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# Molecular Cloning, Expression, Function, Structure and Immunoreactivities of a Sphingomyelinase D from *Loxosceles adelaida*, a Brazilian Brown Spider from Karstic Areas

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## 1. Introduction

*Loxosceles* is the most poisonous spider in Brazil and, at least, three different species of medical importance are known in Brazil (*L. intermedia*, *L. gaucho*, *L. laeta*), with more than 5000 cases of envenomation reported each year. In South Africa, *L. parrami* and *L. spinulosa* are responsible for cutaneous loxoscelism (Newlands et al., 1982). In Australia, a cosmopolitan species, *L. rufescens*, is capable of causing ulceration in humans. In the USA, at least five *Loxosceles* species, including *L. reclusa* (brown recluse), *L. apachea*, *L. arizonica*, *L. unicolor* and *L. deserta* are known to cause numerous incidents (Ginsburg & Weinberg, 1988; Gendron, 1990; Bey et al., 1997; Desai et al., 2000).

Several studies have indicated that sphingomyelinase D (SMase D) present in the venoms of *Loxosceles* spiders is the main component responsible for the local and systemic effects observed in loxoscelism (Forrester et al., 1978; Kurpiewski et al., 1981; Tambourgi et al., 1998, 2000, 2002, 2004, 2005, 2007; van den Berg et al., 2002, 2007; Fernandes Pedrosa et al., 2002; Paixão Cavalcanti et al., 2006, Tambourgi et al., 2010). SMases D hydrolyze sphingomyelin resulting in the formation of ceramide-1-phosphate and choline (Forrester et al., 1978; Kurpiewski et al., 1981; Tambourgi et al., 1998) and, in the presence of  $Mg^{2+}$ , are able to catalyze the release of choline from lysophosphatidylcholine (van Meeteren et al., 2004).

All spider venom SMases D sequenced to date display a significant level of sequence similarity and thus likely possess the same ( $\alpha/\beta$ )<sub>8</sub> or TIM barrel fold (Murakami et al., 2005, 2006). Based on sequence identity, biochemical activity and molecular modelling, a scheme for classification of spider venom SMases D was proposed (Murakami et al., 2006). The class 1 enzymes include SMase I and H13, SMases D from *L. laeta*, which possess a single disulphide bridge and contain an extended hydrophobic loop or variable loop (Murakami et

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al., 2006). All other SMases D, such as SMases P1 and P2 from *L. intermedia* (Tambourgi et al., 1998, 2004), Lr1 and Lr2 from *L. reclusa* and Lb1, Lb2 and Lb3 from *L. boneti* (Ramos-Cerrillo et al., 2004) belong to class 2, which contains an additional intra-chain disulphide bridge that links the flexible loop with the catalytic loop (Murakami et al., 2006). The class 2 enzymes can be further subdivided into class 2a and class 2b depending on whether they are capable of hydrolysing sphingomyelin or not, respectively (Murakami et al., 2006). One representative of class 2b is the isoform 3 from *L. boneti* an inactive SMase D isoform (Ramos-Cerrillo et al., 2004) (Table 1).

Studies on the effect of venoms from synanthropic species of *Loxosceles* spiders have been reported, however, analysis from those living in natural environment have been poorly performed. *Loxosceles* species are present in several different habitats, including the karstic environment, and in Brazil it is the most common troglophile arachnid. The spiders are commonly found on the walls at the entrance of caves, especially in the shady rocky areas.

In order to characterize the venom of *Loxosceles* species living in natural environment, and compare their venom to those of synanthropic species, we have explored the caves of 'Parque Estadual do Alto do Ribeira' (PETAR - Ribeira Valley, SP, Brazil), which is an area of importance to both tourism and scientific research, due to the combination of tropical forest and extensive cave systems. Spiders captured in the caves of PETAR were identified by morphological analysis as *Loxosceles adelaida* Gertsch (1967), which belongs to 'gaucho group' (Gertsch, 1967). This group includes four species: *L. adelaida*, *L. gaucho*, *L. similis* and *L. variegata*. *L. gaucho* belongs to the synanthropic fauna of Brazilian arachnids and is considered an important cause of the loxoscelic accidents in the south-eastern region of the country. Thus, the aims of the present study were to clone and express SMases D from the spider gland of *L. adelaida*, captured in the caves of PETAR (Brazil), to compare the functional activities of the recombinant proteins with toxins from synanthropic species and to investigate the inter- and intra-species cross-reactivities of antibodies raised against the purified recombinant proteins.

SMase Class	<i>Loxosceles</i> sp	SMase D	GenBank	Reference
I	<i>L. laeta</i>	SMase I H13 (SMase II)	AY093599.1 AY093600.1	Fernades-Pedrosa et al., 2002; de Santi-Ferrata et al., 2009 Fernandes-Pedrosa et al., 2002
II	<i>L. intermedia</i>	P1 P2	AY304471.2 AY304472.2	Tambourgi et al., 2004 Tambourgi et al., 2004
	<i>L. reclusa</i>	Lr1 Lr2	AY559846.1 AY559847.1	Ramos-Cerrillo et al., 2004 Ramos-Cerrillo et al., 2004
	<i>L. boneti</i>	Lb1 Lb2 Lb3	AY559844.1 - AY559845.1	Ramos-Cerrillo et al., 2004 Ramos-Cerrillo et al., 2004 Ramos-Cerrillo et al., 2004

## 2. Material and methods

### 2.1 Chemicals, reagents and buffers

Tween 20, bovine serum albumin (BSA), sphingomyelin (SM), choline oxidase, horseradish peroxidase (HRPO) and 3-(4-hydroxy-phenyl) propionic acid were purchased from Sigma Co. (St. Luis, MO). 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Nitroblue Tetrazolium (NBT) were from Promega Corp. (Madison, WI, USA). Goat anti-horse IgG-alkaline phosphatase (GAH/IgG-AP) was from Sigma Co. (St. Luis, MO). Horse serum against SMases D P1 and P2 from *L. intermedia* and SMase D I from *L. laeta* was obtained as previously described (De Almeida et al., 2008). Buffers were: Veronal-Buffered Saline (VBS<sup>2+</sup>), pH 7.4: 10 mM NaBarbitone, 0.15 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>; Phosphate-Buffered Saline (PBS), pH 7.2: 10 mM NaPhosphate, 150 mM NaCl; HEPES-Buffered saline (HBS), pH 7.4: 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>.

### 2.2 Spiders and venoms

*Loxosceles intermedia*, *L. laeta*, *L. gaucho* and *L. adelaida* spiders were provided by Immunochemistry Laboratory, Butantan Institute, Brazil (permission N° 1/35/95/1561-2 was provided by the Brazilian Institute of Environment and Renewable Natural Resources - IBAMA - a Brazilian Ministry of the Environment's enforcement agency). The venoms were obtained (permission N° 01/2009/IBAMA) by electrostimulation by the method of Bucherl (1969) with slight modifications. Briefly, 15–20 V electrical stimuli were repeatedly applied to the spider sternum and the venom drops were collected with a micropipette in PBS, aliquoted and stored at -20°C. The protein content of the samples was evaluated using the BCA Protein Assay Kit (Pierce Biotechnology, MA, USA).

### 2.3 Animals

The adult New Zealand white rabbits weighing approximately 3 kg were supplied by Butantan Institute animal facilities, SP, Brazil. All the procedures involving animals were in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation.

### 2.4 cDNA generation

The venom glands were removed from 50 specimens of *Loxosceles adelaida* 5 days after venom extraction, when the maximum level of RNA synthesis was achieved. Then, the glands were quickly frozen in liquid nitrogen and stored at -80°C until used. The total RNA was extracted from the glands using the Trizol reagent, according to the manufacturer's instructions (Invitrogen, USA). The quality and yield of total RNA were verified by the integrity of 28S and 18S rRNA, through denaturing agarose gel electrophoresis, and spectrophotometrically using the ratio 260/280 nm. The cDNAs were synthesised from 5 µg of total RNA using the cDNA Cycle® Kit for RT-PCR (Invitrogen, USA).

### 2.5 PCR Amplification of Sphingomyelinase D homologue from *L. adelaida*

The SMase D cDNA was amplified by PCR using total reverse transcriptase-PCR products as template. Degenerate primers AdeF1 (5'-G(G/A)ACG(C/A)GC(G/T)GATAA(A/C)CGTCG(A/T)CC-3') and AdeR2 (5'-CTA(A/T/G)TT(C/T)TT(A/G)AA(A/T/G)GTCTCCA(A/T)GG-3'), were designed according to the highly conserved 5'- and 3'-

SMases D P1 and P2 from *L. intermedia* (accession numbers AY304471 and AY304472, respectively) and Smase D I from *L. laeta* (accession number AY093599) (Tambourgi et al, 2004; Fernandes Pedrosa et al, 2002). The PCR protocol included denaturation at 96°C for 3 min, followed by 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C), and extension (2 min at 72°C), and a final extension for 7 min at 72°C. The amplified fragments were purified by low melting using the UltraPure™ Low Melting Point Agarose (Invitrogen, USA) and cloned in a pGEM-T easy vector (Promega, USA). *E. coli* XL1-Blue competent cells (Stratagene, USA) were transformed following the manufacturer's instructions. Positive clones were selected by growing the transformed cells in Luria broth (LB) medium containing 100 µg/ml ampicillin and blue-white color screening. The nucleotide sequence was determined by the dideoxy chain-termination method using the BigDye™ Terminator Cycle Sequence Kit and the ABI 3100 automatic system (Applied Biosystems, USA).

