 2006; da Silveira et al., 2007b; Appel et al., 2008; Vuitika et al., 2013; Wille et al., 2013).

Site-directed mutagenesis of recombinant *Loxosceles* phospholipases D allowed for the production of recombinant molecules with drastically decreased enzymatic activity used as tools to elucidate the major role of the catalytic activity of this enzyme's toxicity (Lee and Lynch, 2005; Kusma et al., 2008; Chaim et al., 2011b; Chaves-Moreira et al., 2009).

In addition, the recombinant toxins allowed for the resolution of the crystal structures of two *Loxosceles* phospholipases D, SMase I from *L. laeta* (class I PLD) and LiRecDT1 from *L. intermedia* (class II PLD) (Murakami et al., 2005; de Giuseppe et al., 2011; Ullah et al., 2011). These studies were fundamental in the understanding of the toxins' catalytic mechanisms.

Recombinant toxins could be used in animal immunization, thus eliminating the need of spiders as a source of venom (Gutiérrez et al., 2011). Recombinant phospholipases D have already been tested as antigens for the development of a polyvalent antivenom, which is effective in the neutralization of the crude venom and for understanding of antigenicity of toxins (Alvarenga et al., 2003; Olvera et al., 2006; de Moura et al., 2011; Mendes et al., 2013).

Although the vast majority of studies focus on producing phospholipases D as recombinant molecules, more recent studies set out to produce other recombinant *Loxosceles* toxins. For instance, a recombinant metalloprotease from *L. intermedia* venom has been produced and characterized (da Silveira et al., 2007a). Additionally, a recombinant hyaluronidase from *L. intermedia* venom was recently produced and used to demonstrate the role of this toxin in the venom (Ferrer et al., 2013). Moreover, a TCTP member-family toxin (Sade et al., 2012) and an Inhibitor Cystine Knot peptide (Matsubara et al., 2013) have also been cloned and expressed and will enable functional and structural studies to further characterize these poorly studied brown spider venom toxins. As will be further discussed below, these recombinant toxins enabled additional insights into loxoscelism and will also be putatively useful as tools for a variety of biotechnological applications.

The evolutionary aspects concerning both *Loxosceles* specimens and phospholipase D toxins have been thoroughly investigated by employing molecular biology techniques, such as cDNA, rRNA and genomic sequencing, analyses of positive selection, structural modeling of amino acid conservation and phylogenetic analyses (Binford et al., 2005, 2008; 2009; Cordes and Binford, 2006; Duncan et al., 2010).

Thus, these molecular biology techniques underlie the recent advances in the understanding of the *Loxosceles* toxins that occurred in the last few decades. Novel technologies not yet applied specifically to the study of *Loxosceles* toxins are promising, such as quantitative PCR, RNA interference and the expression of recombinant toxins in eukaryotic cells. Molecular biology approaches not only expand the knowledge of spider biology and the pathophysiology of loxoscelism but also reveal novel molecules for biotechnological approaches.

6. Highly expressed toxin families

Over the last few years, several studies focusing on the expression profiles of venomous glands of various organisms, such as snakes, scorpions and spiders, have been conducted. As expected, most of the profiles showed the prevalence of the toxin families that have a direct role in the main signs and symptoms observed in envenomation with these animals. In addition, some profiles showed that the highly expressed toxins are mainly involved in the mechanisms of prey capture for feeding purposes (Zhang et al., 2010; Rokyta et al., 2011; Ma et al., 2012).

In *Loxosceles* spider venom, the transcriptome analysis expression profiles of the venomous glands of different species showed different profiles of the highly expressed toxin families. On one hand, Fernandes-Pedrosa et al. (2008) reported the prevalence of transcripts coding for phospholipase D toxins in the *L. laeta* venomous gland. Subsequently, Gremski et al. (2010) affirmed that in the *L. intermedia* venomous gland, transcripts coding for ICK peptides were prevalent. This observation is consistent with the fact that the primary role of brown spider venom, as in all arachnids, is to paralyze or kill envenomed prey. Hypotheses supporting the differences in the expression levels of these toxins have been discussed previously (Gremski et al., 2010).

Both the *L. laeta* and *L. intermedia* transcriptomes exhibit high expression of the phospholipase D (PLDs) and metalloprotease toxin families in the venomous glands. In fact, PLDs (referred to as sphingomyelinases D by Fernandes-Pedrosa et al., 2008) are able to reproduce the major symptoms of loxoscelism. Moreover, a recent study demonstrated that PLDs also possess a potent insecticidal activity (Zobel-Thropp et al., 2012). Thus, based on the known activities of *Loxosceles* PLDs in vertebrates and arthropods, it is not surprising that this toxin family is highly expressed in brown spider venom glands.

Metalloproteases are also highly expressed toxins in *L. laeta* and *L. intermedia* venom glands (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). They comprise a family of venom enzymes and may be involved in the initial digestion of prey. These toxins may also have a role in the hemorrhaging observed in loxoscelism and in the systemic spreading of other toxins in victims (da Silveira et al., 2007a; Trevisan-Silva et al., 2010).

Analyses of venom gland expression profiles reveal a consistent redundancy of transcripts coding for the toxins that are highly expressed (Cidade et al., 2006; Neiva et al., 2009). Functional redundancy in proteins is a rare phenomenon; venoms represent a rare case of this

phenomenon (Morgenstern and King, 2013). Some authors argue that to maintain effective toxins against prey and predators, the genes encoding venom peptides and proteins underwent multiple duplication events. In turn, the duplicated genes acquired related or even novel functions through adaptive evolution (Ma et al., 2012). In fact, because these highly expressed toxins are often related to the venom's main actions, the genes encoding these toxins can be assumed to more likely undergo multiple duplication events, generating redundancy.

6.1. Phospholipases D

The phospholipase D (PLD) family of toxins is the most studied and well-characterized component in the *Loxosceles* species venoms. These molecules have been reported to play an important role in the development of clinical sign and symptoms in loxoscelism. Due to their ability to trigger dermonecrosis *in vivo*, the brown spider PLDs are also known as dermonecrotic toxins (da Silva et al., 2004; Appel et al., 2005; Swanson and Vetter, 2006).

Dermonecrotic toxins are soluble in water or physiological buffers and active enzymes are secreted by the brown spider venom glands. These molecules catalyze the hydrolysis of phospholipids, such as sphingomyelin, at a terminal phosphodiester bond to release choline and produce ceramide 1-phosphate (C1P) (da Silva et al., 2004; Chaim et al., 2011b; Wille et al., 2013). PLDs are also able to hydrolyze lysophosphatydilcholine in a Mg^{+2} dependent-manner (van Meeteren et al., 2004; Chaim et al., 2011b; Horta et al., 2013; Wille et al., 2013). These toxins are proteins which vary in molecular mass from 30 to 35 kDa, and include a signal peptide followed by a propeptide. The amino acid sequences of PLDs are highly conserved (55–99%), especially in the residues around the catalytic cleft. Based on phylogenetic studies, PLDs have been distributed in six different groups of the Loxtox family (*Loxosceles* toxin) (Chaim et al., 2006; Kalapothakis et al., 2007). In the same vein, Binford et al. (2009) have proposed a new nomenclature based on the evolution and phylogenetics of the PLD genes, termed the SicTox family (Sicariidae Toxin).

Gremski et al. (2010) revealed that 9% of the analyzed transcripts from the *L. intermedia* venom gland corresponded to PLDs, comprising 20.2% of all the toxin-encoding ESTs (**Expressed Sequence Tags**), which is a very significant proportion of the toxins. For *L. laeta*, the content of the PLD-encoding transcripts was present at higher levels (16.3% of all ESTs present in database hits). However, the transcriptome analysis for *L. laeta* had methodological limitations due to using only female specimens, which are already known to produce a greater quantity of venom when compared with male spiders (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). Moreover, the *L. intermedia* transcriptome analysis criteria for bioinformatics screening provided new putative isoforms of PLD (Vuitika et al., 2013), which can be included as novel groups in the LoxTox family (Kalapothakis et al., 2007). These data corroborate the findings of Machado et al. (2005), who identified at least 11 PLD isoforms in the venom of *L. gaucho*, termed Loxnecrogin, or data reported by Wille et al. (2013), which showed by 2D electrophoresis at least 25 spots immunologically related to PLD toxins in the *L. intermedia* crude venom.

Several PLD isoforms were also characterized in the venom of other *Loxosceles* species. In *L. reclusa* venom, the native PLDs were present at molecular mass of approximately 32 kDa, and the four active isoforms were characterized as able to induce dermonecrotic lesions, hemolysis, and platelet aggregation (da Silva et al., 2004; Vetter, 2011a, 2011b). Two PLD isoforms, SMase I (32 kDa) and SMase II (35 kDa), were also described in *L. laeta* venom, which experimentally demonstrated complement-dependent hemolysis, dermonecrosis and hydrolysis of sphingomyelin (Fernandes-Pedrosa et al., 2002; de Santi Ferrara et al., 2009). Catalan et al. (2011) reported two new PLD isoforms in *L. laeta*, rLIPLD1 was dermonecrotic and active on sphingomyelin while rLIPLD2 seemed to be inactive; but rLIPLD2 was cloned and expressed and was missing a large portion of the PLD region, i.e., it did not include the initial amino acids of the catalytic site, such as His^{12}. From *L. intermedia* venom, many PLD isoforms have been described, and nine isoforms have already been expressed as recombinant proteins. It has been shown that recombinant isoforms of PLD are able to reproduce most of the toxic effects observed in loxoscelism and antigenic properties of the venom (Kalapothakis et al., 2002; Fernandes-Pedrosa et al., 2002; Chaim et al., 2006; da Silveira et al., 2006; da Silveira et al., 2007b; Appel et al., 2008; Vuitika et al., 2013). Several isoforms of PLD were also very well characterized and cloned from the venom of other *Loxosceles* species (Ramos-Cerrillo et al., 2004; Barbaro et al., 2005; Magalhães et al., 2013).

The PLDs are responsible for a large variety of disturbances in loxoscelism. Both native and recombinant forms of PLDs have been reported to trigger dermonecrotic lesions, an increase in vascular permeability, an intense inflammatory response at the inoculation site and at a systemic level, platelet aggregation, hemolysis, nephrotoxicity, and even lethality in controlled experiments (Cunha et al., 2003; Appel et al., 2005; da Silveira et al., 2006, 2007b; Swanson and Vetter, 2006; Kusma et al., 2008; Senff-Ribeiro et al., 2008; Chaves-Moreira et al., 2009; Tambourgi et al., 2010; Chaim et al., 2011b).

Toxicity to a variety of cell types and structures is often enzyme dependent. At the beginning of the Loxtox protein family characterization, it was thought that these toxins were exclusively able to cleave the head-groups of sphingomyelin, the so-called sphingomyelinases. Further studies have described other substrates to be included as susceptible to catalysis by the PLDs, such as glycerophospholipids and lysophospholipids. Thus, the term phospholipase D for brown spider dermonecrotic toxins is more suitable (Lee and Lynch, 2005; Chaim et al., 2011a; Chaves-Moreira et al., 2011; Wille et al., 2013).

Studies comparing recombinant isoforms with distinct capacities of degrading substrates have shown differences in the intensity of their effects (Gomez et al., 2002; Chaim et al., 2011b; Stock et al., 2012). Several recombinant PLD isoforms from the *Loxosceles* genus were heterologously produced in *Escherichia coli*. These recombinant PLDs are easily obtained in their soluble and active enzyme forms in

large amounts, which provided interesting results concerning the structural and functional properties of the PLDs. For example, critical data examining the putative enzyme mechanism and three-dimensional scaffold were obtained by X-ray crystallography (more details, see Sections 10 and 11). In summary, research into the catalytic site revealed important insights into the enzymatic capabilities of each isoform (Murakami et al., 2005, 2006; de Giuseppe et al., 2011; Ullah et al., 2011). Recently, de Giuseppe et al. (2011) published the crystal structure of LiRecDT1 from *L. intermedia*, indicating that it contained an additional disulfide bond in the PLD structure catalytic loop compared with the previously described PLD from *L. laeta*. These details of PLD molecules can explain the distinct enzymatic behaviors of the venom from different species. PLDs with different structures could have different substrate affinities or enzymatic activities; therefore, these differences could explain the clinical symptoms or severity observed at the local bite site or the systemic effects during envenomation by different species of the *Loxosceles* genus.

Furthermore, there are clear differences in the hydrolytic ability of PLD isoforms within the *Loxosceles* genus (Gomez et al., 2002; Chaim et al., 2011a; Stock et al., 2012). All studies with the named LiRecDTs (isoforms 1–7) showed dermonecrosis at different levels in rabbit skin, consistent with the results of the spectrofluorimetric analysis of sphingomyelin hydrolysis (Appel et al., 2008; Chaves-Moreira et al., 2011; Vuitika et al., 2013). Ribeiro et al. (2007) reported that LiRecDT1 and LiRecDT2 were similar in all functional tests, such as *in vivo* edema or cytotoxicity, while the LiRecDT3 effect was significantly less intense. The amino acid alignment observed paralleled these results: LiRecDT1 and LiRecDT2 were very similar, but LiRecDT3 slightly was different. LiRecDT3 showed some important differences in hydrophobicity at the boundaries of the catalytic site, which can explain its differential performance.

Furthermore, site-directed mutagenesis of His^{12} of LiRecDT1, predicted to play a central role during catalysis, was not sufficient to completely abolish its catalytic activity. Moreover, the LiRecDT1H12A mutant isoform has a drastic reduction in its enzymatic activity, but with no change in the secondary structure, compared with LiRecDT1. Interestingly, the mutant isoform was unable to induce the same level of any activity examined, but the attachment to the cell surface or to mobilized lipids was unaltered (Kusma et al., 2008; Paludo et al., 2009; Chaim et al., 2011b; Wille et al., 2013). Most likely, the other protein domains besides the catalytic cleft were preserved, as they might be relevant for the interaction of the toxin with the cell membrane or lipid substrates. In general, the main value of PLD catalysis can be related to the release of lipid metabolites, which could modulate a wide range of biological events, such as the cell cycle, cell proliferation, cell differentiation processes and cell death (Marchesini and Hannun, 2004; Tani et al., 2007).

