

RESEARCH ARTICLE

Interrogating the Venom of the Viperid Snake *Sistrurus catenatus edwardsii* by a Combined Approach of Electrospray and MALDI Mass Spectrometry

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Abstract

The complete sequence characterization of snake venom proteins by mass spectrometry is rather challenging due to the presence of multiple isoforms from different protein families. In the present study, we investigated the tryptic digest of the venom of the viperid snake *Sistrurus catenatus edwardsii* by a combined approach of liquid chromatography coupled to either electrospray (online) or MALDI (offline) mass spectrometry. These different ionization techniques proved to be complementary allowing the identification a great variety of isoforms of diverse snake venom protein families, as evidenced by the detection of the corresponding unique peptides. For example, ten out of eleven predicted isoforms of serine proteinases of the venom of *S. c. edwardsii* were distinguished using this approach. Moreover, snake venom protein families not encountered in a previous transcriptome study of the venom gland of this snake were identified. In essence, our results support the notion that complementary ionization techniques of mass spectrometry allow for the detection of even subtle sequence differences of snake venom proteins, which is fundamental for future structure-function relationship and possible drug design studies.

Introduction

Snake venoms not only represent rich sources of biologically active peptides and proteins but also serve as versatile platforms for the discovery and development of drug lead substances [1].

Competing Interests: The authors have declared that no competing interests exist.

Significant progress in the investigations of snake venoms has recently been witnessed by different proteomics studies in this field. The combined transcriptome and proteome analysis of the venom of *Cerberus rynchops*, for example, revealed a very low complexity venom composition and a novel snake venom protein family called veficolins; function of veficolins has been hypothesized to be related to the inhibition of platelet aggregation [2]. Likewise, investigations into the venom of the ocellated carpet viper *Echis ocellatus* pointed to a pronounced role of transcriptional and posttranslational mechanisms on determining the final venom composition, as evidenced by a significant divergence between predicted toxin clusters found in the transcriptome and peptide sequences identified in the corresponding venom proteome [3]. A comparative proteome analysis of the venoms of terrestrial *Toxicocalamus longissimus* and a closely related marine species *Hydrophis cyanocinctus* indicates a pronounced reduction of the molecular diversity of the venom components of the marine snake as compared to the venom proteome of its terrestrial relative [4]. The authors reason that molecular economy of the toxin arsenal has been implemented as an evolutionary response to selective pressures from different environmental challenges. To predict possible structure function relationships of the various proteins of the corresponding venom, a complete picture of the sequences of the different protein families and their isoforms is of major importance. Extensive sequence coverage of the venom proteome can be accomplished using a combined approach of electrospray and MALDI ionization mass spectrometry. In the present study, we have used this approach to characterize the venom proteome of the pitviper *Sistrurus catenatus edwardsii* (Desert Massasauga Rattlesnake), a subspecies of *Sistrurus catenatus*, which is primarily encountered in dry and desert grasslands of the southwestern North American prairies [5, 6]. A comparative study of the venom proteomes of four different *Sistrurus* taxa has revealed an overview of the different protein families of the corresponding venoms, as evidenced by BLAST analysis of the detected sequences [7]. The transcriptome of the venom gland of *S. c. edwardsii* has also been characterized and serves as an exhaustive source for protein sequence investigations of the venom proteome [8]. Based on the identification of unique peptides of the corresponding proteins we were able to distinguish ten out of eleven predicted isoforms of serine proteinases and all five predicted metalloproteinase isoforms, together with a disintegrin. We also encountered the snake venom protein families C-type lectin, cysteine rich secretory protein, nerve growth factor, phospholipase A₂, bradykinin-potentiating protein, and L-amino acid oxidase, previously described in the transcriptome of *S. c. edwardsii*. In addition, our analysis revealed the presence of snake venom protein families not detected in the venom gland transcriptome or previous studies, including glutaminyl cyclase, renin-like aspartic protease, and ecto-5'-nucleotidase. These results support the view that an in-depth analysis of the venom proteome is complementary to transcriptomic venom gland studies and will improve our understanding of the interplay of the different venom proteins on the target prey.

Materials and Methods

Venom extraction and Ethics statement

Specimens of *Sistrurus catenatus edwardsii* (Desert Massasauga) were collected in Lincoln County, Colorado, USA under permits granted by the Colorado Division of Wildlife to Stephen P. Mackessy (permits #0456, 06HP456). Venom was extracted manually [9] from 4 adult snakes from the same metapopulation in southeastern Colorado; venoms were pooled, centrifuged and lyophilized. Snakes were then PIT-tagged, returned to the exact locality and released. All procedures were permitted by the University of Northern Colorado Institutional Animal Care and Use Committee as detailed in UNC IACUC protocol #0702. No animals were sacrificed and no suffering of animals occurred during this study.

Tryptic digestion of the venom

Lyophilized crude venom (600 µg) was initially dissolved in 600 µl of ammonium bicarbonate (50 mM) and precipitated with three volumes of ice-cold acetone for 3 h at -20°C. In the following, the sample was spun down at 14000 rpm for 10 min and the pellet brought up in 560 µl of ammonium bicarbonate (50 mM). Afterwards, 12 µl of 1% ProteaseMAX (in 50 mM ammonium bicarbonate) surfactant was added to the sample solution followed by reduction with 8 µl of 0.5 M DTT (at 56°C for 20 min) and alkylation with 16 µl of 0.55 M iodoacetamide at room temperature for 30 min in the dark. Finally, the venom was subjected to digestion (at 37°C for 12 h) by adding 10 µl of trypsin (1 µg/µl in 50 mM acetic acid) and 6 µl of 1% ProteaseMAX surfactant to enhance the enzymatic performance of trypsin.

