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Conformational changes of *Loxosceles* venom sphingomyelinases monitored by circular dichroism

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Abstract

Envenomation by arachnids of the genus *Loxosceles* can induce a variety of biological effects, including dermonecrosis and hemolysis. We have previously identified in *L. intermedia* venom two highly homologous proteins with sphingomyelinase activity, termed P1 and P2, responsible for all these pathological events, and also an inactive isoform P3. The toxins P1 and P2 displayed 85% identity with each other at the amino acid level and showed a 57% identity with SMase I, an active toxin from *L. laeta* venom. Circular dichroism was used to determine and compare the solution structure of the active and inactive isoforms. Effects of pH and temperature change on the CD spectra of the toxins were investigated and correlated with the biological activities. This study sheds new light on the structure–function relationship of homologous proteins with distinct biological properties and represents the first report on the structure–function relationship of *Loxosceles* sphingomyelinases D.

Keywords: *Loxosceles*; Venoms; Circular dichroism; Sphingomyelinase D; Hemolysis; Dermonecrosis

Envenomation by *Loxosceles* spiders, present in temperate and tropical regions of the Americas, Africa, and Europe, can lead to local dermonecrosis but also to serious systemic toxicity [1–3]. In severe cases, the hematologic complications lead to renal failure and death, especially in children [2,3]. Treatment is difficult: antivenoms are not very effective, and the use of corticosteroids or anti-inflammatory medication is controversial [3].

We have recently identified, purified, cloned, and characterized the toxins from *Loxosceles intermedia* (*L. intermedia*) and *Loxosceles laeta* (*L. laeta*) venoms that are responsible for all the local and systemic effects induced by whole venom [4–9]. From *L. intermedia* venom two highly homologous proteins, with M_r 35 kDa, were purified to homogeneity, and shown to be endowed with sphingomyelinase activity. These proteins, termed P1 and P2, were both able to induce dermonecrosis in experimental animals and rendered human erythrocytes (E) susceptible to lysis by complement (C) in vitro. In a mouse model of *Loxosceles* envenomation, we showed that the toxins also induced intravascular hemolysis and provoked a cytokine response, which resembles that seen in endotoxic shock [10]. The *L. intermedia* spider toxins P1 and P2 have probably originated by gene duplication and show a high level of amino acid sequence identity with each other (85%) and with another component in the spider venom. The latter molecule named P3, despite the high degree of identity (86–89%) in N-terminal amino acid sequence with P1 and P2, was ineffective in all activity assays [5].

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The difficulty in obtaining large amounts of *Loxosceles* venom and purified venom components is one of the limiting factors for further functional and structural characterization of the toxins, since, using electrostimulation procedures for venom extraction, it is possible to collect no more than 30 μg of venom protein per spider. We overcame this problem by cloning and functionally expressing the SMase I from *L. laeta* (Accession No. AY093599; [9]), and P1 and P2 *L. intermedia* sphingomyelinase genes (Accession Nos. AY304471 and AY304472, respectively; [6]). These recombinant proteins showed to be endowed with all biological properties ascribed for the whole venom and for the native toxins, demonstrating clearly that the sphingomyelinases are the major or unique toxins responsible for the main symptoms of both systemic and cutaneous forms of loxoscelism.

The ability of expressing large amounts of the recombinant sphingomyelinases now allows us to further investigate the mechanism of pathology induced by the *Loxosceles* venoms. The aim of the present study was to analyze the structure–function relationship of the sphingomyelinases. The characterization of the functionally important domains of these proteins would aid in the design and testing of suitable anti-sphingomyelinase compounds in the development of novel therapies to treat Loxoscelism.

Materials and methods

Spiders and venom. *Loxosceles* spiders were provided by "Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil." The venom was obtained by electro-stimulation as previously described [4,11].

Purification of the native protein P3. The inactive sphingomyelinase isoform P3 was purified from *L. intermedia* venom as described [5].

Sphingomyelinase expression. Recombinant toxins P1, P2 from *L. intermedia* and SMase I from *L. laeta* (further referred to as rP1, rP2, and SMase I) were produced as described previously [6,9]. The protein content of the samples was evaluated by Lowry method [12].

Bioinformatic analysis. Primary sequences of rP1, rP2 [6] P3 [5], and SMase I [9] were aligned using the Clustal W program [13]. The secondary structure prediction was performed using the program PSIPRED [14].