Studies have shown the upregulation of the expression of proinflammatory cytokines/chemokines after the exposure of human fibroblasts to the *L. reclusa* PLD (Dragulev et al., 2007), which hydrolyzes the cell membrane sphingomyelin to ceramide 1-phosphate (C1P) and would lead to a receptor-dependent inflammatory response. This idea challenged the hypothesis that lysophosphatidic acid (LPA) was a preferential product and bioactive metabolite instead of C1P, due to the relative LPC abundance in the plasma as a substrate (van Meeteren et al., 2004, 2007). Recently, Horta et al. (2013) showed that cell death was induced by *L. similis* whole venom (LsV) and especially with a recombinant isoform of *L. intermedia* PLD, recLiD1 (Kalapothakis et al., 2002; Felicori et al., 2006). LPA released by the PLD activity of LsV and recLiD1 was unable to activate LPA receptors in the presence of an LPA1/LPA3 antagonist. This effect was indirectly observed by ELISA assays for IL-6, IL-8, CXCL1, and CXCL2. Moreover, the authors did not find that LPA played a role in the apoptosis induced by LsV or recLiD1 in fibroblast and endothelial cells *in vitro*, which may be related to other LPA-independent stimuli or to C1P acting on the cell membrane receptors, as has been previously described (Horta et al., 2013). The variety of molecular mechanisms triggered by *Loxosceles* PLDs and their lipid metabolites remains open to further investigation as a complex event dependent on the cell types involved, lipid substrate abundance and availability and intracellular signaling cascades. PLDs can serve as biotools for the study of cell–cell communication via cell membranes in the context of inflammation. PLD isoforms have been proposed as potential models for designer drugs or other biotechnological applications (Senff-Ribeiro et al., 2008; Tambourgi et al., 2010; Chaim et al., 2011a). The production of more stable PLD isoforms with enhanced enzymatic activity would greatly contribute to many areas of toxinology and to the complete understanding of the biochemical features of PLDs, their many biological implications and their related molecular mechanisms.

6.1.1. Phospholipase D topology and structure

The amino acid sequence comparisons of spider venom phospholipases D indicate that they contain either 284 or 285 amino acids and display a significant degree of homology (de Santi Ferrara et al., 2009). This single polypeptide chain folds to form a distorted barrel where the inner barrel surface is lined with eight parallel β-strands (termed A–H) linked by short flexible loops to eight α-helices (termed helices 1–8) that form the outer surface of the barrel (Murakami et al., 2005) (Figs. 4 and 5). This structural motif was first observed in the structure of the triose phosphate isomerase (TIM) and is referred to as a TIM barrel or as an $(\alpha/\beta)_8$ barrel. The topology diagram (Fig. 5) presents a structural schematic where the α-helices and β-strands are depicted as cylinders and arrows, respectively, and the central region forms the $(\alpha/\beta)_8$ barrel. The interconnecting loops are primarily hydrophilic and hydrophobic in the upper and lower sections, respectively (Fig. 5). A short β strand (B′) is inserted between strand B and helix 2, and two short helices (3′ and 4′) are inserted between helix 3 and strand D, and helix 4 and strand E. The catalytic loop is stabilized by a disulfide bridge (Cys^{51} and Cys^{57}) and a second disulfide bridge (Cys^{53} and Cys^{201}) is present only in the class II enzymes (de Giuseppe et al., 2011), which links the catalytic loop to the flexible loop to significantly reduce the flexibility of the latter loop, as evidenced by the mean temperature factors (Figs. 4 and 6).

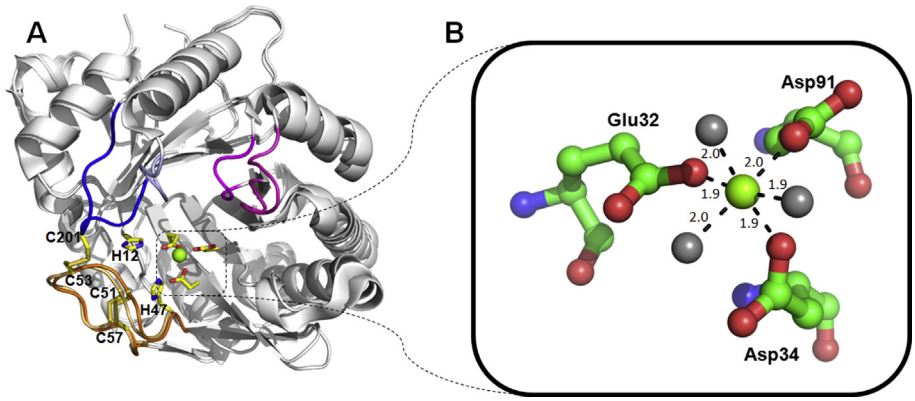

Fig. 4. Structural alignment between the class I and class II phospholipases D. (A) The residues involved in the metal–ion binding and catalysis are highlighted. The catalytic, flexible and variable loops are colored in orange, blue and magenta, respectively. The dark and light colors refer to phospholipase D II and phospholipase D I, respectively. The Mg^{+2} ion is represented by a green sphere. The disulfide bridges are represented as yellow-colored sticks. (B) The coordination sphere of the Mg^{2+} ion. The figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Because the α-helices, β-strands and loops vary in length and character, the barrel with a surface area of 11,254 $Å^2$ is significantly distorted (Fig. 4). The interior of the barrel is densely packed with hydrophobic amino acids, and the short N-terminal section and the C-terminal extension, which contains a short helix (8′), a β-strand (H′) and a random coiled region, serve to cap the torus of the far side of the barrel. The surface loops forming the near side of the barrel are mainly hydrophobic, and a narrow cavity provides access to the catalytic site, which is characterized by a ring of negatively charged amino acids (Murakami et al., 2005; de Giuseppe et al., 2011). The catalytic, variable and flexible loops are located on the same face of the barrel and are colored orange, magenta and blue, respectively (Fig. 4). The catalytic and Mg^{2+} binding sites are located in a shallow depression and contain His^{12}, Glu^{32}, Asp^{34}, Asp^{91}, His^{47}, Asp^{52}, Trp^{230}, Asp^{233}, and Asn^{252}, which are fully conserved in *Loxosceles* PLD isoforms (Figs. 4 and 7) (Murakami et al., 2005; de Giuseppe et al., 2011).

Mutagenesis studies of PLDs (de Giuseppe et al., 2011; Ullah et al., 2011) and its crystal structure (Murakami et al., 2005) indicate the involvement of two histidines that are in close proximity to the metal ion-binding site in the acid-base catalytic mechanism. Based on the structural results, His^{12} and His^{47} of PLD have been identified as the key residues for catalysis and are assisted by a hydrogen bond network that involves Asp^{52}, Asn^{252}, and Asp^{233}. The metal ion is coordinated by Glu^{32}, Asp^{34}, Asp^{91}, and solvent molecules (Fig. 4B).

6.1.2. *Involvement of the Mg^{2+} ion in the phospholipase D catalytic mechanism*

The Mg^{2+} ion is essential for catalysis, and its binding site is completely conserved in all spider venom phospholipases D. Mg^{2+} ion is octahedrally coordinated (with a mean Mg^{2+}–O distance of 1.98 Å) (Fig. 4) equatorially by the carboxylate oxygens of the side chains of Glu^{32} and Asp^{34} and by two tightly bound water molecules and

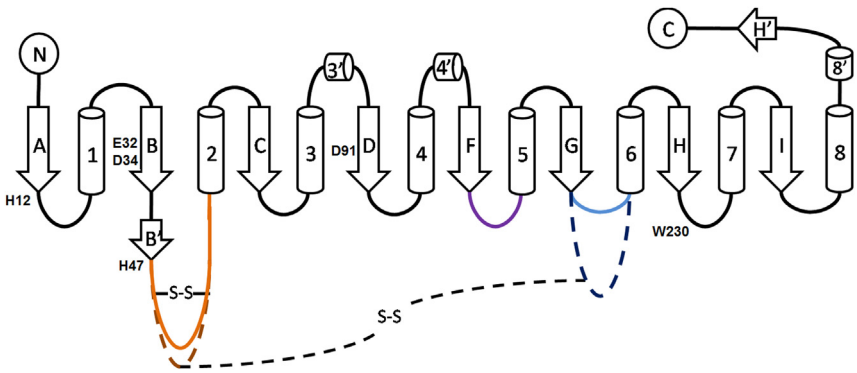

Fig. 5. Structural topology of *L. laeta* phospholipase D I and *L. intermedia* phospholipase D II. The β-strands (arrows) and α-helices (cylinders) forming the $(\alpha/\beta)_8$ barrel are labeled A–H and *1–8*, respectively. The β-strands and α-helices not belonging to the core are designated with a prime. The positions of the catalytic loop B (orange), variable loop E (magenta), flexible loop F (blue), and the disulfide bridge (*S–S*) are indicated. The approximate relative positions of the amino acids involved in the catalysis and Mg^{2+} ion binding are indicated. The dashed line represents the additional disulfide bond in phospholipase D II. Letters N and C represents the positions of amino- and carboxi-terminal domains of proteins. Figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

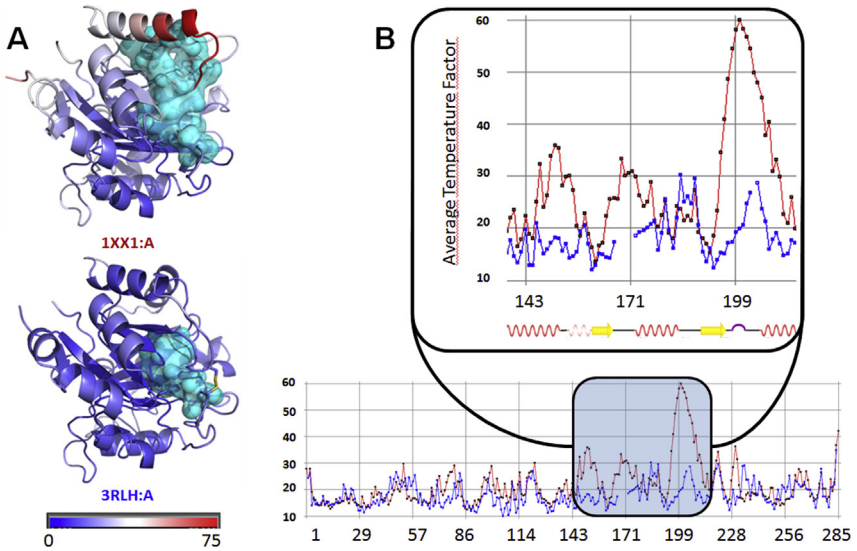

Fig. 6. Protein flexibility by B factor analysis. (A) Ribbon representations of the phospholipase D structures colored to indicate the mean temperature factors as indicated by the bar graph. A light blue surface indicates the cavity volume of the active site. (B) The mean temperature factors (blue, main chain; red, side chain) as a function of the amino acid residues. The inset highlights the flexible region as discussed in the text. 1XX1 and 3RLH are the protein data bank codes for the Class I and Class II enzymes, respectively. Plot performed by MSSP module of BlueStar STING (Neshich et al., 2005). Figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apically by the side-chain carboxylate oxygen atoms of the Asp^{91} and by a water molecule, which is also hydrogen bonded to the $Glu^{32}O^{\varepsilon 1}$ atom. The enzyme structure determined in the presence of a bound sulfate ion (Murakami et al., 2005), which is considered to occupy the position of the substrate phosphate moiety, is coordinated by three solvent molecules, two of which also coordinate the Mg^{2+} ion. The indole ring of Trp^{230} is partially disordered and likely plays a role in stabilizing the choline head group of the substrate.

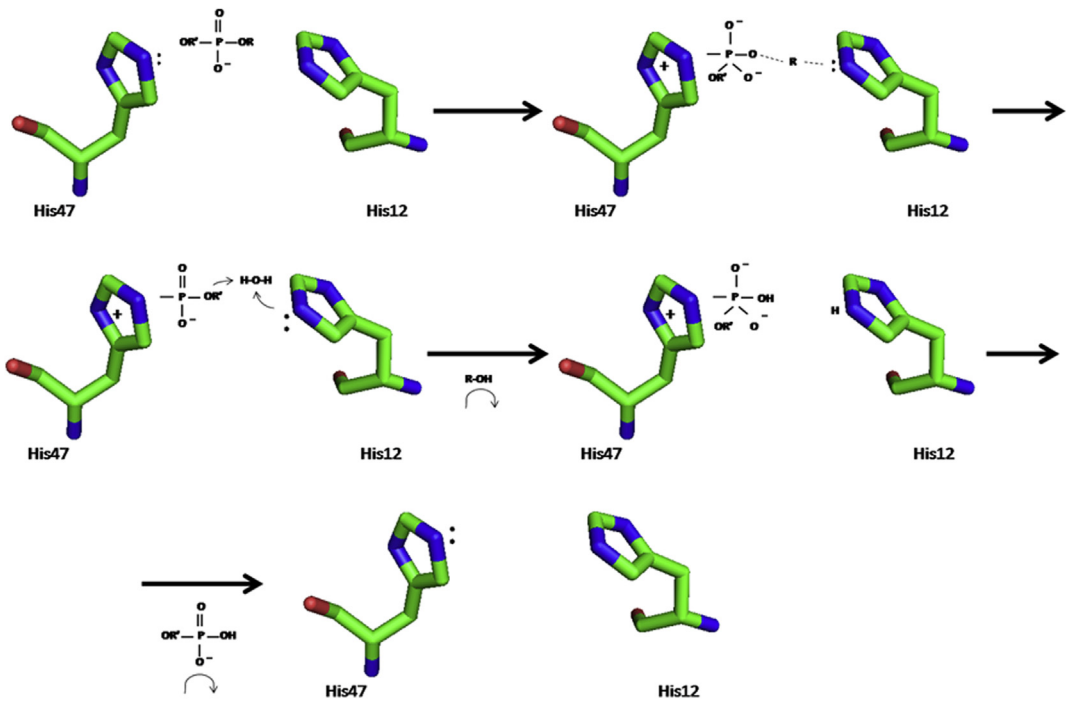

Fig. 7. The reaction mechanism of phospholipase D. The acid-base catalytic mechanism involves His^{12} and His^{47}. R and R′ indicate ceramide 1′-phosphate and choline, respectively.

Based on the crystal structures of phospholipase D, a two-step catalytic mechanism has been suggested where His^{12} and His^{47} play important roles (Fig. 4). In the first step of this mechanism, **His^{47}** plays a role as a nucleophile that initiates the attack on the substrate scissile phosphodiesterase bond, which is followed by the formation of a penta-coordinated intermediate that is subsequently destabilized by the donation of a hydrogen atom by **His^{12}**, leading to the formation of a choline molecule. In the second reaction step, **His^{12}** abstracts a proton from a solvent molecule that then initiates a nucleophilic attack on the stable covalent histidine intermediate, resulting in the formation of the second product, ceramide 1-phosphate, and a return to the initial state. The Mg^{2+} ion is important for the substrate recognition and binding and for further stabilization of the intermediate state in the two-step catalytic mechanism.