Chromatography and mass spectrometry

The tryptic digest was separated on a C-18 reversed phase column (Agilent Zorbax 300SB-C18 1.0 x 150 mm x 3.5 µm) by running a linear gradient from 0% acetonitrile to 72% acetonitrile in 120 min applying a flow rate of 40 µl/min (solvent A contains H₂O / 0.1% TFA, solvent B contains 80% ACN / 0.1% TFA) on a LC Packings Ultimate HPLC system (Dionex, Vernon Hills, IL). During the chromatographic run approximately 130 fractions were manually collected in Eppendorf vials. After reducing the volume in each vial to approximately 1 µl on a Speedvac (Savant SC 110A), samples were spotted onto the MALDI sample plate. Approximately 0.3 µl of the sample solution was mixed with the same volume of a saturated matrix solution (α -cyano-4-hydroxycinnamic acid, (Aldrich, Milwaukee, WI) 10 mg/ml in 50% acetonitrile/0.1% trifluoroacetic acid) on the target plate and allowed to dry at room temperature (dried-droplet method). Raw data for protein identification were obtained on a AB Sciex 5800 (AB Sciex, Foster City, CA) and a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Typically, 1600 shots were accumulated for spectra in MS mode while 3500 shots were accumulated for spectra in MS/MS mode. Up to twenty of the most intense ion signals with a signal to noise ratio above 2 were selected as precursors for MS/MS acquisition excluding common trypsin autolysis and keratin peaks. External calibration in MS mode was performed using a mixture of six singly charged peptides: des-Arg1-Bradykinin ($m/z = 904.468$), angiotensin I ($m/z = 1296.685$), Glu1-fibrinopeptide B ($m/z = 1570.677$), ACTH (1–17 clip) ($m/z = 2093.087$), ACTH (18–39 clip) ($m/z = 2465.199$), and ACTH (7–38 clip) ($m/z = 3,657.929$). MS/MS spectra were externally calibrated using known fragment ion masses observed in the tandem mass spectrum of Glu1-fibrinopeptide B.

Tryptic peptides were also separated on an Easy nLC II (Thermo Scientific) nanoflow HPLC system connected to an LTQ-Orbitrap XL mass spectrometer (Thermo, Bremen, Germany) equipped with a nanoelectrospray ion source. Peptides were initially loaded onto a trap column (100 µm x 2 cm) packed in-house with C₁₈ resin (5 µm, 100 Å pore, Magic C₁₈ AQ, Bruker-Michrom, Auburn, CA) and separated on an RP HPLC column (C₁₈, 75 µm x 30 cm) using a linear gradient from 98% solvent A (H₂O, 0.1% formic acid) to 60% solvent B (ACN, 0.1% formic acid) over 162 min. Precursor scans were performed in the Orbitrap mass detector at a resolution of 60,000 in the mass range of 300 m/z to 1700 m/z , while MS/MS scans were acquired in the linear trap (“high-low”). With an exclusion of singly charged ions, up to ten of the most intense precursor ions were subjected to product ion scans using CID with a normalized collision energy of 35%. Moreover, MS/MS scans were only triggered for precursor ions having a minimum signal threshold of 10,000 counts. Precursors that were selected for MS/MS scans were dynamically excluded for 30 sec from a repeated product ion scan within a ± 10 ppm mass error. Different HPLC separations were performed where the fragmentation of precursor ions was induced using CID only as well as an approach of alternating CID and ETD, in which successively the same precursor ion was fragmented by CID and ETD.

Data analysis

Database searches of the mass spectra acquired on the MALDI mass spectrometers were searched against all entries of NCBIInr (www.ncbi.nlm.nih.gov/index.html) using the Mascot software (www.matrixscience.com) and against an in-house created snake venom database using Mascot (Mascot, version 2.1). The following search parameters were used: No restrictions on species of origin or protein molecular weight, semi-tryptic cleavage products, two tryptic missed cleavages allowed, variable modifications of cysteine (carbamidomethylation) and methionine (oxidation), and pyroglutamate formation at N-terminal glutamine of peptides.

Electrospray data were analyzed using the Peaks (Peaks Studio 5.3) and ProLuCID [10] search engines, respectively, against an in-house created snake venom database (2580 entries). The search parameters of the Peaks search engine were sulfation (serine, threonine), phosphorylation (serine, threonine, and tyrosine), deamidation (glutamine, asparagine), dehydration (serine, threonine) oxidation (methionine, tryptophan, and histidine) and acetylation at N-terminal of peptides, with a maximum number of 3 modifications per peptide allowed. Search parameters of ProLuCID were fixed modification of cysteine (carbamidomethylation), variable modifications of methionine (oxidation), a precursor tolerance of 50 ppm and allowance for semi-tryptic identifications. Peptide spectrum matches obtained by ProLuCID were then validated by the Search Engine Processor with the default parameters previously described [11]. All identified peptides were further manually verified.

Results and Discussion

Envenomation by viperid snakes frequently manifests as a complex medical syndrome dominated by hemorrhagic and inflammatory processes triggered by the combined enzymatic actions of metalloproteinases, serine proteinases and phospholipases A₂ [12–14], as well as by the detrimental effects of C-type lectins (CLP) on platelet function [15]. The snake venom metalloproteinases (SVMP) are classified in four different major groups (PI, PII, PIII, and PIV) based on their final domain composition after posttranslational modification of the corresponding multidomain precursor protein [16, 17]. Functionally, a broad spectrum of biological activities have been attributed to SVMPs, including hemorrhagic, inflammatory and myonecrotic effects [14, 18]. To date, only a few studies have noted the presence of peptides related to the prodomain of SVMPs in the venom [19, 20] and it might be that in most cases the prodomain of the precursor protein is enzymatically removed before its secretion into the venom gland. Only a single (identical) peptide of the prodomain of SVMP isoforms 1 and 3 (Fig 1) was identified, suggesting that the prodomain is proteolytically removed in *S. c. edwardsii* before being exocytosed to the venom gland lumen. The metallo-, disintegrin-, and cysteine rich domains of the four isoforms that belong to the PIII class of metalloproteinases revealed evenly distributed sequence coverage (Fig 1), supporting the view that these domains are efficiently translated. Similarly, we were able to identify tryptic fragments of the predicted [8] PII (isoform 6) and disintegrin of the venom proteome, with sequence coverages of 36% and 71%, respectively (Fig 1). The presence of proteotypic peptides of the corresponding isoforms clearly revealed the existence of these different proteins in the venom proteome (Fig 2). Of further particular note is the presence of protein sequences that went undetected in the transcriptome analysis but could be identified in the venom proteome. These sequences belong to 26 different SVMPs identified as PII and PIIIs and disintegrins of snakes phylogenetically closely related to *S. c. edwardsii* (Table 1). Interestingly, during a recent investigation of the transcriptome and proteome of the cryptic snake *Drysdalia coronoides*, the SVMPs of the venom were initially only identified in the proteome and only the implementation of gene-specific 3'RACE primers of the corresponding signal peptides of the targeted proteins revealed the cDNA sequence [21]. These findings might point to a general difficulty to characterize

