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Isolation and Characterization of a Myotoxic Phospholipase A_2 from the Venom of the Arboreal Snake *Bothriechis* (*Bothrops*) *schlegelii* from Costa Rica

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A new myotoxic phospholipase A_2 was isolated from the venom of the arboreal snake *Bothriechis schlegelii* (formerly *Bothrops schlegelii*) from Costa Rica, by ion-exchange chromatography on CM-Sephadex. *B. schlegelii* myotoxin I is a basic protein (p$I > 9.3$) with a subunit molecular weight of 15 kDa, which migrates as a dimer in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions. This myotoxin is recognized by antibodies generated against *Bothrops asper* myotoxin II (a lysine-49 phospholipase A_2), by both enzyme-immunoassay and gel immunodiffusion, in the latter case with a pattern of partial identity. The toxin induces rapid myonecrosis upon intramuscular injection in mice, as evidenced by the early increase in plasma creatine kinase activity and by direct intravital microscopic observation. *B. schlegelii* myotoxin I also induces edema in the mouse footpad assay and exerts lethal activity (LD_{50} ~2.5 μg/g) upon intravenous injection. The toxin has a low phospholipase A_2 activity (4.2 $\mu Eq \cdot mg^{-1} \cdot min^{-1}$) using egg yolk phospholipids as substrate. It also shows a weak anticoagulant effect *in vitro*. Its N-terminal sequence, SMYELGKMILLETGKNAATSYIAYG, shows 93% homology with both *Bothrops asper* myotoxin II and *B. jararacussu* bothropstoxin I, suggesting that *B. schlegelii* myotoxin I may be a new lysine-49 variant of this family of myotoxic phospholipases A_2.

Key Words: **myotoxin; phospholipase A_2; venom; snake; *Bothriechis; Bothrops.***

Necrosis of muscular tissue is a serious consequence of envenomation by snakebites (1). In envenomations caused by crotalid snakes of the genus *Bothrops* (American lance-headed pit vipers), distributed from Mexico to Argentina, it has been shown that skeletal muscle necrosis develops mainly due to the action of basic toxins with group II phospholipase A_2 (PLA_2)[2] structure (reviewed in Ref. 2). In recent years, several species within the genus *Bothrops* have been reclassified into the new genera *Bothriechis*, *Bothriopsis*, and *Porthidium* (3). The venoms of many of these species contain basic PLA_2 myotoxins (4), which display sequence variability (2, 5). Furthermore, several myotoxin variants or isoforms can exist in the venom of individual specimens, as shown in *Bothrops asper* (6). Given the substantial inter- and intraspecific diversity observed in this family of basic PLA_2s, the biochemical and pharmacological characterization of new variants may add valuable information for the understanding of their structure–function relationship.

Bothriechis schlegelii (formerly *Bothrops schlegelii;* commonly known as "eyelash viper" in the English literature, and "bocaracá" in Costa Rica) causes a number of human envenomations in Costa Rica (7). In earlier studies, it was shown that its venom induces significant myonecrosis in experimental animal models (8, 9). Further studies indicated that this venom contains one or more components antigenically cross-reactive to previously characterized myotoxins from *B. asper* venom (10). In the present work, we describe the isolation and partial characterization of a myotoxic PLA_2 from this venom, named *B. schlegelii* myotoxin I.

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[2] Abbreviations used: PLA_2, phospholipase A_2; PBS, phosphate-buffered saline; CK, creatine kinase; FBS, fetal bovine serum.

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MATERIALS AND METHODS

Isolation of B. schlegelii myotoxin I. *B. schlegelii* venom was a pool obtained from more than 30 adult specimens collected in Costa Rica. After centrifugation, venom was lyophilized and stored at −40°C. Samples of 500 mg were dissolved in 10 ml of 0.05 M Tris–HCl, 0.1 M KCl, pH 7.0 buffer, and applied to a CM-Sephadex C-25 (Pharmacia, Sweden) column (30 × 2.5 cm) equilibrated with the same buffer. After collecting unbound proteins, elution of basic fractions was carried out with a continuous linear gradient toward 0.75 M KCl, 0.05 M Tris–HCl, pH 7.0 buffer (11). Peaks were screened for PLA_2 activity and immunochemical cross-reactivity with rabbit antibodies against *B. asper* myotoxin II (4), as described below. On the basis of these screenings, peak 5 was selected for further analysis. It was rechromatographed identically, dialyzed exhaustively against water, and lyophilized.