### 2.6 Subcloning in expression vector

The cDNA corresponding to the mature *L. adelaida* SMase D was amplified from plasmids containing full length sequence using primer AdeF3 (5'-CTCGAGGCGGATAAACGTCGACCCATATG-3'), which contains a XhoI restriction site (underlined) and AdeR4 (5'-CCAATGGTCAATTCTTGAAAGTCTCC-3'), which includes a restriction site for NcoI (underlined) and a stop codon (in italics and bold).

PCR fragments (approximately 900 bp) were digested with XhoI and NcoI and cloned into the corresponding sites in pRSET-B bacterial expression vector (Invitrogen, USA). The use of the pRSET-B bacterial expression system results in the expression of a recombinant fusion protein, including a 6xHis-tag at the N-terminus and a 26 amino acid linker followed by the mature protein (N-terminal amino acid sequence before the coding sequence of the mature protein: 'MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPSSR').

### 2.7 Recombinant protein expression and purification

Recombinant proteins were produced as described previously (Tambourgi et al., 2004). In brief: pRSETB-*L. adelaida* SMase D cDNA transformed *Escherichia coli* BL21 (DE3) (Invitrogen, USA) cells were inoculated in 500 mL of 2YT/amp and grown overnight at 37°C and induced with IPTG. Recombinant proteins were harvested from the pellet by french-pressure and purified on a Ni (II) Chelating Sepharose Fast Flow column (Pharmacia, Sweden, 1.0×6.4 cm). The fractions containing the recombinant proteins were pooled and concentrated using Centricon-30 (30,000-mw cutoff; Amicon, Inc., USA). Smase D P1 from *L. intermedia* and SMase D I from *L. laeta* were obtained as previously described (Tambourgi et al., 2004; Fernandes-Pedrosa et al., 2002).;The protein content of the samples was evaluated by the BCA protein kit assay, following the manufacturer's protocol (Pierce, USA).

### 2.8 Enzymatic activity

The SMase D enzymatic activity was estimated by determining the choline liberated from lipid substrates, using a fluorimetric assay (Tokumura et al., 2002). Briefly, sphingomyelin (SM – 50 µg) was diluted in 1 mL HEPES-buffered saline (HBS). Samples of the recombinant proteins or venom (2.5 to 20 µg) was added and the reaction was developed for 20 min at 37°C. After incubation, a mixture composed by 1 unit/mL choline oxidase, 0.06 unit/mL of horseradish peroxidase and 50 µM of 3-(4-hydroxy-phenyl) propionic acid in HBS was added and incubated for 10 min. The choline liberated was oxidized to betaine and H<sub>2</sub>O<sub>2</sub>

and this product determined by fluorimetry at  $\lambda_{em}=405$  nm and  $\lambda_{ex}=320$  nm, using 96-well microtiter plates, in a spectrofluorimeter (Victor 3<sup>TM</sup>, Perkin-Elmer, USA).

### 2.9 Electrophoresis and western blotting

Samples of the recombinant SMases D (5  $\mu$ g) or *Loxosceles* venoms (10  $\mu$ g) were solubilised in non-reducing sample buffer, run on 12% SDS-PAGE (Laemmli, 1970) and silver stained. Alternatively, gels were blotted onto nitrocellulose (Towbin et al., 1979). After transfer, the membranes were blocked with PBS containing 5% BSA and incubated with horse serum anti-SMases D from *L. intermedia* (SMases D P1 and P2) and *L. laeta* (SMase D I) (diluted 1:1000) for 1 h at room temperature. Membranes were washed 3 times with PBS/0.05% Tween 20 for 5 min each wash, and incubated with GAH/IgG-AP (1:7500) in PBS/1% BSA for 1 h at room temperature. After washing 3 times with PBS/0.05% Tween 20 for 5 min each wash, blots were developed using NBT/BCIP according to the manufacturer's instructions (Promega).

### 2.10 Normal human serum and erythrocytes

Human blood was obtained from healthy donors. Blood samples drawn to obtain sera were collected without anticoagulant, allowed to clot for 4 h at 4°C and the normal human serum (NHS) was stored at -80°C. Blood samples drawn to obtain erythrocytes for subsequent use as target cells were collected in anticoagulant (Alsever's old solution: 114 mM citrate, 27 mM glucose, 72 mM NaCl, pH 6.1).

### 2.11 Treatment of erythrocytes with *Loxosceles* recombinant proteins/venom

Human erythrocytes were washed and resuspended at 1.5% in VBS<sup>2+</sup> and incubated with different concentrations of the recombinant proteins or venom for 1 h at 37°C. Control samples were incubated with VBS<sup>2+</sup>. The cells were washed, resuspended to the original volume in VBS<sup>2+</sup> and analysed in a haemolysis assay as described (Tambourgi et al., 2000).

### 2.12 Dermonecrotic activity

Samples of *L. adelaida* venom (5  $\mu$ g), SMase P1 from *L. intermedia* (5  $\mu$ g) or SMase D from *L. adelaida* (5  $\mu$ g and 10  $\mu$ g), in PBS, were injected intradermally in the shaved back of adult rabbits. Control sites were injected with equal volume of PBS. The size of the lesions was measured over a period of 16, 24, 48 and 72 h. After 72 h, the animals were euthanized and skin specimens were obtained for histological examination.

### 2.13 Histological analysis

Skin samples were fixed in 4% buffered formalin solution, and then embedded in paraffin. Tissue sections were stained with haematoxylin and eosin and examined for the presence of epithelial necrosis, epithelial slough, dermal infiltrates, haemorrhage and level of collagen dissociation in the dermis and skin muscle fiber degeneration.

### 2.14 Homology molecular modelling and quality analysis

The atomic coordinates of SMase I, a SMase D from *L. laeta* (PDB accession code: 1XX1) was used as 3D-model for restraint-based modelling as implemented in the program MODELLER (Fiser and Sali, 2003). The overall model was improved enforcing the proper stereochemistry using spatial restraints and CHARMM energy terms, followed by conjugate gradient simulation based on the variable target function method (Fiser and Sali, 2003). Ten models were built for the *L. adelaida* SMase D sequence based on the (m)GenThreader

alignment (Lobley et al., 2009) and the model with best global score was selected for explicit solvent MD simulations using the package Yasara (<http://www.yasara.org>) to check its stability and consistency. The overall and local quality analyses of the final model were assessed by VERIFY3D (Eisenberg et al., 1997), PROSA (Wiederstein and Sippl, 2007) and VADAR (Willard et al., 2003). Three-dimensional structures were displayed, analyzed and compared using the program COOT (Emsley and Cowtan, 2004).

### 2.15 Molecular dynamics simulation of the enzyme-substrate complex

The SMase D I structure (PDBid 1XX1) obtained from the Protein Data Base ([www.rcsb.org](http://www.rcsb.org)) and a predicted structure of the sphingomyelin (SM) was manually docked using the sulfate ion from the experimental structure, as a reference of the position of the phosphate moiety of the SM.

The structure was prepared for energy minimization (EM) molecular dynamics (MD) simulation using YASARA program for building missing atoms and hydrogens in the model. The parameters for the force field were obtained from YAMBER3 (Krieger et al., 2004). The pKa values for Asp, Glu, His and Lys residues were predicted. Based on the pH 7.0, the protonation states were assigned according to convention: Asp and Glu were protonated if the predicted pKa was higher than the pH; His was protonated if the predicted pKa was higher than the pH and it did not accept a hydrogen bond, otherwise it was deprotonated; Cys was protonated; Lys was deprotonated if the predicted pKa was lower than the pH and; Tyr and Arg were not modified ([www.yasara.org](http://www.yasara.org)). A simulation box was defined at 15 Å around all atoms of each macromolecular complex. Then, the simulation box was filled with water molecules and Na/Cl counter ions, that were placed at the locations of the lowest/highest electrostatic potential, until the cell neutralization, and the requested NaCl concentration reached 0.9%. A short MD simulation was performed for the solvent adjust, and water molecules were subsequently deleted until the water density reached 0.997 g/mL. A short steepest descent EM was carried until the maximum atom speed dropped below 2200 m/s. Then 500 steps of simulated annealing EM were performed with a target temperature at 0 K. Finally, a 20 ns MD production simulation was performed at 298 K and a non-bonded cutoff of 7.86 Å. A snapshot was saved every 7.5 ps (picoseconds). The graphical analysis was carried out using Visual Molecular Dynamics (VMD) software (Humphrey et al., 1996) and the plots were generated using R ([cran.r-project.org](http://cran.r-project.org)).

Ligand binding analysis was carried out taking into account the potential energy obtained with the current force field for the complex and components:

$$E_{\text{binding}} = (E_{\text{protein}} + E_{\text{ligant}}) - E_{\text{complex}}$$

In our equation, the complex energy is placed after the energy of the components; therefore, higher energies equated to higher affinity between the protein and the substrate.