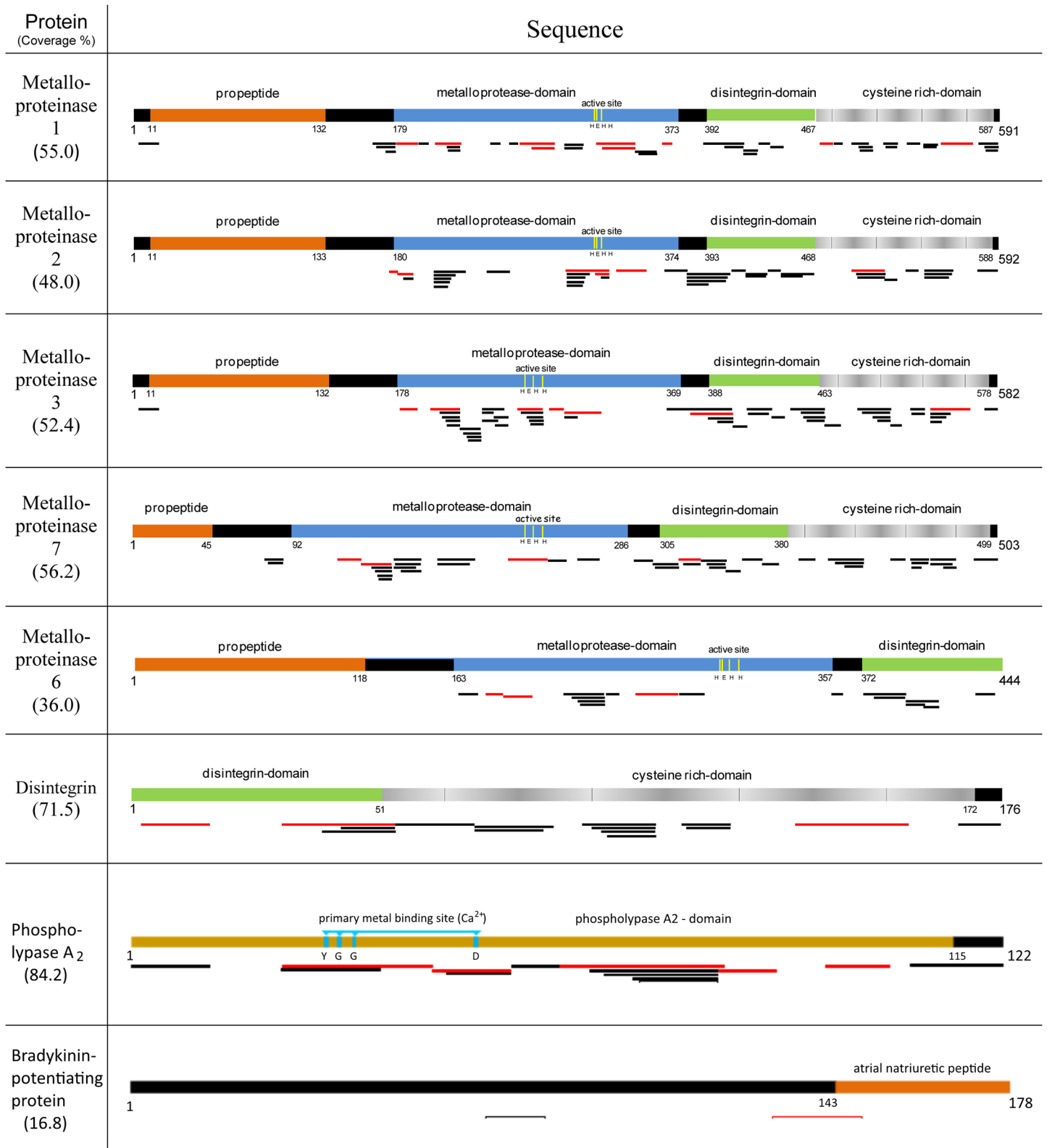


Fig 1. Sequence coverages of some of the predicted venom gland proteins of *S. c. edwardsii* as revealed by the combined approach of MALDI and ESI tandem mass spectrometry. Protein sequences including specific domains are colored by bars; below these, corresponding peptides identified by ESI (black lines) and MALDI (red lines) are indicated.

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6 -----THEKCTALPKGAVQPKYEDAMQYEFK 26
2 MIQVLLVTICLAVFPYQGSSIIILGSGNVNDYEVVYPRKVTALPKGAVQPKYEDALQYELK 60
1 MIQVLLVTICLAAFPYQGSSIIILGSGNVNDYEVVYPRKVTALPKGAAQPKYADAMQYEFK 60
7 -----
5 -----
3 MIEVLLVTICLAVFPYQGSSIIILGSGNVNDYEVVYPRKVTALPKGAVQPNYEDAMQYEFK 60

6 VNGEPVVLHLEKNKGLFSEDYSETHYSPDGREITTYPLVEDHCHYLGRIQNDADSTASLS 86
2 VNGEPVVLHLEKNKGLFSEDYSETHYSPDGRKITTYPSDEHDCYYHGRIQNDADSTASIS 120
1 MNGEPVVLHLEKNKGLFSDYSETHYSPDGRQITTYPMIEDHCYYHGCIQNDADSTASIS 120
7 -----RIENDADSTASIS 13
5 -----
3 VNGESVVLHLEKNKGLFSENYSETHYSPDGREITTYPPAEDHCYYHGRIENDADSTASIS 120