Homogeneity. Toxin homogeneity was assessed by (a) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) (12); (b) PAGE in the presence of urea, using a cathodic buffer system (13); (c) reverse-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C4 column eluted at 1 ml/min with a gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid, using a Varian (Inert 9012) instrument; and (d) cation-exchange HPLC using a Poros (Perseptive Biosystems) column eluted at 3 ml/min with a gradient from 0 to 1 M NaCl in 0.05 M Hepes–NaOH, pH 8.0.

Isoelectric focusing. Isoelectric focusing was performed in 1% IEF–agarose (Pharmacia) containing 12% sorbitol and a 1:1 mixture of pH 3–10 and pH 8–10.5 ampholytes (6.4% v/v). Cathode and anode solutions were, respectively, 0.1 M NaOH and 0.05 M H_2SO_4. Prefocusing was performed for 250 V-h at 750 V, whereas final focusing was at the same voltage for 800 V-h. Isoelectric point marker proteins (Pharmacia, pH range 3–10) were run in parallel with *B. schlegelii* myotoxin I (20 μg).

N-terminal sequencing. *B. schlegelii* myotoxin I was carboxymethylated as previously described (14) and sequence analysis was performed using an Applied Biosystems 470A instrument (Foster City, CA). Homology searches were done in the PIR and Swiss-Prot databases.

Phospholipase A_2 activity. For qualitative screening of fractions, an indirect hemolytic gel diffusion technique was utilized (15). Washed sheep red blood cells (final concentration 1.2% v/v) were mixed at 45°C with 1% agarose in 0.12 M NaCl, 0.04 M sodium phosphate buffer (PBS), pH 7.2, containing 1% egg yolk as a source of phospholipids. The mixture was poured into plastic trays and cooled, and then samples of 15 μl were loaded into wells made in the gel and allowed to diffuse for 16 h at 37°C for the measurement of the lytic halo diameter. To exclude direct hemolytic activity, samples were assayed in parallel gels devoid of egg yolk. For quantitative determination of PLA_2 activity, samples of 0.1 ml were incubated with 1.0 ml of egg yolk suspension (diluted 1:5 in 0.1 M Tris–HCl, 0.01 M $CaCl_2$, pH 8.5) containing 1% Triton X-100, for 15 min at 37°C. Then, the free fatty acids were extracted and titrated according to Dole (16).

Myotoxic activity. Varying amounts of toxin, dissolved in 50 μl of PBS, were injected into groups of five Swiss mice (18–20 g), in their right gastrocnemius muscle. A control group received 50 μl of PBS. After 3 h, tail blood samples were collected into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2.) activity was determined by a colorimetric assay (Sigma No. 520). CK activity was expressed in U/ml, where 1 U results in the phosphorylation of 1 nmol of creatine per minute at 25°C. To determine the time course of CK release *in vivo,* a fixed dose of *B. schlegelii* myotoxin I (80 μg) was injected im into groups of five mice, which were bled after 1, 3, 6, and 24 h for plasma CK determination. In addition, the morphological alterations induced by *B. schlegelii* myotoxin in skeletal muscle were analyzed by intravital microscopy, using the mouse cremaster preparation, as previously described (17). Forty micrograms of toxin, dissolved in 20 μl of PBS, was applied onto the surgically exposed cremaster of anesthetized mice, which was then covered with a thin sheet of Mylar, and the subsequent events were recorded during a 45-min observation period, in three independent experiments.

