

Snake Venomics of *Bitis gabonica gabonica*. Protein Family Composition, Subunit Organization of Venom Toxins, and Characterization of Dimeric Disintegrins Bitisgabonin-1 and Bitisgabonin-2

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The protein composition of the venom of the East African Gaboon viper (*Bitis gabonica gabonica*) was analyzed using RP-HPLC, N-terminal sequencing, MALDI-TOF peptide mass fingerprinting, and CID-MS/MS. In total, 35 proteins of molecular masses in the range of 7–160 kDa and belonging to 12 toxin families were identified. The most abundant proteins were serine proteinases (26.4%), Zn²⁺-metalloproteinases (22.9%), C-type lectin-like proteins (14.3%), PLA₂s (11.4%), and bitiscystatin (9.8%). Other protein classes, that is, bradykinin-potentiating peptides, dimeric disintegrins, Kunitz-type inhibitor, DC-fragments, sv-VEGF, CRISP, and L-amino acid oxidase, comprised between 1.3 and 3.4% of the total venom proteome. Only 11 venom-secreted proteins matched any of the previously reported 22 partial or full-length venom gland transcripts. In addition, venome and transcriptome depart in their relative abundances of different toxin families. The proteomic characterization of purified *B. gabonica gabonica* proteins run under nonreducing and reducing SDS-PAGE conditions revealed their aggregation state and subunit composition. Multimeric proteins included heterodimeric disintegrins, homodimeric sv-VEGF-A, heterodimeric ($\alpha\beta$) and tetrameric ($\alpha\beta$)₄ C-type lectins, and multimeric PIII Zn²⁺-metalloproteinases. Determination of the complete primary structure and subunit composition of the two major dimeric disintegrins, bitisgabonin-1 and bitisgabonin-2, showed that each comprised a distinct RGD- and MLD-bearing subunit and a common, N-terminal-blocked, RGD-containing subunit identical to the disintegrin domain of the PII Zn²⁺-metalloproteinase 4. Cell adhesion inhibition assays showed that bitisgabonin-1 (RGD-RGD) is a potent inhibitor of integrin $\alpha_5\beta_1$, whereas bitisgabonin-2 (MLD-RGD) is a better antagonist of integrins $\alpha_4\beta_1$ and $\alpha_9\beta_1$.

Keywords: *Bitis gabonica* • East African Gaboon viper • snake venom protein families • proteomics • snake venomics • N-terminal sequencing • mass spectrometry • dimeric disintegrins

Introduction

Venoms represent the critical innovation that allowed advanced snakes to transition from a mechanical (constriction) to a chemical (venom) means of subduing and digesting prey larger than themselves, 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,¹ venom thus represents a key adaptation that has played an important role in the diversification of these animals. Venom toxins likely evolved from proteins with a normal physiological function and appear to have been recruited into the venom proteome before the

diversification of the advanced snakes, at the base of the Colubroidea radiation.^{2–5}

Venoms produced by snakes of the family Viperidae (vipers and pitvipers) contain proteins that interfere with the coagulation cascade, the normal homeostatic system and tissue repair, and human envenomations are often characterized by clotting disorders, hypofibrinogenemia, and local tissue necrosis.^{6–8} Although viperid venoms may contain well over 100 protein components,⁹ venom proteins belong to only a few major protein families, including enzymes (serine proteinases, Zn²⁺-metalloproteases, L-amino acid oxidase, group II PLA₂) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, cystatin and Kunitz-type protease inhibitors).^{2,3,7,9,10} However, snake venoms depart from each other in the composition and the relative abundance of toxins. Although the notion that evolutionary interactions

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between snakes and their prey may be responsible for variation in venom composition as a result of strong diversifying selection on venom genes has been controversial,^{11,12} an increasing number of studies strongly support the idea that venom composition variation reflects adaptation for differential utilization of distinct prey types.^{13–17}

In addition to understanding how venoms evolve, characterization of the protein/peptide content of snake venoms also has a number of potential benefits for basic research, clinical diagnosis, development of new research tools and drugs of potential clinical use, and for antivenom production strategies.¹⁸ Within- and between-species heterogeneity of venoms may also account for differences in the clinical symptoms observed in accidental envenomations. To explore the putative venom components, several researchers at various laboratories have carried out transcriptomic analyses of the Duvernoy's (venom) glands of viperid (*Bitis gabonica*,¹⁹ *Bothrops insularis*,²⁰ *Bothrops jararacussu*,²¹ *Bothrops jararaca*,²² *Agkistrodon acutus*,²³ *Echis ocellatus*,²⁴ and *Lachesis muta*²⁵) and colubrid (*Philodryas olfersii*²⁶) snake species. Transcriptomic investigations provide catalogues of partial and full-length transcripts that are synthesized by the venom gland. However, transcriptomes include translated and nontranslated mRNAs, transcripts encoding nonsecreted proteins, housekeeping, and cellular, in addition to toxin, precursor genes. Moreover, toxins may undergo post-translational processing, and this event will not be evident in a transcriptomic analysis. Thus, outlining the full map of native toxins that actually constitute the venom requires a combined biochemical and proteomic approach. To address the need for detailed proteomic studies of snake venoms, we have initiated a project whose long-term goal is a detailed analysis of viperid venomes. To date, we have reported detailed analysis of the protein composition of the venoms from the North American rattlesnakes *Sistrurus miliarius barbouri*,^{10,27} *Sistrurus catenatus* subspecies *catenatus*, *tergeminus*, and *edwardsii*;²⁷ the Tunisian vipers *Cerastes cerastes*, *Cerastes vipera*, and *Macrovipera lebetina*;²⁸ and *Bitis arietans* (Ghana).²⁹ Here, we report a proteomic analysis of *Bitis gabonica gabonica* venom, which complements the study of snake venom gene transcriptional activity (transcriptome) in the same species¹⁹ by showing the relative abundance of the various protein families that are actually secreted into the venoms. This proteomic study was conducted with the double goal of comparing the toxin repertoire, revealed through a proteomic versus a transcriptomic approach, and to elucidate structural features of the venom-secreted proteins like molecular mass, aggregation state, and subunit composition, which require proteomic techniques. A comprehensible catalog of the venom-secreted proteins may contribute to a deeper understanding of the biological effects of the venoms and may also serve as a starting point for studying structure–function correlations of individual toxins.