6.2. Astacins

The astacins are a family of proteases belonging to the metzincin super family, which are grouped with the zinc-dependent metalloproteases (Gomis-Rüth, 2003; Sterchi et al., 2008). Many metalloproteases are characterized by a conserved pentapeptide HEXXH in the active site, which is essential for metal ion coordination and catalysis (Sterchi et al., 2008).

The first report of proteases in *Loxosceles* venom was described in *L. reclusa* venom, which showed protease activity against *Heliothis virescens* and *Musca domestica* larvae, as observed by histochemical techniques (Eskafi and Norment, 1976). In addition, the *L. reclusa* venom protease activity was assayed on L-aminoacyl-β-naphthylamide derivatives and was shown to act more efficiently on L-Leucyl-β-naphthylamide, although other derivatives were also susceptible (Jong et al., 1979).

In *L. intermedia* venom, two metalloproteases were identified, Loxolysin A, a 20–28 kDa protease that degrades the Aα and Bβ chains of fibronectin and fibrinogen, and Loxolysin B, a 30–32 kDa protease with gelatinolytic activity (Feitosa et al., 1998). Similarly, proteolytic enzymes were identified in *L. rufescens* venom: a 23 kDa fibrinogenolytic protease and a 27.5 kDa gelatinolytic protease, which were both inhibited by 1,10-phenantroline (Young and Pincus, 2001). The fibrinogenolytic activity was reported in both *L. reclusa* venom and *L. laeta* venom, which showed the same partial effects that were observed in *L. intermedia* venom (i.e., the degradation of the Aα and Bβ fibrinogen chains) (Zanetti et al., 2002). *L. deserta*, *L. gaucho* and *L. reclusa* venoms were also shown to include metalloproteases (Barbaro et al., 2005). Other extracellular matrix components were also demonstrated as targets for the *Loxosceles* metalloproteases, such as entactin and heparan sulfate proteoglycans (Veiga et al., 2000b, 2001a). Although with the identification of these *Loxosceles* proteases, the proof that proteases are venom components and not contamination derived from gastric contents during venom extraction was reported in two crucial studies of *L. rufescens* venom and *L. intermedia* venom (Young and Pincus, 2001; da Silveira et al., 2002). The proteolytic effect of *L. rufescens* venom discussed above was observed in venom obtained by micro-dissection of the venom glands, a procedure that ensures the absence of gastric contaminants (Young and Pincus, 2001). For *L. intermedia* venom, the protein profile and proteolytic activity were very similar between the venom collected by electrostimulation (possible contamination) and macerated venom glands (free from gastric contaminants) (da Silveira et al., 2002).

A sequence encoding an astacin-like metalloprotease was first identified in a cDNA library of *L. intermedia* venom glands (da Silveira et al., 2007a). Astacin-like proteases (Merops M12A family) have a consensus sequence of 18 amino acids forming the catalytic domain –HEXXHXXGXXHEXXRXDR – in which the three histidines are involved in zinc binding, which is necessary for the catalytic activity. In addition, they have a conserved methionine residue involved in a sequence turn, termed a met-turn (MXY) (Gomis-Rüth, 2003; Sterchi et al., 2008; Gomis-Rüth et al., 2012). The identified *L. intermedia* astacin sequence was named LALP (*Loxosceles* astacin-like protease) and possesses astacin family signatures (catalytic domain and met-turn). LALP was shown to be cytotoxic upon rabbit subendothelial cells and able to hydrolyze fibrinogen and fibronectin (da Silveira et al., 2007a). Astacin family members have been described in prokaryotes and eukaryotes and possess diverse and distinct biological functions. In general, they are expressed in specific tissues of mature organisms, and during embryo development, they are temporally and spatially regulated (Gomis-Rüth, 2003; Mörhlen et al., 2003, 2006). The presence of astacin proteases in animal venoms is rare because LALP was the first report in the literature of an astacin molecule as a constituent of animal venom (da Silveira et al., 2007a).

Recently, two new isoforms of astacin-like proteases were identified in *L. intermedia* venom (named LALP2 and LALP3) and in *L. laeta* venom (LALP4) and *L. gaucho* venom (LALP5). These findings demonstrate that astacin proteases are a family of toxins present in *Loxosceles* venoms and that they are important components of these venoms (Trevisan-Silva et al., 2010, 2013). Corroborating the biological importance of the *Loxosceles* astacin-like proteases, transcriptome analyses showed that astacins are high expressed toxins in *L. laeta* and *L. intermedia* venoms (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). In *L. laeta* venom, astacin's transcripts represents 8% of the total transcripts, and in *L. intermedia* venom, they represent 9.8% of the toxin transcripts, representing the second most expressed toxin in both species (Gremski et al., 2010). Studies concerning the biological activities of *Loxosceles* astacins are essential to improve the knowledge of loxoscelism and to apply these toxins in biotechnology.

6.3. Brown spider venom Inhibitor Cystine Knot (ICKs)

In early the 1990s, many venom peptides from spiders, scorpions, cone snails and sea anemones had their structures solved using various techniques. A great number of cysteine-rich peptides were described, including a common structural motif called the "Inhibitor Cystine Knot" (ICK) (Daly and Craik, 2011). These peptides share a common structure with three disulfide bonds and are composed of three antiparallel β-sheets (Craik et al., 2001; Zhu et al., 2003; Daly and Craik, 2011). Two disulfide bonds

form a ring that is crossed by the third bond, which forms the structural motif ICK. This same structural motif is found in two other families: the Cyclic Cystine Knot (CCK) or cyclotide family and Growth Factor Cystine Knot (GFCK) family (Vitt et al., 2001; Craik et al., 2010; Iyer and Acharya, 2011). This molecular scaffold renders ICK peptides highly resistant to protease action, making them good targets for drug design (Daly and Craik, 2011; King, 2011; Moore et al., 2011). The homology between ICK peptides is usually low, but the distribution of cysteine residues is often conserved. Further studies showed that ICK peptides have an amino acid consensus sequence of $CX_{3-7}CX_{3-6}CX_{0-5}CX_{1-4}CX_{4-13}C$, where X can be any amino acid (Craik et al., 2001). ICK toxins are quite diverse in their biological activity because they can act in voltage-gated sodium, potassium or calcium channels; mechanosensitive channels; nicotinic acetylcholine receptors or ryanodine receptors (Nicholson et al., 2004; Dutertre and Lewis, 2010).

Among spiders, many ICK toxins have been described. One of the most well-studied families is the δ-Atracotoxins (δ-ACTX) family isolated from the venom of Australian funnel-web spiders. δ-ACTX show a similar action to the sea anemone and scorpion ICK toxins, binding at site 3 of the sodium ion channel, which causes neurotransmitter release in the nerve endings and results in the disturbance of the autonomic and somatic nervous systems (Nicholson et al., 2004).

From *Loxosceles* venom, three insecticide peptides named LiTx 1-3 have already been purified (de Castro et al., 2004). These peptides were isolated from *L. intermedia* venom using a combination of chromatography techniques, and their activities were assessed in *Lepidoptera* larvae, resulting in an LD_{50} of 0.90–1.92 μg/g insect. These authors proposed that LiTx 3 may act on **NaV** channels as with other toxins and that LiTx 2 and 3 may act on **NaV** or **CaV** channels. Furthermore, Fernandes-Pedrosa et al. (2008) analyzed the *L. laeta* transcriptome and found that 0.2% of all toxin transcripts matched with the ICK neurotoxin Magi 3 from *Macrothele gigas* (Corzo et al., 2003), which bind at site 3 of **NaV** channels. The transcriptome analysis of the *L. intermedia* venom gland showed that 55.5% of all transcripts putatively encode for toxins that potentially represent insecticide peptides and can be classified as ICK peptides. From the 55.5% of transcripts, 2.3% represent transcripts similar to Magi 3, such as those transcripts described for *L. laeta*. The most abundant venom transcripts found were transcripts similar to LiTx 3 (32%), LiTx 2 (11.4%) and LiTx 1 (6.2%). This transcriptome analysis also described transcripts encoding LiTx 4, another ICK peptide not yet characterized, which represent 3.7% of the toxin transcripts. Recently, it was described the cloning, recombinant peptide production, polyclonal antibody obtention and evaluation of the cross-reactivity of a novel toxin with a great similarity to the ICK family of peptides from *L. intermedia* venom. This peptide was named U2-sicaritoxin-Li1b (U2- SCRTX-Li1b) according to the nomenclature proposed by King et al. (2008) (Matsubara et al., 2013).

The interest in ICK toxin class is due to their targeting of ion channels, which are transmembrane protein complexes regulating ion flux and membrane potential. This ability of ICK peptides to specifically bind to some ion channels provides a great tool not only for electrophysiology and cell biology studies but also for drug design. In addition, the high specificity of spider peptides for insect receptors leads to the proposal of using these peptides for developing novel insecticidal targets or for the development of new biopesticides (Estrada et al., 2007; Dutertre and Lewis, 2010; Klint et al., 2012). Currently, there is no evidence of the involvement of brown spider ICKs in the pathogenesis of spider bites.

7. Low level of expression toxin families

Loxosceles venoms have demonstrated little variation in overall toxin composition (Ramos-Cerrillo et al., 2004; Barbaro et al., 2005; Fernandes-Pedrosa et al., 2008; Gremski et al., 2010; Trevisan-Silva et al., 2010). The high degree of intragenus toxin preservation is evidence of the evolutionary success of the venom formulation and is suggestive of the important functions of some types of toxins (Trevisan-Silva et al., 2010; Corrêa-Netto et al., 2011). However, transcriptome analyses of *L. intermedia* and *L. laeta* venoms indicate some differences in the level of toxin expression in this genus. In the analyses of the *L. laeta* venom gland expression profile, relatively low numbers of transcripts of serine proteases, enzymatic inhibitors, C-type lectin, hyaluronidases, 5′-nucleotidases, chitinases and venom allergens were found (Fernandes-Pedrosa et al., 2008). On the other hand, Gremski and co-workers showed low numbers of transcripts that coded for serine proteases, venom allergen, TCTP (**T**ranslationally **C**ontrolled **T**umor **P**rotein), hyaluronidases and serine proteases inhibitors (Gremski et al., 2010). The hypotheses that may explain these differences in the profile of toxins with low level expression include the different approaches and methodologies employed in the analyses. Additionally, these differences apply to different species, which reinforce the previous data that showed distinct behaviors among the venoms from distinct *Loxosceles* species.

Venom variations occur at all taxonomical levels and can significantly impact the clinical manifestations and efficacy of anti-venom therapies following a spider bite. Cases of incomplete intrageneric antivenom efficacy have been documented, implying a high interspecies venom variation (Casewell et al., 2009). Abundant differences can be observed between the venom compositions of different genera, the venom compositions of different species within a genus and the venom compositions of different individuals within a species (e.g., individuals from different geographical regions). Apparently, the venom composition is subject to strong natural selection pressure as a result of adaptation to specific diets because the primary role of venom is to aid in prey capture (Ruiming et al., 2010). Additionally, toxins with low level of expression do not necessarily possess a low activity. On the contrary, some types of these toxins have been postulated to have a high activity and high stability (Morey et al., 2006; Reitinger et al., 2008; Menaldo et al., 2012; Valeriano-Zapana et al., 2012), and therefore, these molecules would not be synthesized in large amounts. The below sub-items further discuss some of these low level of expression toxin family-members.

7.1. TCTP

The *L. intermedia* venom gland transcriptome analysis described the sequence of a protein identified as a member of the TCTP family and revealed that this TCTP is present at relatively low levels in the venom, only 0.4% of the transcripts that encoded toxins (Gremski et al., 2010). The name TCTP stands for ***T**ranslationally **C**ontrolled **T**umor **P**rotein*, as this protein was described by scientists studying proteins that were regulated at the translational level. The *tumor* is derived from the first TCTP cDNA sequence described, which was obtained from a human mammary tumor (Bommer, 2012). This protein was also shown to be a histamine-releasing factor (HRF) (McDonald et al., 1995) and a fortilin (Li et al., 2001). The *L. intermedia* TCTP was cloned and expressed as a heterologous protein in an *E. coli* expression system. The functional characterization of the recombinant protein, LiTCTP, showed that this toxin caused edema and enhanced vascular permeability (Sade et al., 2012). The cutaneous symptoms of envenomation with *Loxosceles* venoms include erythema, itching and pain. In some cases, *Loxosceles* spider bites can cause hypersensitivity or even allergic reactions. These responses could be associated with histaminergic events, such as an increase in the vascular permeability and vasodilatation. LiTCTP could be related to these deleterious venom actions as it was identified in *L. intermedia* venom (Sade et al., 2012). Another *Loxosceles* TCTP has been described in the venom gland of *L. laeta* by transcriptome analysis (Fernandes-Pedrosa et al., 2008). Recently, a transcriptome analysis revealed a TCTP protein (named GTx-TCTP) in the venom gland and the pereopodal muscle of the tarantula *Grammostola rosea* (Kimura et al., 2012).

Proteins of the TCTP super family have already been described in the gland secretions of many arthropods, such as ixodid ticks, and in the venom gland of the wolf spider (*Lycosa godeffroyi*), where it was described as the principal pharmacological toxin (Mulenga and Azad, 2005; Rattmann et al., 2008). TCTP family members are described as extracellular HRFs and are associated with the allergic reactions of parasites. Among species from the same genus, the TCTPs are completely conserved (Bommer and Thiele, 2004). A LiTCTP phylogeny tree demonstrates the similarities with the TCTPs from ixodid ticks, which were also characterized as HRFs (Mulenga and Azad, 2005; Sade et al., 2012). In the case of the *Loxosceles* venom gland, the TCTP and other constituents of the whole venom are secreted via a holocrine secretion as determined by ultrastructural studies of the *L. intermedia* venom gland (dos Santos et al., 2000; Gremski et al., 2010). TCTP secretion from cells proceeds via an ER/Golgi-independent or non-classical pathway, most likely mediated by secreted vesicles called exosomes (Amzallag et al., 2004; Hinojosa-Moya et al., 2008). TCTP mRNAs do not encode a signal sequence, and no precursor protein has been described; however, a TCTP protein was found in the biological fluid of asthmatic or parasitized patients, in the saliva of ticks (Bommer and Thiele, 2004; Hinojosa-Moya et al., 2008) and in the crude venom of *L. intermedia*. TCTPs represent a large protein family that is highly conserved and ubiquitous in eukaryotes, and they members are widely expressed in various tissues and cell types. TCTP protein levels are highly regulated in response to a wide range of extracellular signals and cellular conditions, which points to an involvement in various participating biological functions at diverse biochemical and signaling pathways. In fact, a wide range of functions and different biochemical roles have already been examined in the TCTP family (Bommer and Thiele, 2004; Choi and Hsu, 2007; Bommer, 2012).