Immunochemical studies. An enzyme immunoassay was utilized to screen *B. schlegelii* venom fractions from the CM-Sephadex for the presence of components antigenically related to *B. asper* myotoxins. One hundred microliters of each fraction, diluted 1:50 in PBS, was adsorbed onto Immulon-2 microplates (Dynatech, Virginia) overnight. After five washings with PBS–0.05% Tween 20, plates were blocked with 150 μl/well of PBS containing 10% fetal bovine serum (FBS) for 1 h. After decanting, 100 μl/well of rabbit antiserum to *B. asper* myotoxin II (diluted 1:1000 in PBS–2% FBS) was incubated for 1 h, followed by five washings and then by 100 μl/well of protein G–horseradish peroxidase conjugate (Sigma, 1:1000) for 1 h. Color was developed with 0.012% H_2O_2 and *o*-phenylenediamine (2 mg/ml in 0.1 M sodium citrate, pH 5.0), stopped with 2 M HCl, and recorded at 490 nm on a Dynatech MR5000 microplate reader. As a control, normal rabbit serum was tested in parallel against each fraction. Finally, enzyme immunoassay readings for each fraction were superimposed onto the chromatographic profile (absorbance at 280 nm) for the identification of cross-reactive peaks (18).

Antigenic cross-reactivity between the final preparation of *B. schlegelii* myotoxin I and purified *B. asper* myotoxins I and II was additionally analyzed by gel immunodiffusion (19) using rabbit anti-*B. asper* myotoxin II serum, in 1% agarose–PBS plates.

Lethality. Varying amounts of toxin, dissolved in 0.1 ml of PBS, were injected by the intravenous route into groups of four mice (16–18 g). Deaths were scored 48 h after injection, and the 50% lethal dose (LD_{50}) was estimated by the Spearman–Karber method (20).

Anticoagulant activity. Platelet-poor plasma from sheep was prepared by centrifuging citrated blood twice at 1000*g* at 5°C. For the assay, 0.5 ml of plasma was incubated with varying amounts of toxin dissolved in 0.1 ml of PBS, for 10 min at 37°C. Then, 0.1 ml of 0.25 M $CaCl_2$ was added and the coagulation time recorded. As a control, plasma aliquots were incubated in parallel with 0.1 ml of PBS.

Edema-forming activity. Groups of five mice (18–20 g) received a subcutaneous injection of varying amounts of toxin, dissolved in 50 μl of PBS, in their right footpad. After 1 h, edema was estimated by measuring the increase in footpad thickness with a low-pressure spring caliper (21). Control mice received an injection of 50 μl of PBS. In another experiment, the time course of edema was determined by injecting a fixed dose of toxin (25 μg) similarly and measuring the footpad thickness at 1, 3, 6, 12, and 24 h. The involvement of different inflammatory mediators in the edema induced by *B. schlegelii* myotoxin I was evaluated by pretreating mice with an intraperitoneal injection of diphenhydramine (25 mg/kg) or indomethacin (10 mg/kg), 1 h before the injection of 5 μg of toxin into the footpad.

RESULTS

Fractionation of crude *B. schlegelii* venom on CM-Sephadex at pH 7.0 resulted in a major unbound peak of acidic components, followed by several smaller peaks of basic components eluted by the salt gradient (Fig. 1A). Of these, peak 5 showed a strong immunochemical cross-reactivity when screened with an antiserum against previously characterized myotoxins from *B. asper* venom (Fig. 1A). PLA_2 activity of the basic fractions concentrated mainly in peaks 5 and 6 (data not shown). Peak 5 was selected for further characterization and rechromatographed under the same conditions. A single symmetrical peak was obtained (Fig.

