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Snake Venomics and Antivenomics of the Arboreal Neotropical Pitvipers *Bothriechis lateralis* and *Bothriechis schlegelii*

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We report the comparative proteomic characterization of the venoms of two related neotropical arboreal pitvipers from Costa Rica of the genus *Bothriechis*, *B. lateralis* (side-striped palm pit viper) and *B. schlegelii* (eyelash pit viper). The crude venoms were fractionated by reverse-phase HPLC, followed by analysis of each chromatographic fraction by SDS-PAGE, N-terminal sequencing, MALDI-TOF mass fingerprinting, and collision-induced dissociation tandem mass spectrometry of tryptic peptides. The venom proteomes of *B. lateralis* and *B. schlegelii* comprise similar number of distinct proteins belonging, respectively, to 8 and 7 protein families. The two *Bothriechis* venoms contain bradykinin-potentiating peptides (BPPs), and proteins from the phospholipase A_2 (PLA_2), serine proteinase, L-amino acid oxidase (LAO), cysteine-rich secretory protein (CRISP), and Zn^{2+}-dependent metalloproteinase (SVMP) families, albeit each species exhibit different relative abundances. Each venom also contains unique components, for example, snake venom vascular endothelial growth factor (svVEGF) and C-type lectin-like molecules in *B. lateralis*, and Kazal-type serine proteinase inhibitor-like proteins in *B. schlegelii*. Using a similarity coefficient, we estimate that the similarity of the venom proteins between the two *Bothriechis* taxa may be <10%, indicating a high divergence in their venom compositions, in spite of the fact that both species have evolved to adapt to arboreal habits. The major toxin families of *B. lateralis* and *B. schlegelii* are SVMP (55% of the total venom proteins) and PLA_2 (44%), respectively. Their different venom toxin compositions provide clues for rationalizing the distinct signs of envenomation caused by *B. schlegelii* and *B. lateralis*. An antivenomic study of the immunoreactivity of the Instituto Clodomiro Picado (ICP) polyvalent antivenom toward *Bothriechis* venoms revealed that L-amino acid oxidase and SVMPs represent the major antigenic protein species in both venoms. Our results provide a ground for rationalizing the reported protection of the ICP polyvalent antivenom against the hemorrhagic, coagulant, defibrinating, caseinolytic and fibrin(ogen)olytic activities of *Bothriechis* (*schlegelii*, *lateralis*) venoms. However, these analyses also evidenced the limited recognition capability of the polyvalent antivenom toward a number of *Bothriechis* venom components, predominantly BPPs, svVEGF, Kazal-type inhibitors, some PLA_2 proteins, some serine proteinases, and CRISP molecules.

Keywords: *Bothriechis lateralis* • *Bothriechis schlegelii* • Side-striped palm pit viper • Eyelash pit viper • Snake venom protein families • Proteomics • Viperid toxins • Snake venomics • Antivenomics • N-terminal sequencing • Mass spectrometry • Kazal-type inhibitor • Polyvalent (Crotalinae) antivenom

Introduction

The suborder of snakes (*Serpentes*) of the reptilian order *Squamata*, named for their scaly skin, includes about 3000 extant species placed in approximately 400 genera and 18 families (http://w.reptile-database.org/). The most generalized phylogenetic view is that the group evolved from a family of terrestrial lizards during the time of the dinosaurs in the Jurassic period, about 200 million years (Myr) ago.[1–3] Advanced snakes (*Caenophidia*) arose in the Oligocene epoch 35−25 Myr ago, but remained a small taxon until the tectonic plates drifted apart from the equator and the cool climate pushed boids to disappear from many ecological niches.[4] Nowadays, snakes are present worldwide except in the far north (Alaska and northern Canada in North America, Greenland, Iceland, Ireland, extreme northern Scandinavia and northern Russia in Eurasia), in the far south (Antarctica and New Zealand) and some small oceanic islands,[5] where they have adapted to a diverse array of habitat types, including terrestrial, arboreal, and aquatic ecosystems.

Colubrids, crotalines, and boids have evolved arboreal habits independently.[6] Adaptations to arboreal habits include the development of cryptic color (mainly green or brown), nar-

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rowed skulls, large eyes and a prehensile tail. The prehensile tail is not only used as an anchor when resting, but also when the snake strikes out to grasp its prey, which commonly consists of small birds, rodents, lizards and frogs. Arboreal snakes are "sit-and-wait" predators, which passively wait for prey at strategic hunting sites, likely selected through chemosensory searching.[7] Among predatory strategies, these snakes (especially juveniles) employ what is known as "caudal luring", which consists in wiggling their tail in worm-like motions to encourage potential prey to move within striking range.[7,8] Arboreal snakes which do not present constriction behavior may feed on small prey and are forced to hold onto it until the prey has succumbed to the venom, and then swallowed. Campbell and Solórzano[9] mentioned that arboreal species such as *Bothriechis* spp. were observed to almost invariably seize and hold their prey. The disadvantages of holding dangerous prey may be outweighed by the disadvantages of releasing and being unable to relocate the prey in their arboreal environment.[10]

The Neotropics are abundant in small arboreal pitvipers. Within Neotropical pitvipers, *Bothriechis* represents a monophyletic basal genus[11,12] comprising eight species of relatively slender arboreal rattleless pitvipers.[13,14] *Bothriechis* species are largely nocturnal, consuming small rodents, frogs, lizards, small birds, and occasionally bats. The name *Bothriechis* is derived from the Greek *bothros*, meaning "pit", and *echis*, meaning "viper", in reference to the prominent heat-sensitive pit that lies between the nostril and the eye on either side of the face. With this receptor, snakes can detect and accurately strike a warm-blooded prey in absolute darkness, guided by the infrared radiation that the prey generates.

Bothriechis lateralis (side-striped palm pit viper, also known as "lora" in Costa Rica) is a pitviper species found in the mountains of Costa Rica and western Panama at 850−980 m altitude. Adult specimens may exceed 100 cm, but are usually less than 80 cm in length.[15] Like other fully arboreal snakes, *B. lateralis* is a relatively slender and lightly built snake and has a prehensile tail. Although *B. lateralis* venom seems to be of moderate toxicity, bites may have dire consequences because the snakes' arboreal habits put them in contact with human's hands, arms and face.

Bothriechis schlegelii (eyelash pit viper, also known as "bocaracá" in Costa Rica), a relatively small species that rarely exceed 75 cm in length found in mesic forest at elevations almost from sea level to 2640 m altitude in Central and South America,[15] also causes a number of envenomation accidents in Costa Rica.[16] However, not much is known about *Bothriechis* venoms except that signs and symptoms generally include localized pain, progressive hemorrhagic edema, and, in some cases, hemorrhagic blisters or hives, ecchymoses, and necrosis.[17,18] It was also shown that the venom of *B. schlegelii* induced significant myonecrosis in experimental animal models.[19,20] Gutiérrez and Chaves[20] studied the venoms of 10 Costa Rican species of pitvipers and found that those of *B. schlegelii* (and *Bothrops asper*) had the greatest myonecrotic activity, whereas the venoms of *B. lateralis* (and *Cerrophidion godmani*) showed the strongest proteolytic activity toward casein.

Venoms represent the critical innovation in ophidian evolution that allowed advanced snakes to transition from a mechanical (constriction) to a chemical (venom) means of subduing and digesting large prey, and as such, venom proteins have multiple functions including immobilizing, paralyzing, killing and digesting prey. Given the central role that diet has played in the adaptive radiation of snakes,[21] venom thus represents a key adaptation in the diversification of these animals. On the other hand, the distinct signs and symptoms of envenomation by snakebites of *B. schlegelii* and *B. lateralis* suggest that these two species possess distinct toxin repertoires. This would be in line with previous proteomic[22–29] and transcriptomic[30–40] analyses showing that snake venom proteins belong to only a few major protein families, though venoms from different species depart from each other in the composition and the relative abundance of their toxins. To address this hypothesis, here we report the proteomic characterization of the venoms of *B. lateralis* and *B. schlegelii*.