6 ACNGLKGYFK-LQGEMYLIEPLKLPDNEAHAVKYENVEKEDEAPKMGCVTQNWESDKLI 145
2 ACSGLKRTFQAQFQEMYLIEPLKLPDSEAHAVFKYENVEKEDEAPKMGCVTQNWESYEPI 180
1 ACNGLKGFHK-LQGEMYLIEPLKLPDSEAHAVKYENIEKEDEAPKMGCVTQNWESYEPI 179
7 ACNGLQGHFK-LQGEMYLIEPLKLPDSEAHAVFKYENVEKEDEAPKMGCVTQNWESYEPI 72
5 -----
3 ACNGLKGFHK-LQGEMYLIEPLELPDSEAHAVFKYENVEKEDEAPKMGCVTQNWKSYPE 179

6 KKASYLDVTAEQRFQ--RHIELVVVADHRMFTKYNLNTVRIWVHEIVNIINVFYRS 203
2 KKASQLVVTAEQQRHLNFRYIELVIVADYRMTKYNRKLTEVKTWVYEVNTLNEIYRY 240
1 KKASQLNLTPEQQAYLDAKYVEFVVLVLDHGMYYTKYKDDLDKIKTRITYEIVNTLNEIYIP 239
7 KKASQSNLTPEQQRYLNAKKYVFLVLDYVVMYLYGRNLTAVRTRMYDTVNVIIPIYHR 132
5 -----
3 IKKPSHLVVTAEQQLTKRYVELVIVADHGMFMKHN---ETLRTWVQMVNTINEIYIP 235

6 LNIHVSLTDLEIWSNQDQINVQSAADTLEAFGDWRETVLLNRISHDNAQLLTAIELDEE 263
2 LYIRVALVGLIEWSNGDLSNVTLSADDTLDSFGWNRKRDLLRRKSHGNAQLLTAIDFNGT 300
1 LNIRVALVRLIEWSNGDLINVSSAANVTLDLFRNRVTDLLRRKSHDNAQLLTTDFDGD 299
7 MNIHVALVGLIEWSNRDKITVQSSPDVTLDFAKWRATDLLRRKSHDNAQLLTGTFNFGP 192
5 -----
3 LNIRFALVGLIEWSNGDKITVQSSAHNTLHLFGNWRKRVLLKRRKHDNAQLLTSIDFDGR 295

6 TIGLAHVSMCDPKLSTGIVQDHSAINLWVAVTMAHELGHNLGMDHDGNCYCDTDSICM 323
2 TIGLAHVASMCELKRSSTGIVQDHSINLLVAVTMAHEMGNLGRHDKKYCTCGGYSICM 360
1 TVGLAYNRMCQPRGSVGVIQEHSTINLLMAVTMAHEMGNLGMSHDQGCCHCGAPSCIM 359
7 TAGLAYLGGICNTMYSAGIVQDHSKIHLVAVIAMAHEMGNLGMHDHKECTCTGTRPCVM 252
5 -----
3 TVGLAHVSSMCNQLSTGIVQDHSAINLWVAVTMAHELGHNLGMDHDIKYCTCGAPACVM 355

6 SEELSEQLSFEFSDCSLNEYQTYLTDHNPQMLNEPLRTDIVS---GNELLEAGEEBCDC 379
2 SAVLSHQASKYFNSCYNQYWNINFYKPCILNEPLRTDIVSPPVCGNELLEAGEEBCDC 420
1 ADRLSHQPTQFSDCSQKRYLINSRQCILNEPLRTDIVSPPVCGNELLEAGEEBCDC 419
7 AGTLCSEASFLSDCSQKREHREFLIKNMPQCILKPLKTDVVSFAVCGNYFVVEGEEBCDC 312
5 -----
3 GNTLSEQPSYGFNSCYNQYQTFIINYNPQCILNEPLRTDIVSPPVCGNELLEAGEEBCDC 415

6 GSPAN---PCCDAATCKLRPGAQCAEGLCCDQCRFIKKGKICRRARGD- NPDDRCTGQSA 435
2 GSPATCQYQCCNATCKLHSHWVECESEGCCEQCIFKGAGTECRAASSECDLPENCTGHS 480
1 GSPANQCNPCDDAATCKLTPGSQCAKGLCCDQCRFKGAGTECRAAKDDCDMDVCTGRSA 479
7 GSPRTCRDPCDDAATCKLRQGAQCAEGLCCDQCRFKGAGTECRAAKDDCDMDVCTGRSA 432
5 GKPGT-----QCEDECCDRQLRRAGTECRPARSECDIAESCTGQSA 43
3 GSPNTRCRDPCDDATCKQHSWVECESEGCCEQCIFKGAGTECRAARSECDIAESCTGQSA 475

6 DCPNRNRFHA----- 444
2 DCPIDLFTDQGPLNNGFYCYNGNCPILYHQCYALFGPNAAEQDSCFDDNRI GNYYGY 540
1 ECPTDRFQRNGHPLNNKGYCYNRTCPMTKNCISFFGSPSATVAKDSCFKTNQK GSSYGY 539
7 EC-IDSFQRNGQPCNNNGYCYNGKCPIMADQCIALFGPGATVSDACQFNRQGNHYGY 431
5 DCPDDEQRNGQPCLNNGYCYNGKCPILDHQCISFFGSSAAVFPDACEFNLYLQGGDFY 103
3 DCPDDDLHRNGQPCLNNGYCYNGMCPIMDHQCIALYGADATVAPDADFNLKQDNFY 535

6 -----
2 CRKENGRKIPCAPEVDKCGRLYCSYNFNGNQLPCVPIYTRDENMGMVEPGTKCGDGKVC 600
1 CRKENGTKIPCEPEDEKCGRLFCFPNPKPKKNICNVIYTPDEIDGMVLGPKCGGRGKVC 599
7 CRKEQNTKIACEPQDVKCGRLYCFNPNPETKPNFCNIYSPNDEDKGMVLPGTKCADGKAC 491
5 CRRENGRI FFCAPQDVKCGRLFCVMPGTGHTISCSQSTYSQNDLDIGMVDLGTCKCDGKVC 163
3 CRREKARIFFCAPQDVKCGRLFCFEG---HYSQYQYS-GDPDFGMVDHGTCKCADGKVC 590

6 -----
2 S-NGQCVDVNIAY 612
1 S-NGHCVDTATAY 611
7 S-NGQCVDVTPY 503
5 NSNRQCVDVNTAY 176
3 K-NRQCVDVTTY 602

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Fig 2. Sequence alignment of the metalloproteinases of the venom of *S. c. edwardsii*. Different colors indicate the unique peptides identified by tandem mass spectrometry of the corresponding proteins.