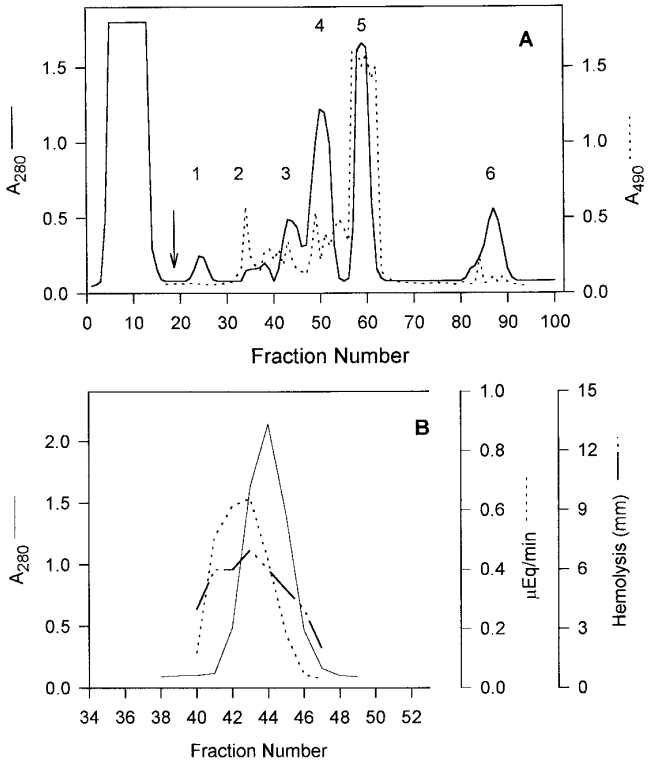

FIG. 1. Isolation of *Bothriechis schlegelii* myotoxin I by ion-exchange chromatography on CM-Sephadex C-25. (A) Elution pattern of crude venom (500 mg) equilibrated with 0.1 M Tris–HCl, 0.1 M KCl, pH 7.0 buffer. The arrow indicates the application of a linear gradient toward 0.1 M Tris–HCl, 0.75 M KCl, pH 7.0 buffer. Protein content of the fractions was monitored at 280 nm (solid line). The immunoreactivity of each fraction probed with a rabbit antiserum to *B. asper* myotoxin II was monitored by enzyme immunoassay (dotted line), as described under Materials and Methods. (B) Rechromatography of peak 5 from (A) on the CM-Sephadex (solid line) and analysis of phospholipase A_2 activity (dotted line) and indirect hemolytic activity (dashed line) of the fractions.

1B). This protein appeared as a single band of 15 kDa in SDS–PAGE, after reduction by 2-mercaptoethanol (Fig. 2A). In the absence of reducing agent and heating, it migrated as a single band of an apparent mass of 21 kDa (not shown). In cathodic urea–PAGE the protein also appeared as a single component, with a mobility identical to a major band of the basic components of crude venom (Fig. 2B). By both analytical RP-HPLC (Fig. 3A) and cation-exchange HPLC (Fig. 3B), the purified toxin eluted as a single, symmetrical peak. By isoelectric focusing, the toxin appeared as a single band near the cathode, with an isoelectric point above that of trypsinogen (p*I* 9.3) (not shown).

The N-terminal amino acid sequence of *B. schlegelii* myotoxin I, compared to those of related proteins, is shown in Fig. 4. In gel immunodiffusion, the toxin was immunoprecipitated by the rabbit antiserum to *B. asper* myotoxin II, with a pattern of partial identity to *B. asper* myotoxins I and II (Fig. 2C).

The toxin induced a dose-dependent myonecrosis upon im injection in mice, as evidenced by the significant increase in plasma CK activity (Fig. 5A). CK levels increased since the first hour after injection, reached a maximum at 3 h, and declined to normal after 6 h (Fig. 5B). At 3 h, the plasma CK activity represented a 12-fold increase over control levels, at a dose of 80 µg (Fig. 5A).

By intravital microscopy, damage to skeletal muscle fibers was evidenced as soon as 4–6 min after exposure to the toxin. The first observed effect, starting within the first minute, was the induction of slow, transient contractions of the muscle fibers, which significantly distorted the whole tissue and its microvasculature. These contractions ceased after about 30–60 s, and then evident damage to the muscle fibers followed. The damaged fibers developed initially small lesions which were gradually enlarged by the slow retraction of the myofibrils in opposite directions. This retraction finally produced a complete transverse rupture of the fiber, with a general disorganization of intracellular contents in the lesion borders and their vicinity. This process was sometimes repeated at several points along a given muscle fiber, leaving at the end several ruptured seg-