Treatment of snakebite envenomations is based on the intravenous administration of monovalent or polyvalent antivenoms. Several antivenoms are produced in Latin America using different venoms in the immunization schemes.[41] Each of these antivenoms is effective against envenomations by snake venoms not included in the immunization protocol, demonstrating the high degree of immunological cross reactivity between Central and South American crotaline snake venoms. The polyvalent antivenom manufactured at the Instituto Clodomiro Picado (ICP, University of Costa Rica) using a mixture of three snake (*B. asper*, *Crotalus durissus durissus*, *Lachesis stenophrys*) venoms has been reported to neutralize the lethality and the hemorrhagic, caseinolytic, coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of a number of venoms of Central and South American snakes.[42–44] A second aim of this study was the evaluation of the immunoreactivity of the ICP polyvalent anti-(*Crotalinae*)-venom toward *Bothriechis* venoms, that is, to investigate which specific protein species within *B. lateralis* and *B. schlegelii* venoms are effectively recognized and immunoprecipitated by the ICP polyvalent snake antivenom.

Experimental Section

Isolation of Venom Proteins. Crude venoms of *B. lateralis* and *B. schlegelii* were pooled from specimens collected in Costa Rica and kept at the serpentarium of the Instituto Clodomiro Picado, University of Costa Rica in San José. For reverse-phase HPLC separations, 2−5 mg of crude, lyophilized venom was dissolved in 100 μL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation in an Eppendorff centrifuge at 13 000*g* for 10 min at room temperature. Proteins in the soluble material were separated using an ETTAN LC HPLC system (Amersham Biosciences) and a Lichrosphere RP100 C_{18} column (250 × 4 mm, 5 μm particle size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B) (5% B for 10 min, followed by 5−15% B over 20 min, 15−45% B over 120 min, and 45−70% B over 20 min). Protein detection was at 215 nm and peaks were collected manually and dried in a SpeedVac (Savant). The relative abundances (% of the total venom proteins) of the different protein families in the venoms were estimated from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks.

Characterization of HPLC-Isolated Proteins. Isolated protein fractions were subjected to N-terminal sequence analysis (using a Procise instrument, Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program[45] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de. The mo-

lecular masses of the purified proteins were determined by SDS-PAGE (on 12 or 15% polyacrylamide gels) and by electrospray ionization (ESI) mass spectrometry using an Applied Biosystems QTrap 2000 mass spectrometer[46] operated in Enhanced Multiple Charge mode in the range m/z 600−1700.

In-Gel Enzymatic Digestion and Mass Fingerprinting. Protein bands of interest were excised from a Coomassie Brilliant Blue-stained SDS-PAGE and subjected to automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing grade bovine pancreatic trypsin (Roche) using a ProGest digestor (Genomic Solutions) following the manufacturer's instructions. A total of 0.65 μL of the tryptic peptide mixtures (total volume of $\sim$ 20 μL) was spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (Swiss-Prot accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450−3300 Da).

Collision-Induced Dissociation Tandem Mass Spectrometry (CID-MS/MS). For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization (ESI) mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems)[46] equipped with a nanospray source (Protana, Denmark). Doubly- or triply charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q_0 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1, unit resolution; Q1-to-Q2 collision energy, 30−40 eV; Q3 entry barrier, 8 V; LIT (linear ion trap) Q3 fill time, 250 ms; and Q3 scan rate, 1000 amu/s. CID spectra were interpreted manually or using a licensed version of the MASCOT program (http://www.matrixscience.com) against a private database containing 927 viperid protein sequences deposited in the Swiss-Prot/TrEMBL database (Knowledgebase Release 12 of July 2007; http://us.expasy.org/sprot/; 212 in Swiss-Prot, 715 in TrEMBL) plus the previously assigned peptide ion sequences from snake venomics projects carried out in our laboratory.[22–25,27,28] MS/MS mass tolerance was set to ±0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively.

Variation in Venom Composition between *Bothriechis* Taxa. We used similarity coefficients to estimate the similarity of venom proteins between taxa. These coefficients are similar to the bandsharing coefficients used to compare individual genetic profiles based on multilocus DNA fingerprints.[47] We defined the Protein Similarity Coefficient (PSC) between two species "a" and "b" in the following way: PSC_{ab} = [2(no. of proteins shared between a and b)/(total number of distinct proteins in a + total number of distinct proteins in b)] × 100. We judged two proteins (listed in Tables 1–3) as being different when they met one or more of these criteria: (1) had different N-terminal sequences and/or distinct internal peptides sequences (derived from MS/MS data) corresponding to homologous regions; 2) had different peptide mass fingerprints; 3) were of different sizes (judged by MALDI-TOF MS or SDS-PAGE) (For these comparisons, two proteins were judged to differ in size if they differed by more than our estimate of the 95% confidence interval for particular sizing techniques (0.01% for ESI-QTrap MS; 0.4% for MALDI-TOF MS derived masses, and + 1.4 kDa for SDS-PAGE-determined masses)); or (4) eluted in different reverse-phase HPLC peaks.

Antivenomics. We have coined the term "antivenomics" for the identification of venom proteins bearing epitopes recognized by an antivenom using proteomic techniques. Polyvalent (Crotalinae) antivenom (batch 3980506LQ, protein concentration of 55 mg/mL; expiry date: May 2009) was produced at ICP by immunizing horses with a mixture of equal amounts of the venoms of *B. asper*, *C. durissus durissus*, and *L. stenophrys* obtained from adult specimens kept in captivity at the ICP serpentarium.[48] Whole immunoglobulins were purified by caprylic acid precipitation.[49] Two milligrams of whole venom was dissolved in 70 μL of 20 mM phosphate buffer, pH 7.0, mixed with 4 mg of purified polyvalent antivenom IgGs, and incubated with gentle stirring for 1 h at 37 °C. Thereafter, 6 mg of rabbit anti-horse IgG antiserum (Sigma) in 350 μL of 20 mM phosphate buffer, pH 7.0, was added, and the mixture was incubated for another 1 h at 37 °C. Immunocomplexes were precipitated by centrifugation at 13 000 rpm for 30 min in an Eppendorf centrifuge and the supernatant was submitted to reverse-phase separation as described for the isolation of venom proteins. HPLC-fractions were characterized as described above. The control sample was subjected to the same procedure except that antivenom IgGs were not included in the reaction mixture.

Western Blot. The occurrence within the Polyvalent (Crotalinae) antivenom produced at ICP of antibodies directed against antigenic determinants exhibited by *B. schlegelii* and *B. lateralis* venom proteins, which may or may not be immunoprecipitated by the antivenomics approach described above, was investigated by Western blot analysis. To this end, the reverse-phase HPLC chromatographic fractions were electrophoresed in SDS-(10%)polyacrylamide gels under nonreduced conditions followed by electrotransfer to nitrocellulose membranes as described[50] using a Bio-Rad minitransfer cell operated at 150 mA during 90 min. To assess transfer efficiency, the nitrocellulose membranes were previsualized by reversible Ponceau-S Red stain. Then, the membranes were incubated in 1% bovine serum albumin in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) for 30 min at room temperature to block reactive sites, and the membranes were subsequently incubated overnight at 4 °C with either ICP polyvalent antivenom or normal equine serum, diluted 1:1000 (for *B. schlegelii*) or 1:2000 (for *B. lateralis*) in PBS. After washing four times with PBS containing 0.1% albumin and 0.05% Tween-20, the membranes were incubated with an anti-horse IgG−alkaline phospatase conjugate (Sigma) diluted 1:1000 (for *B. schlegelii*) or 1:2000 (for *B. lateralis*), during 2 h at room temperature. Finally, membranes were washed four times as above, and color development was performed by adding BCIP/NBT (Chemicon) substrate.