Experimental Section

Isolation of Venom Proteins. For reverse-phase HPLC separations, 2–5 mg of crude venom of *B. gabonica gabonica* (Latoxan Serpentarium, Rosans, France) was dissolved in 100 μ L of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN), and insoluble material was removed by centrifugation in an Eppendorff centrifuge at 13 000g 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 C₁₈ column (250 \times 4 mm, 5 μ m particle

size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and ACN (solution B) (5% B for 5 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 Speed-Vac (Savant). The relative abundances (percentage of the total venom proteins) of the different protein families in the venom 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 (2–5 mg/mL in 100 mM ammonium bicarbonate, pH 8.3, containing 5 M guanidinium hydrochloride) were reduced with 1% (v/v) 2-mercaptoethanol for 15 min at 85 °C and alkylated by addition of 4-vinylpyridine at 5% (v/v) final concentration and incubation for 15 min at room temperature. Pyridylethylated (PE) proteins were freed from reagents using a C18 Zip-Tip pipet tip (Millipore) after activation with 70% ACN and equilibration in 0.1% TFA. Following protein adsorption and washing with 0.1% TFA, the PE-proteins were eluted with 10 μ L of 70% ACN and 0.1% TFA and 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 program³⁰ implemented in the WU-BLAST2 search engine at <http://www.bork.embl-heidelberg.de>. The molecular masses of the purified proteins were determined by SDS-PAGE (on 12–15% polyacrylamide gels) and by MALDI-TOF mass spectrometry using an Applied Biosystems Voyager-DE Pro mass spectrometer operated in linear mode. To this end, isolated proteins (2–5 mg/mL in 100 mM ammonium bicarbonate, pH 8.3, containing 5 M guanidinium hydrochloride) were reduced with 10 mM 2-mercaptoethanol for 15 min at 65 °C and alkylated by addition of iodoacetamide at 50 mM final concentration and incubation for 1 h at room temperature. For MALDI-TOF analysis, the carbamidomethylated (CM) proteins were freed from reagents using a C18 Zip-Tip pipet tip (Millipore), as above, and equal volumes (0.5 μ L) of the protein solution and the matrix (sinapinic acid, Sigma, saturated in 50% ACN and 0.1% TFA) were mixed on the MALDI-TOF plate. The mass calibration standard consisted of a mixture of the following proteins (isotope-averaged molecular masses in daltons): bovine insulin (5734.6), *Escherichia coli* thioredoxin (11674.5), horse apomyoglobin (16952.6), *E. coli* N-acetyl-L-glutamate kinase (NAGK) (27159.5), *Pyrococcus furiosus* carbamoyl-phosphate synthetase (PFU) (34297.4), *Parkia platycephala* seed lectin (PPL) (47946), and bovine serum albumin (66431). NAGK, and PFU and PPL were generous gifts of Dr. Vicente Rubio (Instituto de Biomedicina de Valencia, Valencia, Spain) and Dr. Benildo S. Cavada (Universidade Federal de Ceará, Fortaleza, Brazil), respectively. The other proteins were purchased from Applied Biosystems.

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, alkylation with iodoacetamide, and digestion with sequencing-grade bovine pancreas trypsin (Roche) using a ProGest digester (Genomic Solutions) following the manufacturer's instructions. The tryptic peptide mixtures were dried in a Speed-Vac and redissolved in 5 μ L of 70% ACN and 0.1% TFA. Digests (0.65 μ L) were 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% ACN 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).

CID MS/MS. For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems)³¹ 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 Q₀ 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 the on-line form of the MASCOT program at <http://www.matrixscience.com>.

Amino Acid Sequence Determination of Dimeric Disintegrins Bitisgabonin-1 and Bitisgabonin-2. Fractions BG-11 (bitisgabonin-1) and BG-14 (bitisgabonin-2) [0.1–0.5 mg/mL in 100 mM ammonium bicarbonate, pH 8.3, containing 6 M guanidinium hydrochloride] were reduced and ethylpyridylated (as above), and the PE-subunits were isolated by reverse-phase HPLC using a Lichrosphere RP100 C₁₈ column (250 × 4 mm, 5 μ m particle size) developed at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and ACN (solution B). The subunits were denoted “A” or “B” according to their elution order. The isolated PE-subunits were initially characterized by N-terminal sequencing and electrospray ionization mass spectrometric analysis using a QTrap mass spectrometer. The primary structures of the gabonin-1 and gabonin-2 subunits were deduced from CID-MS/MS analysis of peptides obtained by proteolytic digestion with endoproteinase Lys-C (Boehringer Mannheim) for 18 h at 37 °C using an enzyme/substrate ratio of about 1:100 (w/w).

Cell Adhesion Inhibition Assays. The inhibition of the adhesion of K562 cells (which express integrin $\alpha_5\beta_1$), Jurkat cells (expressing integrin $\alpha_4\beta_1$), and α 9SW480 cells (SW480 cells transfected with integrin $\alpha_9\beta_1$) labeled with 5-chloromethylfluorescein diacetate (CMFDA) to fibronectin and VCAM-1 immobilized on a 96-well microtitre plate was assayed as described.³² Briefly, fibronectin (10 μ g/mL) (Sigma) and VCAM-1 (3 μ g/mL) (provided by Dr. P. Wainreb, Biogen, Inc.) were immobilized on a 96-well microtiter plate (Falcon, Pittsburgh, PA) in 20 mM phosphate buffer containing 150 mM NaCl overnight at 4 °C, and the wells were subsequently blocked with 1% BSA. The cells were labeled with fluorescein by incubation at 37 °C for 15 min with 12.5 μ M CMFDA in Hank's Balanced Salt Solution (HBSS) containing calcium and magnesium. Cells were freed from unbound ligand by washing with the same buffer. Labeled cells (1 × 10⁵/sample) were added to the wells in the presence or absence of increasing concentrations of bitisgabonin-1 and bitisgabonin-2 and incubated at 37 °C for 30 min. Unbound cells were removed by aspiration, the wells were washed, and bound cells were lysed by adding 0.5% Triton X-100. In parallel, the standard curve was prepared in the same

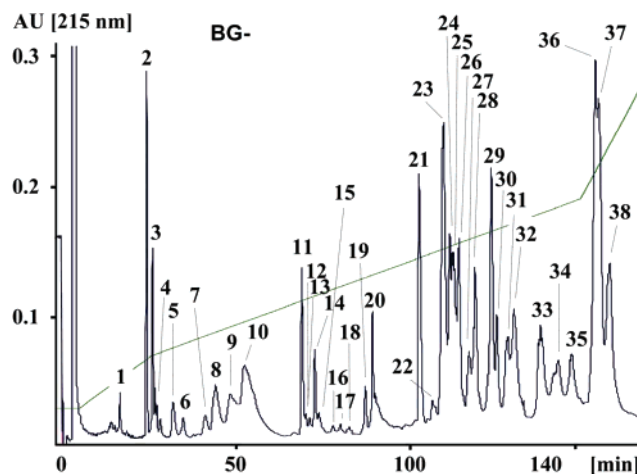


Figure 1. Reverse-phase HPLC separation of the *B. gabonica gabonica* venom proteins. Chromatographic conditions were isocratically (5% B) for 10 min, followed by 5–15% B for 10 min, 15–50% B for 140 min, and 50–70% for 10 min. Fractions were collected manually and characterized by N-terminal sequencing, MALDI-TOF 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.

plate using known concentrations of labeled cells. The plates were read using an FLx800 fluorescence plate reader (Bio-Tek, Winooski, VT) at an excitation wavelength of 485 nm using a 530 nm emission filter.