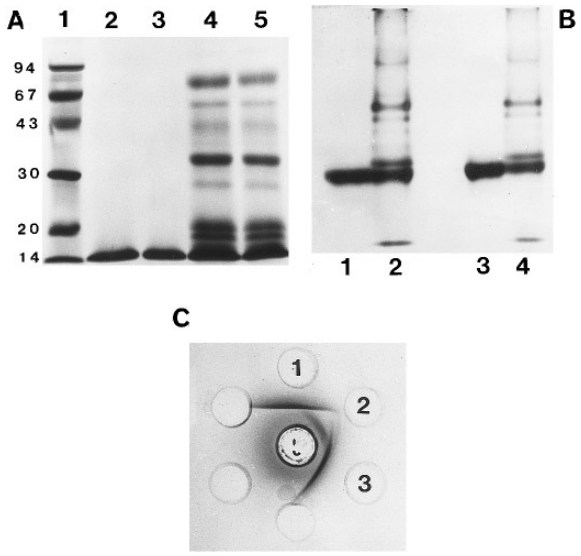

FIG. 2. Electrophoretic and immunodiffusion analyses of *Bothriechis schlegelii* myotoxin I. (A) Polyacrylamide gel (12%) electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) of reduced *B. schlegelii* myotoxin I (8 µg, lanes 2 and 3) and crude venom (40 µg, lanes 4 and 5), stained with Coomassie brilliant blue R-250. Molecular weight markers (kDa) are in lane 1. (B) Urea–PAGE (12%) of *B. schlegelii* myotoxin I (10 µg, lane 1; 8 µg, lane 3) and crude venom (50 µg, lane 2; 40 µg, lane 4). Cathode is at the bottom. Coomassie stain. (C) Agarose gel immunodiffusion of (1) *B. asper* myotoxin I, (2) *B. schlegelii* myotoxin I, and (3) *B. asper* myotoxin II, against rabbit anti-*B. asper* myotoxin II serum (S). Toxin concentration was 0.5 mg/ml in all cases. Wells were filled with 35 µl of tests solutions. Coomassie stain.

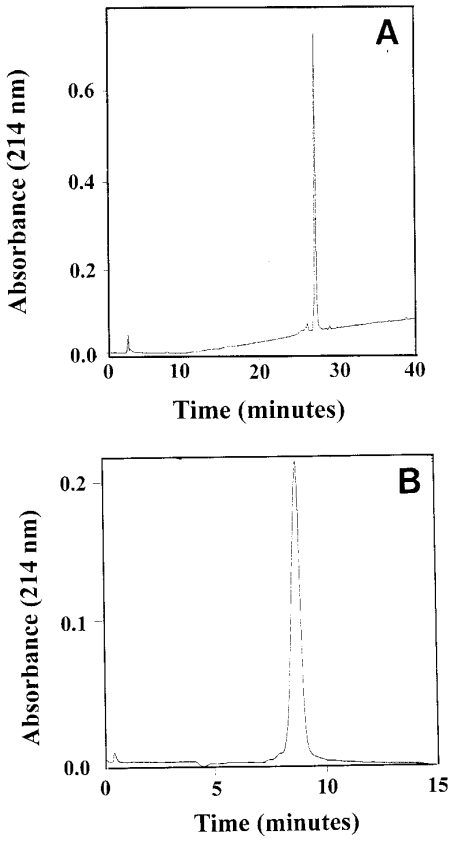

FIG. 3. HPLC analyses of *Bothriechis schlegelii* myotoxin I. (A) RP-HPLC of myotoxin (20 μg) on a C4 column, eluted at 1 ml/min with an acetonitrile gradient: 0–5 min, 100% A; 5–35 min, 0–60% B; 35–40 min, 60–80% B. Buffer A was 0.1% trifluoroacetic acid, and buffer B was 0.1% trifluoroacetic acid in 100% acetonitrile. Detection was at 214 nm. (B) Cation-exchange HPLC of myotoxin (600 μg) on a Poros column, eluted at 3 ml/min with a NaCl gradient: 0–3 min, 100% A; 3–13 min, 0–50% B. Buffer A was 50 mM Hepes–NaOH, pH 8.0, and buffer B was 50 mM Hepes–NaOH, 2 M NaCl, pH 8.0. Detection was at 214 nm.

ments separated by spaces apparently devoid of cellular material. No hemorrhagic foci developed throughout the observation period.

B. schlegelii myotoxin I exerted a low, but detectable PLA_2 activity on egg yolk phospholipids (4.2 μEq·mg^{-1}·min^{-1}). When individual tubes collected from the CM-Sephadex rechromatography of the toxin were analyzed for both PLA_2 and indirect hemolytic activities, and then superimposed to the protein content profile of the fractions, a slight displacement to the left was observed (Fig. 1B).