Results And Discussion

Characterization of the Venom Proteomes of *B. lateralis* and *B. schlegelii*. The protein composition of the venoms of *B. lateralis* and *B. schlegelii* was investigated using our snake venomics approach,[51] including fractionation of the crude venoms by reverse-phase HPLC (Figures 1 and 2), analysis of each chromatographic fraction by SDS-PAGE (Figures 3 and 4), N-terminal sequencing, and tryptic peptide MALDI-TOF

Table 1. Assignment of the reverse-phase fractions of *Bothriechis lateralis* venom, isolated as in Fig. 1, to protein families by N-terminal Edman sequencing, mass spectrometry, and collision-induced fragmentation by nESI-MS/MS of selected peptide ions from in-gel digested protein bands (separated by SDS-PAGE as in Fig. 2)[a]

HPLC fraction Bl-	N-terminal sequence	molecular mass	peptide ion *m/z*	peptide ion z	MS/MS-derived sequence	protein family
1	N.D.		538.8	2	NPPAGPDVGPR	Bradykinin-potentiating peptide
2	N.D.		472.0	2	$[NPPAGPDVGP]^{Na+}$	Bradykinin-potentiating peptide
3, 4	n.p.					
5	N.D.		855.8	2	(212.2)PGNPPAGPDVGPRXR	Bradykinin-potentiating peptide
6	N.D.		352.7	2	PPPISPP	Bradykinin-potentiating peptide
7	N.D.		621.8	2	ZKWDPPPISPP	Bradykinin-potentiating peptide
8	N.D.	24■/13▼ kDa	685.1	2	ZVMPFMEVYSR	svVEGF
9	SLLQFREMIKEETGK	14037				PLA_2
10	SLLQFREMITKMTGK	13926				PLA_2
11	VIGGDECNINEHR	48 kDa■/▼	616.6	2	KVNDEDEQTR	Serine proteinase
			522.6	2	VVYPENVPK	
			756.8	2	VIGGDECNINEHR	
12	SVDFDSESPRKPEIQ	24586			CRISP	
13	IIGGDECNINEHRSL	38 kDa■/▼				Serine proteinase
14	IIGGDECNINEHRFL	38 kDa■/▼				Serine proteinase
15	IIGGDECNINEHRSL	38 kDa■/▼				Serine proteinase
16	IIGGDECNINEHRSL	26 kDa■/▼	538.6	2	SPDEDEQTR	Serine proteinase
17	IIGGDECNINEHRSL	27 kDa■/▼	538.6	2	SPDEDEQTR	Serine proteinase
18	(V/I)(V/I)IGGDECNINEHR(S/F)L	27 kDa■/▼				Serine proteinase
19	VIGGDECNINEHRFL	27.5 kDa■/▼				Serine proteinase
20	VIGGDECNINEHRFL	26 kDa■/▼				Serine proteinase
21	Heterogeneous	26■/13−16▼ kDa	718.8	2	(229)QGXEQDTNYXK	C-type lectin-like
22	Heterogeneous	56 kDa■/▼	758.3	2	ETDYEEFXEXAR	L-amino acid oxidase
			583.1	2	KFWEDDGXR	
		26■/13−16▼ kDa	640.6	3	DCPSDWSSYEGHCYR	C-type lectin-like
			718.8	2	(229)QGXEQDTNYXK	
		26 kDa■/▼	756.9	2	VIGGDECNINEHRFL	Serine proteinase
23, 24	DDPRNPLEECFRETD	56 kDa■/▼	758.3	2	ETDYEEFXEXAR	L-amino acid oxidase
			583.1	2	KFWEDDGXR	
25	N.D.	56 kDa■/▼	758.3	2	ETDYEEFXEXAR	L-amino acid oxidase
		46 kDa■/▼	657.8	2	YXEXAXXADHR	PIII-metalloproteinase
		38 kDa■/▼	647.2	2	XNXXDYEVCR	Serine proteinase
		26■/13−16▼ kDa	640.6	3	DCPSDWSSYEGHCYR	C-type lectin-like
			718.8	2	(229)QGXEQDTNYXK	
		26 kDa■/▼	756.9	2	VIGGDECNINEHRFL	Serine proteinase
26	Blocked	52 kDa■/▼	579.1	2	KIPCAPEDVK	PIII-metalloproteinase
			670.2	2	YVEXFVXVVDQR	
			526.9	2	GNYYGYCR	
27	Blocked	96 kDa■/48▼kDa	715.2	2	XAXVDNEXWSNR	$(PIII\text{-metalloproteinase})_2$
			657.8	2	YXEXAXXADHR	
		23 kDa■/▼	555.9	2	TXDSFGEWR	PI-metalloproteinase
28	Blocked	52 kDa■/▼	502.1	2	GKGDFYCR	PIII-metalloproteinase
			641.2	2	XYDDNTQPCR	
		46 kDa■/▼	657.8	2	YXEXAXXADHR	PIII-metalloproteinase
			673.3	3	(451.2)ECESGDCCDQCR	
		28 kDa■	555.9	2	TXDSFGEWR	PI-metalloproteinase
		26 kDa■/▼	555.9	2	TXDSFGEWR	PI-metalloproteinase
29	Blocked	23 kDa■/▼	555.9	2	TXDSFGEWR	PI-metalloproteinase
30	Blocked	96 kDa■/48▼kDa	715.2	2	XAXVDNEXWSNR	$(PIII\text{-metalloproteinase})_2$
			657.8	2	YXEXAXXADHR	
30, 31	Blocked	42 kDa■/▼	664.7	2	YIELVIVADHR	PIII-metalloproteinase
			673.3	3	(451.2)ECESGDCCDQCR	

[a] X, Ile or Leu; Z, pyrrolidone carboxylic acid. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated. Molecular masses were determined by electrospray-ionization (±0.02%) or MALDI-TOF (*) (±0.2%) mass spectrometry. Apparent molecular mass determined by SDS-PAGE of non-reduced (■) and reduced (▼) samples; n.p., nonpeptidic material found. M and m, denote mayor and minor products within the same HPLC fraction.

mass fingerprinting (Tables 1 and 2). Protein fractions showing single electrophoretic band, molecular mass, and N-terminal sequence were straightforwardly assigned by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) to a previously reported