Circular dichroism measurements. CD spectra were recorded using a JASCO J-810 spectropolarimeter with a Peltier system to control the cell temperature (Japan Spectroscopic, Tokyo, Japan). Each spectrum represented the average of eight accumulations recorded between wavelengths of 190 and 260 nm, with a 0.2 nm resolution, a bandwidth of 0.5 nm, a response time of 4 s, sensitivity of 100 mdeg, and a scan speed of 20 nm/min in cell of path length of 0.2 and 1 mm. All spectra were corrected for background by the subtraction of the buffer blank. The CD intensities were expressed as molar ellipticity, [deg cm^2/dmol], with the unit (deg cm^2/dmol). The percentages of the different secondary structures (α-helix, β-sheet, β-turn, and random coil) were estimated (prediction errors on the range of 190–260 nm were 5%) using the Selcon 3 program [15].

Normal human serum and erythrocytes. Human blood was obtained from healthy donors. Blood samples drawn to obtain sera were collected without anticoagulant and allowed to clot for 2 h at room temperature, while the normal human serum (NHS) was stored at −80 °C. Blood samples drawn to obtain E for subsequent use as target cells were collected in anticoagulant (Alsever's solution: 114 mM citrate, 27 mM glucose, and 72 mM NaCl, pH 6.1).

Treatment of E with the recombinant sphingomyelinases P1, P2, and SMase I. Erythrocytes were washed and resuspended at 2% in VBS^{++} and incubated with the recombinant proteins rP1, rP2, and SMase I (2.5 μg/mL), which had previously been incubated at different temperature and pH conditions for 30 min at 37 °C and pH 7.4. Control samples were incubated with VBS^{++} (Veronal buffered saline: 10 mM Na barbitone, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4). The cells were washed three times, resuspended to the original volume in VBS^{++}, and analyzed in a hemolysis assay.

Hemolysis assays. One hundred microliters of 2% E pre-incubated with VBS^{++}, rP1, rP2, and SMase I was mixed with 100 μl NHS (1/2 in VBS^{++}). Background or total cell lysis was evaluated by incubation of E with VBS^{++} or H_2O, respectively. After incubation for 1 h at 37 °C, unlysed cells were spun and the absorbance of the supernatant was measured at 414 nm and expressed as percentage of lysis. Mean and standard deviations were determined from duplicate samples.

Dermonecrotic activity. Two hundred microliters of the recombinant proteins P1, P2, and SMase I (2.5 μg/mL), pre-treated or not at different temperature and pH conditions, was injected intradermally in the shaved back of adult rabbits. Control sites were injected with an equal volume of control solution PBS (phosphate-buffered saline: 10 mmol/L Na phosphate and 150 mmol/L NaCl, pH 7.2). The size of the lesions was measured 24 h after injection.

Results

CD spectra of the active recombinant sphingomyelinases P1, P2, SMase I, and of the inactive isoform P3

The CD spectra of the active sphingomyelinases rP1, rP2, SMase I, and also of the inactive isoform P3 were recorded at 37 °C in the 190–260 nm region. Deconvoluted CD data were obtained using the Selcon 3 program [15]. rP1, rP2, and SMase I showed very similar secondary structure compositions (Figs. 1A–C). Table 1 shows the different fractions of secondary structure elements for these recombinant proteins. These experimental results were consistent with the bioinformatic data for the active sphingomyelinases (Table 2). The spectral deconvolution of the native protein P3, whose N-terminal region is highly homologous (86–89%: in 30 amino acids of the N-terminal region) to P1 and P2, but which was inactive in all biological assays [5], showed a remarkable decrease of α-helix fraction (22%), while β-sheet (18.9%) and random coil content (44%) increased in comparison to the active sphingomyelinase isoforms; β-turn content was 12.6% (Fig. 1D; Table 1).

Effect of pH on the secondary structure of rP1, rP2, and SMase I monitored by CD

In order to understand rP1, rP2, and SMase I behavior as a function of pH, CD spectra of these recombinant proteins were measured over a wide range of pH values (2.0–9.5) at 37 °C. As shown in Fig. 2, rP1, rP2, and SMase I CD spectra were dependent on the pH.