Results and Discussion

Characterization of the *B. gabonica gabonica* Venome. The crude venom of *B. gabonica gabonica* was fractionated by reverse-phase HPLC (Figure 1), followed by analysis of each chromatographic fraction by SDS-PAGE (Figure 2), N-terminal sequencing, and MALDI-TOF mass spectrometry (Table 1). Many reverse-phase peaks corresponded to essentially pure proteins (Figure 2). Although only 23 *B. gabonica* venom protein entries (comprising both partial and full-length ETS-derived sequences) are annotated in the nonredundant database UniProtKB/TrEMBL (Release 33.4 of July 25, 2006), HPLC fractions that yielded unambiguous N-terminal sequences could be classified into known protein families using a BLAST amino acid similarity search (Table 1), indicating that representative members of each of these families are present in the protein sequence banks. SDS-PAGE-separated protein bands of fractions containing ambiguous or blocked N-termini or mixtures of components were excised, subjected to in-gel tryptic digestion, and identified by combination of MALDI-TOF mass fingerprinting and peptide ion sequencing by CID-MS/MS. As expected from the rapid amino acid sequence divergence of venom proteins by accelerated evolution,^{15,32–35} with a few exceptions, the product ion spectra did not match to any known protein using a licensed MASCOT search program and a database constructed with all reported viper venom proteins and EST-derived sequences from transcriptomic projects. The CID-MS/MS spectra were therefore manually interpreted and the deduced peptide ion sequences submitted to BLAST sequence similarity searches. This approach allowed us to assign unambiguously almost all (98.8%) of the isolated venom fractions to protein families (Table 1). Supporting the view that venom proteomes are mainly composed of proteins belonging to a few protein families,^{2,3,10,28} the proteins found in the venom

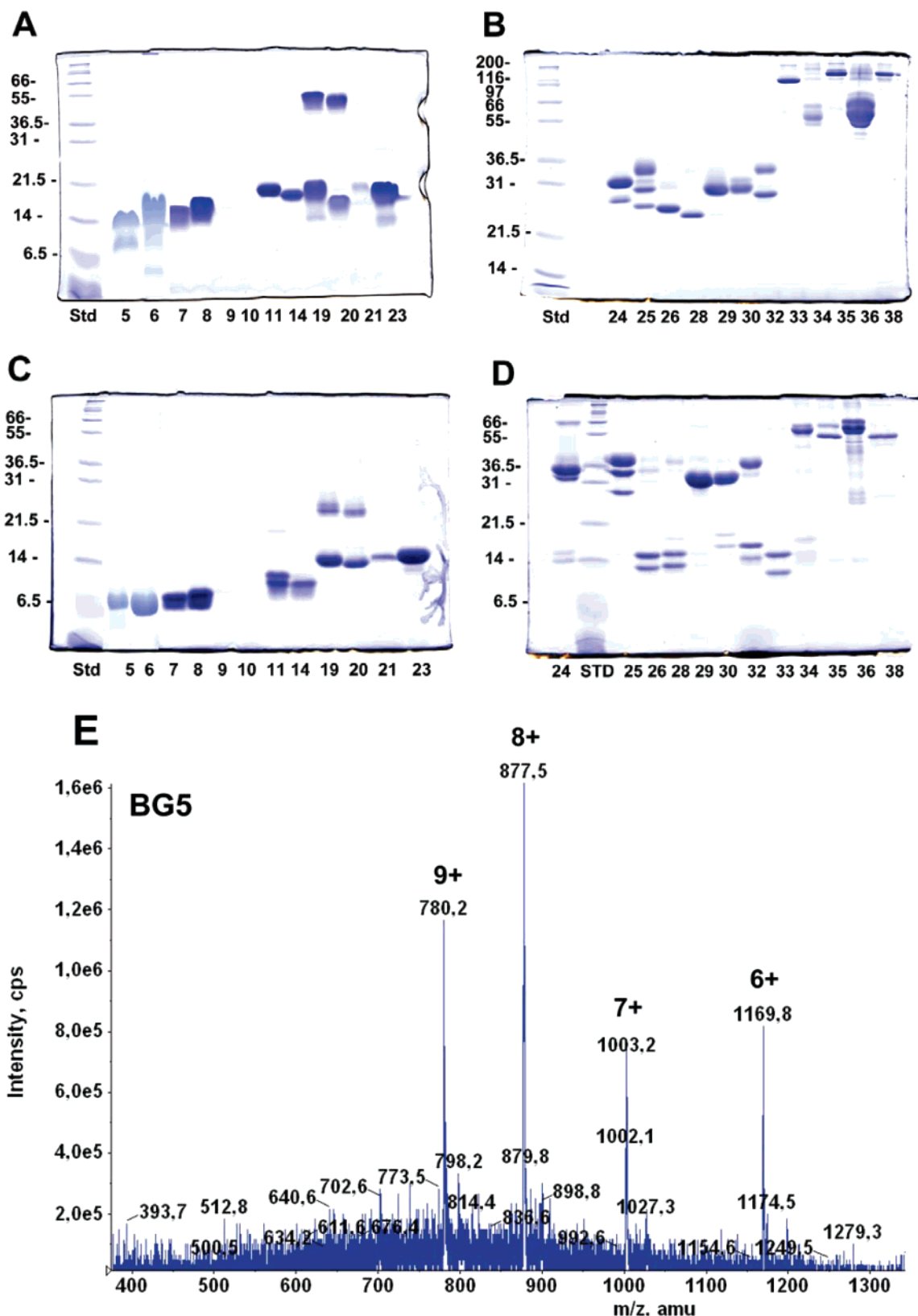


Figure 2. SDS-PAGE of reverse-phase separated *B. gabonica gabonica* venom fractions. (A–D) SDS-PAGE showing the protein composition of the reverse-phase HPLC-separated venom protein fractions run under nonreduced (A and B) and reduced (C and D) conditions. Molecular mass markers (in kDa) are indicated at the left side of each gel. Protein bands were excised and characterized by mass fingerprinting and CID–MS/MS. The results are shown in Table 1. (E) Electrospray-ionization mass spectrometric characterization of native (nonreduced) Kunitz-type inhibitor-1 isolated in fraction BG5, from which a molecular mass of 7012.6 ± 0.3 Da was calculated.

of *B. gabonica gabonica* cluster in 12 different families (bradykinin-potentiating peptides, dimeric disintegrin, Kunitz-type

inhibitor, cystatin, DC fragment, VEGF, PLA₂, serine protease, cysteine-rich secretory proteins (CRISP), C-type lectins, L-amino