Table 2. Assignment of the Reverse-Phase Fractions of *B. schlegelii* Venom, Isolated as in Figure 2, to Protein Families by N-Terminal Edman Sequencing, Mass Spectrometry, and Collision-Induced Fragmentation by nESI-MS/MS of Selected Peptide Ions from in-Gel Digested Protein Bands (separated by SDS-PAGE as in Figure 2)[a]

			peptide ion			
HPLC fraction Bs-	N-terminal sequence	molecular mass	*m/z*	*z*	MS/MS-derived sequence	protein family
1	N.D.		549.1	2	ZKKWPPGHH	Bradykinin-potentiating peptide
2	N.D.		665.1	2	APAAPHRLPK	Bradykinin-potentiating peptide
3	N.D.		608.1	2	TPPAGPDVGPR(171.2)	Bradykinin-potentiating peptide
4, 5	n.p.					
6	N.D.		598.1	2	ZWSHKGWPPRPQIPP	Bradykinin-potentiating peptide
7	N.D.		702.6	2	ZKKWPPGHHIPP	Bradykinin-potentiating peptide
8	N.D.		573.2	2	(351.3)EGNPDAPP	Unknown
9	N.D.		622.6	2	ZKWDPPISPP	Bradykinin-potentiating peptide
10	MLVNCLGYPTLFCFGKPLPHCASDGKKYPNRCRFCF	6170				Kazal-type serine proteinase inhibitor
11	EEEKIPVDCTGFPKKACTLEYEPHCASD	6673				Kazal-type serine proteinase inhibitor
	EKIPVDCTGFPKKACTLEYEPHCASDGNTYPNR	6415				Kazal-type serine proteinase inhibitor
	GDGCFGLKLDRIGSMSGLGC●	1983.8				C-type natriuretic peptide
12, 13	EEEKIPVDCTGFPKKACTLEY	6543				Kazal-type serine proteinase inhibitor
	EKIPVDCTGFPKKACTLEYEP	6283				Kazal-type serine proteinase inhibitor
14	SMYELGKMILLETGK	13554	574.2	2	LTGCNPLTDR	PLA_2
15	N.D.	14 kDa■/▼	574.2	2	LTGCNPLTDR	PLA_2
16	SMYELGKMILLETGK	18 kDa■/▼	574.2	2	LTGCNPLTDR	PLA_2 myotoxin II [P80963]
			709.6	2	TIVCGENKPCLK	
			917.6	2	NAATSYIAYGCNCGVGR	
		13551	574.2	2	LTGCNPLTDR	PLA_2 myotoxin II [P80963]
			709.6	2	TIVCGENKPCLK	
			917.6	2	NAATSYIAYGCNCGVGR	
17	DLLQFREMIKKMTGKEPVVSYA	13620				PLA_2
18	NLLQFNKMIKIMTRK	13787	581.8	2	TDIYSYSWK	N6-PLA_2 [AY355168]
			735.7	2	VAAVCFGANLGTYK	
			753.6	2	SGVIICGEGTPCEK	
			789.1	2	CCFVHDCCYEK	
			671.8	3	SYMFYPDFLCTEPSEK	
			1014.8	3	NGIPYYSSYGCYCGWGGQGGPLDATDR	
19	HLMQFEGMIMRIAGR	18 kDa■/▼	735.7	2	VAAVCFGANLGTYK	PLA_2
			753.6	2	SGVIICGEGTPCEK	
			789.1	2	CCFVHDCCYEK	
			671.8	3	SYMFYPDFLCTEPSEK	
		13798	917.6	2	NAATSYIAYGCNCGVGRH	PLA_2
20	DLLQFREMIMKMTGKEPAISYAFYGCFCGLGGHGKPKDATD	13545				PLA_2
21	HLMQFEGMIMTIAGRSGIWYYG	18 kDa■/▼	487.3	2	WYFYPAK	
			548.6	2	DNXDTYDNK	PLA_2(~AAW92117)
			752.8	2	CCFVHDCCYGK	
			867.6	2	HLMQFEGMIMTIAGR	
			885.9	2	EVCECDKNAAXCFR	
		13748	917.6	2	NAATSYIAYGCNCGVGRH	PLA_2
22	SVDFDSESPRKPEIQ	24610	569.1	2	SVDFDSESPR	CRISP
			597.9	2	EXVDXHNSXR	
			768.9	2	MEWYPEAAANAER	
23	VVGGDECNINEHRSLVVLF	33 kDa■/▼	749.7	2	VVGGDECNINEHR	Serine proteinase

Table 2. Continued

HPLC fraction Bs-	N-terminal sequence	molecular mass	peptide ion *m/z*	peptide ion *z*	MS/MS-derived sequence	protein family
			634.8	2	XNXXDHEVCR	
24	IIGGDECNINEHRFL	38, 33 kDa▼	634.8	2	XNXXDHEVCR	Serine proteinase
	DLLQFEGMIMTIAGR	13698	885.9	2	EVCECDKNAAICFR	PLA_2 [AAW92117]
			847.9	2	DLLQFEGMIMTIAGR	
			487.3	2	YWFYPAK	
			601.2	2	YCQEESEPC	
			752.8	2	CCFVHDCCYGK	
		12 kDa■/▼	917.6	2	NAATSYIAYGCNCGVGRH	PLA_2
25, 26	VIGGDECNINEHRSL	32 kDa■/▼	756.6	2	VIGGDECNINEHR	Serine proteinase
27	VVGGDECNINEHRFL	32 kDa■/▼	749.8	2	VVGGDECNINEHR	Serine proteinase
			714.8	2	SLPSSPPSVGSVCR	
			601.8	2	XMGWGSXTPXK	
28	VIGGDECNINEHRSL	32 kDa■/▼	756.6	2	VIGGDECNINEHR	Serine proteinase
29	DDRNPLEECFQETDYE	48 kDa■/▼	647.2	2	EGWYANXGPMR	L-amino acid oxidase
			626.2	2	SAGQXYQESXR	
30	V(I/F)GGDECNINEHRFL	32 kDa■/▼				Serine proteinase
	TPPQPHQRYVE	>110 kDa■/▼				$(PIII\text{-metalloproteinase})_n$
31	Blocked	98 kDa■/48 kDa▼	655.8	2	XYCFPNSPANK	$(PIII\text{-metalloproteinase})_2$
32	Blocked	58 kDa■/▼	572.2	2	XYCFPNSDK	PIII-metalloproteinase
			657.3	2	(244.2)YQTYXXNR	
33	Blocked	52 kDa■/▼	500.9	2	GKGNFYCR	PIII-metalloproteinase
			686.8	2	YXEDFXFTXGR	
34	N.D.	23 kDa■/▼	663.8	2	YXEXVXVADHR	PI-metalloproteinase
		14 kDa■/▼	917.3	2	NAATSYIAYGCNCGVGRH	PLA_2

[a] X, Ile or Leu; Z, pyrrolidone carboxylic acid. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated. Molecular masses were determined by electrospray-ionization (±0.02%) or MALDI-TOF (*) (±0.2%) mass spectrometry. Apparent molecular mass determined by SDS-PAGE of non-reduced (■) and reduced (▼) samples; n.p., nonpeptidic material found. M and m, denote mayor and minor products within the same HPLC fraction; ●, contains an intramolecular disulphide bond.

Table 3. Overview of the Relative Occurrence of Proteins (in Percentage of the Total HPLC-Separated Proteins) of the Different Families in the Venoms of *B. lateralis* and *B. schlegelii*

	% of total venom proteins	
protein family	*B. lateralis*	*B. schlegelii*
Bradykinin-potentiating peptides	11.1	13.4
svVEGF	0.5	–
Kazal-type inhibitors	–	8.3
PLA_2	8.7	43.8
CRISP	6.5	2.1
Serine proteinases	11.3	5.8
L-amino acid oxidase	6.1	8.9
C-type lectin-like	0.9	–
Zn^{2+}-metalloproteinases	55.1	17.7

protein or to a known protein family. Thus, the bradykinin-potentiating peptides found in fractions Bs1−3, Bs6−7, Bs8 and Bl6−7, and the C-type natriuretic peptide in Bs11 (Tables 2 and 3) are identical to peptides from *Lachesis muta* Q27J49 identified by MALDI-TOF MS in the crude venom of a specimen kept in captivity at the serpentarium of the Fundação Ezequiel Dias (Belo Horizonte, Brazil).[52] In addition, *B. schlegelii* PLA_2 molecules isolated in fractions Bs16, Bs18, Bs21/Bs24 (Table 2) may correspond to Lys-49 myotoxin II [P80963, Mcalc: 13534 Da],[53,54] the basic N6-PLA2 [AY355168, Mcalc: 13786 Da],[55] and the acidic PLA_2 [AAW92117, Mcalc: 13699 Da],[56] respectively. These three PLA_2 toxins represent the only protein sequences from any *Bothriechis* species reported to date in the literature.