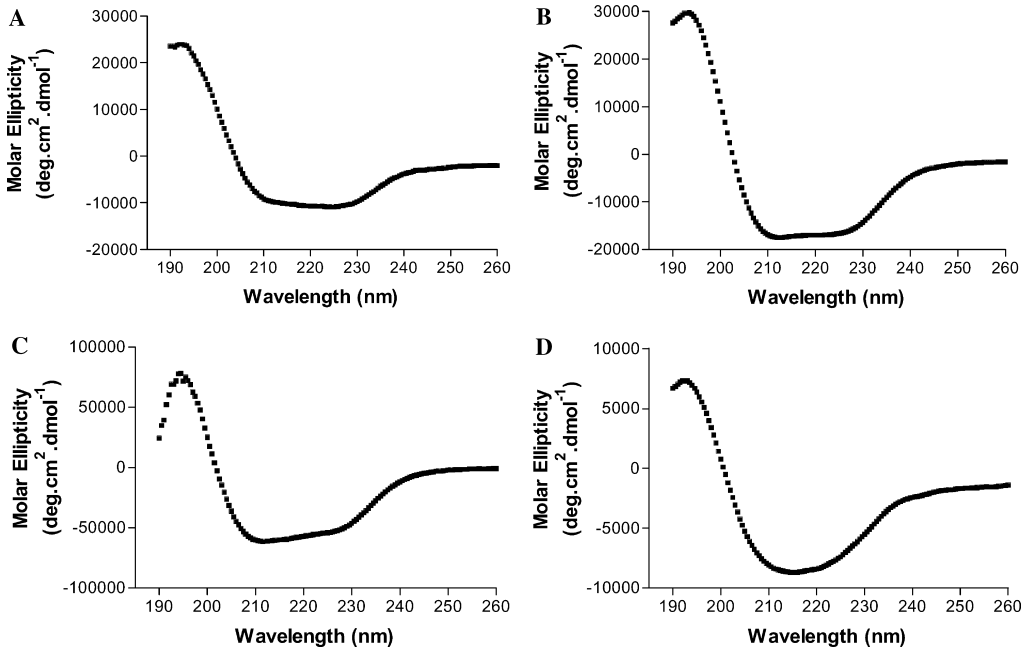

Fig. 1. CD spectra of rP1 (A), rP2 (B), SMase I (C), and P3 (D) proteins. The CD spectra of rP1 (53 μM), rP2 (60 μM), SMase I (165 μM), and native protein P3 (20 μM) were obtained in 10 mM sodium phosphate buffer, pH 7.4.

Table 1
Secondary structure content of rP1, rP2, SMase I, and native P3 determined by circular dichroism

Proteins	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
rP1	32.1	16.2	13.7	38.7
rP2	30.0	16.9	15.4	36.2
SMase I	38.0	13.8	10.6	38.0
P3	22.0	18.9	12.6	44

Table 2
The rP1, rP2, and SMase I α-helix and β-sheet content: comparison of CD data (experimental) and bioinformatic analyses

Recombinant protein	Circular dichroism		Bioinformatic	
	α-helix	β-sheet	α-helix	β-sheet
rP1	32.1	16.2	35.3	14.0
rP2	30.0	16.9	36.8	11.4
SMase I	38.0	13.8	38.2	11.0

These data indicate that the secondary structures of these proteins are affected by acidification and alkalization of the solution. Extremely acidic or alkaline environments (pH 2.0 or 9.5) induced a considerable reduction of the regular conformations, suggesting that rP1, rP2, and SMase I do not preserve their structure at acidic and alkaline pH. These conformational changes were irreversible (data not shown).

Effect of temperature on the secondary structure of rP1, rP2, and SMase I monitored by CD

To monitor the thermal behavior of rP1, rP2, and SMase I at pH 7.4, CD spectra were recorded as a function of temperature between 37 and 80 °C. The regular structure of rP1, rP2, and SMase I decreased upon increase of the temperature (Fig. 3). At high temperature (80 °C), these proteins show a CD spectrum typical of an unfolded polypeptide chain. To investigate if the refolding of rP1, rP2, and SMase I would occur upon cooling, the temperature was increased to 80 °C and reduced abruptly to 37 °C, and the conformational changes were monitored by CD. Fig. 4 shows that the structural changes induced by these conditions were irreversible to all Lox-SMases.