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the relatively large SVMP sequences fully in the transcriptome of the venom gland without the use of amplification techniques. However, it is worth mentioning that a recent in-depth transcriptome analysis of the venom of the eastern diamond rattlesnake *Crotalus adamanteus* produced full-lengths SVMP sequences by using next-generation sequencing including the Illumina technology [22]. The high abundance of metalloproteinases in the venom of *S. c. edwardsii* is in line with a previous transcriptome analysis [8] and point to an explicit role of accelerated evolution on the development of distinct metalloproteinase isoforms [23].

Snake venom serine proteinases (SVSPs) primarily affect the hemostatic system of prey organisms and often show fibrinogenolytic and fibrinolytic activities. Since this is similar to the action of thrombin on fibrinogen, SVSPs have also been known as thrombin-like enzymes (TLEs) [24, 25]. In addition, SVSPs also act on kininogen and platelet receptors [26]. While most SVSPs exist as monomers, dimeric forms have been detected in the venom of the viper *A. b. brevicaudus* [27]. One of these is brevinase, a heterodimeric enzyme with a covalent disulfide link between the two monomers. The analysis of the transcriptome of the venom gland of *S. c. edwardsii* predicted the presence of eleven SVSP-specific isoforms. Previous studies on the protein coding region of SVSPs in pitvipers have noted a trend towards accelerated evolution of this protein family, a result which is also found in the venom of *S. c. edwardsii*, as evidenced by the ratio (0.99) between non-synonymous and synonymous substitutions of the exon sequences of the mRNA transcripts [8]. Such sequence variations are likely related to different pharmacological activities. The combined electrospray and MALDI ionization analysis of the proteome resulted in the identification of ten out of eleven predicted SVSP isoforms, as evidenced by the detection of unique peptides (Fig 3) with a sequence coverage between 35% (isoform 8) and 82% (isoform 1). Again, we observed the presence of additional peptides that match sequences of SVSPs from related snake venoms (*e. g. C. adamanteus*) and that were not identified in the transcriptome analysis of *S. c. edwardsii* (Table 1). Taken together, these additional ten identified isoforms raises the total number of isoforms of SVSPs to a total number to twenty, reinforcing the idea that this protein family has evolved in an accelerated manner, producing an elevated number of isoforms.

Phospholipase A₂ (PLA₂s) are functionally characterized by their multiple pharmacological activities such as cardiotoxic, neurotoxic, myotoxic, antiplatelet and anticoagulant effects [28, 29]. They enzymatically cleave the second ester bond of the glycerol ester and represent one of the most extensively studied snake venom families. The venom proteome revealed the presence of only one PLA₂ protein (Fig 1), consistent with the transcriptome analysis of the venom gland [8]. However, an additional single peptide that showed sequence identity to a PLA₂ from *S. c. tergeminus* (Table 1) was also found. It appears that, contrary to other species, where PLA₂s are present in multiple isoforms, the venom of *S. c. edwardsii* was not under evolutionary pressures selecting for the evolution of a pronounced diversity of PLA₂s.

The cysteine-rich-secretory proteins (CRISP) are widely distributed in snake venoms, particularly in Viperidae and Elapidae. The biological activity of some is related to the inhibition of the cyclic nucleotide-gated ion channels as well as L-type Ca²⁺ and BK_{Ca} K⁺ channels [30]. For example, triflin and ablomin (from the pitviper *Gloydius blomhoffii*) block L-type Ca²⁺ channels that lead to contraction of smooth muscle [31, 32]. However, scientists are only beginning to understand the full scope of biological and pharmacological effects of this protein family. We identified the predicted CRISP protein in the venom of *S. c. edwardsii* (60% coverage) along with peptides that match part of the CRISP protein Catrin (gi 28972959) from *C. atrox*

Table 1. Identification of proteins of the venom of *S. c. edwardsii* not predicted from the transcriptome analysis.