Table 1. Assignment of the Reverse-Phase-Isolated Fractions of *B. gabonica gabonica* Venom to Protein Families by N-Terminal Edman Sequencing, Mass Spectrometry, and Collision-Induced Fragmentation by Nano-electrospray-MS/MS of Selected Peptide Ions from In-Gel-Digested Protein Bands^a

HPLC fraction BG-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
1	Blocked		413.7	2	ZEDXSPR	Unknown
2-3	n.p.					
4			440.1	2	(249.1)RPYVP	Metalloprotease fragment
5			487.1	2	ZRPGPEXPP	Bradykinin-potentiating peptide (BPP)
	N.D.	7012.6 [§]	504.8	2	FYYSVSK	Kunitz inhibitor-1 [Q6T6T5] (26-87)
			780.3	2	GNSNNFESMDECR	Fragment from BPP precursor (similar to AAP83422 68-73)
6			381.1	2	QPWPHP	Kunitz inhibitor-1 [Q6T6T5] (28-90)
	N.D.	7088.1 [§]	780.3	2	GNSNNFESMDECR	Kunitz inhibitor-2 [Q6T6S5] (25-86)
			433.2	2	TCVASATR	Kunitz inhibitor-2 [Q6T6S5] (25-85)
7	KKRPDFCYLPADTGP	6951.3 [§]	615.7	3	FTYGGCHGNANNFETR	BPP
		7066.6 [§]				
8	KKRPDFCYLPADTGP	6998.2 [§]	615.7	3	FTYGGCHGNANNFETR	Metalloprotease 4 [Q6T271]
			658.3	3	KFTYGGCHGNANNFETR	Gabonin-1 [Q6T6T3]
9			644.2	2	ZWERPGPEXPP	
10			712.3	2	(247.3)WERPGPEXPP	
11	NSAHPCCDPVTCKPK	15183.6 [§]	554.0	2	FLRPGTVCR	
			672.6	2	GDWNDDFCTGR	
			767.6	2	AWEHCISGPCCR	
			667.6	2	GEHCISGPCCR	
			651.5	3	GDSLHDIYCTGVTPDCPR	
12	N.D.	15 kDa [■]	554.0	2	FLRPGTVCR	
			672.6	2	GDWNDDFCTGR	
			767.6	2	AWEHCISGPCCR	
13	N.D.	14 kDa [■]	554.0	2	FLRPGTVCR	
			672.6	2	GDWNDDFCTGR	
			767.6	2	AWEHCISGPCCR	
14	NSAHPCCDPVTCKPK	15111.4 [§]	554.0	2	FLRPGTVCR	
			672.6	2	GDWNDDFCTGR	
			767.6	2	AWEHCISGPCCR	
			667.6	2	GEHCISGPCCR	
			512.8	2	FLNAGTICK	
			728.8	3	TMLDGLNDYCTGVTPDCPR	
15	N.D.	15 kDa [■]	672.6	2	GDWNDDFCTGR	
			767.6	2	AWEHCISGPCCR	
16,17	SPPVCGNELLEVGE	23 kDa [■]				
18	HLVQFGRMISYMGTN	14234 [*]				
19	NLLEFGKMIKKEETGF	13827 [*]	550.9	2	TDMYGYHR	
	Blocked	46 [■] / 23 [▼] kDa	773.9	2	NKDNHCEPCSER	
			652.3	2	DNHCEPCSER	
			580.6	2	QQGEVIPFLK	
			758.8	3	LKPFQSQHHPVSVFQHSK	
20	HLEQFGNMIDHVSGR	13966.4 [§]	561.9	2	VAAICFGNRR	
			753.9	2	CCFVHDCCYGK	
			695.8	3	ELCECDRVAICFGNRR	
			860.8	3	WTSYNYEFQNGDIICGDEDPR	
			773.9	2	NKDNHCEPCSER	
			569.1	2	VAAICFANRR	
21	Blocked	46 [■] / 23 [▼] kDa				
	NLFQFGNMINYMTGASVDYI	13525.8 [§]				
	YGCYCGWG					
	KVGGGLYPRKVMDEP	13201.3 [§]	750.8	2	RIVEAQSQVSVGVK	
			672.2	2	IVEAQSQVSVGVK	
			540.7	2	MELAKTCK	
24	VIGGAECNINEHRFL	32 kDa [■]	734.6	2	VIGGAECNINEHR	
			552.0	2	TLCAGVLEGGK	
			749.5	2	KSAHIAPLSLPSSPPSVGSVCR	
		26 kDa [■]	580.5	2	FHCAGTLLNK	
			595.6	2	WDKDIMLIR	
			632.5	2	NVQNEDEEMR	
			757.6	2	VTYDPVPHCANIK	
			795.2	2	NVQNEDEEMRVPK	
25	VIGGAECNINEHRSL	34.5 kDa [■]	567.9	2	VTDFTPWIR	
			734.8	2	VIGGAECNINEHR	
			749.8	2	KSAHIAPLSLPSSPPSVGSVCR	
		29 kDa [■]	668.3	2	HXAPXSPPSPPR	
			600.6	2	VFDYNDWXX	
25		27 kDa [■]	569.6	2	WDFDSESPR	
			756.2	2	MEWYAEAAANAER	
			636.3	3	KPEIQNEIVDLHNSLR	
26	N.D.	26 kDa [■]	846.9	2	EEADFVAQLISDNIK	
27	VIGGAECKIDGHRSL	37.5 kDa [■]	615.8	2	VYDYTDWR	
			459.3	2	FFCXSSR	
28	N.D.	25 kDa [■]	846.9	2	EEADFVAQLISDNIK	
			587.9	2	(171.3)NFVWXGXR	
29	VIGGAECKIDGHRSLALLAY	27 kDa [■]				
30	VIGGAECNINEHRSL	35 kDa [■]	667.1	3	SIYDPVPHCADINILDR	
31	DEGCLPDWSS(R/H)EGYC	28 kDa [■]				
32	VIGGDECDINEHPSL	32.5 kDa [■]	590.6	2	VFDYXPWIK	
			715.4	2	SLPSSPPSVGSVCR	
			538.4	2	XYDYSVCR	

Table 1. (Continued)