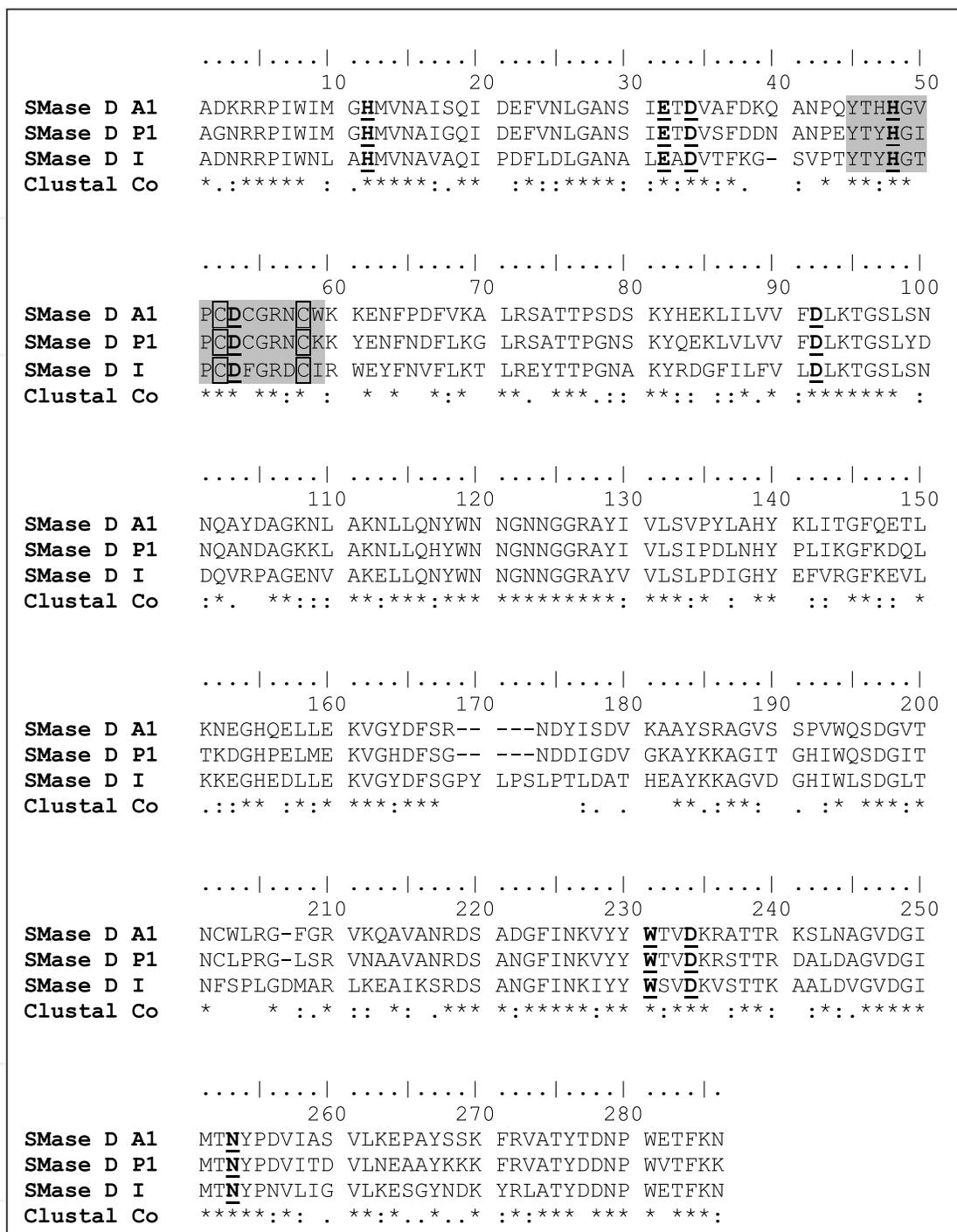
## 3. Results

### 3.1 Identification and characterization of *L. adelaida* recombinant protein

Analysis of *L. adelaida* SMase D clone revealed that the mature cDNA covers an open reading frame of 843 nucleotides encoding 280 amino acid residues (GenBank JN202927). The complete nucleotide sequence of this cDNA clone and the deduced amino acid sequence are shown in Figure 1A. Sequence analysis revealed that SMase D from *L. adelaida* exhibits similarity with previously characterized class 2 SMase D and shares 75% and 59% homology with the sequences of *L. intermedia* SMase P1 and *L. laeta* SMase I, respectively (Figure 1B).

1	GCG	GAT	AAA	CGT	CGA	CCC	ATA	TGG	ATC	ATG	GGA	CAT	ATG	GTT	AAC	45
1	A	D	K	R	R	P	I	W	I	M	G	H	M	V	N	15
46	GCC	ATC	TCT	CAA	ATA	GAC	GAA	TTT	GTC	AAC	CTT	GGG	GCG	AAT	TCC	90
16	A	I	S	Q	I	D	E	F	V	N	L	G	A	N	S	30
91	ATC	GAA	ACG	GAC	GTG	GCT	TTC	GAT	AAG	CAA	GCC	AAT	CCT	CAA	TAC	135
31	I	E	T	D	V	A	F	D	K	Q	A	N	P	Q	Y	45
136	ACG	CAT	CAC	GGG	GTC	CCA	TGC	GAT	TGT	GGA	AGG	AAT	TGT	TGG	AAA	180
46	T	H	H	G	V	P	C	D	C	G	R	N	C	W	K	60
181	AAG	GAG	AAC	TTC	CCA	GAC	TTC	GTA	AAA	GCT	CTA	CGA	AGT	GCT	ACA	225
61	K	E	N	F	P	D	F	V	K	A	L	R	S	A	T	75
226	ACC	CCC	AGT	GAT	TCC	AAA	TAT	CAT	GAA	AAA	CTG	ATC	TTA	GTT	GTG	270
76	T	P	S	D	S	K	Y	H	E	K	L	I	L	V	V	90
271	TTC	GAC	CTA	AAA	ACG	GGT	AGT	CTT	TCC	AAC	AAT	CAA	GCC	TAC	GAC	315
91	F	D	L	K	T	G	S	L	S	N	N	Q	A	Y	D	105
316	GCA	GGA	AAG	AAT	TTG	GCA	AAA	AAT	CTC	CTT	CAA	AAT	TAC	TGG	AAC	360
106	A	G	K	N	L	A	K	N	L	L	Q	N	Y	W	N	120
361	AAT	GGC	AAT	AAT	GGT	GGA	AGA	GCA	TAC	ATA	GTG	TTA	TCA	GTA	CCA	405
121	N	G	N	N	G	G	R	A	Y	I	V	L	S	V	P	135
406	TAC	CTT	GCA	CAT	TAT	AAA	TTG	ATA	ACA	GGA	TTT	CAA	GAA	ACG	CTT	450
136	Y	L	A	H	Y	K	L	I	T	G	F	Q	E	T	L	150
451	AAA	AAC	GAG	GGA	CAT	CAA	GAA	TTG	TTG	GAG	AAA	GTT	GGA	TAC	GAC	495
151	K	N	E	G	H	Q	E	L	L	E	K	V	G	Y	D	165
496	TTC	TCC	AGG	AAT	GAC	TAC	ATC	AGC	GAT	GTT	AAG	GCA	GCT	TAT	AGT	540
166	F	S	R	N	D	Y	I	S	D	V	K	A	A	Y	S	180
541	AGA	GCT	GGA	GTA	TCA	AGT	CCC	GTG	TGG	CAA	AGC	GAC	GGT	GTC	ACC	585
181	R	A	G	V	S	S	P	V	W	Q	S	D	G	V	T	195
586	AAC	TGT	TGG	CTA	CGT	GGT	TTT	GGT	CGT	GTG	AAG	CAA	GCT	GTG	GCA	630
196	N	C	W	L	R	G	F	G	R	V	K	Q	A	V	A	210
631	AAT	AGA	GAC	TCC	GCG	GAC	GGA	TTC	ATT	AAC	AAA	GTA	TAC	TAT	TGG	675
211	N	R	D	S	A	D	G	F	I	N	K	V	Y	Y	W	225
676	ACA	GTG	GAT	AAA	CGC	GCA	ACA	ACT	AGA	AAA	TCA	CTT	AAT	GCT	GGA	720
226	T	V	D	K	R	A	T	T	R	K	S	L	N	A	G	240
721	GTT	GAC	GGT	ATA	ATG	ACC	AAT	TAC	CCC	GAT	GTA	ATT	GCT	AGT	GTA	765
241	V	D	G	I	M	T	N	Y	P	D	V	I	A	S	V	255
766	CTC	AAG	GAA	CCT	GCT	TAC	AGT	TCA	AAA	TTC	AGG	GTC	GCC	ACA	TAC	810
256	L	K	E	P	A	Y	S	S	K	F	R	V	A	T	Y	270
811	ACG	GAT	AAT	CCT	TGG	GAG	ACT	TTC	AAG	AAT	TGA					
271	T	D	N	P	W	E	T	F	K	N	*					

A

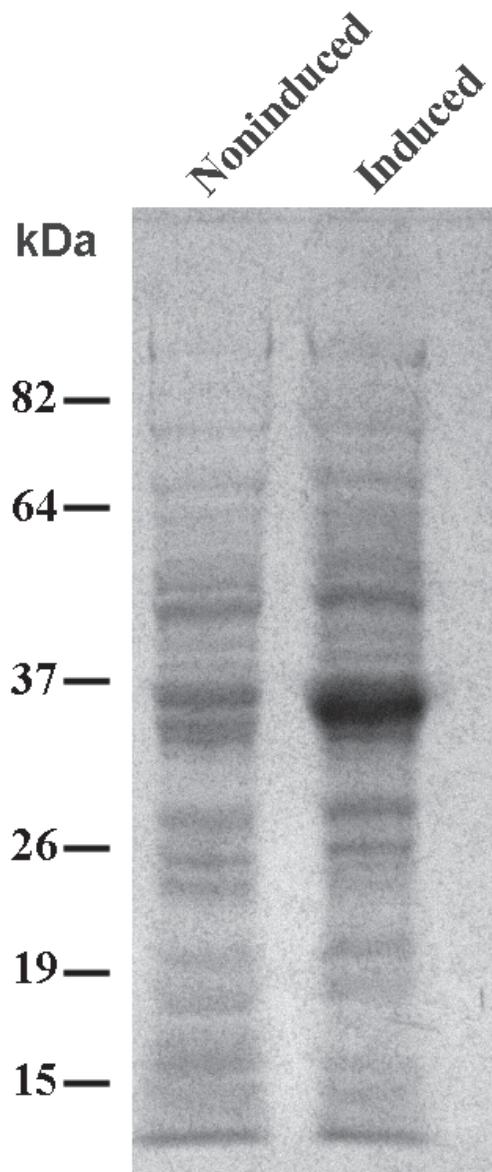


B

Fig. 1. Sequence of *L. adelaida* SMase D A1. [A] Complete cDNA sequence of *L. adelaida* SMase D A1 and its deduced mature protein sequence. The primers used in the subcloning are underlined. The nucleotide residues are numbered in the 5' to 3' direction. [B] Alignment of the complete deduced amino acid sequences of *L. adelaida* SMase D A1 and SMase D P1 of *L. intermedia* and SMase D I of *L. laeta*. The conserved residues forming the catalytic pocket are underlined and in bold letters. Open boxes indicate the two conserved cysteine residues in SMases D. The catalytic loops (residues 45-59) are shaded gray. Asterisks indicate identical amino acid residues.