Protein family	Taxonomy	gi	Sequence	charge	m/z	score
PI-Metalloproteinase	Agkistrodon contortrix laticinctus	1098019	QWVHQIVNTINEIYR	2	958	34.93
PII-Metalloproteinase	Bothrops jararaca	123911605	LTTGSQCAEGLCCDQCK	1	1987.75	130
			DSCSCGANSCIMSATVSNEPSSR	1	2476.95	154
PII-Metalloproteinase	Echis ocelatus	320579329	YVQLVIVADHSMVTK	1	1703.83	24.89
PII-Metalloproteinase	Crotalus adamanteus	338855314	YHFVANR	1	906.46	53
			NSVGIVQDHR	1	1124.59	48
PII-Metalloproteinase	Glodius saxatilis	31322301	YNSNLDTIR	2	548.28	54.37
PII-Metalloproteinase	Crotalos atrox	258618062	VALIGLEIWSSGELSK	1	1702.97	0.65
			YYTEVGEDCDGPPANCQNPCCDAATCK	1	3312.36	80
PIII-metalloproteinase	Botrox atrox	205278807	SVGIVQDHGK	2	347.19	65.47
			HELGHNLGMDHDR	1	1546.71	94
			SYQFSDCSQNDHLR	1	1757.76	0.53
			YLISHTPQCILNEPLR	2	977.53	75.23
PIII-metalloproteinase	Trimeresurus gramineus	172044536	AQCGEGLCCDQCR	2	1613.61	0.32
			SCAGQSADCPTDDFHR	2	912.37	73.56
			AGEDCDGSPANPCCDAATCK	1	2258.73	32.37
PIII-Metalloprotease	Glodius halys	4106007	AAGDTLEAFGDWR	2	704.84	63.91
			LRPGQQCAEGLCCDQCR	1	2107.9	0.54
PIII-Metalloprotease	Agkistrodon psicivorus leucostoma	258618068	YLIDNRPPCILNK	2	808.45	41.89
			NLQGQGNFYCR	1	1356.66	46
PIII-Metalloproteinase	Atractaspis microlepidota andersoni	6007789	DTLDSFEEWR	2	649.3	52.74
PIII-metalloprotease	Bothrops jararaca	209870468	HDNAQLLTAIDFN _{GR}	2	843.43	49.11
PIII-metalloprotease	Viridovipera stejnegeri	123900232	QLLTAIDFDGPTIGR	2	808.95	66.65
			LHSWVECESGECCEQCR	1	2226.88	0.61
PIII-Metalloproteinase	Trimeresurus flavoviridis	82217336	QGNYYGYC _R	1	1222.5	30.19
PIII-Metalloproteinase	Bothrops jararacussu	123889624	YSEDLDFGMVDHG _{TK}	1	1729.7	36.49
PIII-Metalloproteinase	Echis coloratus	297593938	VTLNSFG _{EW} R		1208.58	30.30
PIII-Metalloproteinase	Echis carinatus sochureki	297593788	LHSWVECESGEC _{CDQCK}		2183.82	38.67
PIII-Metalloproteinase	Bothrops jararaca	82219706	SEC _{DIAESCTGQSAD} CPTDDFKR	1	2649.01	214
PIII-Metalloproteinase	Crotalus atrox	75570463	SEC _{DIAESCTGQSAD} CPTDDFHR	1	2658.05	185
PIII-Metalloproteinase	Crotalus adamanteus	338855314	YEGDKTEI _{CSR}	1	1357.58	32
			MAHELGHNLGIDHDR	1	1714.76	51
PIII-Metalloproteinase	Trimeresurus flavoviridis	344925813	HSVGVQDHGK	1	1176.59	44
PIII-Metalloproteinase	Crotalus adamanteus	338855316	LDVMVAVTMAHELAH	1	1636.80	125
PIII-Metalloproteinase	Crotalus adamanteus	338855326	YSELDLYGMVDHG _{TK}	1	1729.73	114
			LFCKFNFP _{CQYK}	1	1766.79	72
			LHSWVECESGEC _{CEQCK}	1	2197.83	108
PIII-Metalloproteinase	Crotalus adamanteus	338855330	PKC _{ILNEPLR}	1	1239.65	64
			TDIISPPVCGNELLEAGEE _{CDGSPR}	1	2875.28	120
			CTGQSAE _{CPTDDFHR}	2	890.86	57.51
disintegrin	Gloydus shedaoensis	91680863	YFVEVGEE _{CDGLPAHC}	1	2041.84	0.54
			SEC _{DIAESCTGQSAE} CPTDDFHR	1	2672.07	185
disintegrin	Crotalus atrox	327507705	GDW _{NDTCTGQSAD} CPR	1	1954.75	131
Serine proteinase	Crotalus adamanteus	338855332	AAYPEFGLPATSR	2	690.36	66.03
Serine proteinase	Bothrops jararaca	82233395	LDSPVSDSEHIAPL	2	740.38	42.25

(Continued)

Table 1. (Continued)

Protein family	Taxonomy	gi	Sequence	charge	m/z	score
Serine proteinase	<i>Viridovipera stejnegeri</i>	82242793	IIGGDEÇNIDEHR	2	764.36	56.68
C-type lectin	<i>Sistrurus miliarius</i>	21530567	GLQQGTNYHK	2	382.53	72.72
			FCSEQAEGGHLVSISSSEEA	1	2239	0.54
			WSDGSSVSYENWIEAESK	1	2073.83	125
C-type lectin	<i>Sistrurus miliarius</i>	21530570	DCPSGWSSYDQHCYR	1	1917.74	118
			YD VWIGLR	2	511.28	60.77
			WSDGSSVNYENLIK	2	806.4	75.31
C-type lectin	<i>Sistrurus miliarius</i>	21530564	DFDCPSDWYAYDQYCYR	1	2323.83	144
			FTSMWIGLK	1	1082.57	64
			LASHSSEEEAFV GK	1	1603.81	0.52
C-type lectin	<i>Sistrurus miliarius</i>	21530573	TWDDAESFCYQHR	1	1815.69	95
			QNQYYVWIGLR	1	1439.75	53
			ETEFLQWYNTDCEEK	1	1991.88	84
C-type lectin	<i>Crotalus adamanteus</i>	338855278	YEDWAEESYCVYFK	1	1888.79	92
C-type lectin	<i>Crotalus durissus terrificus</i>	82129809	WSDGSSVNYENLLK	2	806.40	75.31
			QNKYVWIGLR	1	1439.75	46
			ETEFLQWYNTDCEEK	1	1991.86	75
L-amino acid oxidase	<i>Bothrops neuwiedi pauloensis</i>	195927838	GNPLEECFR	2	561.26	59.52
			NGLSATS NPK	2	495.25	45.52
L-amino acid oxidase	<i>Demansia vestigiata</i>	118151720	YPVKPSEK	2	474.27	42.82
L-amino acid oxidase	<i>Viridovipera stejnegeri</i>	34014953	LSAAVVLGAGHEVTLEASER	1	2244.2	0.56
L-amino-acid oxidase	<i>Naja kaouthia</i>	124015192	QNDYEEFLEIAK	2	749.86	59.53
L-amino-acid oxidase	<i>Crotalus atrox</i>	124106294	TPYQFQHFSEALTAPFK	1	2012.03	80
CRISP	<i>Crotalus atrox</i>	28972959	EDKYTNCK	1	1057.43	53
			SLVQQAGCQDK	2	617.31	75.45
			MEWYPEAANAER	1	1537.68	105
			SGPPCGDCPSACDNLCTNPCTK	1	2525.08	152
CRISP	<i>Vipera nikolskii</i>	215262114	GNVDFDES PR	1	1263.55	74
Venom nerve growth factor	<i>Bothrops asper</i>	186659795	NPNPVPTGCR	2	556.28	52.27
Venom nerve growth factor	<i>Cryptophis nigrescens</i>	123907150	HWNSYCTTTQTFVK	1	1773.81	96
Phospholipase A2	<i>Sistrurus catenatus tergeminus</i>	45934756	LDTYTYSEENGEIICGGDDPCKK	1	2664.18	124

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[33]. Both proteins share about 87% sequence identity, with pronounced variations located primarily at the C-terminus. In addition, a peptide that matches a CRISP protein from another viperid species was also encountered.