HPLC fraction BG-	N-terminal sequencing	isotope-averaged molecular mass	peptide ion <i>m/z</i>	<i>z</i>	MS/MS-derived sequence	protein family
		29 kDa■	845.3	2	XTGKFXTHFWIGXR	C-type lectin-2 [AAR06852.1]
			587.9	2	(171.3)NFVWXGXR	16 kDa▼
			844.6	2	ITGKFFITHFWIGLR	
			846.7	2	EEADFVAQLISDNIK	
			667.8	3	TWLNILCGDDYPFVCK	
			591.3	2	WTDGSNVIYK	
			626.3	3	FCSEQGN SGHLVSIQSK	
			516.9	2	TTDNQWLR	14 kDa▼
33	DFQCPFGWSAYGQHCY DFECPSEWRPFDQHCYRAFD- -EPKRSADAEKF	120 kDa■	655.6	3	GSHLLSLHNI AEADFLK	C-type lectin (14 kDa▼)
			940.1	2	FCSEQGN SGHLVSIQSK	C-type lectin-2 [AAR06852.1]
			774.1	2	NCFGLEKETEYR	(16 kDa▼)
			870.6	2	SSPDYVVMGLWNQR	
			846.7	2	EEADFVAQLISDNIK	
34	Blocked	58 kDa▼	667.8	3	TWLNILCGDDYPFVCK	
			552.3	2	NGDGFYCR	PIII-metalloprotease
			534.3	2	QCISLFGSR	
	N.D.	56 kDa▼	770.1	2	(261.4)CESVANXGXR	
			534.2	2	NPLEEFCR	L-amino acid oxidase
			743.6	2	EADYEEFLEIAR	
			618.6	2	SAEGLFEESLR	
			748.1	3	IFFAGEYTANAHGWIDSTIK	
35	Blocked	160 kDa■				
		58 kDa▼	770.1	2	(261.4)CESVANXGXR	PIII-metalloprotease
		52 kDa▼	534.3	2	QCISLFGSR	PIII-metalloprotease
			566.3	2	XANDYGYCR	
			649.3	2	NPCQXYSPR	
			719.0	2	LYCFDNLPEHK	
			764.6	2	ATVAQDACGFNR	
36, 37	Blocked	56 kDa▼	878.3	2	(261.3)VEGEDCDCGSPR	
			616.1	2	SAECPXDGNXR	PIII-metalloprotease
			792.1	2	NFCESVDN XGXR	
		52 kDa■	616.1	2	SAECPXDGNXR	PIII-metalloprotease
			514.4	2	IPCAPQDVK	
			552.3	2	NGDGFYCR	
			522.6	2	(187.1)XXQVNDR	
			629.1	2	GNNYFYCR	
			764.9	2	GIVAEDACGFNR	
			718.9	2	LYCFDNLPEHK	
38	Blocked	48 kDa■	616.1	2	SAECPXDGNXR	PIII-metalloprotease
		160 kDa■	727.2	2	LFCVEPSTGNSIK	PIII-metalloprotease-3
		(52 kDa▼)	487.8	2	NGYCYNGK	[Q6T270]
			671.3	3	SYSSQDDPDYGMVDFGTK	

^a Only peptides whose complete sequences could be unambiguously determined by MS/MS are listed. X, Ile or Leu; Z, pyrrolidone carboxylic acid. Unless other stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses were determined by MALDI-TOF (*) ($\pm 0.2\%$) or by electrospray-ionization (\$) ($\pm 0.02\%$) mass spectrometry. Apparent molecular mass determined by SDS-PAGE of nonreduced (■) and reduced (▼) samples; n.p., non peptidic material found. Previously reported proteins are identified by their databank accession codes.

Table 2. Overview of the Relative Occurrence of Proteins (in Percentage of the Total HPLC-Separated Proteins) of the Different Families in the Venom of *B. gabonica gabonica*^a

protein family	% of total venom proteins	
	<i>B. g. gabonica</i>	<i>B. arietans</i>
Bradykinin-potentiating peptide	2.8	-
Dimeric disintegrin	3.4	-
Long disintegrin	-	17.8
Kunitz-type inhibitors	3.0	4.2
Cystatin	9.8	1.7
DC-fragment	0.5	-
VEGF	1.0	-
PLA ₂	11.4	4.3
Serine proteinase	26.4	19.5
CRISP	2.0	-
C-type lectin	14.3	13.2
L-amino acid oxidase	1.3	-
Zn ²⁺ -metalloproteinase	22.9	38.5
Unknown peptides	1.2	0.9

^a For comparison, the composition of the venom of *B. arietans*²⁹ is shown.

acid oxidase (LAO), and Zn²⁺-dependent metalloproteases), whose relative abundance is listed in Table 2. In comparison with the related snake from the same genus, *B. arietans*, the composition of the venom of *B. gabonica gabonica* appears to

be much more complex. Despite this, and although the East African Gaboon viper does indeed produce prodigious amounts of venom, its toxicity, weight for weight, is rather low compared to other poisonous snakes.³⁷ In mice, the LD₅₀ is 0.8–5.0 mg/kg intravenous (iv), 2.0 mg/kg intraperitoneal (ip) and 5.0–6.0 mg/kg subcutaneous (sc).³⁶ With average length of 1.3 m and weight of 7–10 kg, *B. gabonica gabonica*, is among the largest of the vipers. This pitless viper is native to high rainfall areas of West, Central, and East Africa. It is noted for its docile nature, and this may account for the very few reported bites in the literature. In particular, no documented deaths have been attributed to *B. gabonica gabonica* bites (<http://www-surgery.ucsd.edu/ent/DAVIDSON/Snake/Gabonica.htm>), perhaps due to the fact that their range is mainly limited to rainforest areas. In contrast, the Puff Adder (*B. arietans*) (average length 90 cm, weighting over 6.0 kg), common throughout most of southern Africa except for mountain tops, true desert, and dense forest, is also common around human settlements. *B. arietans* is a very dangerous snake that is responsible for a large proportion of the venomous snakebites in sub-Saharan Africa, where it is indigenous. Its venom is one of the most toxic of any viper. The LD₅₀ values in mice vary: 0.4–2.0 mg/kg iv, 0.9–3.7 mg/kg ip, and 4.4–7.7 mg/kg sc.³⁶ Studies have shown that more than 50% of severe envenomations in humans if left

untreated result in death (<http://www-surgery.ucsd.edu/ent/DAVIDSON/Snake/Arietans.htm>).

In line with a previous study on *S. catenatus* subspecies,²⁷ an implication of our results is that here does not appear to be a simple relationship between levels of venom complexity and toxicity. The high degree of differentiation in the venom proteome among congeneric taxa emphasizes unique aspects of venom composition of even closely related species of venomous snakes and points to a strong role for adaptive diversification via natural selection as a cause of this distinctiveness. *B. arietans*' diet includes mammals, birds, amphibians, and lizard, whereas *B. gabonica* feeds on a variety of birds and mammals, such as doves, many different species of rodents, hares, and mongooses. Given the central role that diet has played in the adaptive radiation of snakes,¹ venom may thus represent a key adaptation that has played an important role in the diversification of these animals. However, because species-specific effects of venom components are largely unknown, it is difficult to assign a functional role unequivocally to the variation we observed in *Bitis* venoms.