TCTP proteins have already been described as calcium-binding proteins (Graidist et al., 2007) and as proteins that interact with the cytoskeleton by binding to and stabilizing microtubules (Bazile et al., 2009). The involvement of TCTP in the mitotic spindle has also been shown, and TCTP is now considered a regulator of mitosis (Burgess et al., 2008). The crucial role of TCTP has also been described in early development. The loss of TCTP expression in mice leads to increased spontaneous apoptosis during embryogenesis and causes lethality (Chen et al., 2007; Susini et al., 2008). TCTP can be described as a multifunctional protein due to the high number of protein partners and the several areas/pathways of cell metabolism where it is involved (Amson et al., 2013a).

The downregulation of TCTP has been implicated in biological models of tumor reversion (Tuynder et al., 2002, 2004), and the protein is the target of various anticancer drugs (Efferth, 2005; Telerman and Amson, 2009; Amson et al., 2013b).

Studying LiTCTPs can elucidate the biological aspects of loxoscelism, especially those aspects related to the histaminergic symptoms. Moreover, LiTCTP investigation can provide new insights regarding the TCTP family and its different functions. LiTCTP is a promising subject for study in toxinology and in immunological, allergenic and experimental oncology.

7.2. Hyaluronidases

Hyaluronidases are a group of enzymes that degrade hyaluronic acid (HA) and, to a limited extent, chondroitin, chondroitin sulfate (CS) and dermatan sulfate (DS). HA is a ubiquitous component of the vertebrate extracellular matrix where it fills the space between cells and acts as a lubricant and a barrier to the penetration of foreign particles (Markovic-Housley et al., 2000).

This type of matrix-degrading enzyme are found in many animal venoms, such as lizards, scorpions, spiders, bees, wasps, snakes and stingrays (Girish and Kemparaju, 2005; Kemparaju and Girish, 2006; Magalhães et al., 2008). These enzymes are always reported as “spreading factors” in the venoms due to their ability to degrade extracellular matrix components and to increase the diffusion of other toxins from the inoculation site (Kemparaju and Girish, 2006).

Wright et al. (1973) were the first to describe hyaluronidase activity in the *Loxosceles* genus. This work was performed with *L. reclusa* venom, and the purified enzymes were estimated to have molecular mass of 33 and 63 kDa (the 63 kDa protein is thought to be a 33 kDa-dimer) by SDS-PAGE. These enzymes exhibited activity against HA

and CS types A, B, and C. (Wright et al., 1973). Although *Loxosceles*-derived hyaluronidases alone are not able to produce necrosis, they are thought to be an important factor in the spread of these venoms. The detection by HA-substrate SDS-PAGE of a hyaluronidase of 32.5 kDa from *L. rufescens* venom has been reported (Young and Pincus, 2001). In *Loxosceles* envenomation, the presence of edema, erythema and necrosis is common, which indicates extracellular matrix disturbances.

Barbaro et al. (2005) found hyaluronidase activity on hyaluronic acid in a 44 kDa protein in *L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta and L. reclusa* venoms. Shortly after, da Silveira et al. (2007c) showed that *L. intermedia* venom contained at least two hyaluronidase isoforms. This venom demonstrated lysis of both HA and CS substrates at 41 and 43 kDa. These authors also showed, using biochemical assays, that the hyaluronidases from this venom are pH-dependent endo-β-N-acetyl-D-hexosaminidase hydrolases. *L. intermedia* venom was also able to degrade HA in rabbit skin (da Silveira et al., 2007c). A proteomic study also corroborated the presence of hyaluronidases in *Loxosceles* venoms (dos Santos et al., 2009).

Analyzing the transcriptome of *L. laeta*, Fernandes-Pedrosa et al. (2008) found 4 clones within 1 cluster with similarity to the hyaluronidase from *Bos taurus* (gb|AAP55713.1), which represented 0.13% of the total transcriptome. In addition, Gremski et al. (2010) demonstrated a unique partial sequence in the *L. intermedia* transcriptome with similarity to hyaluronoglucosaminidase 1 from *Rattus norvegicus* (gb|EDL77243.1). Recently, the first recombinant hyaluronidase from the *Loxosceles* venom was produced from *L. intermedia* venom gland cDNA (Dietrich's Hyaluronidase). The recombinant toxin was expressed in *E. coli* and had a molecular mass of approximately 45 kDa. Hyaluronidase activity of this recombinant toxin was detected on HA and CS after refolding *in vitro*. An assessment of dermonecrosis *in vivo* showed that Dietrich's Hyaluronidase increased the macroscopic erythema, ecchymosis and dermonecrotic effect induced by the recombinant dermonecrotic toxin (LiRecDT1) a phospholipase D homologue in rabbit skin. This work confirmed the hypothesis that hyaluronidase acts as a spreading factor in *Loxosceles* venoms (Ferrer et al., 2013).

HA levels are markedly increased during embryogenesis, inflammation, malignant transformation, and wound healing and whenever fast tissue turnover and remodeling is required. The occurrence of various diseases related to HA metabolism suggest that the level of HA must be tightly controlled (Markovic-Housley et al., 2000; Girish and Kemparaju, 2007). The process of degradation of glycosaminoglycans from connective tissues is related to bacterial pathogenesis, the spread of toxins and venoms, fertilization processes, and cancer progression (Hynes and Walton, 2000; Girish et al., 2004; Girish and Kemparaju, 2007; Lokeshwar and Selzer, 2008). Therefore, the identification and characterization of hyaluronidase inhibitors could be important in the development of new drugs and biotechnological tools to be applied in the above-mentioned fields (Botzki et al., 2004; Barla et al., 2009).

7.3. Serine proteases

Serine proteases were first identified in *Loxosceles* venom as zymogens activated by trypsin (Veiga et al., 2000a). In Veiga et al. (2000a), using zymography assays **with venom previously incubated with exogenous proteases**, trypsin was shown to activate two gelatinolytic molecules of 85 and 95 kDa in *L. intermedia* venom. Among the various protease inhibitors assayed, only serine protease inhibitors were able to inactivate these enzymes. The activity of the assayed *L. intermedia* serine proteases were optimal in a pH range of 7.0–8.0, and no enzymatic activity was observed on hemoglobin, immunoglobulin, albumin, fibrinogen or laminin, suggesting the specificity of their proteolytic actions.

At the time, no previous descriptions of proteases that behaved as zymogens had been described for spider venoms. However, as this feature had already been reported for several snake venom proteases, the authors suggested that trypsin treatment could specifically degrade the pro-peptide domains of the zymogen molecules and release the active proteases. As this activation was only observed after treatment with trypsin, even though various proteases were assayed, it was suggested that the hydrolysis of zymogen molecules of *L. intermedia* serine proteases was specific because trypsin hydrolyzes peptide bonds immediately after a lysine or arginine (Veiga et al., 2000a).

Consistent with the results of the Veiga et al. (2000a) study, Machado et al. (2005) also found high molecular mass proteins at 85–95 kDa in 2-DE gels. These protein spots were also detected in *L. laeta* and *L. gaucho* venoms.

The transcriptome analysis of the *L. laeta* venom gland revealed twelve clusters that grouped fourteen ESTs putatively assigned as serine proteases coding sequences (Fernandes-Pedrosa et al., 2008). All clusters are similar to serine proteases described in arthropods, such as ticks, spiders and crabs. Shortly thereafter, a proteome study of *L. intermedia* venom described five peptide sequences similar to snake venom serine proteases (dos Santos et al., 2009).

A transcriptome analysis of the *L. intermedia* venom gland putatively assigned five transcripts as serine proteases (Gremski et al., 2010). The ESTs were grouped into two clusters with no sequence similarity with each other. One of the sequences significantly aligned with an arthropod serine protease that was most likely synthesized as an inactive precursor (Nene et al., 2007). The other cluster was similar to a serine protease sequence of the spider *Lycosa sigoriensis* venom gland (Gremski et al., 2010; Zhang et al., 2010). As previously mentioned, *L. intermedia* venom demonstrated serine proteolytic activity at two high molecular mass proteins, suggesting that two or more molecules in the venom exhibit these particular characteristics (Veiga et al., 2000a). Thus, it is not surprising that both transcriptome and proteome studies described distinct sequences coding for serine proteases (Fernandes-Pedrosa et al., 2008; dos Santos et al., 2009; Gremski et al., 2010). The latter *Loxosceles* venom proteome study noted that the serine proteases in venoms have also been related to complement activation (dos Santos et al., 2009). In fact, various studies have related the involvement of complement system factors in the pathological events triggered by

Loxosceles venom, such as hemolysis and dermonecrosis (Lane and Youse, 2004; Tambourgi et al., 2005). However, this feature is currently associated specifically with venom phospholipases D.

Venom serine proteases, in addition to their contribution to prey digestion, can play an important role in local tissue destruction and interfere in blood coagulation and fibrinolysis (Veiga et al., 2000a; Kini, 2005; Devaraja et al., 2010). In fact, venom serine proteases may possess thrombin-like, fibrinogenase and plasminogen-activating activities, and they are molecules with the potential to be novel diagnostic or anti-thrombotic agents (Muanpasitporn and Rojnuckarin, 2007). Snake venom serine proteases, in turn, have been used to determine fibrinogen levels in the presence of heparin (Reptilase® time, Funk et al., 1971) and to remove fibrinogen in samples for thrombin-dependent tests (Mullin et al., 2000). In addition, recombinant Ancrod®, a thrombin-like serine protease from the *Agkistrodon rhodostoma* viper, improves the outcomes after cerebral stroke in humans (Liu et al., 2011), and Defibrase®, from the *Bothrops* spp., is clinically beneficial in ischemic stroke (Guo et al., 2006). Thus, further studies concerning *Loxosceles* serine proteases are imperative for the development of potential novel therapeutic agents.

7.4. Serine protease inhibitors

Proteinaceous inhibitors of proteolytic enzymes comprise the largest group of naturally occurring enzyme inhibitors. Their vast structural diversity is detailed in the MEROPS database of peptidase inhibitors (available in http://merops.sanger.ac.uk/inhibitors/). Recent work comprehensively listed 91 families of protease inhibitors grouped based on their homology. Some families of the serine and cystein protease inhibitors stand out for their high frequency, such as the Kazal and Kunitz-type inhibitor families (e.g., I1 – I3 **peptidase inhibitor families**), serpins (e.g., I4 **peptidase inhibitor family**) and cystatins (e.g., I25 **peptidase inhibitor family**) (Rawlings et al., 2012).

The first report of the presence of protease inhibitors in *Loxosceles* venom glands was made in 2008 by Fernandes-Pedrosa and colleagues, who performed a transcriptome analysis of venom glands of female *L. laeta* spiders. The sequences that matched these molecules were described as “enzymatic inhibitors” and represented 0.6% of the total number of sequences analyzed.

Some transcripts of the *L. laeta* cDNA library are related to serine (or cysteine) protease inhibitors of diverse species, which have been characterized and have been shown to have different functions and activities, such as an *in vitro* anticlotting activity and *in vivo* antithrombotic and anticoagulant activities related to the inhibition of Factor-Xa. A proprotein-convertase (PC) inhibitor sequence of *Branchiostoma lanceolatum* (emb|CAD68157.1) was also listed as similar to some of the *L. laeta* ESTs (Bentele et al., 2006; Fernandes-Pedrosa et al., 2008). This serpin, termed Bl-Spn1, inhibits the proprotein processing proteases PC1/3 and furin (Bentele et al., 2006). Analyses of some of the ESTs from the *L. laeta* cDNA library revealed a similarity with a cystatin sequence from the tick *Boophilus microplus* (gb|ABG36931.1) that was found to inhibit the human cathepsin L and vitellin degrading cysteine endopeptidase (VTDCE). More recently, the venom of the brown spider *L. intermedia* was subjected to proteomic analysis through the MudPIT proteomic strategy, and approximately a dozen peptides were found to be similar to protease inhibitors (dos Santos et al., 2009). Three sequences showed similarity with an inhibitor of *Oryza sativa* from the cystatin super family (P20907) (Kondo et al., 1990; dos Santos et al., 2009). On the other hand, other peptides sequenced in this proteomic analysis are related to the Kunitz-type inhibitors (dos Santos et al., 2009). Finally, the *L. intermedia* proteome revealed some peptides related to an inhibitor from the serpin super family of protease inhibitors (P07385) (dos Santos et al., 2009). Thus, it is possible that *L. intermedia* venom contains proteases inhibitors belonging to different groups (i.e., the serpins, Kunitz-type and cystatin super families).

A transcriptome analysis of the venom glands of *L. intermedia* identified an EST similar to protease inhibitors from the serpin family (Gremski et al., 2010). This EST sequence is related to mammalian and arthropod serpins, such as the human neuroserpin and the *Ambliomma americanum* tick and *Tachypleus tridentatus* horseshoe crab serpins (Gremski et al., 2010).

The function of protease inhibitors in *L. intermedia* venom has been suggested to be related to the protection of the toxin integrity (dos Santos et al., 2009). Some authors that have described serine protease inhibitors in different venoms (snakes, spiders and scorpions) propose that one of the physiological roles of these molecules is to resist prey proteases to protect their venom protein toxins (Zupunski et al., 2003; Yuan et al., 2008; Zhao et al., 2011). In addition, these inhibitors may generate a synergistic effect with other neurotoxins, as suggested by other authors (Yuan et al., 2008; Zhao et al., 2011). Because the proteases are involved in several physiological processes, they represent excellent therapeutic targets. Thus, the protease inhibitors arising from venoms are potential candidates to mediate certain biological processes. The Kunitz-type protease inhibitor isolated from ***Pseudonaja textilis*** venom, **textilinin-1**, was submitted to a preclinical developmental program and has been shown to be equally effective as aprotinin, an anti-fibrinolytic agent that reduces the blood loss associated with cardiac surgery, but with an enhanced safety profile (Flight et al., 2005).

Certain serpins are able to reduce the excess protease activity and consequent damage associated with inflammatory diseases. SERP1 from the myxoma poxvirus, for example, inhibits human coagulation and fibrinolytic proteases and has been shown to have potent anti-inflammatory effects in the treatment of human inflammatory diseases induced by vascular injuries. Therefore, *Loxosceles* protease inhibitors emerge as compounds with potential therapeutic and biotechnological applications, which, in turn, depend on the further characterization of their biochemical and biological features.