Induction of C-dependent hemolysis by rP1, rP2, and SMase I: effect of pH and temperature induced structural changes

In previous studies we have shown that lysis of E induced by *Loxosceles* sphingomyelinases is dependent on the activation of complement (C) alternative and classical pathways (4, 5, 7–10). To investigate the effects of structural changes induced by pH change on the induction of C-dependent hemolysis by Lox-SMases, E were incubated with the recombinant proteins (2.5 μg/mL) pre-treated at wide range of pH values (2.0, 5.5, 7.4, 8.5, and 9.5) for 30 min at 37 °C. Cells were then assessed for C-susceptibility in a hemolysis assay. Fig. 5A shows that the structural changes induced by change of the pH values from pH 7.4 (the optimum pH for activity), and monitored by CD (Fig. 2), are reflected in the ability of the sphingomyelinases to induce C-dependent hemolysis.

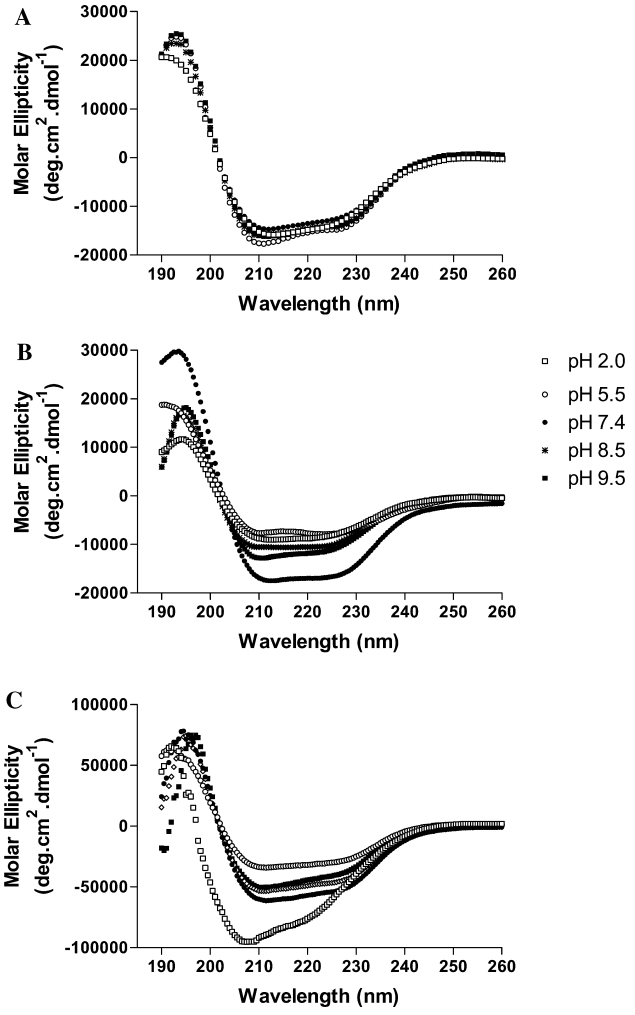

Fig. 2. CD spectra of rP1 (A), rP2 (B), and SMase I (C). The recombinant proteins were subjected to various experimental conditions of pH: 2.0 (□); 5.5 (○); 7.4 (●); 8.5 (✱); and 9.5 (■), at 37 °C.