The snake C-type lectin or C-type lectin-like protein families (snaclecs [34]) usually form disulfide linked homo- or hetero-dimers which are organized in oligomers to form larger quaternary protein complexes [35]. They affect the haemostatic system by interfering with coagulation factors or platelet activation [15]. The analysis of the proteome of *S. c. edwardsii* led to the identification of three isoforms of C-type lectins, as evidenced by the detection of sequence-specific unique peptides with sequence coverages between 67% (isoform 1) and 24% (isoform 3). Interestingly, however, the presence of peptide sequences identical to those of six C-type lectin proteins from the closely related rattlesnakes *Sistrurus miliarius*, *Crotalus adamanteus*, and *Crotalus terrificus* were also noted (Table 1). This brings the number of C-type

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5 -----MVLIRVLANLLILQLSYAQKSSELVIGGDECNINEHRSLALVYI 44
9 -----MVLIRVLANLLILQLSYAQKSSELIIGGEECIINEHRFLVALYS 44
4 -----MVLIRVLANLLILQLSYAQKSSELVVGGECCNINEHRSLVFFN 44
8 -----MVLIRVLATLLILQLSYAQKSSELVVGGRPCNINEHRSLVLFN 44
3 -----MVLIRVLANLLILQLSYAQKSSELVIGGDECNVNEHRSLVAIFN 44
1 -----MVLIRVLANLLILQLSYAQKSSELVVGGECCNINEHRFLVALYH 44
2 -----MVLIRVLANLLILQLSYAQKSSELIIGGDECNINEHRFLVALYS 44
11 -----MVLIRVLANLLILQLSYAQKSSELVVGGECCNINEHRSLVLVYS 44
6 -----SSELVIGGDECNINEHRFLVALYH 24
10 -----MVLIRVLANLLILQLSYAQKSSELIIGGEECNINEHRFLVALYH 44
7 MVSQVKANNLLHRIEAMALIRLLVNLILQLSYAQKSSELVIGGDECNVNEHRFLALLYS 60

5 TNG--FLCGGTLINQEWVLTAAHCDRGNMLIFFGVHRLKGLNKDAQTRVAKEKFCPNRK 102
9 FKSKRFHCSGTLINQEWVLTAAHCNRKNIIRIKLGHSTKVTNEDVQTRVPKPKKFFCLSSK 104
4 STNF--LCAGTLINQEWVLTAAHCDSKNFQMLQGVHSHKVLNEDEQTRDPKEKFCPNRK 102
8 SSGF--LCAGTLINQEWVLTAAHCDIKNFQIQLVHSHKVRNEDEQTRDPREKFFCLGSK 102
3 STGF--FCAGTLINQEWVLTAAHCDSTNFQMKLVHSHKVSNEDEQTRNPKEKFFCPD-K 101
1 SRSKTFLCGGTLNNEEWVLTAAHCDRRNMQIKLGMHSTVENEDEQTRVPKEKFFCLSSK 104
2 DGNQ---CGGTLINQEWVLTAAHCEGNMKIHLGMHSHKVPNKDKQTRVPKEKFFCVSSK 101
11 DGIQ---CGGTLINQEWVLTAAHCDGKRMKLVQFGLHSHKVPNKDKQTRVPKEKFFCLSSK 101
6 SRSKTFLCGGTLNNEEWVLTAAHCNRVFLYIRLGMHNKNVNFDDQRRYPKPKKYFFRCHN 84
10 SRSKTFLCGGTLNNEEWVLTAAHCNRVFMYIRLGMHNKNVIFDDDHIRYAKEKYFFRCRN 104
7 DK---FQCGGTLINQEWVLTAAHCNRHMYIFLGVHNSVQYDDEQRRFPKPKKYFCLSSR 117

5 KNDEKDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGATSPNVTLPGVPH 162
9 TYTRWDDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGATSPNVTLPGVPH 164
4 KDDKDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGINSPTKETYPDVP 162
8 TNNEWKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 162
3 KDDVLDKDIMLIKLDSPVNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 161
1 NYTLWDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGRISPNKETYPDVP 164
2 NYTFWDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 161
11 NNKEWKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 161
6 NFKTRDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 144
10 NFKTRDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 164
7 NYTKGDKDIMLIRLDRPVSNSSTHIAPISLPSNPPSVGVSCHIMGWGTSSTKEIYPNVP 177

5 CANINILDHEVCQAAYPS--LTATSRTLCAILEGGKSSCDGDSGGPLICNGEIQQIVSW 220
9 CANINILDHEVCQAAYPS--LTATSRTLCAILEGGKSSCDGDSGGPLICNGEQFQIVSW 222
4 CANINILHHEVCQAAYPS--LTATSGTLCAILEGGKDACGGDSGGPLICNGEIQQIVSG 220
8 CANINILDYAVCRAAAYPW--WPVTTRILCAILEGGKSDCGDSDGGPLICNGEIQQIVSW 220
3 CANINILDDAVCQPPYPE--LPATSRRLCAILEGGKDTCCGDSGGPLICNGEQFQIVFY 219
1 CANINILNVEVCVAAYPEYMLPATSRRLCAILEGGKSDCKGDSGGPLICNGEQFQIVSW 224
2 CVNINILNYSVCRAAAYPEYGLPATSRRLCAILEGGKDTCCGDSGGPLICNGEQFQIVSW 221
11 CANINILDQAVCREIYPG--LLEKSRVLCAGILEGGKDTCCGDSGGPLICNGEIQQIVSW 219
6 CANINILLDYEVCRAAHPQFLPATSRRLCAILEGGKDTCCGDSGGPLICNGEQFQIVSW 204
10 CANINILLDYEVCRAAYS--SLPATSRVLCAGILEGGKDTCCGDSGGPLICNGEQFQIVSW 222
7 CANINILDYEVCRAAAYG--GSPATSRRLCAILEGGKSDCGDSDGGPLICNGEQFQIVSW 235