Proteomic versus Transcriptomic Comparison of *B. gabonica gabonica* Venom. Comparison of the protein composition of the venom of *B. gabonica gabonica* determined using a proteomic (this work) and a transcriptomic approach¹⁹ shows clear differences, both in the relative occurrence of protein families (expressed as percentages of the total HPLC-separated proteins) (Figure 3) and in the identity of the polypeptides of each protein family. Notably, only 11 out of the 35 distinct proteins identified in this work in *B. gabonica gabonica* venom correspond to proteins reported in the transcriptome of the same species.¹⁹ This low degree of accordance between the proteomic and the transcriptomic approaches clearly indicate that the cDNA library lacked many transcripts encoding venom-expressed proteins. In particular, the major PIII-metalloproteinase(s) in *B. gabonica gabonica* venom, isolated in fractions 36 and 37 (Figures 1 and 2), is(are) not represented in any cluster of venom gland cDNA-assembled contigs (Table 1). In addition, among the distinct serine proteinase isoenzymes characterized in the venom (HPLC fractions 24, 25, 27, 29, 30, and 32), only the 26 kDa band of fraction 24 (Figure 2) was identified as the single serine proteinase (serine protease-1 [Q6T6S7]) reported in the *B. gabonica gabonica* transcriptome.¹⁹ Similarly, PLA₂ molecules found in fractions 19 and 21, as well as C-type lectin-like proteins other than C-type lectin-2 (fractions 32 and 33, Table 1) and the CRISP molecule found in the 23 kDa band from fraction 25 (Figures 1 and 2, and Table 1), have not been described in the *B. gabonica gabonica* transcriptome.¹⁹ Alternatively, in some cases, the lack of correspondence between the proteomic and the transcriptomic data may be due to the availability of only a small region of cDNA-deduced protein sequence. Thus, the amino acid sequence of the doubly charged tryptic ion of *m/z* 534.2, NPLEECFR, of the L-amino acid oxidase identified in the 56 kDa SDS-PAGE band of fraction 34 (Figure 2, Table 1) matches residues 5–12 of the N-terminal sequence of the ~56 kDa SDS-PAGE band BG-6 (LADDKNPLE-EXFRESSYEEFL in Figure 1 of ref 19). It could correspond to the L-amino acid oxidase (LAO) found in contigs 165, 181, and 182¹⁹ for which only the N-terminal sequence and a 60-residue internal fragment (Swiss-Prot/TrEMBL Q6T627) are available. However, the NPLEECFR sequence is highly conserved in many snake venom L-amino-acid-oxidases, and the lack of sequence coverage from other parts of the molecule impedes an unambiguous assignment. Furthermore, the peptides characterized

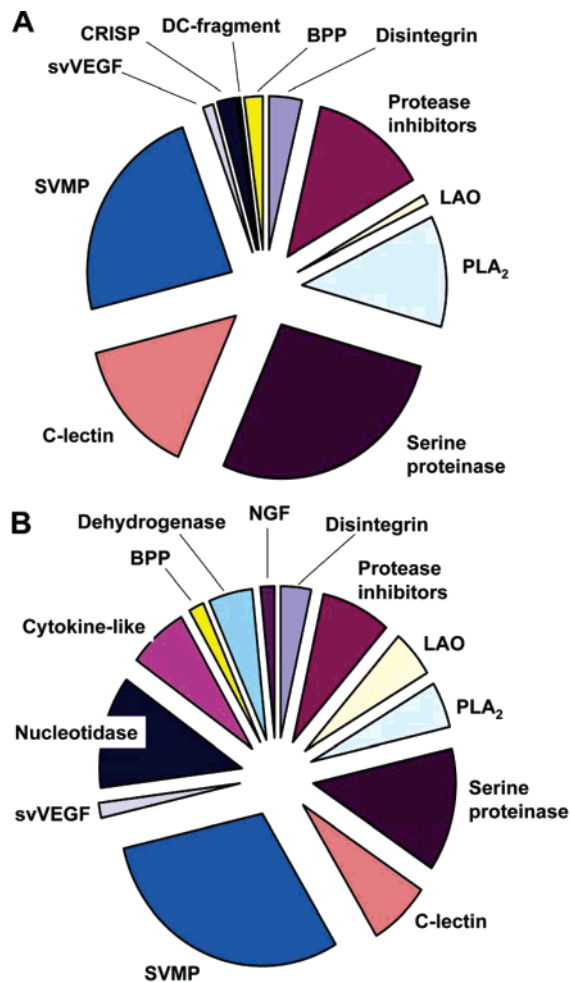


Figure 3. Proteomics versus transcriptomics. Comparison of the protein composition of the venom of *B. gabonica gabonica* determined using a proteomic (this work) and a transcriptomic approach.¹⁹ BPP, bradykinin-potentiating peptide; LAO, L-amino acid oxidase; PLA₂, phospholipase A₂; SVMP, snake venom metalloproteinase; svVEGF, snake venom vascular endothelial growth factor; CRISP, cysteine-rich secretory protein; DC-fragment, disintegrin-like and cysteine-rich fragment derived from PIII-SVMP; NGF, nerve growth factor.

in venom fractions 4–6, 9, and 10 (Table 1) exhibit large sequence similarity to regions ⁴⁸QGGWPRGPGEIPP⁶⁰ and ⁶⁸QNWPH⁷³ of bradykinin-potentiating and C-type natriuretic peptides precursors from *B. jararacussu* [AAP83422], *B. jararaca* [BAA12879], and *B. insularis* [AAM09691] and are thus likely secreted into the venom by proteolytic processing of a BPP/CNP precursor.³⁸ In this respect, contig number 188 of the reported *B. gabonica gabonica* transcriptome¹⁹ (GenBank accession code AY434452) encodes a four residue coding sequence (CDS) (GLGC) and a 587 bp sequence similar to the 3' untranslated region (UTR) of *Agkistrodon blomhoffi* bradykinin-potentiating peptide (GenBank AB020810). The GLGC sequence corresponds to the highly conserved last four C-terminal residues of the open reading frame (ORF) of a bradykinin-potentiating and C-type natriuretic peptides precursor³⁸ from which, we hypothesize, peptides 4–6, 9, and 10 (Table 1) are released.

Transcripts encoding putative secreted proteins which could not be found in our proteomic analysis of *B. gabonica gabonica* venom include nucleotidases, dehydrogenases, a prolyl isomerase

(Q6T268), a two-Kunitz protease inhibitor (Q6T269), metalloproteinase-2 (Q6T6S6), three hypothetical proteins (Q6T6S8, Q6T6S9, and Q6T6T0), a nerve growth factor (Q6T6T1), a cytokine-like protein (Q6T7B4), and C-type lectins 3 and 1 (Q6T7B5 and Q6T7B7, respectively).