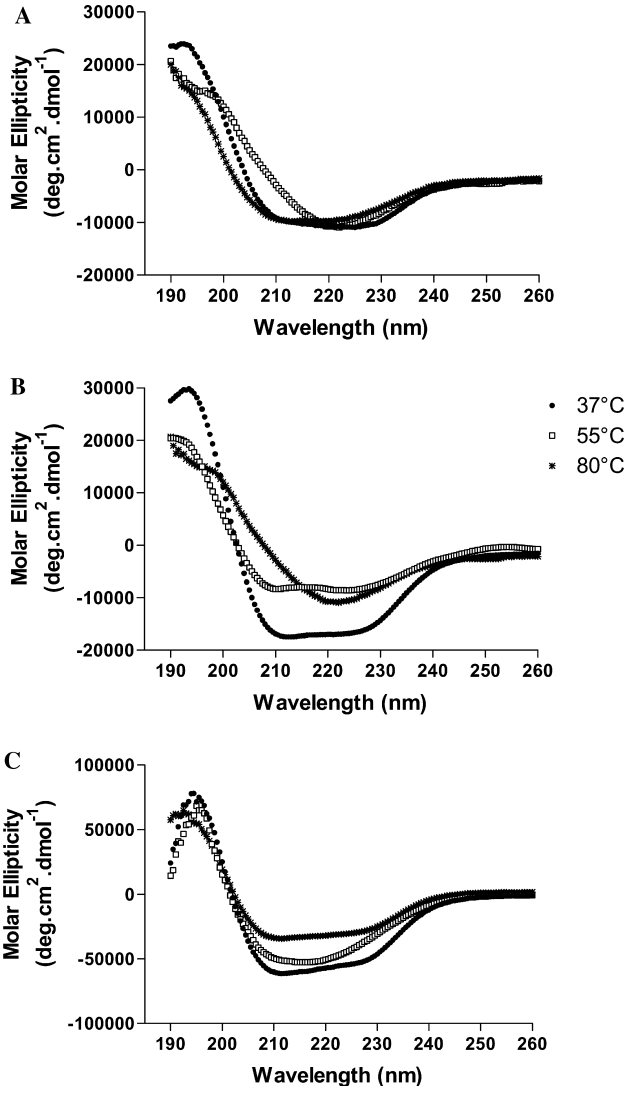

Fig. 3. CD spectra of rP1 (A), rP2 (B), and SMase I (C). The recombinant proteins were progressively heated: 37 °C (●), 55 °C (□), and 80 °C (✱), at pH 7.4.

Similarly, when the recombinant proteins were pre-incubated at different temperatures, concomitant with the loss of secondary structure as shown in Fig. 3, a loss of C-dependent hemolysis was observed (Fig. 5B). After the heating at 80 °C, rP1 and rP2 partially preserved the ability of causing C-dependent hemolysis, while SMase I was completely inactivated (Fig. 5B).

Induction of dermonecrosis by rP1, rP2, and SMase I sphingomyelinases: effect of pH and temperature induced structural changes

The effects of structural changes induced by pH or temperature alteration on the ability of the Lox-SMases to induce dermonecrosis were evaluated in a rabbit model. The animals received buffer, rP1, rP2 or SMase I pre-treated for 30 min at a wide range of pH or temperature values. After pH variations, typical loxoscelic lesions developed at all toxin inoculation sites, however, a significant decrease in the size of the dermonecrotic lesion at the inoculation sites of the toxins pre-treated at pH values distinct from 7.4 (the optimum pH for the activity these proteins) was observed (Fig. 6A). The dermonecrotic activity of rP2 showed to be most sensitive to pH change.

Pre-incubation of the Lox-SMases at higher temperatures also reduced their ability to induce dermonecrosis. While only a partial reduction in the size of the lesion was observed after pre-incubation at 55 °C, the dermonecrotic activity was completely destroyed by heating to 80 °C (Fig. 6B).

Discussion

The toxins responsible for the local and systemic effects of *Loxosceles* venoms are unusual SMases D that