In vitro, B. schlegelii myotoxin I showed a low anticoagulant activity on sheep platelet-poor plasma (Fig. 6). On the other hand, it was devoid of procoagulant activity and exerted lethal activity upon intravenous injection, with an estimated LD_{50} of 2.5 μg/g. It also induced a significant edema in mice, at doses as low as 2.5 μg/footpad (Fig. 7A). The edema was of rapid onset and was sustained for at least 6 h, then gradually resolving (Fig. 7B). Pretreatment of mice with diphenhydramine or indomethacin did not modify significantly the course of footpad edema induced by 5 μg of the toxin (data not shown).

DISCUSSION

The isolation and properties of a myotoxic PLA_2 from the venom of *B. schlegelii* are presented in this study. Earlier reports on the ability of this venom to induce myonecrosis (8, 9), together with the observation of its antigenic cross-reactivity with antibodies against previously characterized myotoxic PLA_2s from *B. asper* (10), prompted the search for this new myotoxin. Cation-exchange chromatography at pH 7.0 confirmed the presence of several basic proteins in the venom of *B. schlegelii*, one of which showed a strong antigenic cross-reactivity when probed with antibodies to *B. asper* myotoxin II. This protein was further purified and appeared homogeneous by several analytical criteria, including SDS–PAGE, urea–PAGE for basic proteins, RP-HPLC, and cation-exchange HPLC.

Biochemical analyses indicated that *B. schlegelii* myotoxin I is a basic protein (pI > 9.3), with a subunit molecular weight of 15 kDa, occurring as a dimer. Its N-terminal amino acid sequence shows highest homology to snake venom myotoxins belonging to the family of so-called Lys-49 PLA_2s. This suggests that *B.*

Name (Reference)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	%Homology
B. schlegelii myotoxin I		**S**	M	Y	E	**L**	G	**K**	**M**	**I**	L	L	**E**	**T**	**G**	**K**	N	A	A	T	S	**Y**	I	A	**Y**	**G**	-
B. asper myotoxin II	(38)	**S**	L	F	E	**L**	G	**K**	**M**	**I**	L	Q	**E**	**T**	**G**	**K**	N	P	A	K	S	**Y**	G	A	**Y**	**G**	93
B.jararacussu bothropstoxin I	(39)	**S**	L	F	E	**L**	G	**K**	**M**	**I**	L	Q	**E**	**T**	**G**	**K**	N	P	A	K	S	**Y**	G	A	**Y**	**G**	93
A. p. piscivorus Lys-49	(40)	**S**	V	L	E	**L**	G	**K**	**M**	**I**	L	Q	**E**	**T**	**G**	**K**	N	A	I	T	S	**Y**	G	S	**Y**	**G**	91
T. mucrosquamatus Lys-49	(41)	**S**	L	I	E	**L**	G	**K**	**M**	**I**	F	Q	**E**	**T**	**G**	**K**	N	P	V	K	N	**Y**	G	L	**Y**	**G**	67
T. flavoviridis BP-I	(30)	**S**	L	V	Q	**L**	W	**K**	**M**	**I**	F	Q	**E**	**T**	**G**	**K**	E	A	A	K	N	**Y**	G	L	**Y**	**G**	65

FIG. 4. Amino-terminal sequence of *Bothriechis schlegelii* myotoxin I and related class II phospholipases A_2.