Fractions showing heterogeneous or a blocked N-terminal residue were analyzed by SDS-PAGE and the bands of interest were subjected to automated reduction, carbamidomethylation, and in-gel tryptic digestion followed by sequencing of selected doubly- and triply charged tryptic peptide ions by collision-induced dissociation tandem mass spectrometry (Tables 1 and 2). As expected from the rapid amino acid sequence divergence of venom proteins evolving under accelerated evolution,[57−62] with a few exceptions, the

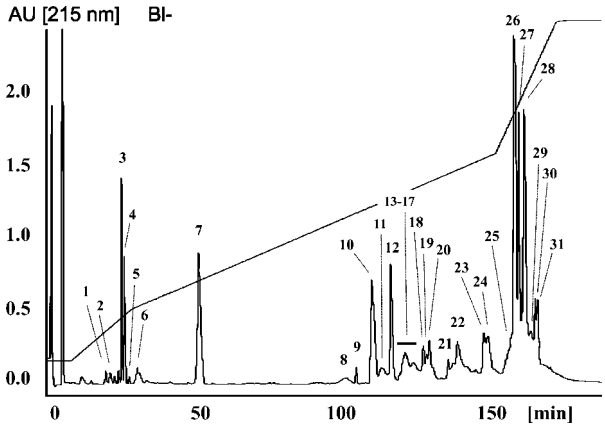

Figure 1. Reverse-phase HPLC separation of the venom proteins from *B. lateralis*. Two milligrams of *B. lateralis* venom was applied to a Lichrosphere RP100 C_{18} column, which was then developed with the following chromatographic conditions: isocratically (5% B) for 10 min, followed by 5−15% B for 20 min, 15−45% B for 120 min, and 45−70% B for 20 min. Fractions were collected manually and characterized by N-terminal sequencing, ESI mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly- or triply charged peptide ions. The results are shown in Table 1.

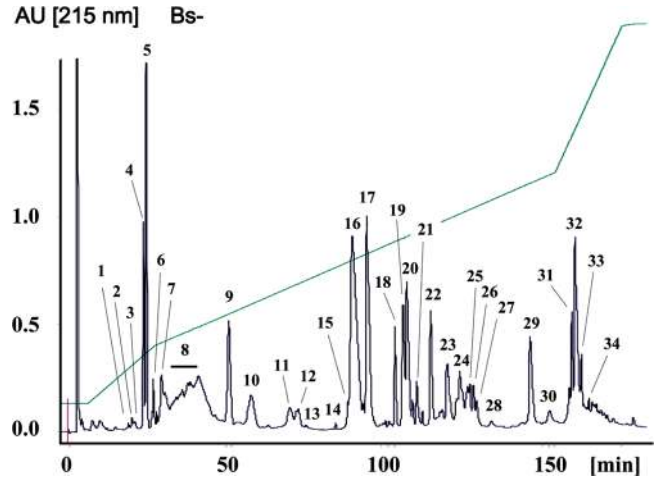

Figure 2. Reverse-phase HPLC separation of the venom proteins from *B. schlegelii*. Two milligrams of *B. schlegelii* venom was applied to a Lichrosphere RP100 C_{18} column, which was then developed as in Figure 1. Fractions were collected manually and characterized by N-terminal sequencing, ESI mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly- or triply charged peptide−ions. The results are shown in Table 2.

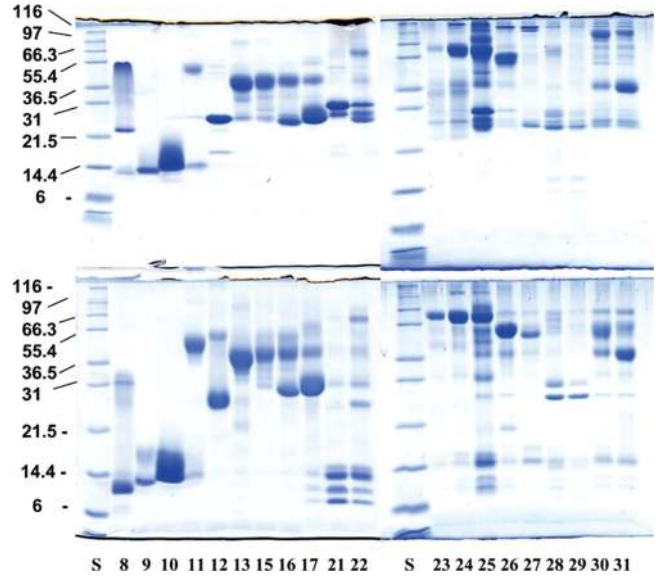

Figure 3. SDS-PAGE of reverse-phase separated fractions from the venom of *B. lateralis*. SDS-PAGE showing the protein composition of the reverse-phase HPLC separated venom protein fractions run under nonreduced (top panels) and reduced (bottom panels) conditions. Molecular mass markers (S, in kDa) are indicated at the left of each gel. Track numbering correspond to HPLC fraction numbering shown in Figure 1. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS. The results are shown in Table 1.

product ion spectra did not match any known protein using the ProteinProspector (http://prospector.ucsf.edu) or the MASCOT (http://www.matrixscience.com) search programs. Although the lack of any complete snake genome sequence is a serious drawback for the identification of venom proteins, high-quality MS/MS production spectra usually yielded sufficient *de novo* amino acid sequence information derived from almost complete series of sequence-specific b- and/or y-ions to unambiguously identify a homologue protein in the current databases by BLAST analysis. The

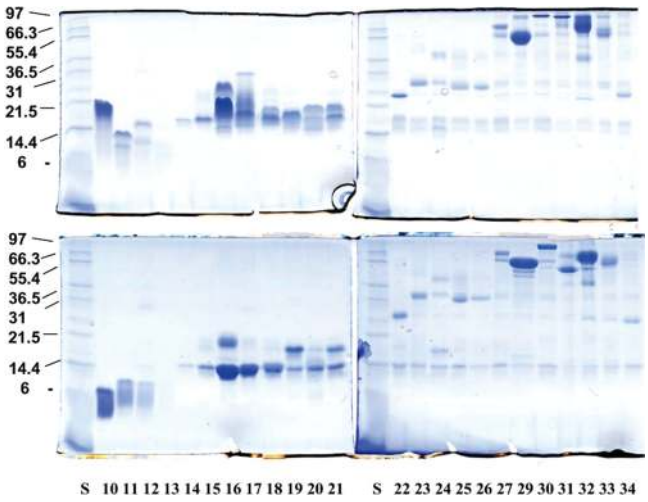

Figure 4. SDS-PAGE of reverse-phase separated fractions from the venom of *B. schlegelii*. SDS-PAGE showing the protein composition of the reverse-phase HPLC separated venom protein fractions run under nonreduced (top panel) and reduced (bottom panel) conditions. Molecular mass markers (S, in kDa) are indicated at the left of each gel. Track numbering correspond to HPLC fraction numbering shown in Figure 2. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS. The results are shown in Table 2.