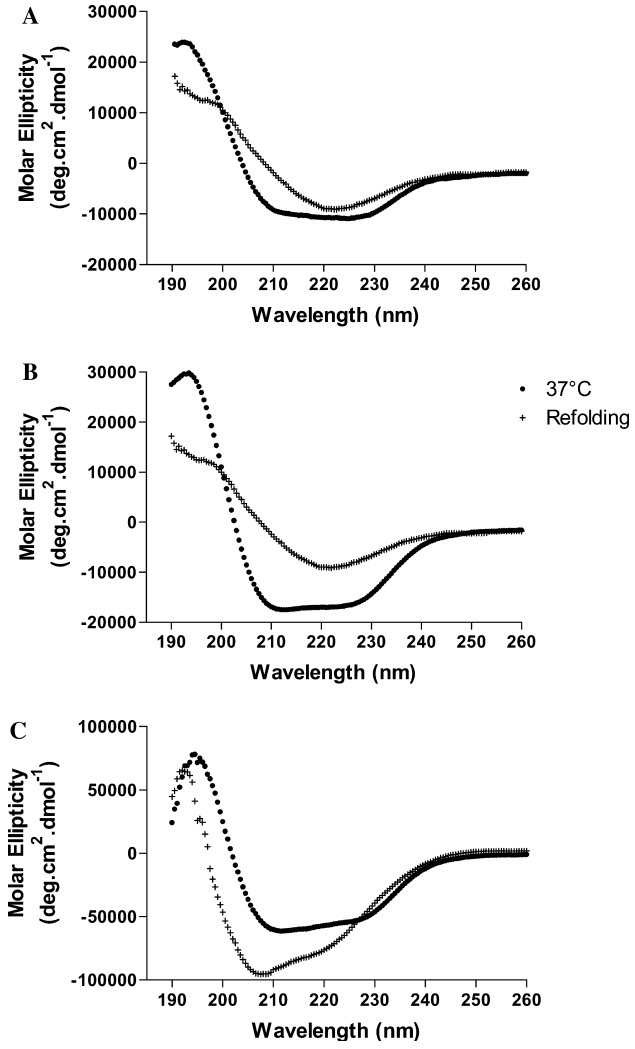

Fig. 4. CD spectra of rP1 (A), rP2 (B), and SMase I (C). The recombinant proteins were heated to 37 °C (●) or to 80 °C and cooled abruptly to 37 °C (+).

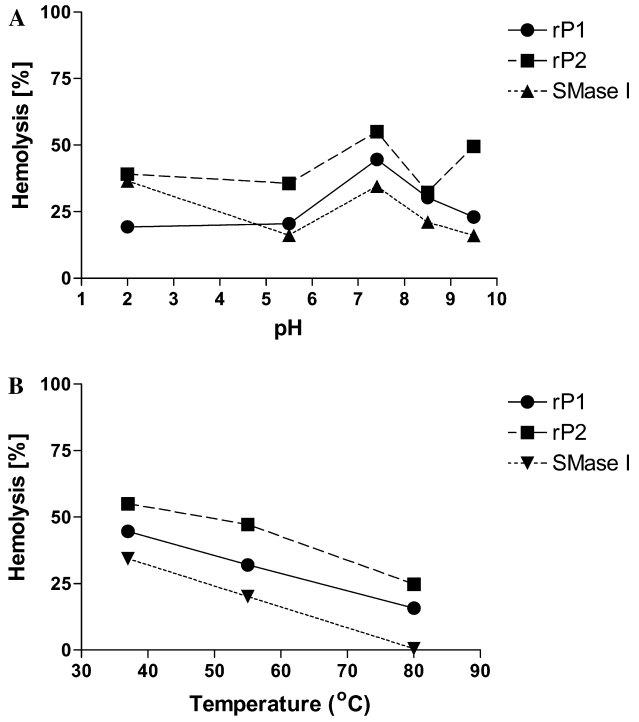

Fig. 5. pH and temperature dependence on the ability of rP1, rP2, and SMase I to induce of complement susceptibility. Erythrocytes were incubated with rP1, rP2, and SMase I (2.5 μg/mL) pretreated at different pH and temperatures. After incubation for 1 h at 37 °C, unlysed cells were spun down; the absorbance of the supernatant was measured at 414 nm and expressed as percentage of lysis.

convert sphingomyelin (SM) in the outer leaflet of the plasma membrane to ceramide 1-phosphate (*N*-acylsphingosine 1-phosphate) [5,9,16,17]. Recent evidence suggests that branching pathways of sphingolipid metabolism mediate either apoptotic or mitogenic responses depending on the cell type and the nature of the stimulus [18]. Events involving SM metabolites and ceramide in particular include proliferation, differentiation, and growth arrest as well as the induction of apoptosis.

Spider SMases D can also catalyze the release of choline from lysophosphatidylcholine (LPC), but not from phosphatidylcholine, in the presence of Mg^{2+} [19]. LPC is an abundant phospholipid in plasma, where it is tightly bound to albumin; removal of its choline headgroup yields lysophosphatidic acid, a potent lipid mediator with numerous biological activities in many different cells [20,21].

In this study, we have analyzed the structure–function relationship of the recombinants SMases P1 and P2, and the native protein P3 from *L. intermedia* venom, and SMase I from *L. laeta* venom. These recombinant Loxosceles SMases D (Lox-SMases D: P1, P2, and SMase I) show high identity with each other (85% between P1 and P2, and 57% between P1/P2 and SMase I).