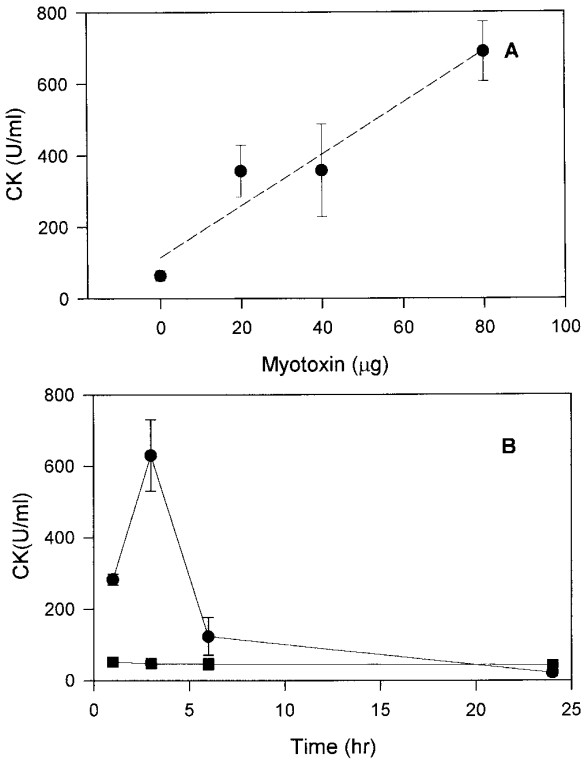

FIG. 5. Myotoxic activity of *Bothriechis schlegelii* myotoxin I in mice. (A) Plasma creatine kinase (CK) activity 3 h after the intramuscular injection of varying amounts of toxin in the gastrocnemius. Results are presented as means ± SD (n = 5). (B) Time course of the plasma CK increase after the intramuscular injection of 80 μg of toxin (●) or phosphate-buffered saline (■). Results are presented as means ± SD (n = 5).

schlegelii myotoxin I might also be a Lys-49 PLA_2. However, some amino acid residues of this toxin show differences when compared to Lys-49 myotoxins described so far. Of positions 5 and 11, which in Lys-49 PLA_2s are invariantly occupied by Leu and Gln, respectively, only Leu-5 is conserved in *B. schlegelii* myotoxin I, since Gln-11 is replaced by Leu. In addition, the conserved Gly-22 is replaced by Ile, while Met-2 and Tyr-3 would represent new amino acid variants in this group, if future sequence studies confirm *B. schlegelii* myotoxin I to be a Lys-49 PLA_2. At least two natural variants of Asp-49 other than Lys have been described, including Ser-49 and Arg-49, from the venoms of *Vipera ammodytes* (22) and *Trimeresurus mucrosquamatus* (23), respectively.

Elucidation of the complete sequence of *B. schlegelii* myotoxin I would be helpful to understand the structure–function relationship of these basic PLA_2s. The myotoxic site of these proteins has not been conclusively identified, despite some experimental evidence suggesting the participation of their N-terminal α-helix (24, 25) and C-terminal cationic/hydrophobic sequence (26). *B. schlegelii* myotoxin I has the conserved Lys-7, a residue which might be important for the expression of myotoxicity, on the basis of PLA_2 sequence comparisons (27).

Lys-49 PLA_2s are unable to catalyze phospholipid hydrolysis due to critical amino acid substitutions in their calcium-binding loop (28). However, in some cases, Lys-49 proteins that exhibit a very low catalytic activity have been purified, and there has been a debate on whether this low activity is an intrinsic property of these proteins or a slight contamination with enzymatically active Asp-49 PLA_2s present in the same venoms (2, 28–34). In the case of *B. schlegelii* myotoxin I, a low PLA_2 activity was clearly detectable, despite its N-terminal sequence strongly suggesting that it may be a Lys-49 variant. While a combination of analytical electrophoretic and chromatographic techniques failed to show a contaminant, the PLA_2 activity of fractions obtained after CM-Sephadex rechromatography of the toxin evidenced a slight peak displacement when superimposed onto the protein profile. This result would favor the hypothesis of a minor contaminant being responsible for the low PLA_2 activity of the preparation, but the possibility of an intrinsic activity cannot be excluded. Molecular cloning and expression of snake venom Lys-49 myotoxins will be invaluable in clarifying this controversy on their catalytic activity.

The spectrum of pharmacological activities of *B. schlegelii* myotoxin I is similar to that of other basic PLA_2s isolated from *Bothrops* spp. venoms (2) and includes the ability to induce rapid skeletal muscle necrosis at the site of injection, to increase local microvascular permeability (leading to significant edema without hemorrhage), and to cause death when injected by the intravenous route, albeit at relatively high doses.