The theoretical molecular mass for the putative recombinant mature protein of *L. adalaida* is 31.545 kDa, with a pI = 8.85. Expression of *L. adalaida* SMase D resulted in an approximately 36 kDa recombinant His6-tagged protein, clearly visible by SDS-PAGE in the bacterial cell extracts (Figure 2A). The recombinant protein was purified from the soluble fraction of cell lysates by Ni<sup>2+</sup>-chelating chromatography and eluted from the resin in extraction buffer containing 0.8 M imidazole at greater than 95% purity (Figure 2B).

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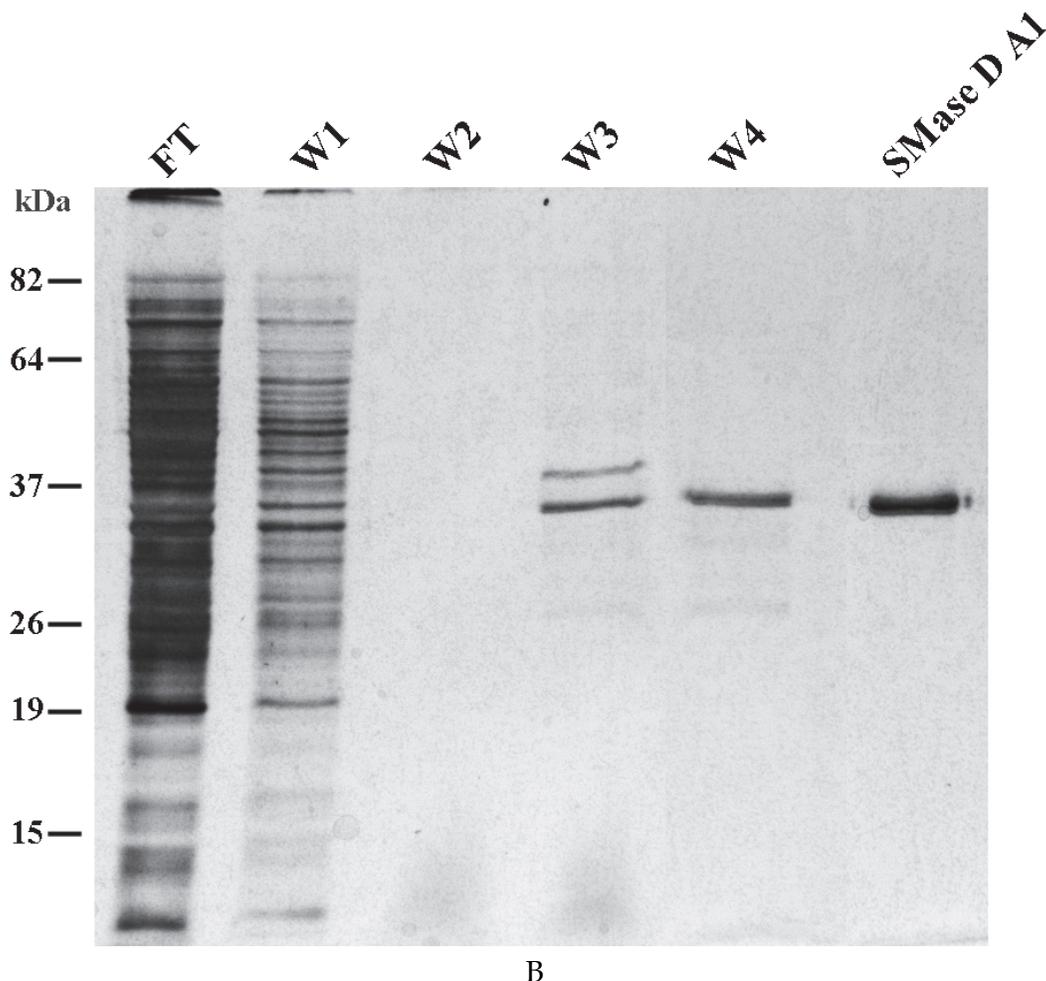


Fig. 2. Expression and purification of recombinant SMase D A1 of *L. adelaida*. [A] Extracts of *E. coli* BL21(DE3) transformed with the plasmid pRSET B clone A1 were induced or not with IPTG for 2 h. The cells were collected by centrifugation, resuspended in buffer extraction and disrupted by French-pressure. [B] The supernatant was loaded onto Ni(II)-Chelating Sepharose column, the flowthrough was collected (FT), the column was washed (W1 - W4), and the recombinant protein was eluted with the elution buffer. Samples were separated by SDS-PAGE (12% gel) under reducing conditions and stained by silver.

### 3.2 Enzymatic activity

The ability of the *L. adelaida* recombinant SMase D protein to degrade sphingomyelin was investigated and compared with the activity of the previously characterized active recombinant isoform from *L. intermedia* gland, SMase D P1, and with *L. adelaida* crude venom. Figure 3 shows that the crude *L. adelaida* venom, as well as the recombinant *L. adelaida* SMase D A1 and Smase D P1 proteins present significant sphingomyelinase activity as shown by the breakdown of the substrate, being the activity of *L. adelaida* SMase D protein lower than that of the *L. intermedia* SMase P1. However, *L. adelaida* recombinant SMase D activity was approximately ten times higher than that of the crude *L. adelaida* venom. Since the recombinant *L. adelaida* protein was endowed with sphingomyelinase activity, we named it as SMase D A1.

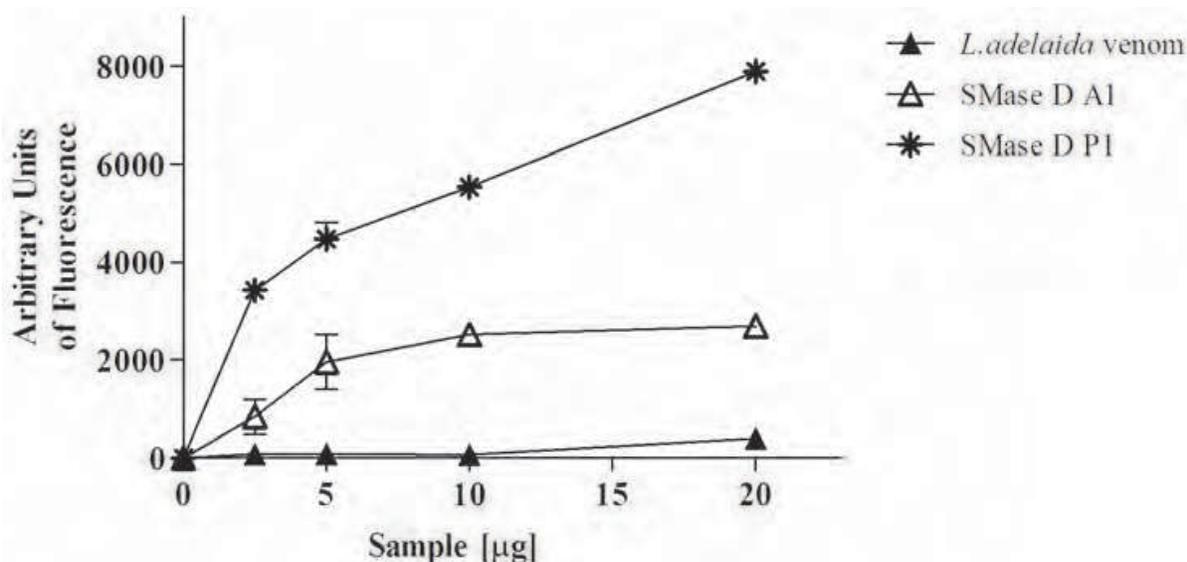


Fig. 3. Sphingomyelinase activity of *L. adelaida* SMase D recombinant protein. Sphingomyelin (50  $\mu\text{g}$ ) was incubated with increasing amounts of buffer, *L. adelaida* or *L. intermedia* recombinant SMases D or crude *L. adelaida* venom. After 30 min at 37°C, the formed choline was oxidized to betaine and determined fluorimetrically. Results are representative for two separate experiments expressed as mean of duplicates +/-SD.