7.5. Venom allergen

Hypersensitivity reactions from arthropod stings include immediate reactions, such as local swelling,

generalized urticaria and anaphylaxis. The pathogenesis is, in many cases, most likely an IgE-mediated reaction. Delayed reactions are also possible, for example, local papules or bullous, hemorrhagic reactions, disseminated papules, generalized papular urticaria and general systemic symptoms, such as fever, myalgia and lymphadenopathy. In some studies, deposits of complement components and immunoglobulins have also been found (Arlian, 2002; Bircher, 2005).

With the exception of bee and wasp venom allergies, the immediate type allergic reactions to arthropod stings and bites, such as mosquitoes, flies, ticks, moths, caterpillars and spiders, are rare (Bircher, 2005). Indeed, allergic reactions to the *Loxosceles* genus have been postulated in only a few cases (Donepudi et al., 2005; Robb et al., 2007; Makris et al., 2009; Lane et al., 2011). In approximately 25% of the published loxoscelism cases, a fine macular or papular eruption develops over the entire body (Pippirs et al., 2009). Cases of AGEP (Acute Generalized Exanthematous Pustulosis) following bites by *L. reclusa* and *L. rufescens* have also been reported (Makris et al., 2009; Lane et al., 2011).

The pathogenesis of AGEP is not clear, but it is a rare and severe cutaneous reaction usually triggered by drugs and viruses (Makris et al., 2009). Of note, several studies have shown that *Loxosceles* venom stimulates the release of large amounts of IL-8 and GM-CSF, in addition to other cytokines, such as the growth-related oncogene and protein-1 (Gomez et al., 1999). This release of IL-8 and GM-CSF could contribute to the development of AGEP following *Loxosceles* envenomation (Lane et al., 2011). In addition, the ability of this venom to evoke inflammatory events was partially reduced in compound 48/80 –pretreated animals, suggesting that mast cells may be involved in these responses. Pre-treating mice with receptor antagonists of histamine (prometazine and cetirizine) and of serotonin (methysergide) significantly attenuated the edema and vascular permeability induced by toxins (Paludo et al., 2009).

Corroborating the hypothesis that *Loxosceles* venom may cause allergic reactions, two transcriptome studies on *Loxosceles* venom glands found sequences similar to allergen-like toxins from other venoms. In the case of *L. laeta*, transcripts similar to venom allergen III (sp|P35779|VA3_SOLRI) represented 0.6% of the total sequences. The similarity of the putative amino acid sequence of an allergen from *L. laeta* with known venom allergen III sequences includes the presence of conserved cysteine residues (Fernandes-Pedrosa et al., 2008). Data from work with the cDNAs of the *L. intermedia* venom gland showed that some messages encode for venom allergens that are cysteine-rich molecules. These RNA messages are poorly expressed: two ESTs are grouped in one cluster representing 0.2% of the toxin-encoding transcripts. These transcripts putatively encode for allergens that show significant similarity to allergens from another spider genus (*Lycosa sigoriensis*), scorpion species (*Opisthacanthus cayaporum*) and some mite allergens (*Ixodes scapularis* and *Argas monolakensis*) (Gremski et al., 2010). In addition, an *L. intermedia* venom proteomic study also reported the presence of a putative allergenic protein similar to a mite allergen (dos Santos et al., 2009).

Some of the allergens have been characterized, and a few of them have been synthesized through recombinant techniques (Bircher, 2005). An isoform of a recombinant allergen from *L. intermedia* venom was cloned and had a calculated molecular mass of approximately 46.2 kDa and a predicted hydrophobic import signal (24 residues) to the endoplasmic reticulum (Ferrer, V.P. and de Mari, T.L. personal communication, 2013). With the availability of allergen sequences and purified recombinant allergens, allergen-specific cellular immune responses were investigated, and *in vivo* animal models based on defined and clinically relevant allergens were established (Valenta et al., 2011). In this context, the crystal structures from some recombinant allergens derived from insect venoms (wasps, bees, and fire ants) have been important in the search for specific or cross-reacted epitopes (Henriksen et al., 2001; Hoffman, 2008; Padavattan et al., 2008; Borer et al., 2012). Additionally, recombinant allergens were applied for *in vivo* provocation testing in allergic patients with the aim of comparing their biological activity to natural allergens and to explore their usefulness for *in vivo* diagnosis (Schmid-Grendelmeier and Crameri, 2001; van Hage-Hamsten and Pauli, 2004). These studies confirmed the biological equivalence of most of the recombinant allergen preparations with the corresponding natural allergens, indicating that the recombinant allergens can substitute for natural allergen extracts for *in vivo* applications (Valenta et al., 2011). In this way, the allergen-like toxin from *L. intermedia* venom may be a useful tool for investigating the underling mechanisms of allergic responses following spider bites involving this venom and might serve biomedical purposes in this area.

8. Modulation of cell and tissue structures by brown spider venom toxins

Brown spider venom toxins have been implicated in a number of histological changes following spider bites or experimental envenomation under laboratory conditions (Ospedal et al., 2002; da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006). The first and most characteristic tissue/cellular changes observed after brown spider venom exposure is the massive infiltration of inflammatory cells into the dermis and the generation of inflammatory mediators near the bite site or toxin injection (Ospedal et al., 2002; Domingos et al., 2003; Barbaro et al., 2010). Recombinant brown spider phospholipases D reproduce the above-mentioned histological changes (Chaim et al., 2006; da Silveira et al., 2006, 2007b; Ribeiro et al., 2007; Appel et al., 2008).

Although the modulation of leukocyte activity is demonstrated by the massive infiltration of skin structures, *Loxosceles* venom apparently has no direct stimulatory effects on leukocytes in culture, and leukocyte activation represents an indirect effect triggered by the endothelial cells of blood vessels exposed to the venom toxins. This hypothesis is supported by data from cell culture assays using human umbilical vein endothelial cells (HUVEC) treated with *L. reclusa* crude venom. The results pointed to a potent endothelial cell agonist activity of the venom, which stimulated the endothelial cell expression of E-

selectin and the secretion of the granulocyte macrophage colony-stimulating factor and interleukin-8, which resulted in a dysregulated inflammatory response (Patel et al., 1994). The treatment of HUVEC with *L. deserta* crude venom leads to the expression of a growth-related oncogene and to the synthesis and secretion of the monocyte chemoattractant protein-1 and interleukin-8 (Desai et al., 1999; Gomez et al., 1999). In addition, *L. deserta* venom evokes the expression of vascular endothelial growth factor (VEGF) in human keratinocytes (Desai et al., 2000), suggesting that VEGF may contribute to the endothelial activation observed after brown spider envenomation. Additional data from histopathological findings revealed that *L. intermedia* venom acts *in vivo* (intradermally injected) on rabbit vessel endothelial cells, which causes an endothelial-leukocyte adhesion, a massive transmigration of leukocytes across the endothelium, vessel instability, the degeneration of blood vessels and vascular leakage (Veiga et al., 2001b; Ospedal et al., 2002; Zanetti et al., 2002). Exposing cultured rabbit aorta endothelial cells (RAEC) to *L. intermedia* crude venom evokes the disadhesion of the cells and the degradation of heparan-sulfate proteoglycans, nidogen/entactin and fibronectin (Veiga et al., 2001a; Paludo et al., 2006). Moreover, the direct binding of the venom toxins on the endothelial cell surface has also been reported, which induces drastic morphological changes (Paludo et al., 2006). These data are supported by the internalization of the toxins following endothelial cell treatment with the *L. intermedia* crude venom, the involvement of endocytic vesicles and the final homing of toxins to lysosomes, culminating in cell death by anoikis (Nowatzki et al., 2010).

The direct binding of a recombinant *L. intermedia* phospholipase D on the surface of RAEC has also been reported, as well as the catalytic activity of this toxin to degrade RAEC membrane detergent-extracts, which generates important bioactive lipids and cell morphological changes (Chaim et al., 2011b). Additionally, by using cultured human fibroblasts exposed to a recombinant phospholipase D isoform from the *L. reclusa* venom, an upregulation of the human cytokines genes IL-6, IL-8, CXCL1, CXCL2 that are important inflammatory activators has been demonstrated (Dragulev et al., 2007). The authors postulated that together with the endothelia, the fibroblasts in the dermis also mediate the dysregulated leukocyte activation involved in dermonecrosis and are an additional cellular target for the venom toxins.

Other cells targeted by *Loxosceles* venom toxins are erythrocytes. The hemolytic activity evoked by *Loxosceles* venom was first demonstrated using clinical and laboratory observations from spider bite victims, some of which had a lethal outcome. These observations included elevated creatine kinase levels, hemoglobinuria, bilirubinuria, proteinuria, jaundice, acute hemolytic anemia, reticulocytosis, and shock (Lung and Mallory, 2000; França et al., 2002; Zambrano et al., 2005; de Souza et al., 2008; McDade et al., 2010; Malaque et al., 2011). The hemolytic activity is a conserved event because it has also been reported for the *L. similis*, *L. gaucho*, *L. laeta*, *L. reclusa* and *L. intermedia* venoms (da Silva et al., 2004; Silvestre et al., 2005; Zambrano et al., 2005; McDade et al., 2010; Malaque et al., 2011). The lysis of erythrocytes is associated with two distinct mechanisms, which are defined as direct (Chaves-Moreira et al., 2009, 2011) and complement-dependent hemolysis (Tambourgi et al., 2002, 2005, 2007; Pretel et al., 2005). Various studies have already reported that spider envenomation induces the activation of the alternative complement pathway facilitating complement-mediated hemolysis (da Silva et al., 2004; Swanson and Vetter, 2006). Tambourgi et al. (2002, 2005) evaluated the mechanism of hemolysis caused by *L. intermedia* venom and proposed the involvement of phospholipases D in the activation of an endogenous metalloprotease, which then cleaves glycophorins and causes erythrocyte susceptibility to lysis by human complement. In another study, the functional changes of rabbit erythrocytes were described following exposure to *L. gaucho* crude venom. The results pointed to an alteration of red cell function using an osmotic fragility test and greater deformability after venom exposure (Barretto et al., 2007).

Chaves-Moreira et al. (2009) compared the hemolytic activities of a recombinant active phospholipase D from *L. intermedia* venom (LiRecDT1) (Chaim et al., 2006) and its mutated version (LiRecDT1H12A) (Kusma et al., 2008). They demonstrated the involvement of a direct molecular mechanism dependent on the catalytic activity of phospholipase D in hemolysis, strengthening previous data that reported the participation of dermonecrotic toxins in red blood cell lysis. Furthermore, the metabolism of membrane phospholipids, such as sphingomyelin and lysophosphatidylcholine, and the influx of calcium mediated by an L-type channel in human erythrocytes have been shown to be involved in hemolysis (Chaves-Moreira et al., 2011).

Along with erythrocytes, platelets represent another target of brown spider venom. Data indicating platelets as a target were first described in biopsies of animal models exposed to crude venom, which reported the intravascular coagulation and thrombosis inside the dermal blood vessels. The hypothesis of platelet involvement is also strengthened by the findings of the infiltration and aggregation of inflammatory cells in the generated thrombus. In addition, clinical laboratory analysis of envenomed victims often reveals thrombocytopenia (Ospedal et al., 2002; da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006). Cellular changes in the bone marrow and peripheral blood of rabbits caused by *L. intermedia* venom include the marrow depression of megakaryocytes correlated with the thrombocytopenia in the peripheral blood observed in the early envenomation (da Silva et al., 2003). Similar results were reported by using *L. gaucho* venom, where a decrease in the platelet count in rabbit peripheral blood after venom and/or purified phospholipase D exposure, without platelet aggregation and no signs of platelet lysis, was observed. The activation of platelets after venom exposure is supported by the increased expression of ligand-induced binding site 1 and P-selectin (Tavares et al., 2004, 2011).

By studying recombinant venom phospholipases D and using human platelet-rich plasma, *in vitro* platelet aggregation activity has been reported (da Silveira et al., 2006, 2007b; Appel et al., 2008), strengthening the hypothesis of venom activity on platelets. However, the molecular pathway by which the toxins cause platelet aggregation is

not fully understood. In the case of phospholipases D, catalysis involvement is expected, such that it would generate a broad range of bioactive lipids on the platelet membrane and finally inducing aggregation.

Renal structures and kidney cells represent other targets for brown spider venom toxins. Renal injury was previously described in clinical and laboratory data from injured patients (da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006). Clinical case reports have indicated a direct correlation of renal damage and hemolysis induced by different *Loxosceles* venoms (Zambrano et al., 2005; Abdulkader et al., 2008; de Souza et al., 2008; Hubbard and James, 2011; Malaque et al., 2011). By studying experimental-induced kidney injury in rats exposed to *L. gaucho* crude venom, Lucato et al. (2011) concluded that this acute injury was a consequence of the impaired renal blood flow associated with the systemic rhabdomyolysis. Nevertheless, animal models or cultured MDCK epithelial cells exposed to *L. intermedia* crude venom or a recombinant phospholipase D have also provided evidence for a direct renal cytotoxicity (Luciano et al., 2004; Chaim et al., 2006; Kusma et al., 2008). Using mice exposed to a recombinant phospholipase D and a mutated isoform exhibiting decreased phospholipase activity, is has been shown that *in vivo* nephrotoxicity and *in vitro* MDCK cytotoxicity depends on the catalytic activity of the phospholipases (Kusma et al., 2008).

The activities of *Loxosceles* venom have also been demonstrated in other tissues and organs, such as the heart and liver. Dias-Lopes et al. (2010) showed cardiotoxic effects in mice administered *L. intermedia* venom. These effects were observed through the increase of the levels of markers associated with heart lesions, indicating that the venom antigens can reach the heart tissue and, thus, lead to cardiac dysfunction. Christoff et al. (2008) reported changes in histological and biochemical aspects of the liver in rats injected with *L. intermedia* crude venom. In the venom-treated group, the plasma levels of enzymes, such as alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl-transferase and lactate dehydrogenase, increased. Furthermore, histopathological changes indicating hepatic lesions were also observed.

Finally, the extracellular matrix and associated constituents represent a key histological structure targeted by *Loxosceles* venom toxins, as observed by the histopathological findings of hemorrhage into the dermis at the bite site, fibrin deposition inside the blood vessels, defective wound healing after bites, gravitational lesion spreading and the systemic dissemination of toxins (da Silva et al., 2004; Swanson and Vetter, 2006). Previous work also described the proteolytic activities of *L. intermedia* venom on gelatin, fibronectin and fibrinogen (Feitosa et al., 1998; Veiga et al., 2001a; Zanetti et al., 2002; Paludo et al., 2006), the disruption of EHS basement structures, the degradation of entactin/nidogen and the hydrolysis of heparan sulfate proteoglycan from endothelial cells (Veiga et al., 2001a, b) and more recently the involvement of a hyaluronidase in the noxius activity of venom (Ferrer et al., 2013) (See **topic** 7.2).