The morphological characteristics of myonecrosis in-

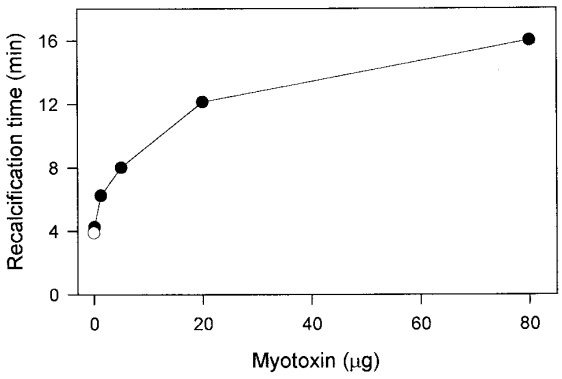

FIG. 6. Anticoagulant activity of *Bothriechis schlegelii* myotoxin I *in vitro*. Sheep platelet-poor plasma was incubated for 10 min at 37°C with different amounts of myotoxin (●), and then the recalcification time was determined after the addition of $CaCl_2$. Recalcification time of the phosphate-buffered saline control is indicated by (○). Results represent means of duplicates, where SD is smaller than the symbol size.

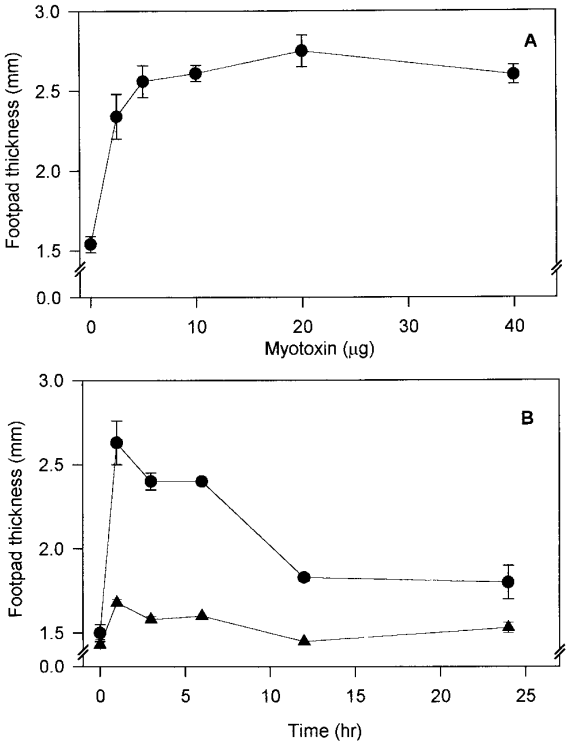

FIG. 7. Edema-forming activity of *Bothriechis schlegelii* myotoxin I in mice. (A) Footpad edema induced by varying doses of myotoxin after 1 h. Results are presented as means ± SD (n = 5). (B) Time course of the footpad edema induced by 25 μg of myotoxin (●) or phosphate-buffered saline control (▲).

duced by this toxin are indistinguishable from those previously described for *B. asper* myotoxin II using intravital microscopy (17), suggesting a common mechanism of action. In addition, the time course of CK release to plasma, as well as the myotoxic potency, as estimated by the CK increase, is similar to other *Bothrops* myotoxins (2). It has been proposed that these myotoxic PLA_2s induce acute muscle cell damage by first affecting the integrity of their plasma membrane, thereby causing hypercontraction and other intracellular alterations leading to cell death (2).

The edema induced by *B. schlegelii* myotoxin I was of rapid onset and was sustained for at least 6 h. Pretreatment of mice with diphenhydramine or indomethacin alone did not modify the course of edema, suggesting that neither histamine nor prostaglandins, individually, play a prominent role in this effect. The strong edema-forming activity of this toxin, in spite of its low PLA_2 activity, suggests that the mechanism of this effect is independent of enzymatic phospholipid hydrolysis. This is in agreement with the observation that *B. asper* myotoxin II, which lacks PLA_2 activity, induces edema (11, 17). Direct effects of *B. asper* myotoxin II on cultured endothelial cells, including increased interleukin-6 production (B. Lomonte, unpublished data) and rapid cytolysis (35), have been described.

It has been proposed that the anticoagulant activity of *Bothrops* myotoxic PLA_2s on platelet-poor plasma depends on their ability to penetrate bilayers and on enzymatic phospholipid degradation (2, 36, 37). In the case of *B. schlegelii* myotoxin I, our present findings support this hypothesis, since it has low anticoagulant and PLA_2 activities.

The molecular basis of the myotoxic action of PLA_2s from snake venoms is still largely unsolved. Further characterization of more natural myotoxin variants, in combination with the study of genetically engineered mutants, will provide valuable clues in this field of research.

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