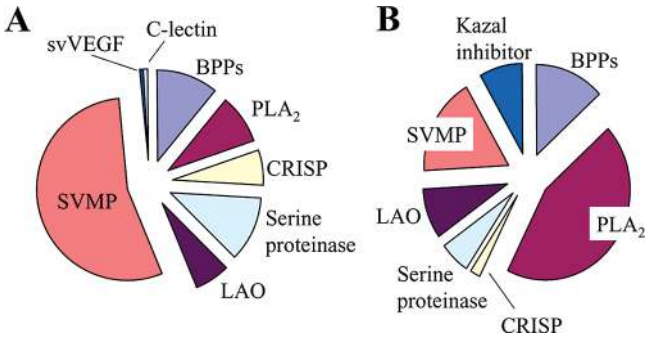

Figure 5. Overall protein composition of *Bothriechis* venoms. Comparison of the protein composition of the venoms of *B. lateralis*(A) and *B. schlegelii*(B). BPP, bradykinin-potentiating; PLA_2, phospholipase A_2; CRISP, cysteine-rich secretory protein; C-lectin, C-type lectin-like protein; SVMP, snake venom metalloproteinase; LAO, L-amino acid oxidase; svVEGF, snake venom vascular endothelial growth factor.

outlined snake venomics approach[51] allowed us to assign unambiguously to known protein families all the isolated venom toxins representing more than 0.05% (w/w) of the total venom proteins (Tables 1 and 2). In line with an array of previous snake venomics studies,[22–29] the proteins found in the venoms of *B. lateralis* and *B. schlegelii* belong to only a few families which are listed in Table 3. The venom proteomes of *B. lateralis* and *B. schlegelii* comprise similar number of distinct proteins belonging, respectively, to 8 and 7 types of toxins. The two *Bothriechis* venoms share bradykinin-potentiating peptides, and proteins from the PLA_2, serine proteinase, L-amino acid oxidase, CRISP, and Zn^{2+}-dependent metalloproteinase families, albeit each species exhibits distinct relative abundances (Figure 5, Table 3). On the other hand, each venom also contains unique components, for example, svVEGF and C-type lectin-like molecules in *B. lateralis*, and Kazal-type serine proteinase inhibitor-like proteins in *B. schlegelii*.

B. schlegelii represents the sister taxon to all other *Bothriechis* species, whereas *B. lateralis* segregates with *Bothriechis bicolor* and *Bothriechis marchi* in the most recently isolated clade from a common ancestor.[63] Comparison of their venom proteomes shows that the two *Bothriechis* species sampled have diverged to a point where only few components (BPP Bl7 and Bs9; CRISP Bl12 and Bs22; and PIII-SVMP Bl28 and Bs33) display identical, or highly similar, structural features, such as N-terminal sequence, molecular mass, and peptide mass fingerprint. Using a similarity coefficient, we estimate that the similarity of the venom proteins between these two *Bothriechis* taxa may be <10%. This measure may give only a maximum estimate of the similarity between the two venoms, since some of the proteins that we judge to be highly similar would be found to differ at one or more of the evaluation criteria if more complete information were available. Furthermore, the large divergence between the two *Bothriechis* species is also highlighted in Figure 5. The major toxin families of *B. lateralis* and *B. schlegelii* are SVMP (55% of the total venom proteins) and PLA_2 (44%), respectively. The high degree of differentiation in the venom proteome among congeneric taxa emphasizes unique aspects of venom composition of related species of *Bothriechis* snakes and points to a strong role for adaptive diversification via natural selection as a cause of this distinctiveness, whose possible physiological significance is discussed below.

A Novel Type of Venom Protein in *B. schlegelii*. N-terminal sequence of the polypeptides isolated in fractions Bs10−13 display 57−75% similarity (48−62% identity) with the N-terminal region of ovomucoid third domains from a large number of avian species,[64–66] including the absolute conservation of cysteine residues and the spacing between them. Structurally, these protein domains belong to the Kazal-type proteinase inhibitor fold (family PD000417 in the ProDom protein domain family database, http://prodom.prabi.fr/prodom/current/html/home.php). However, though a number of Kazal-like domains harbor serine proteinase inhibitor activity, these protein scaffolds are also present in the extracellular part of a number of proteins, which are not known to be proteinase inhibitors. Clearly, experimental evidence is needed to assess the biological activity of the Kazal-type proteins in this venom.

Noteworthy, Kazal-type inhibitor-like proteins have not been previously found in any snake venom reported to date. The fact that Kazal-type inhibitors are not represented among the restricted set of protein families that appear to have been recruited into the venom proteome in an early stage of the evolution of the venom systems in lizards and snakes[67–70] indicates either that the repertoire of ancestral toxin families is still incomplete or that recruitment of Kazal-type proteins represents a more recent event in ophidian radiation. Multigene phylogenetic reconstructions strongly support monophyly of New Word crotalines.[71,72] The diversity of New World pitvipers arose over a period of 15−30 million years from a single invasion of an Old World Eurasian species of the Gloydius-Ovophis clade, which probably occurred during the early Tertiary or late Cretaceous via the Bering Land Bridge.[72] All phylogenies indicate an early cladogenetic event splitting New Word pitvipers into temperate (*Agkistrodon*, *Crotalus*, and *Sistrurus*) and Neotropical (bothropoid genera and *Lachesis*) groups.[13] The lack of evidence for the occurrence of Kazal-type proteins (or messengers) in the venom proteomes and transcriptomes of species of genera *Agkistrodon*,[34,35] *Sistrurus*,[22,24] *Bothrops*,[26,31–33] and *Lachesis*,[37] would support the hypothesis

of the recruitment event of these proteins along the speciation of the Neotropical pitviper clade. Whether the occurrence of Kazal-type proteins in *B. schlegelii* venom represents a genus-specific recruitment event or are more widely distributed in other snake clades deserves further investigation. On the other hand, the presence of Kazal-type proteins in the venom of the basal species, *B. schlegelii*, could indicate a species-specific expression of this class of proteins, which may have played a role in the early adaptive radiation of *Bothriechis* snakes, loosing this function along the speciation of *B. lateralis*.

Clues for Understanding the Envenomation Profiles of *B. lateralis* and *B. schlegelii*. Documentation of human accidents by *Bothriechis* snakebites is scarce. Indeed, we were unable to find any report on the pathophysiology of human envenomation by *B. lateralis*. On the other hand, initial symptoms of *B. schlegelii* snakebite include localized pain, progressive hemorrhagic edema, and, in some cases, hemorrhagic blisters or hives, ecchymoses, and necrosis.[17,18,73] In mice, the venom of *B. schlegelii* shows a higher toxicity than that of *B. lateralis* (intravenous LD_{50} of 2 mg/kg for the former and 4.8 mg/kg for the latter).[74] Compared to a number of *Bothrops* and *Bothriopsis* venoms, *B. schlegelii* venom exhibited higher LAO activity.[75] This observation can now be rationalized by the high L-amino acid oxidase content of *B. schlegelii* venom (Table 3). Other studies have shown that *B. schlegelii* venom inflicts significant myonecrosis in experimental animal models.[19,20] Rabbits injected with *B. lateralis* venom developed a conspicuous edema and hemorrhage in the muscle where venom was administered, but no histological evidence of myonecrosis was found.[76] These distinct signs of envenomation caused by *B. schlegelii* and *B. lateralis* venoms might be explained by the large amounts of PLA_2 in the former and the high SVMP content of the latter (Table 3). Myotoxic PLA_2 molecules account for most of the muscle necrosis that results from envenomation by crotaline snakes.[77,78] On the other hand, SVMPs display a wide range of biological activities, including hemorrhagic, fibrin(ogen)olytic, degradation of extracellular matrix components, and activation of prothrombin and Factor X, resulting in extensive local tissue damage and local and systemic hemorrhage.[79,80]