9. *Loxosceles* spider toxin immunology and perspectives for development of a new generation of antivenoms

Spiders and other venomous animals contain a complex mixture of biologically active substances developed to block the vital physiological and biochemical functions of the victims. Antidotes prepared from animal anti-sera are effective against all species of *Loxosceles* spiders; these antivenoms are less complex than other spider or snake antivenoms because the major toxic components of these spider venoms are proteins known as dermonecrotic toxins, sphingomyelinases D (SMases D), the phospholipase D family and Loxtox proteins (Kalapothakis et al., 2007; Binford et al., 2009; Wood et al., 2009). Although significant progress has been made in immunological studies of these groups of toxins, few medical and university centers are dedicated to this subject of research. Novel approaches based on epitopes and mimotopes selected from microarray peptides (Spot-synthesis) or from phage-displayed random peptide libraries have generated information sufficient to develop a new generation of antivenoms for therapeutic or vaccines purposes. The immunological investigation of the phospholipase D of *Loxosceles* spiders was mainly performed with LiD1, a dermonecrotic toxin from the venom of the *L. intermedia*, by Chávez-Olórtegui and his collaborators (Kalapothakis et al., 2002; Araújo et al., 2003; Felicori et al., 2006, 2009; Dias-Lopes et al., 2010). The cDNA encoding this protein was shown to display a similarity with the genes of the known *Loxosceles* phospholipase D toxins (Kalapothakis et al., 2007). The recombinant protein rLiD1 was strongly recognized by anti-*L. intermedia* crude venom and was also able to generate reactive antibodies against the native dermonecrotic proteins and whole *L. intermedia* venom. Using these antibodies and overlapping synthetic peptides covering the whole (LiD1) sequence, regions with an epitope function were revealed. The N-terminal (residues 13–27), central (residues 31–45, 58–72, 100–114, and 160–174) and C-terminal (residues 247–261) parts of the protein have been shown to contain continuous epitopes with neutralizing potential. The antibodies elicited by these epitopes were found to protect against the dermonecrotic-, hemorrhagic- and edema-forming activities induced by LiD1 and whole venom. To visualize the three-dimensional position of the experimentally determined epitopes, the LiD1 protein was modeled by homology using the solved structure of phospholipase D from *L. laeta as a template*. The localization of the epitopes in the context of the three-dimensional structure of the dermonecrotic protein is shown in Fig. 8. As shown, most of epitope regions determined were localized in the α-helix-loop regions. However, 3 of the 8 α-helix regions were not antigenic (Felicori et al., 2009).

Studies using monoclonal antibodies raised against the toxins of the *L. intermedia* whole venom revealed that one antibody (LimAb7) recognized several venom proteins, including LiD1 (Alvarenga et al., 2003). Because LimAb7 reacts with rLiD1, the LiD1 epitope recognized by LimAb7 was mapped. None of the overlapping peptides that

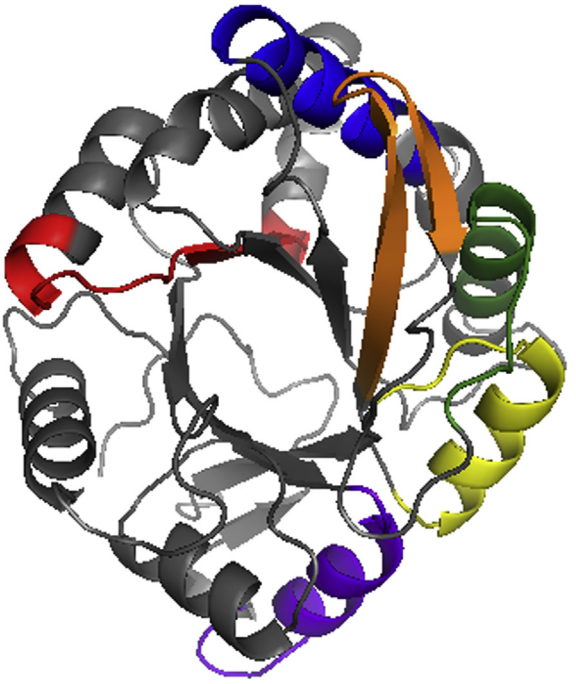

Fig. 8. LiD1 structural model indicating the position of the selected epitopes that reacted with the horse anti-*L. intermedia* venom serum. In yellow, the N-terminal epitope (residues 13–27); in orange, the central epitope (residues 31–45); in green, the central epitope (residues 58–72); in blue, the central epitope (residues 100–114); in red, the central epitope (residues 160–174); and in purple, the C-terminal epitope (residues 247–261). Figure color codes refer to the on-line images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

covered the sequence of LiD1 were recognized, indicating that the epitope is not continuous but rather, discontinuous (de Moura et al., 2011). Consequently, the phage-display technique was used, and this method allowed for the identification of mimotopes without homology between the amino acid sequences of the phage-selected peptides and the sequence of LiD1 (de Moura et al., 2011). The potential epitope regions in LiD1 based on the amino acid sequences of the selected mimotopes and on the 3D LiD1 protein model were predicted, and the MIMOP residues C^{197}, Y^{224}, W^{225}, T^{226}, D^{228}, K^{229}, R^{230}, T^{232} and Y^{248} were identified as being the putative epitope bound by LimAb7 (de Moura et al., 2011).

In a recent study, a chimeric protein (rCpLi) expressing the epitopes of LiD1 previously defined as residues 25–51 and 58–72 and a conformational epitope identified by the phage display technique were generated by cloning the respective synthetic genes in a pET 26b vector. ELISA and immunoblot assays revealed that the mini-protein displayed antigenic activity against the antibodies of the anti-individual epitopes. Anti-*Loxosceles* sp. crude venoms also reacted with rCpLi. Because the protein is non-toxic, it is considered to be an important immunogen target for vaccines against this dangerous regional spider (Mendes et al., 2013).

10. Biotechnological use of brown spider venom components

Spider venoms are mixtures of several hundred biologically active proteins, glycoproteins and peptides that act synergistically as an adaptation to defend against predators and to paralyze and kill insect prey. Because these toxins are active on different cells and tissue structures and effectively modulate distinct physiological responses in insects and vertebrates, they are potential models to study the design of pharmacological tools, drugs and/or biochemical, immunological and cell biology reagents.

The first biotechnological application of brown spider venom constituents consisted of the antiserum-based products used for the therapy of spider bite victims. For example, anti-arachnid serum (obtained using the venom of *L. gaucho*) was produced by the Butantan Institute, São Paulo, Brazil; anti-*Loxosceles* serum using the *L. intermedia*, *L. gaucho and L. laeta* crude venoms was produced by the Production Center of Immunobiologic Products, Parana, Brazil; and anti-*Loxosceles* serum raised against *L. laeta* venom was produced by the National Institute of Health (Peru) (Roodt et al., 2002; da Silva et al., 2004; Pauli et al., 2009). With the recombinant brown spider venom toxins available, a new generation of loxoscelic antiserum could be produced directly by using the antigenic active recombinant toxins from different *Loxosceles* species or by enriching the crude venom with biologically active recombinant molecules to increase antibody production and venom neutralization. In fact, a recombinant phospholipase D was used to produce an anti-*Loxosceles* serum that was able to neutralize the toxic effects induced mainly by the *L. intermedia* and *L. laeta* or slightly weaker the activity of *L. gaucho* venoms (de Almeida et al., 2008). Additional recombinant antigens should be used as antigenic sources for vaccines or antivenom development (See **topic** 10).

Another *Loxosceles* venom-based product is named ARACHnase (Hemostasis Diagnostics International Co., Denver, CO, USA). It consists of plasma containing *L. reclusa* crude venom that mimics the presence of a lupus anticoagulant and should be a useful positive control for lupus anticoagulant testing (McGlasson et al., 1993).

Based on their properties, brown spider recombinant phospholipases D could be used as putative models for the application in the different areas of cell biology, immunology, pharmacology and biochemistry. They can be used, for example, as reagents for biochemical lipid research protocols by generating bioactive lipids, such as ceramide-1-phosphate from the hydrolysis of sphingomyelin and lysophosphatidic acid from the hydrolysis of lysoglycerophospholipids (Lee and Lynch, 2005; Chaim et al., 2011b). In addition, they may be applied in cell biology studies that investigate the biological activities triggered by ceramide-1-phosphate, lysophosphatidic acid and their derived molecules, such as the control of cell proliferation, death, differentiation and migration (Anliker and Chun, 2004; Chalfant and Spiegel, 2005). *Loxosceles* recombinant phospholipases D or mutated isoforms (Kusma et al., 2008; Chaim et al., 2011b; Mendes et al., 2013) could also be used as immunological adjuvant molecules for stimulating

immunogenicity because they can modulate inflammation and stimulate the production of cytokines in different cell models. Recombinant phospholipases D could be used as standard laboratory reagents to investigate platelet aggregation, platelet receptor(s), and related molecular pathways. In addition, these recombinant proteins could be used as reagents to induce hemolysis, possibly establishing a new model of hemolysis dependent on phospholipase D and bioactive lipids. Furthermore, recombinant phospholipases D could be used as reagents applied in the diagnosis of loxoscelism because a clinical laboratory diagnosis is currently unavailable. Because brown spider venom phospholipases D are strong antigenic molecules and are highly expressed molecules in crude venom (Ribeiro et al., 2007; Gremski et al., 2010; Wille et al., 2013), recombinant phospholipases D could be used as antigens for generating polyclonal or monoclonal antibodies for the diagnosis of Loxoscelism (de Moura et al., 2011).

ICK peptides have been studied as potential insecticidal bioactive toxin molecules, and recombinant brown spider ICK toxins (Matsubara et al., 2013) could be used as substitutes for chemical defense products as well as in transgenic agricultural models, **if further studies show that they specifically act upon insect channels**. In addition, brown spider ICK peptides could also be useful reagents to probe ion channel structures and functions, as previously described for other similar molecules (Dutertre and Lewis, 2010; Klint et al., 2012).

Recombinant *Loxosceles* hyaluronidase (Ferrer et al., 2013) could be used as a reagent in the biochemical studies of glycosaminoglycan hydrolases and as a tool to design **specific** inhibitors to reduce the spread of venom and toxins **retaining the activity of native hyaluronidases**. Moreover, because hyaluronidases are involved in bacterial pathogenesis, fertilization, and cancer progression, recombinant brown spider hyaluronidase could be utilized to generate hyaluronidase inhibitors that regulate several pathological events involving the balance between the anabolism and catabolism of HA. Finally, it could be used as an adjuvant molecule to increase drug absorption through increased tissue permeabilization (da Silveira et al., 2007c; Ferrer et al., 2013).

Recombinant brown spider venom astacins (da Silveira et al., 2007a; Trevisan-Silva et al., 2010) could be used as tools in the study of extracellular matrix remodeling, for the generation of proteolytic inhibitors and as direct thrombolytic agents for the treatment of vascular diseases.

Finally, other brown spider venom constituents also have putative biological applications. These include serine protease inhibitors, which could be useful agents for the investigation of general proteolysis, and recombinant TCTP (Sade et al., 2012), which could be used to study tumor cell behavior in experimental oncology, to study cell proliferation mechanisms, in the screening of anticancer drugs and as a model for allergy screening. Recently, the N-terminal fragment of TCTP (MIIYRDLISH) was shown to function as a **P**rotein **T**ransduction **D**omain (PTD), which is cell-penetrating peptide. This new feature of TCTP is being studied in drug delivery systems development (Maeng et al., 2013). Detailed data on the putative biotechnological use of brown spider venom toxins have been provided in Senff-Ribeiro et al. (2008) and Chaim et al. (2011a).

11. Future directions

Although many scientific studies have been published in recent years examining the brown spider venom and loxoscelism that have brought insights and improved the knowledge base regarding these topics, there are several opened questions still to be answered, and the challenges and opportunities for researchers are enormous. The use of combined data from molecular biology techniques, bioinformatics, proteomic studies, transcriptome analysis, and the expression of recombinant toxins will open great possibilities in this field.

The challenges concerning brown spiders and loxoscelism can be divided into clinical and basic research. The first clinical challenge is the production of a new generation of antisera using purified recombinant brown spider bioactive and antigenic competent toxins individually or by enriching crude venoms with recombinant toxins. Such antisera would be monospecific and may be used at lower doses, thereby decreasing the deleterious side effects of serum therapy, but with the same or higher efficiency in neutralizing the noxious venom activities.

A second clinical challenge hinges on the improvement of treatment of the injured victims. Currently, loxoscelism treatment is empirical and based on clinical signs, as described above (**topic** 4). A molecular comprehension of brown spider venoms and the mechanism by which the toxins trigger their effects, together by obtaining recombinant toxins and toxin 3D structural/biological data, will open to the possibility of a rational design of synthetic inhibitors directed at the specific venom toxins involved in the local and systemic effects. In addition, inhibitors for the receptors or cellular molecules involved in the metabolism of the bioactive lipid mediators generated by phospholipases D could also produce novel and powerful tools for the treatment of loxoscelism. Another clinical hurdle is the development of a clinical, *in vitro* diagnostic for loxoscelism with the sensitivity and specificity for different brown spider species. This diagnostic would be based on low-invasive molecular biology techniques and would provide sensitivity and specificity for different brown spider species using blood or even urine taken from exposed victims.

Finally, because loxoscelism is a public health problem around the world, the development of rational biological control methods, which are currently not available, is necessary to decrease the number of spiders and domiciliary infestations in the endemic regions. Similar methods are currently used for agricultural plagues, utilizing bacteria, fungus or other natural predators (Boyer et al., 2012; Lockett et al., 2012) to replace the pesticides currently used as a nonspecific biological control method and which cause environmental and human hazards.

The future of basic research on brown spider venoms and/or loxoscelism represents a remarkable challenge. Although considerable growth in this field has occurred, a great number of molecules in brown spider venoms remain

unidentified or their biological effects and mechanisms have not been described, especially for toxins with low level of expression and/or novel toxin isoforms of previously described. In addition, a genomic project focusing on *Loxosceles* species is a rational future direction that will bring novel insights for brown spider biology and loxoscelism and that will create access for several novel research tools.