Nucleotidases, dehydrogenases, and prolyl isomerase have not been reported in snake venom proteomes and may thus represent venom gland cellular housekeeping proteins. Lack of correlation between gene transcriptional (transcriptome) and translational (proteome) activities has been often noticed. The occurrence of nonvenom-secreted toxins suggests that these messengers could exhibit an individual or a temporal expression pattern over the lifetime of the snake. Sex-based individual variation of snake venom proteome among *B. jararaca* siblings have been reported.³⁹ In addition, ontogenetic variations have been noticed in the venom proteomes of other snakes, that is, *Crotalus viridis viridis*, *Crotalus viridis oreganus*,¹³ *Bothrops atrox*,^{40,41} and *Bothrops asper*,⁴² and might represent a common phenomenon in many other species. The venom gland used for the transcriptomic survey¹⁹ was obtained from a single snake held in captivity at the Kentucky Reptile Zoo (Slade, KY), while the venom that we have used for the proteomic study reported here represents the pooled venom milked from an unknown number of snakes at Latoxan Serpentarium. Alternatively, the nonvenom-secreted or very low-abundance (<0.05% of the total venom proteins) toxins may play a hitherto unrecognized physiological function in the venom gland, or may simply represent a hidden repertoire of orphan molecules which may eventually become functional for the adaptation of snakes to changing ecological niches and prey habits. Clearly, although further work is needed to clarify this point, overall, our results emphasize the relevance of detailed proteomic studies for a thorough characterization of the venom composition.

Multimeric Proteins in *B. gabonica gabonica* Venom. SDS-PAGE analysis, under nonreducing and reducing conditions, of the HPLC-separated protein fractions (Figure 2) revealed the existence of a number of multimeric proteins in *B. gabonica gabonica* venom. Fractions 5 and 6 (isoforms of Kunitz inhibitor-1) and 7 and 8 (isoforms of Kunitz inhibitor-2) (Table 1) exhibited apparent molecular masses of 14–16 and 7–8 kDa when analyzed in nonreduced (Figure 2A) or reduced (Figure 2C) SDS-PAGE, respectively. However, electrospray ionization mass spectrometry showed that the isoforms of both Kunitz-type inhibitors 1 and 2 had native (nonreduced) molecular masses of about 7 kDa (see Figure 2E as example) (Table 1). Hence, the distinct electrophoretic behavior of the Kunitz-type inhibitors in nonreduced (migrating as ~14 kDa proteins) (Figure 2A, lanes 5–8) versus reduced gels (apparent molecular masses of ~7 kDa) (Figure 2C, lanes 5–8) may be regarded as artificial or as indicating the existence of noncovalent dimers.

Fractions 11–15 contained dimeric disintegrins which share a subunit comprised of the disintegrin domain of the PII-metalloprotease 4 [Q6T271]. Indeed, the PII-metalloprotease-4 disintegrin domain appeared to form part of all the different dimeric disintegrins present in *B. gabonica gabonica* venom (Table 1). Sharing of subunits may represent an effective mechanism of structural and functional diversification of dimeric disintegrins.³² The structural and functional characterization of the major dimeric disintegrins (BG11 and BG14) (Figure 2A,C) is described below.

Fractions 19 and 20 contained each a distinct monomeric PLA₂ molecule (14 kDa) (Table 1) and isoforms of the homodimeric vascular endothelial growth factor A (VEGF-A or vascular permeability factor A, 165 residues (19.3 kDa) per subunit) [P83906].¹⁹

C-type lectin-like proteins (CLPs) were identified in fractions 26, 28, 32, and 33 (Table 1). The proteins recovered from the HPLC peaks 26, 28, and 32 (25–29 kDa, Figure 2B) exhibit dimeric ($\alpha\beta$) structures commonly observed in CLPs from viperid venoms.^{42,43} In contrast, the C-type lectin-like protein in fraction 33 had a native apparent molecular mass (120 kDa) of the protein (Figure 2B) and, upon reduction of disulphide bonds, released two subunits of 16 and 14 kDa (Figure 2D), indicating that the quaternary structure of this CLP is composed of a tetrameric arrangement of $\alpha\beta$ dimers (i.e., $[(\alpha\beta)_4]$). So far, this type of multimeric CLPs has been characterized from venoms of only a few pit vipers, including the habu snake *Protobothrops* (formerly *Trimeresurus*) *flavoviridis*,⁴⁴ the brown spotted pitviper *Protobothrops mucrosquamatus*,⁴⁵ the South American rattlesnake *Crotalus durissus terrificus*,^{46,47} and the Chinese Moccasin *Deinagkistrodon acutus*.⁴⁸ The existence of a $(\alpha\beta)_4$ CPL in the pitless viper *B. gabonica gabonica* (Viperinae) venom shows that this type of quaternary structures is not restricted to species of genera from the Crotalinae subfamily. In addition, CLPs found in fractions 32 and 33 share the subunit CPL-2 [AAR06852.1] (Table 1), highlighting the molecular economy developed during venom evolution for generating structural and functional diversification within toxin families.³²

Multimeric (160 kDa) PIII-metalloproteases were characterized in fractions 35 and 38 (Figure 2, panels B and D, Table 1). Upon reduction of disulfide bonds, the metalloprotease in fraction 35 released 56 and 52 kDa subunits, which yielded different MALDI-TOF mass fingerprints, suggesting thus that this metalloprotease may represent a heterodimeric (or heterotrimeric) molecule. In contrast, the metalloprotease in fraction 38 ran under reducing conditions as a single 52 kDa electrophoretic band, which was identified by MS/MS analysis as PIII-metalloprotease-3¹⁹ [Q6T270]. Hence, we conclude that the 160 kDa protein of fraction 38 is a homo-oligomer (dimer or trimer) of PIII-metalloprotease-3.

Characterization of Dimeric Disintegrins Bitisgabonin-1 and Bitisgabonin-2. For establishing the primary structure and subunit composition of the major dimeric disintegrins isolated in HPLC fractions 11 (BG11, 15183 Da) and 14 (BG14, 15111 Da), the proteins were reduced and pyridylethylated, and the subunits were purified by reverse-phase HPLC. Electrospray ionization mass spectrometry of the reduced disintegrins showed that BG11 was built by subunits of 7419.4 and 7769.3 Da. The molecular masses of the reduced subunits of BG14 were 7344.9 and 7769.6 Da. Combination of Edman degradation and collision-induced dissociation of endoproteinase Lys-C-derived peptides (Figure 4) showed that BG11 was a heterodimer of gabonin-1 [Q6T6T3] and the disintegrin domain of the PII-metalloprotease-4 [Q6T271]. The C-terminal residues of the gabonin-1 subunit could not be confirmed. The calculated isotope-averaged molecular mass of gabonin-1 (7346.3 Da) differs by 73 Da from the experimentally determined molecular mass (7419.4), indicating either a C-terminal modification or the presence of a C-terminal alanine amino acid instead of the expected leucine from the DNA sequence [Q6T6T3].