Severe envenomation by *B. asper*, *Bothrops punctatus*, *Porthidium nasutum*, and *B. schlegelii* snakebites in Colombia were characterized by local necrosis, local and systemic bleeding, and/or hypotension or renal failure.[81] The toxin composition of the venoms of *B. asper* and *P. nasutum* have not been reported. However, preliminary results from our laboratories indicate that these venoms, like the *B. schlegelii* venom, contain a large amount of bradykinin-potentiating peptides (BPPs, Table 3). BPPs have been described as snake venom inhibitors of angiotensin-converting enzyme, a dipeptidylcarboxypeptidase expressed in endothelial, epithelial and neuroepithelial cells, which converts inactive angiotensin I into the potent vasoconstrictor angiotensin II, and degrades bradykinin into bradykinin (1−7) or bradykinin (1−5).[82] BPPs prevent the hypertensive effect of the angiotensin II and potentiate the hypotensive effect of the circulating bradykinin. C-natriuretic peptides elicit natriuretic, diuretic, and vasorelaxant activities. Vasodilatation and hypotension contribute synergistically to overall venom toxicity evoking the rapid diffusion of toxic substances in the circulatory system and a hypotensive shock, which is a major cause of death of the prey or victim of viper snakebites. Rapid immobilization due to BPP-induced hypotension and other mechanisms may represent an adaptation

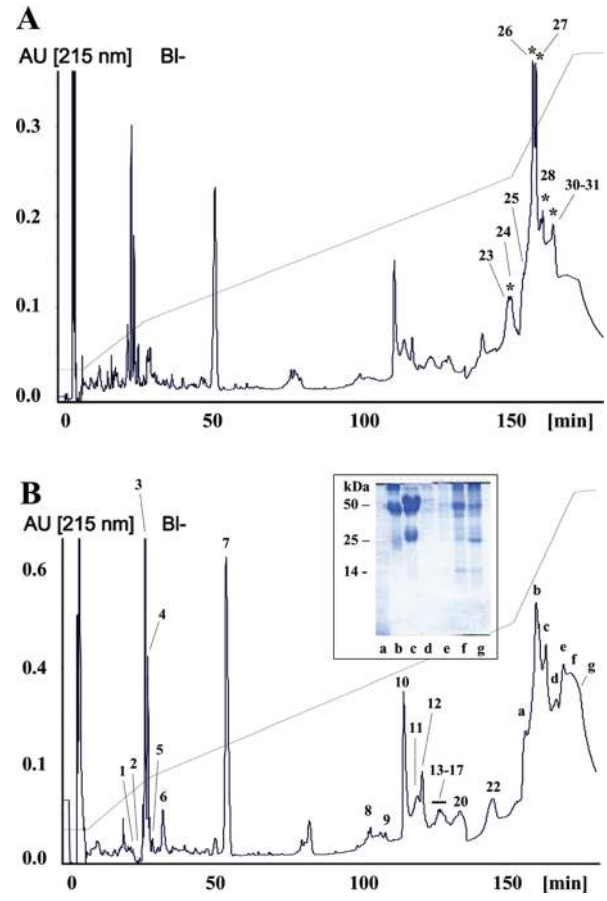

Figure 6. Immunodepletion of *B. lateralis* venom proteins by the polyvalent antivenom produced at Instituto Clodomiro Picado (ICP). Panels A and B show, respectively, reverse-phase separations of soluble *B. lateralis* venom proteins recovered after incubation of the venom with rabbit anti-horse IgG antiserum (control sample), and with polyvalent (*Crotalinae*) antivenom followed by rabbit anti-horse IgG antiserum and immunoprecipitation. HPLC fractions labelled with asterisks in panel A were quantitatively immunodepleted from the venom sample incubated with the polyvalent antivenom, whereas fractions labelled in panel B with numbers (following the same numbering as in Figure 1) were not affected at all (1−12, 20), or were only partially recognized (13−17, 22), by the antivenom (see Figure 7). The insert in panel B shows an SDS-PAGE analysis of β-mercaptoethanol-reduced fractions a−g. CID-MS/MS of tryptic peptide ions from these fractions (601.7^{2+}: LSVPTSEWQR; 545.6^{2+}: VVSTLPIAHQDWLR; 621.6^{3+}: TTPAVLDSDGSYFLYSK) identified them as rabbit IgGs.

of *Bothriechis* species, and possibly other arboreal "sit-and-wait" predators for outweighting the threat of holding large, dangerous prey, and for restraining it from escaping during the swallowing process.

Antivenomics: *Bothriechis* Venom Components Immunodepleted by the Polyvalent ICP Antivenom. Panel B of Figure 6 displays a reverse-phase separation of *B. lateralis* venom proteins recovered in the soluble fraction after incubation of the venom with polyvalent (*Crotalinae*) antivenom followed by immunoprecipitation with rabbit anti-horse IgG antiserum. In parallel, the immunoreactivity of the reverse-phase separated fractions from a nontreated venom sample or a control sample (venom incubated with PBS followed by rabbit anti-horse IgG antiserum and centrifugation), shown in panel A of Figure 6, was assessed by Western blotting (Figure 7). Both approaches

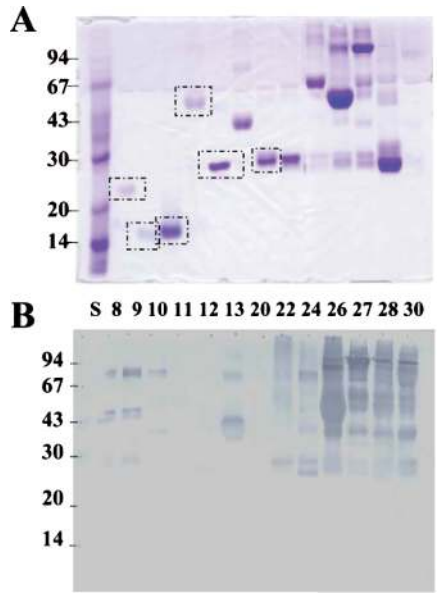

Figure 7. Western blot analysis of the reactivity of polyvalent antivenom with *B. lateralis* venom proteins. Venom proteins separated by reverse-phase HPLC as in Figure 6 were electrophoresed in SDS-(10%)polyacrylamide gels under nonreduced conditions (A), electrotransferred to nitrocellulose membranes, and probed with the ICP polyvalent antivenom (B). Protein bands 8−12, 20 not recognized by the antiserum are highlighted in panel A.

yielded essentially the same information: fractions 1−12 and 20 were not recognized by antivenom antibodies; fractions 13−22, although displaying immunoreactivity toward the antivenom, were only partially immunodepleted from the venom; and fractions 23−31 were quantitatively immunodepleted from the venom and show strong immunoreactivity by Western blotting.

L-Amino acid oxidase and the different PI- and PIII SVNPs represent the major immunodominant proteins in *B. lateralis* venom, whereas serine proteinases and C-type lectin-like molecules were only partially immunoprecipitated from the venom. Together, these proteins represent about 73% (SVMPs + LAO, 61%) of the total *B. lateralis* venom components. On the other hand, components not recognized by the antivenom comprise all the bradykinin-potentiating peptides, the single svVEGF and CRISP proteins, and the two PLA_2 molecules found in *B. lateralis* venom, as well as a 48 kDa serine proteinase (Table 1). Together, these components represent about 27% of the total venom proteins (Table 3).