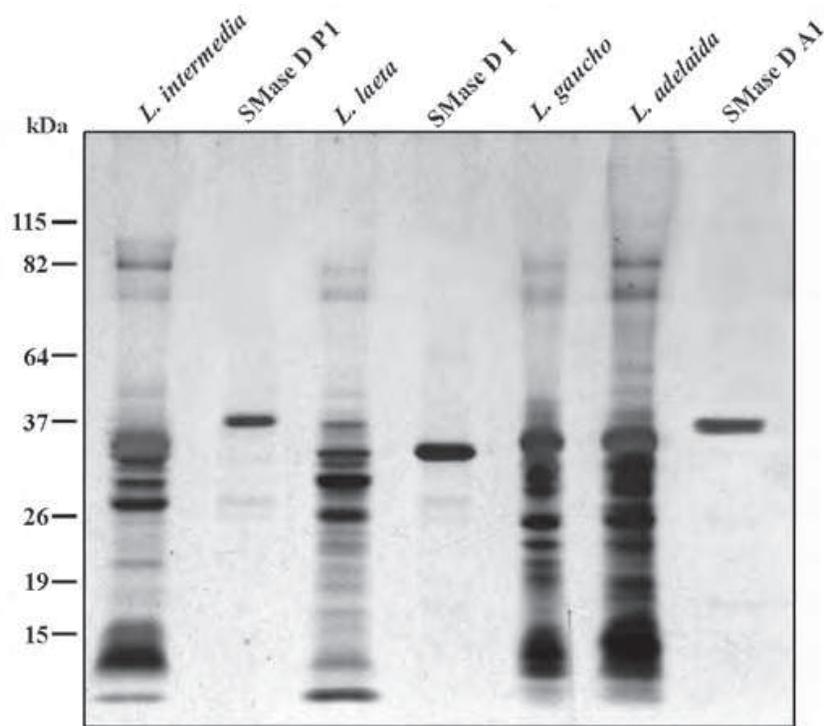
### 3.3 Cross-reactivities of SMase D A1 of *L. adelaida*

The protein profile from the *Loxosceles* spp venoms and the recombinant SMases D were analyzed by SDS-PAGE followed by silver staining. Figure 4A shows that the venoms from *L. intermedia*, *L. laeta*, *L. gaucho* and *L. adelaida* differ in composition, number and intensity of bands. It can also be observed that the recombinant proteins were purified to homogeneity and that P1 and A1 exhibit similar molecular weight. SMase I presents Mr of approximately 33 kDa, and SMases P1 and A1, around 37 kDa and 36 kDa, respectively.

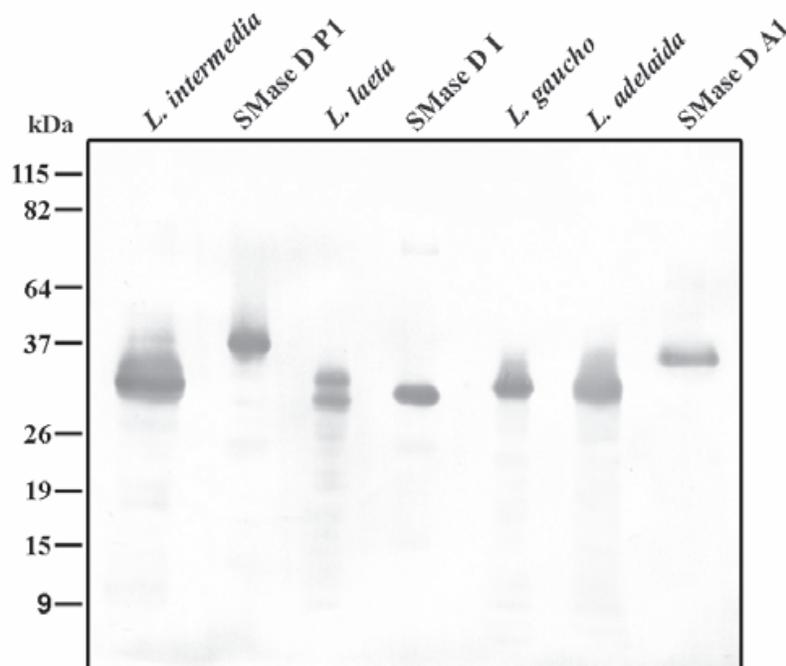
In order to analyze the inter- and intra-species cross-reactivities, horse polyclonal antiserum raised against a combination of the SMases D P1 and P2 from *L. intermedia* and Smase D I from *L. laeta*, were used in western blotting reactions. Figure 4B shows that the antiserum was able to recognize the purified recombinant proteins, SMases D A1, I and P1, as well as bands of approximately 35 kDa in the *Loxosceles* spp spider venoms. The slightly higher Mr of the recombinant proteins is attributed to the extra N-terminal tag, which increased the size of *L. adelaida* SMase A1, *L. intermedia* SMase P1 and *L. Laeta* SMase I proteins by, approximately, 4 kDa, 3 kDa and 1 kDa, respectively.

### 3.4 *L. adelaida* recombinant SMase D induces haemolysis and dermonecrosis

Sphingomyelinases D isolated from *Loxosceles* spider venoms have been shown to be able to transform human erythrocytes in activators of the complement system (Tambourgi et al., 1995, 1998, 2000). In order to assess whether the recombinant SMase A1 could also induce Complement-dependent haemolysis, erythrocytes were incubated with increasing amounts of the SMases A1, P1 or crude venom. Although the activity of SMase A1 was relatively lower compared with the SMase P1 and *L. adelaida* venom, as shown in Figure 5, *L. adelaida* recombinant SMase A1, as well as SMase P1 and *L. adelaida* venom were able to render erythrocytes susceptible to lysis by autologous Complement.



A



B

Fig. 4. SDS-PAGE and Western blotting analysis of the recombinant mature *L. adelaida* SMase D protein. Purified recombinant proteins, *L. intermedia* SMase D P1, *L. laeta* SMase D I and *L. adelaida* SMase D A1, and *L. intermedia*, *L. laeta*, *L. gaucho* and *L. adelaida* venoms were compared. [A] Samples were separated by SDS-PAGE (12% gel) under non-reducing conditions and silver stained. [B] Purified recombinant proteins and venoms were run on 12% SDS-PAGE gel under non-reducing conditions and western blotted using horse antiserum raised against recombinant SMases D P1 and P2 and anti-*L. laeta* SMase I.

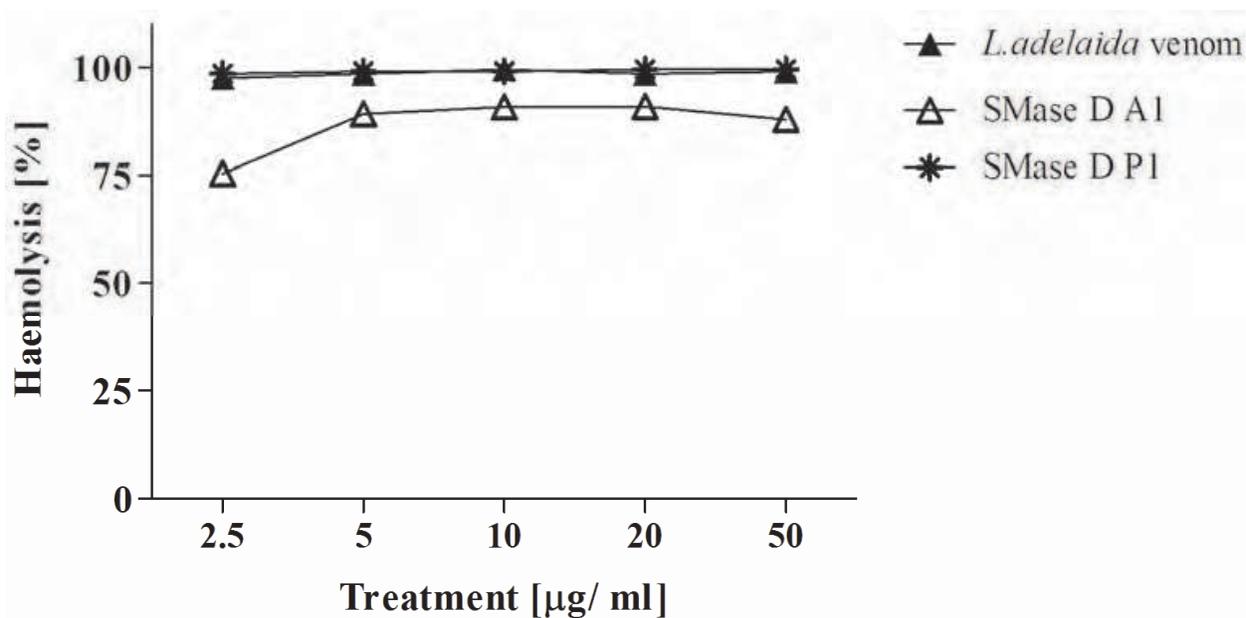


Fig. 5. Induction of haemolysis by *L. adelaida* SMase D A1. Erythrocyte pre-treated with different amounts of *L. adelaida* or *L. intermedia* recombinant SMases D or crude *L. adelaida* venom or VBS<sup>2+</sup> were incubated with NHS. After incubation for 1h at 37°C, the absorbance of the supernatant was measured at 414 nm and expressed as percentage of lysis. Results are representative for three separate experiments and expressed as mean of duplicates +/-SD.