Another current challenge for the brown spider venom toxinologist is the access to purified recombinant toxins in models other than bacteria. Currently, all recombinant brown spider venom toxins obtained have been produced in bacteria, an inexpensive expression model system that is simple to manipulate. However, because it does not generate co- and post-translational modifications, such as N-glycosylation and disulfide bonds, bacteria model systems often produce recombinant molecules in their unfolded form, with incorrect conformations, water insolubility, and with no biological function. The synthesis of brown spider venom recombinant toxins using alternative expression models with additional features that optimize and refine this process, such as the yeast *Pichia pastoris*, the insect *Drosophila* Schneider cells and mammalian systems, is an immediate challenge.

Another future direction is to obtain native brown spider venom toxins by developing primary cultures of the venom secretory cells. Similar approaches have been successfully established for other venomous animals. Examples include culturing the secretory cells from the venom glands of snakes *Crotalus durissus terrificus* and *Bothrops jararaca* (Duarte et al., 1999; Yamanouye et al., 2007) and from the *Phoneutria nigriventer* spider (Silva et al., 2008). These cultures could produce and secrete sufficient amounts of native toxins to be useful for biological and biotechnological evaluation. The use of mass spectrometry analysis and other proteomic protocols, such as 2-DE, N-terminal amino acid sequencing and high efficiency chromatography, provide great promise for detailed studies of brown spider venoms and hemolymph proteins and peptides. To date, only two studies have addressed this topic: Machado et al. (2005) described eleven isoforms of the phospholipase D toxin in *L. gaucho* venom, and dos Santos et al. (2009) identified 39 proteins in *L. intermedia* venom. There are no reports of this type of study for hemolymph.

Additionally, in the near future, novel data will provide information related to the tridimensional toxin structures, which will require the experimental co-crystallization of putative ligands or substrates to recombinant toxins. The tridimensional analysis of brown spider venom toxins will be critical to elucidate the location of the catalytic sites and sites that interact with natural substrates or ligands, and especially to show how the toxins interact with cell structures. These analyses will allow for the development of synthetic ligands, analogs, or inhibitors.

Finally, an attractive and practically unknown model for studying *Loxosceles* spiders is the analysis of hemolymph contents and its relationship to venom toxins and loxoscelism. From such analysis, natural inhibitors and/or other important molecules could be discovered, which would add great value to the field of toxinology.

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Conflict of interest statement

None.

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Structural Insights into Substrate Binding of Brown Spider Venom Class II Phospholipases D

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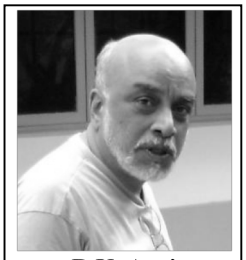

R.K. Arni

Abstract: Phospholipases D (PLDs), the major dermonecrotic factors from brown spider venoms, trigger a range of biological reactions both *in vitro* and *in vivo*. Despite their clinical relevance in loxoscelism, structural data is restricted to the apo-form of these enzymes, which has been instrumental in understanding the functional differences between the class I and II spider PLDs. The crystal structures of the native class II PLD from *Loxosceles intermedia* complexed with *myo*-inositol 1-phosphate and the inactive mutant H12A complexed with fatty acids indicate the existence of a strong ligand-dependent conformation change of the highly conserved aromatic residues, Tyr 223 and Trp225 indicating their roles in substrate binding. These results provided insights into the structural determinants for substrate recognition and binding by class II PLDs.

Keywords: Loxoscele, *myo*-inositol-1-phosphate, phospholipase D, sphingomyelin.

INTRODUCTION

The crude venoms of spiders of the *Loxosceles* species contain approximately 20% (w/w) phospholipases D (PLD) [1, 2] which induce dermonecrosis *in vivo* and are hence referred to as dermonecrotic toxins since they play key roles in the development and manifestation of the observed clinical symptoms of loxoscelism [3, 4]. Most PLDs hydrolyze both cell membrane lipids such as sphingomyelin (SM) and plasma lysophosphatidylcholine (LPC) into ceramide-1-phosphate (C1P) and lysophosphatidic acids (LPAs), respectively, thereby generating a receptor-dependent inflammatory response and bioactive metabolites and are hence, also referred to as Sphingomyleinases D (SMase D) [5, 6]. More recently, recombinant PLDs from *Loxosceles arizonica* were shown to possess the ability to catalyze the transphosphatidylation of SM to generate cyclic ceramide phosphate (CC(1,3)P) and to catalyze LPC to generate palmitoyl cyclic phosphatidic acid (CPA). The biological properties of cyclic phosphates are vastly different from their monoester counterparts, and these molecules might be relevant for the observed pathology of Brown spider envenomation [7]. In addition, the PLD from *L. reclusa* was shown to upregulate the expression of pro-inflammatory cytokines after exposure to the venom or toxin. Collectively, these functional findings have emphasized the importance of brown spider PLDs in lipid [8] metabolism, cell signaling, meiosis, and vesicle trafficking [9], providing insights into novel pharmacological properties of these toxins.

Sequence alignment and structural superpositioning of PLDs indicate that the amino acids essential for metal-ion binding and catalysis are strictly conserved (Fig. **1**) and that they can be grouped into two major structural classes [10]. The class I members, represented by the SMase I from *L. laeta*, possess a single disulphide bridge and contain an extended hydrophobic loop [10], whereas class II members possess an additional disulphide bridge that links the catalytic loop to the flexible loop significantly modifying the shape and volume of the active-site cleft. A subgroup, class IIb PLD, where the amino acids forming the metal-ion binding and catalytic sites are strictly conserved have been shown to be catalytically inactive against PLD substrates. Murakami *et al.* (2006) proposed that the microenvironment around the entrance to the active site becomes hydrophilic with the substitution of two residues, G95N/K in *L. laeta* and *L. spinulosa,* respectively, and P134E for *L. laeta*. This change in the polarity of the interface might affect the charge balance at the Mg^{2+}-binding site and results in repulsive forces that are detrimental to substrate binding (Fig. **1**). Currently, structural data of spider PLDs complexed with substrates and substrate analogues, which would be useful to understand the structural determinants for substrate recognition and binding, is not available.

We have solved the crystal structures of the recombinant wild-type and H12A mutant PLD from *Loxosceles intermedia* referred to as the dermonecrotic toxin 1 (LiRecDT1)

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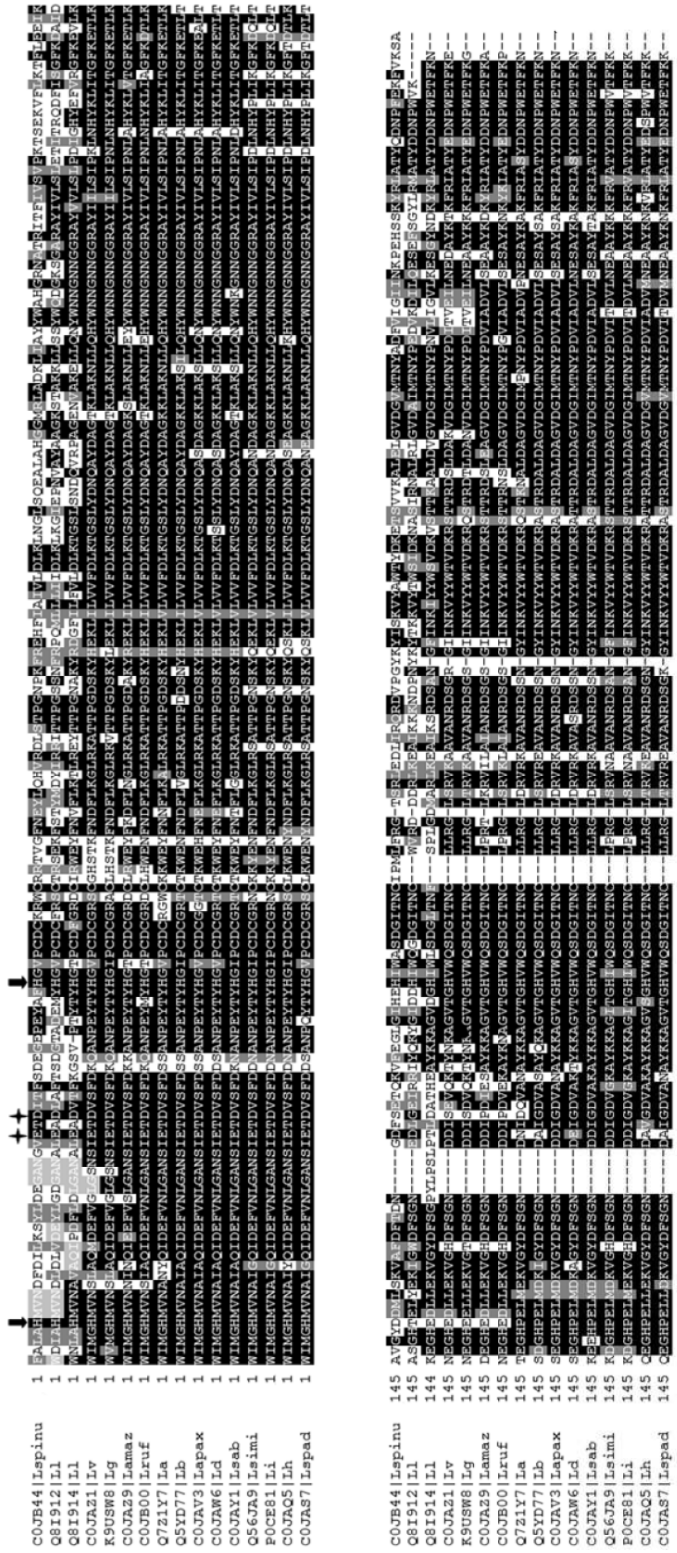

Fig. (1). Alignment of the complete deduced amino acid sequences of PLD class I and class II from different *Loxosceles*. The N-terminus amino acid position of the mature toxins is located at position 1. The conserved catalytic histidines residues are marked with black arrows. Residues responsible for Mg^{2+} ion coordination are marked with black stars. The following sequences were used to build this alignment and were obtained from Uniprot database: *Loxosceles spinulosa (ID:* C0JB44); *Loxosceles laeta* (ID: Q8I912); *Loxosceles laeta* (ID: Q8I914): *Loxosceles variegata* (ID: C0JAZ1); *Loxosceles gaucho* (ID: K9USW8); *Loxosceles amazonica* (ID: C0JAZ9); *Loxosceles rufescens* (ID: C0JB00); *Loxosceles arizonica* (ID: Q7Z1Y7); *Loxosceles boneti* (ID: Q5YD77); *Loxosceles apachea* (ID: C0JAV3); *Loxosceles deserta* (ID: C0JAW6); *Loxosceles sabina* (ID: C0JAY1); *Loxosceles similis* (ID: Q56JA9); *Loxosceles intermedia* (ID: P0CE81); *Loxosceles hirsuta* (ID: C0JAQ5); *Loxosceles spadicea* (ID: C0JAS7).

complexed with *myo*-inositol and octanoic acid, respectively. LiRecDT1, a class II enzyme [11] demonstrates PLD head-group specificity by recognizing and cleaving common phospholipids such as lysophosphatidylcholine (LPC), sphingomyelin (SM) or lysoPAF, but is unable to efficiently hydrolyze phosphatidylcholine (PC) [3,11]. These complexes enable us to delineate residues relevant for substrate binding such as the conserved Trp225 (Trp230 in the numbering scheme of Murakami *et al.*, [10]) and Tyr223, and demonstrate, for the first time, that a PLD can possibly hydrolyze phosphatidyl-inositol monophosphate (PiP). In the light of this finding, we suggest that this PLD from *Loxosceles intermedia* could participate in other signaling pathways related to inflammation and proliferation events by releasing PiP.

MATERIALS AND METHODS

Expression and Purification

Mature dermonecrotic toxin from *L. intermedia* (LiRecDT1) was cloned into a pET-14b vector (Novagen, madison, USA) and expressed in *Escherichia coli* BL21(DE3)pLysS cells (Invitrogen) as described previously [12]. Expression was induced by the addition of 0.5 mM IPTG (isopropyl β-$_D$-thiogalactoside) during 3.5 h at 30 ºC after the cell culture had reached a value $O.D_{550\ nm}$ of 0.5. The cell suspension was disrupted by six 10 s-cycles of sonication. Lysed material was centrifuged (20,000 x g, 20 min) and the supernatant was incubated with 1 ml Ni^{2+}-NTA agarose beads for 1 h at 4 ºC. The suspensions were loaded onto a column and the packed gel was washed with 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole. The recombinant protein was eluted with 10 ml of the above buffer, which additionally contained 250 mM imidazole and 1 ml fractions were collected and analyzed by 12.5% SDS-PAGE. Fractions were pooled and dialyzed against phosphate buffer saline (PBS).

Site-Direct Mutagenesis

The wild-type LiRecDT1 gene was used as a template for site-direct mutagenesis. The residue His12 was mutated to alanine (LiRecDT1-H12A) using the mega-primer strategy. His12 is one of the two catalytic residues and its truncation to alanine yielded an inactive enzyme as demonstrated previously [13]. The H12A protein was produced and purified as described for the wild-type protein.

Crystallization

LiRecDT1 was crystallized by vapor diffusion in sitting drops using a Cartesian HoneyBee 963 system (Genomic solutions) at 18 ºC as described earlier [13]. The crystal used for data collection was obtained from a mixture of 2 µl of protein solution (17 mg ml^{-1}) and 2 µl of the reservoir solution equilibrated over 1 ml of reservoir solution (0.1 M Tris-HCl pH 7.5, 40% (v/v) PEG 200).

X-Ray Data Collection and Processing

LiRecDT1 crystals were directly flash-cooled in a 100 K nitrogen-gas stream. X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light laboratory (Campinas, Brazil). The data were indexed, integrated and scaled using DENZO and SCALEPACK programs from HKL-2000 package [14]. Data collection and refinement statistics are summarized in Table **1**.

Table 1. Data collection and refinement statistics.