Our study of the secondary structures of the Lox-SMases shows that under physiological conditions the contents of secondary structure elements of rP1, rP2, and SMase I as measured by circular dichroism (Figs. 1A–C; Table 1) are very similar and are very close to the predicted structure for these proteins (Table 2). These proteins show an α/β-fold as well as the PLD family members [22]. Among PLD family, only a few members have known 3D structure. Detailed bioinformatic, including search similarity and fold recognition (http://geno3d-pbil.ibcp.br) [23] to Lox-SMases D, did not produce significant matches to these few PLDs with known structures.

However, the X-ray analysis of the PLD member isolated from the bacteria *Streptomyces* sp. [24], which presents similar structure secondary element content to Lox-SMases (data not shown), revealed two rotated α-helices in the center of the molecule distant from the active site and that these helices provide the necessary

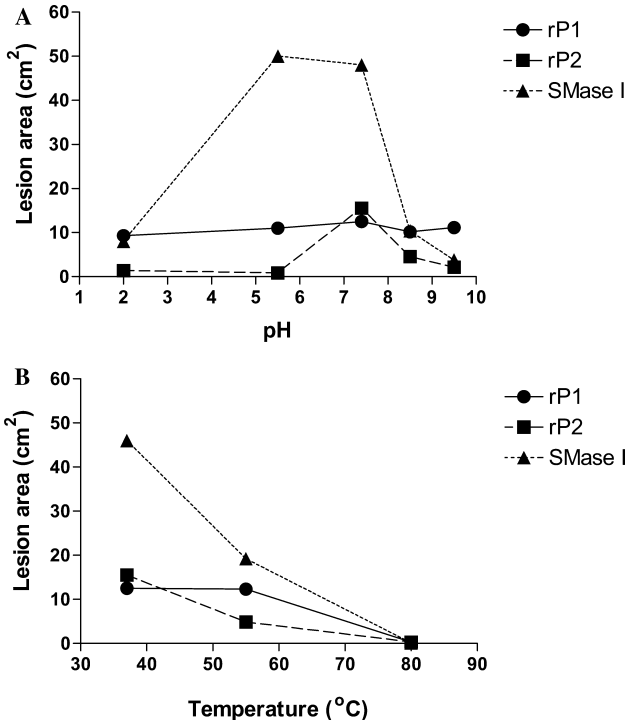

Fig. 6. pH and temperature dependence of rP1, recP2, and SMase I to induce dermonecrotic lesion. The animals received buffer or the recombinant proteins rP1, rP2, and SMase I pretreated at different pH and temperatures. The areas of the dermonecrotic lesions were determined 24 h after injection.

scaffolding or framework for keeping the active site in an open and accessible conformation so that substrate can enter. In other words, these results give us an insight into the structural importance of α-helix element to activity and binding to substrate for the PLDs.

The Lox SMase D isoform P3, which has the same molecular weight as P1 and P2 and shows a high degree of homology in the amino terminal region, was inactive in all biological assays [5]. Under physiological conditions, where rP1, rP2, and SMase I showed maximal activity, the CD spectra of P3 showed a secondary structure pattern significantly different from those of these active recombinant proteins. The most remarkable difference was the decrease between 27% and 43% in the α-helix content (Table 1). Therefore, the destabilization of α-helices in P3 could be affecting the interaction of the putative active site of this protein with the substrate and may explain why P3 does not display any biological activity. This also suggests a positive correlation between α-helical content and biological activity of the Lox-SMases D. This was confirmed by the correlation between the loss of α-helical content induced by either pH change or increase in temperature (decrease in negative intensity of the spectrum around 208 and 222 nm) and the ability of the active recombinant Lox-SMases D to induce complement-dependent hemolysis and dermonecrosis (Figs. 5 and 6). Change in pH induced a variable alteration of the biological activities, e.g., while at pH 5.5, rP2 kept its capacity to induce hemolysis, it lost its capacity to induce dermonecrosis. This suggests that these activities are not only dependent on folding of these proteins but that conditions that changes the net charge of the protein may increase the hemolytic activity, while this does not seem to modify the dermonecrosis inducing activity. Temperature increase reduced the biological activities of the Lox-SMases D in all cases, with the exception of the relatively heat resistance of the dermonecrosis inducing ability of rP1.

In conclusion, our study of the secondary structure of Lox-SMases shows that the toxins responsible for the two main clinical manifestations of Loxoscelism, e.g., dermonecrosis and complement-dependent hemolysis, are α/β hydrolases, as well as the PLD members and that the α-helical structural elements may be important for their biological activities.

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