The PII-metalloprotease-4 disintegrin domain was found also associated with gabonin-2 [Q6T6T2] in BG14. The shared

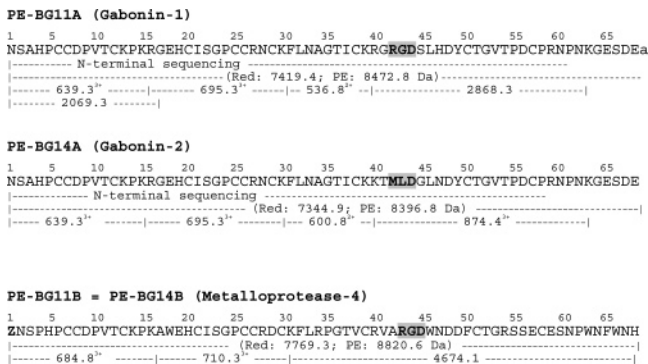


Figure 4. Primary structure of dimeric disintegrin subunits. The amino acid sequences of the subunits of bitisgabonin-1 and bitisgabonin-2 (fractions BG-11 and BG-14 of Figure 1, respectively) were established by combination of automated Edman degradation of the reverse-phase-separated pyridylethylated (PE-) subunits and CID-MS/MS of endoproteinase Lys-C-derived peptides. The shared subunit of bitisgabonin-1 and bitisgabonin-2 (PE-BG11B = PE-BG14B) is N-terminally blocked (Z = pyrrolidone carboxylic acid) and is identical to the disintegrin domain of metalloprotease 4 [Q6T271].

subunit contained a blocked N-terminal residue (pyrrolidone carboxylic acid), which explains the reason native BG11 and BG14 yielded single sequences by Edman degradation (Table 1).

Dimeric disintegrins BG11 and BG14, hitherto called bitisgabonin-1 and bitisgabonin-2, bear, respectively, RGD/RGD and MLD/RGD integrin binding motifs in their A- and B-subunits. The inhibitory effects of bitisgabonin-1 and bitisgabonin-2 on the adhesion of K562, Jurkat, and $\alpha 9$ -SW480 cells to immobilized ligands are shown in Figure 5 and Table 3. As expected from their integrin binding motifs,^{23,49} bitisgabonin-1 was a potent inhibitor of the adhesion of the RGD-dependent integrin $\alpha_5\beta_1$ to immobilized fibronectin, whereas bitisgabonin-2 preferentially inhibited the adhesion of the $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins to VCAM-1 and acted also as a strong antagonist of $\alpha_5\beta_1$.

Concluding Remarks. Our study highlights the relevance of detailed proteomic studies for a thorough characterization of the protein composition of snake venoms and supports the hypothesis that snake venom proteomes may be composed of proteins belonging to only a few protein families. However, the protein family profile and the relative abundance of each protein group in venoms of recently evolved congeneric taxa are not conserved, emphasizing the uniqueness of the venom composition of even closely related, yet ecologically distinct, venomous snake species, that is, *B. gabonica gabonica* (high rainfall areas) and *B. arietans* (savannah and grasslands).

The combination of classical biochemical analyses (reverse-phase HPLC, Edman degradation, and SDS-PAGE) and proteomic techniques revealed the subunit composition of multimeric venom proteins and provided a comprehensive catalog of secreted proteins, which may contribute to a deeper understanding of the biology and ecology of *B. gabonica gabonica*, and may also serve as a starting point for studying structure-function correlations of individual toxins. In humans, systemic envenomation is characterized by immediate abrupt hypotension, subsequent cardiac damage, and dyspnoea,^{37,50} and blistering, bruising, swelling, and necrosis may be extensive. The individual venom components responsible for these effects have not been isolated, but it seems likely that brady-

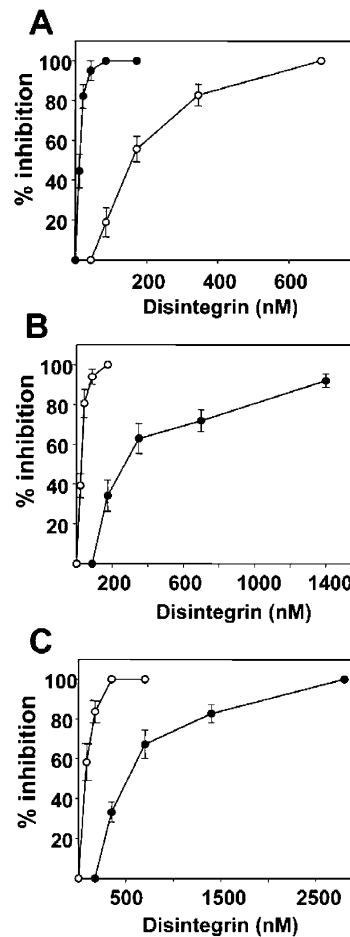


Figure 5. Inhibitory effect of BG11 (bitisgabonin-1) and BG14 (bitisgabonin-2) on the adhesion of K562, Jurkat, and $\alpha 9$ SW480 cells to immobilized ligands. The adhesion of CMFDA-labeled K562 cells to fibronectin (A), Jurkat cells to VCAM-1 (B), and $\alpha 9$ SW480 cells to VCAM-1 (C) was assayed in the presence or absence of increasing concentrations of dimeric disintegrins bitisgabonin-1 (filled circles) and bitisgabonin-2 (open circles). The error bars represent the standard deviation (SD) from three duplicated experiments.

Table 3. Inhibitory Activities of Dimeric Disintegrins Bitisgabonin-1 (BG11) and Bitisgabonin-2 (BG14) on the Adhesion of Cell Lines Expressing Defined Integrins to Immobilized Ligands^a

cell line	integrin receptor	ligand	IC ₅₀ (nM)	
			bitisgabonin-1	bitisgabonin-2
K562	$\alpha_5\beta_1$	FN	12.4 ± 3.1	167 ± 12
Jurkat	$\alpha_4\beta_1$	VCAM-1	312 ± 41	32.6 ± 6.9
$\alpha 9$ -SW480	$\alpha_9\beta_1$	VCAM-1	528 ± 82	78 ± 21

^a The data represent the means for three experiments. FN, fibronectin; VCAM-1, vascular cell adhesion molecule-1.

kinin-potentiating peptides may cause peripheral vasodilation and a drop in blood pressure. Zn²⁺-metalloproteinases, causing swelling and a widespread damage to microvasculature, along with edema-inducing PLA₂ enzymes and serine proteinases, altering hemostasis, may lead to pulmonary edema and dyspnoea.

In addition to addressing how venoms evolve, characterization of the relative abundance and structural organization of snake venom toxins may also have a number of potential

benefits for basic research, clinical diagnosis, development of new research tools, and drugs of potential clinical use, as well as for rational antivenom production strategies.

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