Similar results were obtained with the venom of *B. schlegelii* (Figure 8). In Western blots, the polyvalent antivenom recognized, although very weakly, proteins Bs 13−17 (PLA_2 molecules) and with higher avidity Bs 29−34 (LAO and SVMPs). The latter toxins were quantitatively immunodepleted from the venom, whereas PLA_2 Bs-17 was only partly immunoprecipitated (Figure 8). These proteins represent about 30% of the total *B. schlegelii* venom proteins. The bradykinin-potentiating peptides, Kazal-type inhibitors, and the CRISP protein within *B. schlegelii* venom were not recognized by antivenom IgGs by neither approach (Western blot or immunoprecipitation in solution). A previous work showed that the polyvalent ICP antivenom was able to neutralize the indirect hemolytic activity of PLA_2 molecules of crude *B. lateralis* and *B. schlegelii* venoms, albeit requiring a relatively high antivenom concentration.[83]

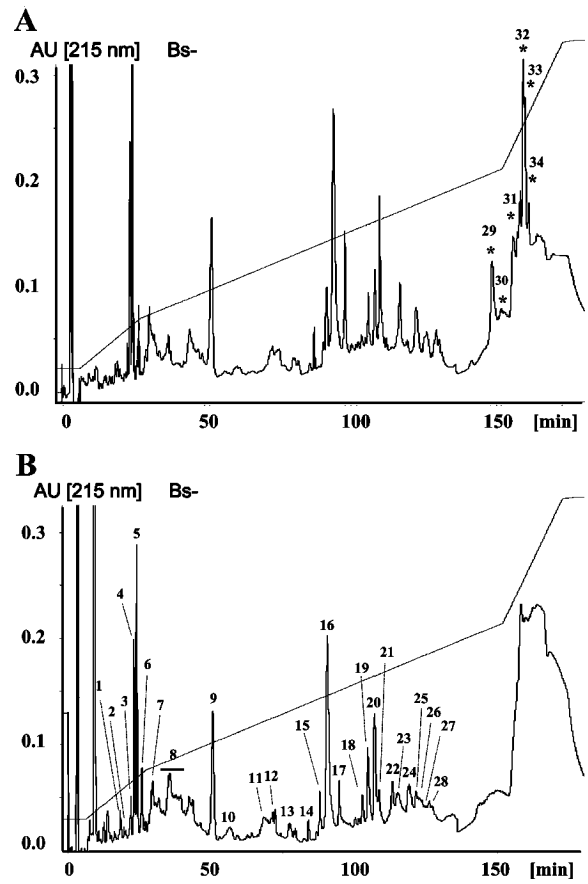

Figure 8. Immunodepletion of *B. schlegelii* venom proteins by the ICP polyvalent antivenom. Panels A and B show, respectively, reverse-phase separations of soluble *B. schlegelii* venom proteins recovered after incubation of the venom with rabbit anti-horse IgG antiserum (control sample), and with the ICP polyvalent antivenom followed by rabbit anti-horse IgG antiserum and immunoprecipitation. HPLC fractions labelled with asterisks in panel A were quantitatively immunodepleted from the venom sample incubated with the antivenom, whereas fractions labelled in panel B with numbers (following the same numbering as in Figure 2) were not affected (1−16, 18−28), or were only partially immunodepleted (Bs17), by the antivenom. Nonlabeled late-eluting peaks correspond to rabbit IgG molecules, as in Figure 6.

Differences in the employed methodologies may explain variations in immunorecognition/neutralization of PLA_2s between the previous report and the present investigation.

Considering the central role that venom has played in the adaptive radiation of snakes and the underlying diversifying selection pressure to maintain high levels of variation in venom genes through accelerated evolution,[21,62] it is not surprising that the ICP polyvalent antivenom, raised against the venoms of *B. asper*, *C. durissus durissus*, and *L. stenophrys* binds to, and immunoprecipitates, a limited set of *Bothriechis* venom components. The structure of the nonrecognized venom proteins may have diverged to an extent where the antivenom does not display significant affinity toward some *Bothriechis* venom proteins. In line with this hypothesis, snake venom serine proteinase and PLA_2 genes are known to be members of large multigene families.[58,60,61] Positive Darwinian selection and neofunctionalization has been shown to be common in snake venom PLA_2 genes.[84] On the other hand, the bradykinin-potentiating peptides (BPPs) NPPAGPDVGPR (Bl-1) and ZK-

WDPPPISPP (Bl-7) (Table 1), and TPPAGPDVGPR (Bs-3), ZWSHKGWPPRPQIPP (Bs-6), ZKKWPPGHHIPP (Bs-7) and ZKWDPPISPP (Bs-9) (Table 2) are conserved in the sequence of the BPP- and C-natriuretic peptide precursor molecule [Q27J49] from *L. stenophrys*,[85] suggesting that at least the short BPPs may escape the immune response of the hyperimmunized horses, probably since their low molecular mass (M_r) values are below the general limits for immunogenicity.

Our results reveal a limitation of the ICP polyvalent antivenom in binding to, and thus neutralizing, the low M_r venom protein components. The failure of snake antivenoms to recognize specific components of the venom used for hyperimmunization has been previously reported.[86,87] A study on the spectrum of venom proteins from the Australian snakes *Pseudonaja affinis affinis*, *Pseudonaja textilis* and *Pseudonaja nuchalis* recognized and neutralized by a brown snake antivenom,[87] raised in horse against the venom of *P. textilis* by the Australian Commonwealth Serum Laboratories (CSL), highlighted a deficiency in the interaction between this antivenom and protein constituents of the *Pseudonaja* sp. snakes of 6 kDa to <32 kDa, predominantly PLA_2 proteins and α-neurotoxins.

Concluding Remarks

The aims of the present work were to characterize through a snake venomics approach[51] the protein composition of the arboreal neotropical pitvipers *B. lateralis* and *B. schlegelii*, and to investigate which protein species within these snake venoms are effectively recognized and immunoprecipitated by the ICP polyvalent antivenom. For the latter aim, we have coined the term "antivenomics". A major aim of the combined venomic and antivenomic studies is the understanding of the antivenom specificity as a first step toward the development of improved antivenoms with a more complete toxin neutralization profile. The venomics results reveal that the major toxin families of *B. lateralis* and *B. schlegelii* are SVMP (55% of the total venom proteins) and PLA_2 (44%), respectively, providing clues for rationalizing the distinct signs of envenomation caused by these *Bothriechis* species. In addition, the antivenomic characterization of the venoms provides a ground for the reported protection of the ICP polyvalent antivenom against the lethal, hemorrhagic, coagulant, defibrinating, fibrin(ogen)olytic and caseinolytic activities of *Bothriechis* (*schlegelii*, *lateralis*) venoms,[42–44] which are associated with serine proteinases and/or SVMPs.[88] Our study also predicts the limited neutralization capability of the polyvalent antivenom toward a number of *Bothriechis* venom components, predominantly BPPs, svVEGF, Kazal-type inhibitors, some PLA_2s, some serine proteinases, and CRISP molecules. The potential therapeutic implications of these findings are presently unknown since the biological effects of the nonimmunoprecipitated venom fraction have not been studied.

Snakebite envenomation constitutes a highly relevant public health hazard in Central and South America which has an impact in terms of mortality and morbidity. Antivenoms should ideally neutralize all venom components playing a role in the pathophysiology of envenomation. Antivenoms can be improved by using proteomic and molecular approaches, such as the selection of candidate epitopes for structure-based design of antitoxin antibodies.[89] In addition, the design of DNA constructs encoding a string of relevant and widespread distributed epitopes has been successfully used for immunization.[90] Current antivenoms contain an immunoglobulin pool of unknown antigen specificity, including antibodies to nontoxic components and non-neutralizing antibodies against toxin epitopes. Therefore, the venomic and antivenomic tools employed in this work may become useful for a more precise knowledge of the most relevant and abundant venom components and for the identification of toxins not recognized by antivenoms, thus, paving the way for the design of more effective immunotherapeutics.

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