The ability of *L. adelaida* recombinant SMase A1 to induce dermonecrotic lesions was tested by injecting rabbits with 5 µg of the toxin. The animals received buffer, as negative control, and 5 µg of SMase D P1 or venom as positive controls. A typical loxoscelic lesion, as revealed by the presence of oedema, oerythema and mild tenderness, developed in the skin area injected with the recombinant proteins or venom within a few hours of injection and approximately 24 h post injection, necrosis with gravitational spread and scar were observed at the inoculation site (Figure 6A). Figure 6B shows that the dermonecrotic action of *L. adelaida* SMase D A1 is dose dependent, but that this activity was less intense than that exhibited by the crude venom and SMase D P1.

Histological analysis of the skin samples obtained from PBS inoculated animals showed a thin epidermis and a normal pattern for the collagenous area and muscle fibers (Figure 7, panels A/A1/A2). Despite the *L. adelaida* recombinant SMase D A1 has exhibited lower dermonecrotic activity than the Smase D P1, skin samples obtained from the recombinant proteins or venom inoculation sites showed a thin epidermis, dissociation of the collagenous fibers due to the oedema, degeneration of muscle fibers, moderate haemorrhage in the superficial dermis and intense neutrophil infiltration in the deep dermis and musculature (Figure 7).

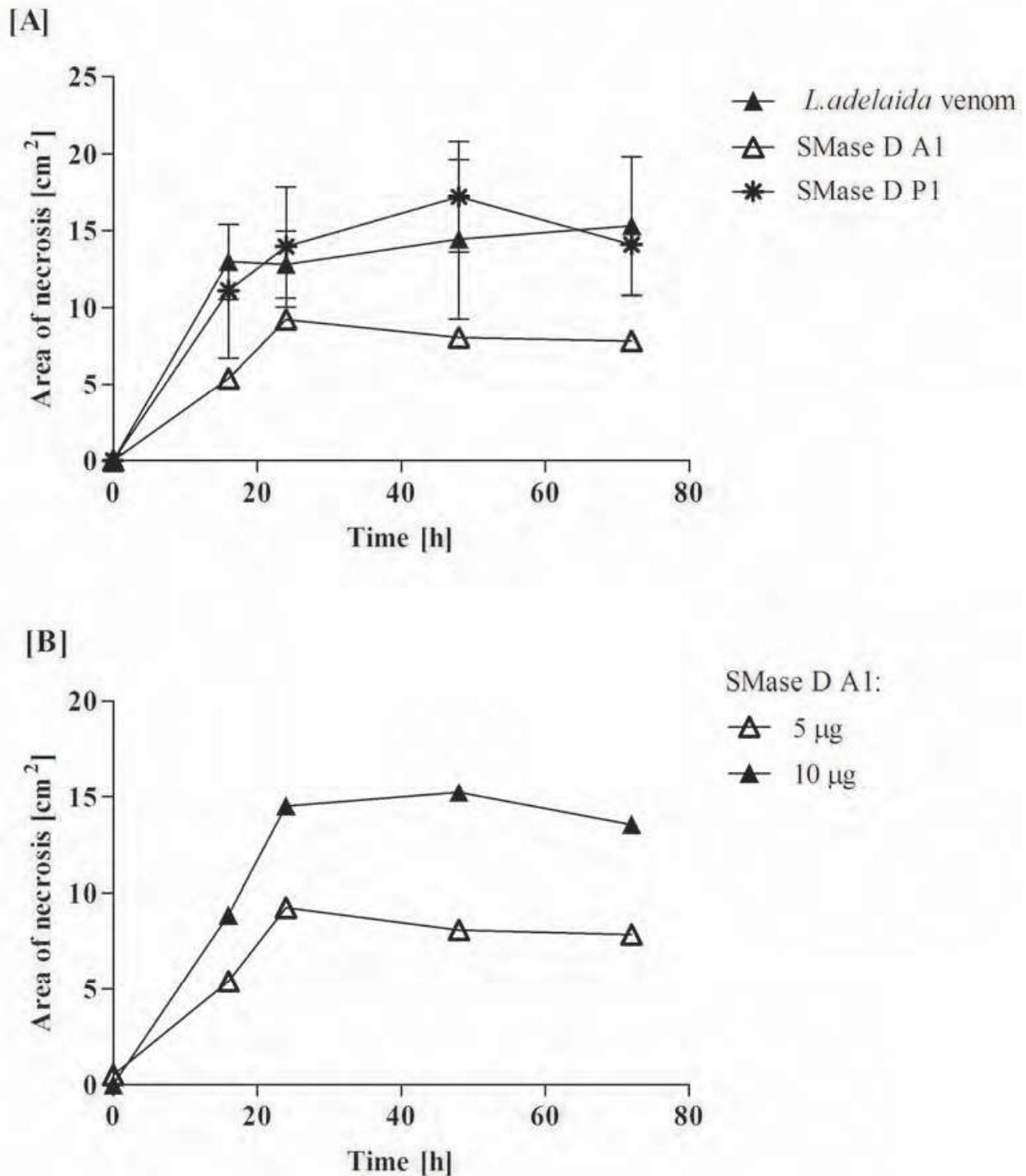


Fig. 6. Induction of dermonecrosis by *L. adelaida* SMase D recombinant protein. [A] Adult rabbits were injected intradermally with 5 µg of *L. adelaida* or *L. intermedia* recombinant SMases D or crude *L. adelaida* venom. The animals received buffer for negative control reactions. The areas of the dermonecrotic lesions were determined 16, 24, 48 and 72 h after injection. Results are representative for three separate experiments and expressed as mean of duplicates +/-SD. [B] Samples of 5 µg or 10 µg of SMase D A1 were injected intradermally in rabbits and the areas of the dermonecrotic lesions were determined 16, 24, 48 and 72 h after injection.

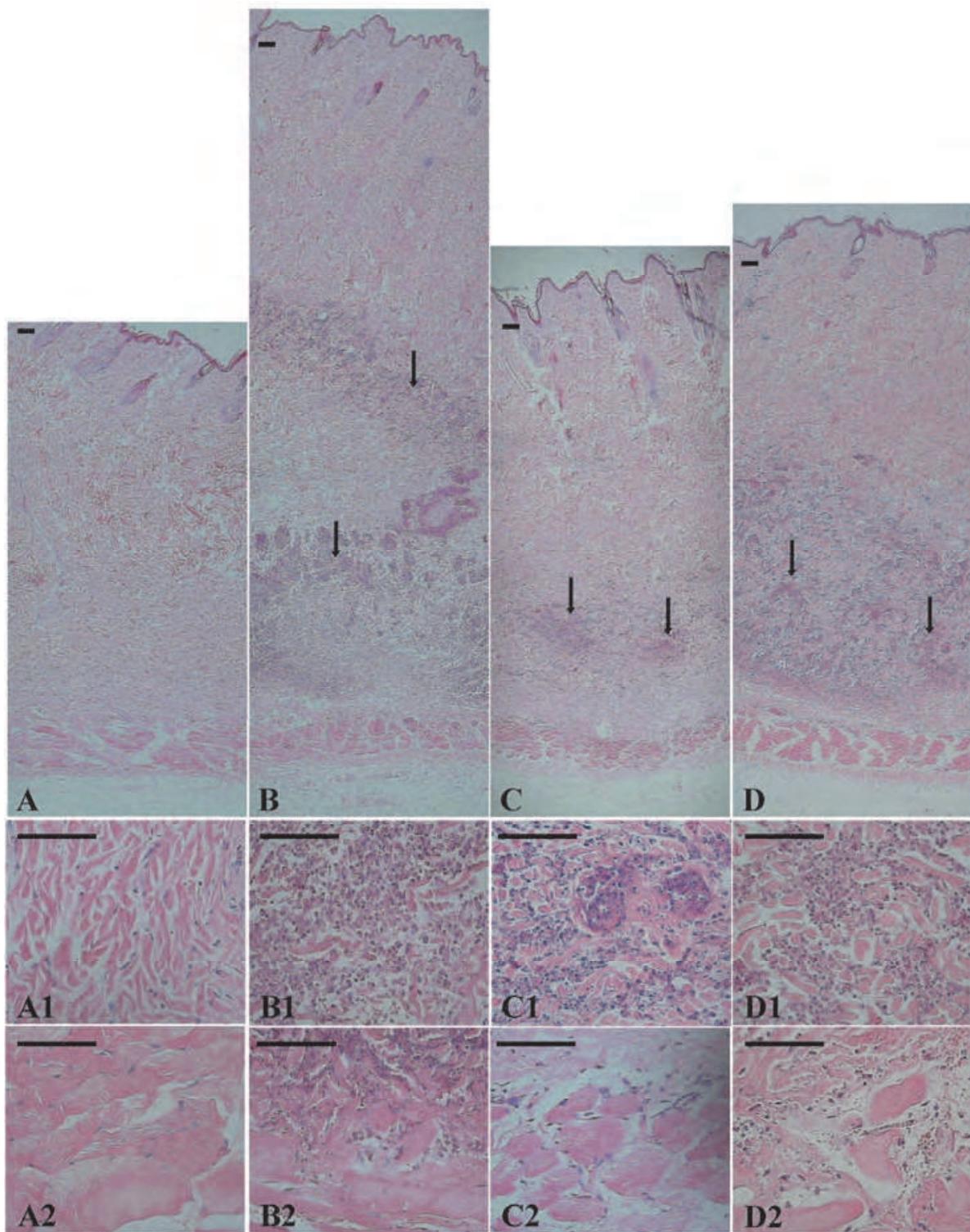


Fig. 7. Histological analysis. Analysis of skin of rabbits injected with 5  $\mu\text{g}$  *L. adelaida* or *L. intermedia* recombinant SMases D or crude *L. adelaida* venom. Control sites were injected with an equal volume of phosphate-buffered saline (PBS). Panels correspond to the panoramic view of skin sections from rabbits injected with PBS [A], *L. adelaida* venom [B], purified *L. adelaida* SMase D A1 [C] and *L. intermedia* SMase D P1 [D]. Arrows indicate areas of leukocyte infiltration. Panels A1/2–D1/2 show details of the collagenous area of the dermis of the same sections. Bars at the top of each panel indicate 100  $\mu\text{m}$ .