Data Collection	SMase Native	SMase H12A
Radiation source	W01B-MX2 (LNLS, Brazil)	
Wavelength (Å)	1.458	1.458
Detector	MarMosaic 225	
Space group	$P12_11$	$P12_11$
Unit-cell parameters (Å)	*a*=49.81, *b*=49.30, *c*=56.30	*a*=49.76, *b*=49.34, *c*=56.15
Resolution (Å)	30.0-1.72 (1.76-1.72)	24.4-1.6 (1.66-1.63)
R_{merge} (%)	12.1 (49.4)	7.3 (37.0)
$I/\delta(I)$	9.3 (2.4)	19.5 (2.9)
Completeness (%)	99.9 (99.8)	95.4 (74.1)
No. of measured reflections	28074	
No. of unique reflections	25218 (4040)	47089 (3241)
Average B-factor (Å)	19	
R_{factor} (%)	18.3	17.7
Free R_{factor} (%)	22.3	21.6
R.m.s. deviations		
Bond lengths (Å)	0.008	0.019
Bond angles (º)	1.34	2.03
Ramachandran plot (%)		
Most favored	99.3	99.3
Additionally allowed	0.7	0.7
Nº of atoms		
Protein atoms	2241	2241
Water molecules	149	87
Magnesium ions	1	1
PEG	2	-
IPD	1	-
Glycerol	1	1
Fatty acid	7	3

† $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i(hkl) - (I(hkl))| / \Sigma_{hkl} \Sigma_i I_i (hkl)$, where $(I(hkl))$ is the mean intensity of the observations $I_i(hkl)$ of reflection *hkl*. Values in parentheses are for the highest resolution shell.

Structure Determination and Refinement

The structures of LiRecDT1 and mutant LiRecDT1 H12A were determined by molecular replacement using the program MOLREP [15] and the atomic coordinates of the PLD from *L. intermedia* as the template (PDB code 3RLH; [11]). Refinement was carried out using cycles of REFMAC5 [16] interspersed with visual inspection of the electron density maps and manual rebuilding with COOT [17]. One magnesium ion (Mg^{2+}), one *myo*-inositol molecule in the active site, two polyethylene glycol (PEG), one glycerol (GOL), and seven fatty acids with different numbers of carbon atoms were constructed in the difference Fourier map of LiRecDT1. One magnesium ion (Mg^{2+}), three fatty acids, and one glycerol (GOL) molecule were modeled in the map of LiRecDT1 mutant. The refinements of both structures converged to crystallographic residuals of 18.3% and 17.7% (22.3% and 21.6% R_{free}) for structures of the WT and mutant enzymes respectively. No Ramachandran plot outliers were present. Data collection and structure refinement statistics are summarized in Table **1**.

Molecular Dynamics Simulation

The missing hydrogen atoms were added to the phophatidylinositol fragment from the crystal structure in order to optimize the ligand geometry by Conjugate Gradient Method by the Avogrado 1.0.3 software [18]. The gradient converged after 131 steps using the GAFF force field [19] and the convergence criteria of 10^{-4}. The structure present in the last step of the optimization process was used to initiate geometry optimization with Gaussian 09 [20]. The theory level HF/6-31G* was used to optimize the structure and to calculate the ESP. RESP charges and force field parameters based on GAFF were obtained using antechamber and parmcheck suite implemented in AMBER 11 [21]. Molecular dynamics simulations using the AMBER 11 package were performed in three different protonation states for His12 and His47. In the first state, both ND1 and NE2 nitrogens were protonated in His12 and His47, in the second state, both ND1 and NE2 were protonated in His12 and only ND1 in His47. For the third state, ND1 and NE2 were protonated in His47 and only ND1 in His12. The parameters previously obtained by antechamber were used for *myo*-inositol and AMBER 99SB parameters were applied to the protein. All systems were neutralized using Cl ions if necessary, and placed in a rectangular box of TIP3P [22] water extended 10 Å away from any protein atom. Restrained minimizations runs were done with 10.000 steps of steepest descent followed by 10.000 steps of conjugate gradient applying a constant of 50 kcal/mol.A^2 to protein-*myo*-inositol complex. More 10.000 steps of steepest descent followed by 10.000 steps of conjugate gradient were done allowing solvent and solute to relax. The systems were heated for 100ps by a Langevin thermostat [23] from 0 to 300 K maintaining a restrain constant to the solute of 50 kcal/mol.A^2. After that, 300 *ps* of equilibration with constant pressure of 1 atm, without restraints, was performed by nearly 25 *ns* for production. Long range interactions were treated using the particle mesh Ewald method [24], van der Waals and short-range electrostatic interactions were calculated within a 12 Å cutoff. Bonds containing hydrogen atoms were constrained by using the SHAKE algorithm [25]. The time step of 2 *fs* was used during heating, equilibration and simulation.

RESULTS AND DISCUSSION

The spider venoms PLDs are phylogenetically related to the glycerophosphoryl diester phosphodiesterases [10] and possess a conserved TIM-barrel fold with a metal-ion binding site (Fig. **2**). The crystal structures of the wild-type LiRecDT1 and the H12A mutant, both in complex with ligand bound to the active site, were determined at 1.7 and 1.6 Å resolution, respectively.

LiRecDT1 in Complex with Myo-Inositol-1-Phosphate

The *myo*-inositol-1-phosphate molecule is located in the active-site cleft of LiRecDT1 interacting with the two catalytic His residues (His12 and His48) and with Asp92, Lys94 and Tyr223. The phosphoryl group is hydrogen bonded to the Tyr223 side chain, whereas the *myo*-inositol moiety is stabilized by interactions with His12, His48, Asp92 and Lys94 (Fig. **3a**) and by a hydrophobic coplanar stacking interaction with the conserved aromatic residue Trp225. Lys94 is also highly conserved plays a crucial role in orienting the bound substrate and moderating the charge during catalysis. To evaluate the importance of these amino-acid residues in substrate binding, the mutants H12A, H48A, K94A and Y223A were constructed by molecular modeling and the minimized models were analyzed Table **S1** (Supplementary material). As expected, these mutations were detrimental for *myo*-inositol 1-phosphate binding with significant changes in the hydrogen bond pattern. Moreover, the ligand binding was evaluated by molecular dynamics simulations varying the protonation states of the two catalytic residues (Fig. **S1**). According to the H^{++}server [26], His12 is charged and His48 is neutral. This protonation state maintains the ligand in a more stable conformation as indicated by MD simulations, corroborating previous studies in which the mechanism of action was proposed [10]. Interestingly, in the absence of the metal ion Mg, with charge +2, the protonation state of His12 changes from charged to neutral as shown by H++Server. This supports the concept that the metal ion may play a crucial role in determining the proper pKa of the catalytic residues. The surprising finding of the complex LiRecDT1 with *myo*-inositol-1-phosphate not only permitted the identification of residues most likely to be involved in substrate binding of a class II PLD, but also suggested an important functional role of this toxin. *Myo*-inositol is a precursor for the phosphatidyl-inositol signaling pathway, which is important and not widely encountered in the plasma membrane. Furthermore, phosphatidyl-inositol phosphate (PiP) is a key regulator of diverse cellular processes in eukaryotic cells, expanding the possibilities of biological activities for spider class II PLDs, beyond SM and LPC degradation.

H12A Mutant in Complex with Octanoic Acid

The crystal structure of the H12A mutant was refined at resolution 1.63 Å (Table **1**) and is very similar to the wild-type protein with a root-mean-square (r.m.s) deviation for all Cα atoms in the range of 0.3 to 0.4 Å. As expected no electron density was observed for the imidazole side-chain of the

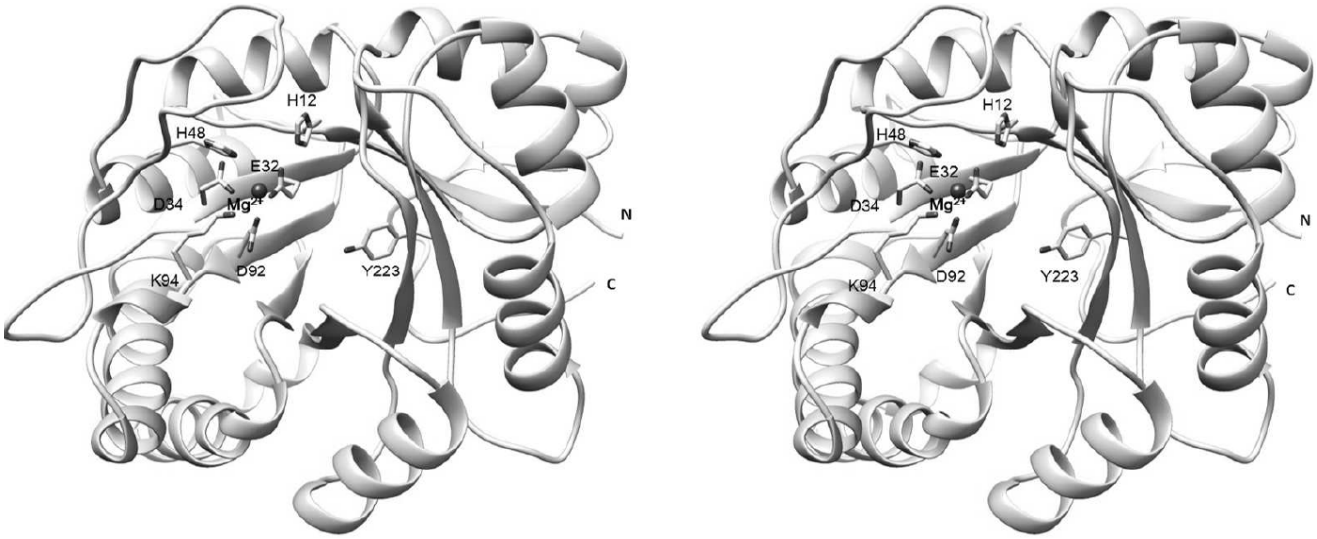

Fig. (2). Stereo view of phospholipase D from *Loxosceles intermedia* model in ribbon view. The magnesium ion is highlighted. Figures were produced using the programs Chimera [29].

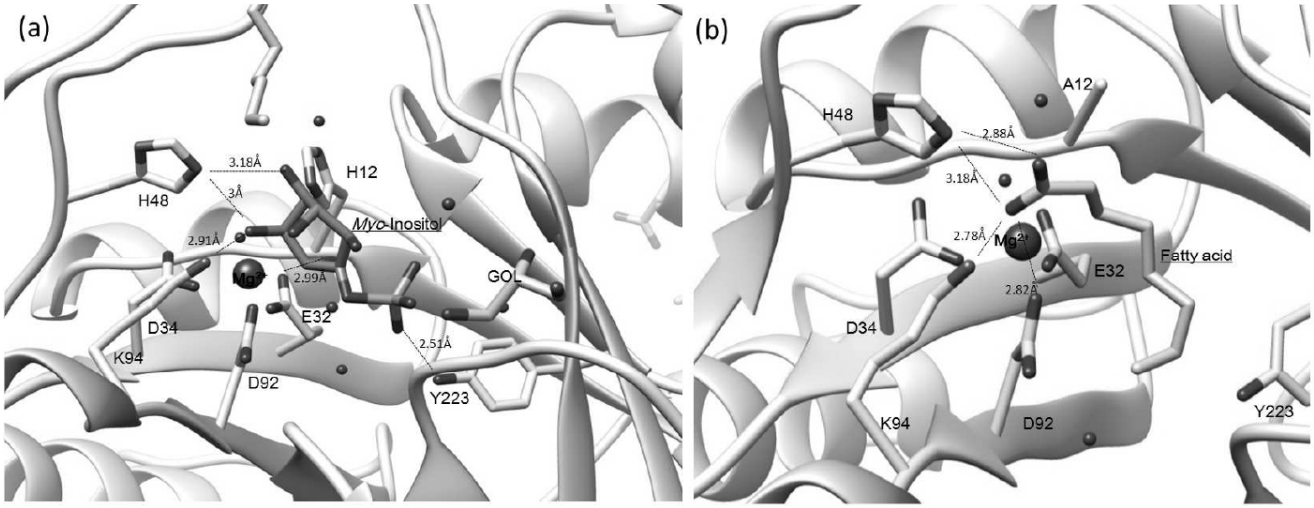

Fig. (3). Ribbon representation of the interactions of the ligands within the PLD binding pocket. (**a**) Representation of the WT PLD structure with the relative position of the *myo*-inositol 1 phosphate and residues mentioned in the text are shown. (**b**) Representation of the mutant PLD structure and the octanoic acid within the active site. Residues mentioned in the text are shown as sticks colored by atom type. Magnesium ions are colored in red. Figures were produced using the programs Chimera [29].

residue His12 and a fatty acid ligand was found in the active-site cleft. The octanoic acid, comprising an eight-carbon chain, was hydrogen bonded to the catalytic His48, interacting with the conserved Lys94 in a similar mode as observed for the *myo*-inositol-1-phosphate (Fig. **3b**). This finding is in agreement with functional studies in which LiRecDT1 is able to cleave a variety of lipidic compounds encountered in cell membranes.

CONCLUSION

The two recombinant proteins, LiRecDT1 [27], and the mutated toxin LiRecDT1-H12A [13], from *L. intermedia* spider venom were crystallized and their structures were determined with *myo*-inositol-1-phosphate and octanoic acid bound at the active site, respectively. The interaction of the *myo*-inositol 1-phosphate with PLD from *L. intermedia* in the electron density map was very clear for the entire ligand ring but was characterized by poor density for the fatty acid, suggesting the presence of multiple conformations and no solvent-mediated contacts between LiRecDT1 and the ligand were observed.

The molecular dynamic data obtained with *L. intermedia* PLD (wild-type and mutated) in complex with different ligands, provided evidence for the binding of *myo*-inositol 1-phosphate and fatty acid and indicates that derivatives of *myo*-inositol 1-phosphate can be used as inhibitors for this class of enzyme. Our results suggest the probable existence of alternate signaling pathway for the observed pathogeny. The metabolite phosphatidic acid is a bioactive compound and has been implicated among others, in cell proliferation and differentiation, cell transformation, signaling and seems to regulate membrane trafficking events, and is also implicated in causing tissue damage during inflammation [28].

LIST OF ABBREVIATIONS

A	=	Alanine
Asp	=	Aspartic acid
C1P	=	Ceramide-1-phosphate
CC(1,3)P	=	Cyclic Ceramide Phosphate
CPA	=	Cyclic Phosphatidic Acid
E	=	Glutamic acid
fs	=	Femtosecond
G	=	Glycine
GOL	=	Glycerol
H/His	=	Histidine
His/H	=	Histidine
IPTG	=	Isopropyl β-$_D$-thiogalactoside
K/Lys	=	Lysine
LPC	=	Lysophosphatidylcholine
Mg^{2+}	=	Magnesium
N	=	Asparagine
P	=	Proline
PBS	=	Phosphate Buffer Saline
PC	=	Phosphatidylcholine
PEG	=	Polyethylene Glycol
PiP	=	Phosphatidyl-inositol Monophosphate
PLD	=	Phospholipase D
r.m.s	=	Root-mean-square
SM	=	Sphingomyelin
Trp	=	Tryptophan
WT	=	Wild-Type
Y/Tyr	=	Tyrosine

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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