### 3.5 Overall structure description

MD analysis of *L. adelaida* SMase D A1 *in silico* model converged to a RMSD of 1.17 Å and showed a stable behavior over the simulation. Global and local stereochemical assessment indicated a very good model for comparative structural analyses. SMase D A1 displays a typical TIM ( $\alpha/\beta$ )<sub>8</sub>-barrel fold and its active-site cleft, formed by the metal-binding site (Asp, Glu and Asp) and the two catalytic histidines, is furthermore surrounded by the catalytic loop (residues 46-60), variable loop (residues 167-175), flexible loop (residues 196-203) and other short hydrophobic loops (Figure 8). Based on the current classification of SMases D (Murakami et al., 2006), SMase A1 belongs to class II containing an additional disulphide bridge (Cys53–Cys201), which connects the catalytic loop to flexible loop (Figure 8). This feature, not present in class I SMases D, diminishes significantly the active-site volume and also alters the inherent flexibility exhibited by the flexible loop. Beyond that, all the structural features observed in class I SMases D are fully conserved and details concerning the action mechanism are well described in Murakami et al., 2005 and Murakami et al., 2006.

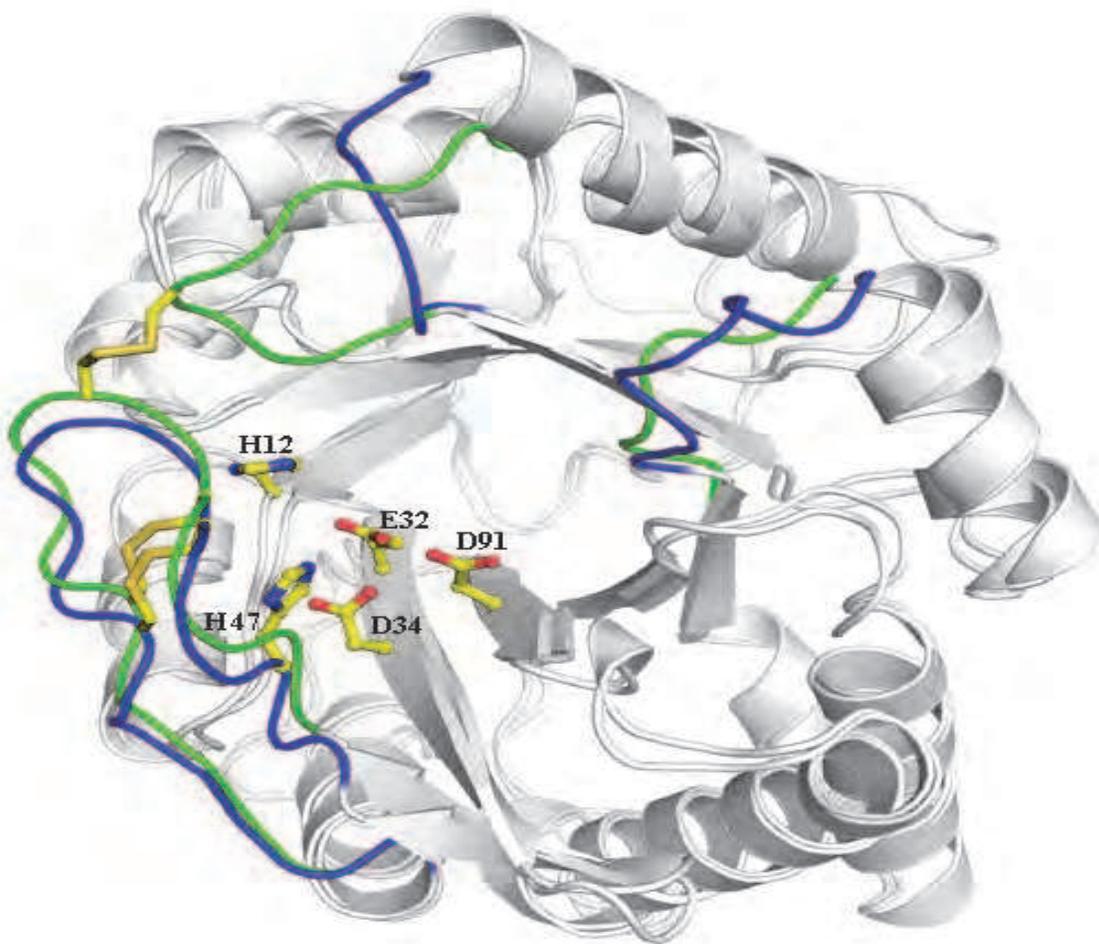


Fig. 8. Structure superposition of Smase D I (class I) and *L. adelaida* SMase D A1 (class II). The residues involved in metal-ion binding and catalysis are presented in atom colors (PDB code: 1XX1). Differences in the catalytic, flexible, and variable loops in *L. laeta* SMase D I (blue) and *L. adelaida* SMase D A1 (green).

### 3.6 Sphingomyelin-binding mode to SMases D

Despite the structure of a SMase D member has been solved, there is neither structural nor biophysical data relating the binding mode of the sphingomyelinase (SM) into the active-site cleft of SMases D. Thus, in order to shed light into the structural determinants for recognition and binding of SM by SMases D, a MD simulation was performed using the crystal structure of the SMase D I and the SM docked into enzyme taking into account the crystallographic position of the sulphate ion, which provides a good notion how the phosphate group from SM is oriented in the active-site cavity. As observed in Figure 9, the RMSD of the protein is low ( $\sim 1.5$  Å), whereas the SM shows a higher variation in the first 3000 ps ( $\sim 5.15$  Å) and after that become more stable ( $\sim 3$  Å). Although, the aliphatic tails of the substrate exhibit high flexibility as observed by RMSD analysis over the simulation, the polar head is stable. The binding analysis showed that the substrate-enzyme interaction increases during the simulation and it stabilizes around 291 Kcal/mol (Figure 9). In 10 ns of MD simulation, the model achieved a stable conformation with some fluctuations in the aliphatic tails as expected (Figure 9). The choline head is buried in the active-site cleft making van der Waals contacts with Trp230, which is conserved among SMases D (Figures 10A-B). The phosphate moiety is coordinated by the magnesium ion as expected, forming a tetrahedral cage of the ion along with the three acidic residues (Figure 10B). The residue Lys93, which is also highly conserved, is found in a distance range that permits to interact with the carbonyl oxygen of the sphingosine backbone. Val89, Ser132, Asp164, Ser166, Pro134, Pro168, Tyr169, Leu170, Leu198, Tyr228 and Met250 are also participating in the coordination of the substrate (Figure 10B). These data corroborates with previous crystallographic studies, whose suggested the importance of Lys93, Trp230 and other aromatic residues in the recognition and interaction of the substrate.

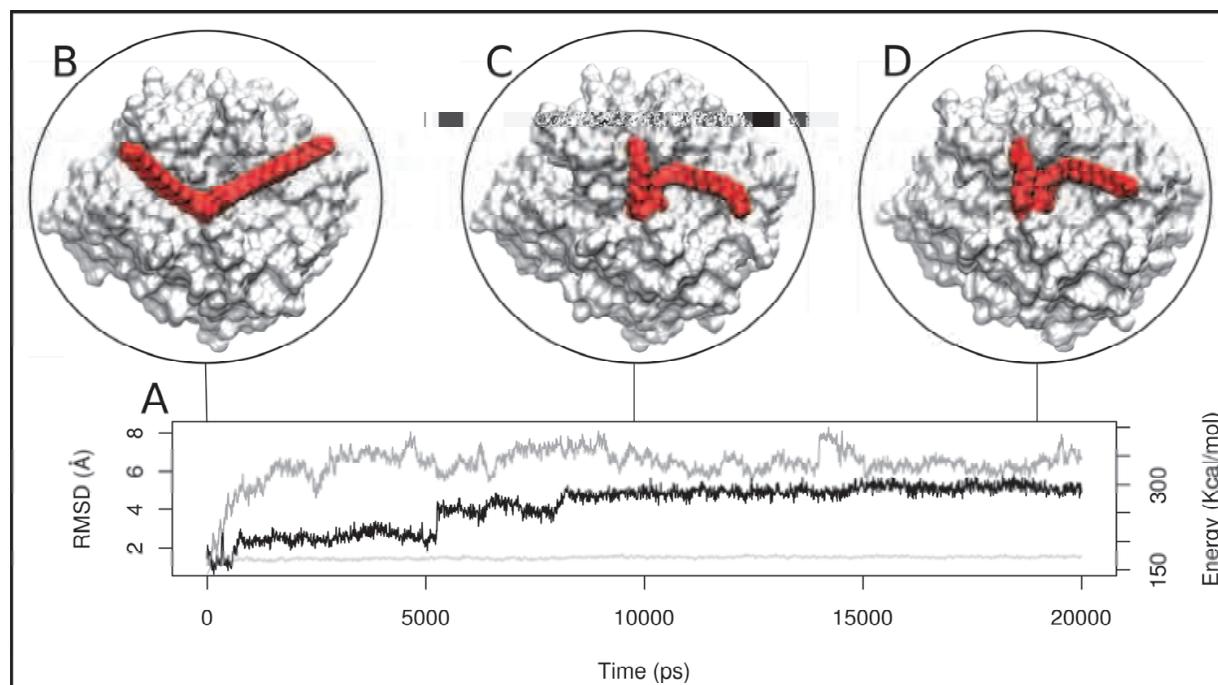


Fig. 9. MD simulation of SM/SMase D I complex. [A] RMSD of the protein (light gray) and the substrate (dark gray). MD structural frames in 0 ns (initial state) [B], 10 ns [C] and 20 ns (final state) [D].

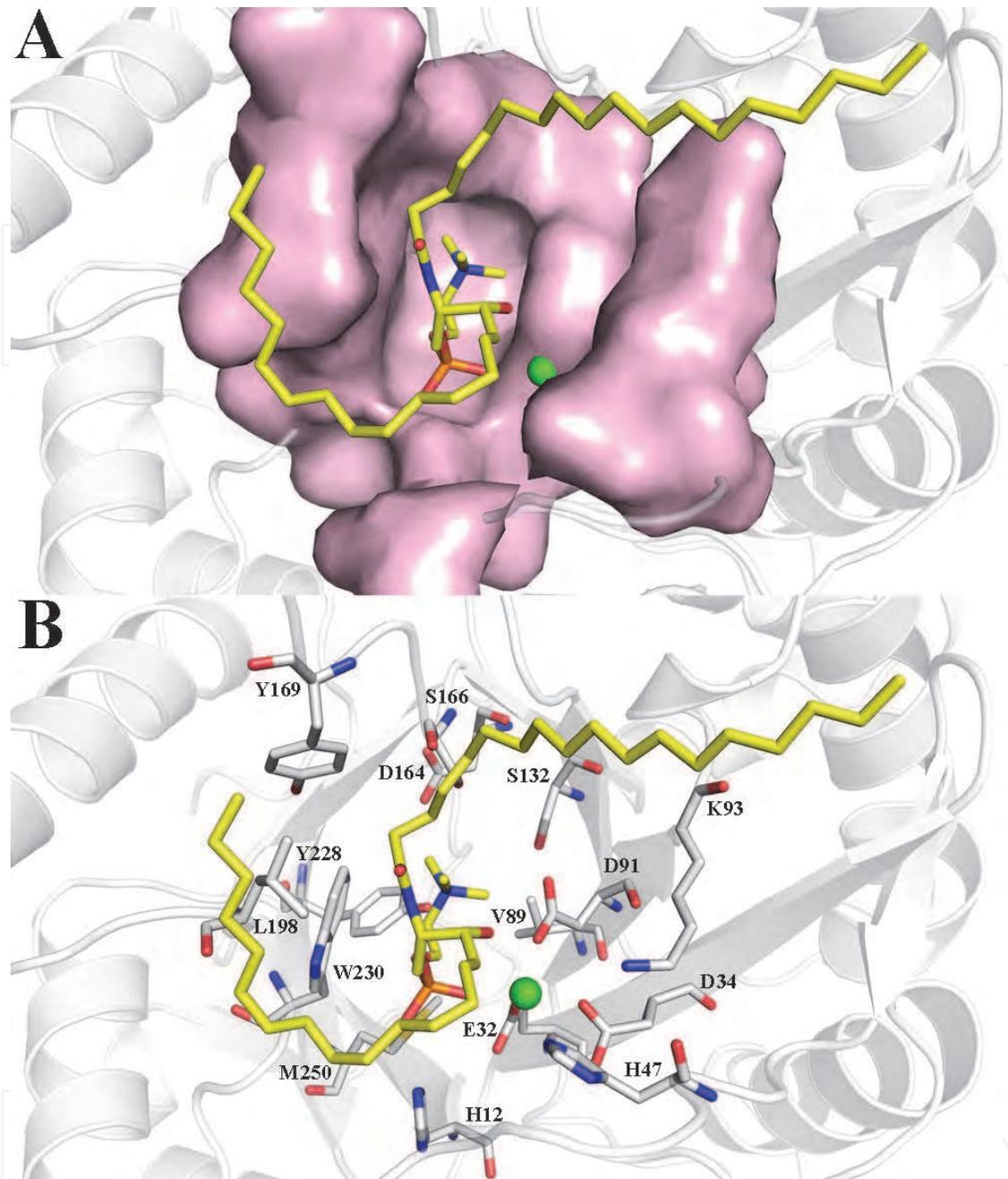


Fig. 10. Binding mode of sphingomyelin in SMase D I. [A] Surface representation of the active-site cavity with the substrate. [B] Schematic representation of the residues involved in the sphingomyelin (SM) interaction.

#### 4. Discussion

Previously, we have characterized the biochemical and biological properties of *L. adelaida* venom and evaluate the toxic potential of envenomation by this non-synanthropic *Loxosceles* species (Pretel et al., 2005). The biological activities of the *L. adelaida* venom was compared to that of *Loxosceles gaucho*, a synanthropic species of medical importance in Brazil. *L. adelaida* venom showed a similar potential to induce haemolysis, dermonecrosis and lethality as *L. gaucho* venom. Thus, showing that the troglophile *Loxosceles* species, *L. adelaida*, commonly

found in the complex of caves from PETAR, is potentially able to cause envenomation with the same gravity of those produced by synanthropic species.

Since various studies have indicated that sphingomyelinase D present in the venoms of *Loxosceles* spiders is the main component responsible for the local and systemic effects observed in loxoscelism, we have cloned and expressed a SMase D from the spider gland of *L. adelaida* to compare the functional activities of the recombinant protein with toxins from synanthropic species and to investigate the inter- and intra-species cross-reactivities of antibodies raised against the purified recombinant proteins.

The *L. adelaida* SMase D A1 cDNA exhibits similarity with previously characterized class 2 SMase D, *i.e.*, the SMase P1 from *L. intermedia*. Both sequences show the residues of the active site pocket, *i.e.*, His<sup>12</sup>, Glu<sup>32</sup>, Asp<sup>34</sup>, Asp<sup>91</sup>, His<sup>47</sup>, Asp<sup>52</sup>, Trp<sup>230</sup>, Asp<sup>233</sup>, and Asn<sup>252</sup>, which are essential for the metal-ion binding of SMases D (Mg<sup>2+</sup> is coordinated by Glu<sup>32</sup>, Asp<sup>34</sup>, Asp<sup>91</sup>), and for acid-base catalytic mechanisms (His<sup>12</sup> and His<sup>47</sup> play key roles and are supported by a network of hydrogen bonds between Asp<sup>34</sup>, Asp<sup>52</sup>, Trp<sup>230</sup>, Asp<sup>233</sup>, and Asn<sup>252</sup>) (Murakami et al., 2005, and Figures 1, 8 and 10). The importance of histidine residues was also demonstrated by Lee et al. (2005) through site-directed mutagenesis of a *Loxosceles reclusa* recombinant SMase D isoform.

Antiserum produced against the recombinant SMases D P1, P2, from *L. intermedia*, and I, from *L. laeta* was highly cross-reactive against *L. adelaida* SMase A1, and also exhibit a high level of recognition to SMases present in *Loxosceles adelaida* and *L. gaucho* venoms. These data suggest that SMases D from *Loxosceles* species analyzed share the main immunogenic epitopes. This also means that this antivenom is of potential benefit to patients being bitten, not only by the spiders of the *Loxosceles* species, which the antiserum was raised against, but also by *L. adelaida*.

We show here that the SMases D A1 and P1 in spite of their cross-reactivity, being able to induce a typical dermonecrotic reaction, exhibit differences in their toxic potential, being the lesions produced by *L. adelaida* SMase A1 smaller than that induced by SMase P1. These enzymes were also able to transform erythrocytes into activators of the autologous complement system, as demonstrated by increase of lysis susceptibility in the presence of complement. But, again, SMase D P1 was more active than A1.

Based on sequence and structural similarities, the SMases D can be grouped into two classes depending on the presence of an additional disulphide bridge between the catalytic loop and flexible (Murakami et al., 2006). *L. adelaida* SMase D A1 is a class II member and conserves all structural features for catalytic activity upon sphingomyelin. MD simulations indicated the binding mode of SM in the SMase I, a class I member that already has its crystallographic structure solved (Murakami et al., 2005) and they demonstrated the role exerted by Trp<sup>230</sup> in the orientation of choline head, the magnesium ion in the coordination of the phosphate group and other aliphatic residues in the stabilization of substrate at the active site.

In conclusion, we have cloned, expressed and biochemically and structurally characterized a new sphingomyelinase D from *Loxosceles adelaida* spider and shown that it displays all the functional characteristics of whole venom. The recombinants toxins, representing different classes of SMase D molecules, will allow us to further characterize the functionally important domains of these proteins. The identification of the active site(s) would aid in the design and testing of suitable anti-sphingomyelinase compounds in the development of novel therapies to treat loxoscelism.

## 5. Acknowledgements

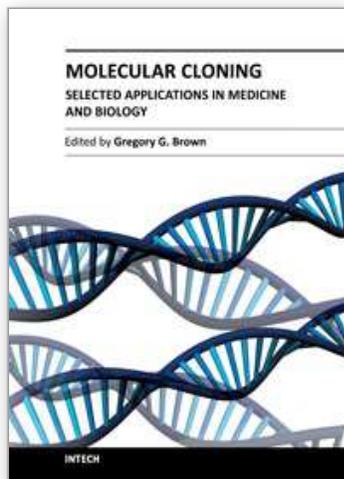
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