5 GGDICAQPREPGVYTKVFDYTDWIQNI IAGNTDATCPP 258
9 GPDTCQAQPKPSLYTKVFDHLDWIQSI IAGNKTVCNCP 260
4 GEHPCGQVLTGPFYTTVFDYIDWIQSI IAGNTDATCPP 258
8 GAHPCGRRLNPGFYTKVFDYIDWIQSI IAGNTTVCNCP 258
3 GAHPCGQALKPGVYTKVFDYNDWIQSI IAGNTAATCPP 257
1 GDDPCGYVLPALYTKVFDHLDWIQSI IAGNTDATCPP 262
2 GSPNCGYVREPGLYTKVFDHLDWIQSI IAGNTDATCPP 259
11 GGDPCAQSHVPALYIKVFDYTEWIQSI IAGNTAATCPP 257
6 GDGSCAQPKPALYSKVFHLDWIQNI IAGNTTVCNCP 242
10 GDGSCAQPKPALYSKVFHLDWIQNI IAGNTTVCNCP 260
7 GADTCAQPRAPGFYTNVFDYTDWIQSI IAGNTDATCPP 273

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Fig 3. Sequence alignment of the serine proteinases of the venom of *S. c. edwardsii*. Different colors indicate the unique peptides identified by tandem mass spectrometry of the corresponding proteins.

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lectins in the venom of *S. c. edwardsii* to a total of nine isoforms and might indicate a prominent role of this protein family to the envenomation of prey by *S. c. edwardsii*.

Snake venom L-amino acid oxidases (SV-LAAOs) catalyze the oxidative deamidation of amino acids and, besides effects on platelet aggregation, may induce apoptosis in prey [36]. We identified the predicted SV-LAAO (65% coverage) from *S. c. edwardsii* and also found five additional SV-LAAOs with sequence identities related to viperid and elapid snakes; an investigation of the biological functions of these isoforms in the venom could illuminate a broader role for SV-LAAOs in envenomation.

Bradykinin-potentiating peptides (BPPs) inhibit the activity of angiotensin I-converting enzyme (ACE) by repressing both the generation of the hypertensive peptide angiotensin II as well as the degradation of the hypotensive peptide bradykinin [37]. The result of these synergistic actions is a significantly reduced blood pressure in envenomated animals [38]. The transcriptome investigation of *S. c. edwardsii* revealed only one singleton (transcript abundance 0.28%) encoding for BPPs. This low abundance is in line with the modest sequence coverage (16.8%, Fig 1) [8] obtained via proteome analysis and it appears that contrary to other pitvipers such as *Bothrops* and *Lachesis*, BPPs play a minor role in envenomation by *S. c. edwardsii*.

Vascular endothelial growth factors from snake venoms (VEGF-F) bind specifically the kinase insert domain-containing receptor (KDR) and thereby induce low blood pressure as well as proliferation of vascular endothelial cells [39, 40]. In the venom of *S. c. edwardsii* we identified the predicted VEGF-F together with two peptides that showed homology to VEGF-Fs from the viper *B. asper* (Central America) and the elapid *C. nigrescens* (eastern small-eyed snake; coastal eastern states of Australia). Again, there appears to be greater diversity in the proteome of *S. c. edwardsii* than was previously observed.

Several protein families were identified in the current study which were not found in the previous transcriptome analysis of the venom gland of *S. c. edwardsii*. The cyclization of N-terminal glutamine by glutaminyl cyclase (QC) is an important posttranslational process in the modification of a variety of proteins including hormones and cytokines [41], and this modification is found in many venom proteins, including SVMPs and some colubrid three-finger toxins [42]. The formation of pyroglutamine at the N-terminal likely protects proteins from enzymatic degradation and induces conformational changes to improve receptor binding [43]. Recently, glutaminyl cyclase was found in the venom gland of colubrid snakes (*Boiga*) and the authors propose that this modification might lead to increased stability of venom components against exopeptidase degradation and therefore indirectly contributing to venom toxicity [44]. The QC encountered in the venom of *S. c. edwardsii* might have similar functions (Table 2). Interestingly, recent proteomic studies of the venoms of rattlesnakes of the *Crotalus species*, which is related to *S. c. edwardsii*, also revealed the presence of glutaminyl cyclase [45–47].

We also found in the venom proteome three peptides that showed identity to ecto-5'-nucleotidase (5' NT) from *Gloydus blomhoffi* venom. Nucleotidases from different snake venoms have been functionally related to the inhibition of platelet aggregation [48, 49]. A study using mouse and human blood revealed that 5' NT from *Crotalus atrox* inhibits platelet aggregation via the production of increased levels of extracellular adenosine [50].

Renin-like aspartic protease was described for the first time in the venom gland transcriptome of *Echis ocellatus*, a viperid snake found in West Africa [51]. Based on the confident identification of a single peptide we confirm the presence of this enzyme in the proteome of the venom of *S. edwardsii*. To date, there are no further descriptions in the literature on the potential function of this protein, and it would be interesting to investigate the biological implications of this enzyme, especially on venom potency.

Phospholipase B (PLB) cleaves ester linkages from both the sn-1 and sn-2 positions of glycerophospholipids. Recently, a PLB has been identified for the first time in the venom of the

Table 2. Identification of protein families of the venom of *S. c. edwardsii* not predicted from the transcriptome analysis.

Protein family	Taxonomy	gi	Sequence	charge	m/z	score
Ecto-5'-nucleotidase	Gloydius blomhoffi	211926756	SSGNPILLNK	2	521.80	73.51
			ETPVLSNPGPYLEFR	1	1718.83	95
			LTAVLPFGGTFDLLQIK	1	1834.08	0.58
Glutamyl cyclase	Gloydius blomhoffi	15991080	LIFFDGEEAFVR	2	721.88	75.11
			TFSNIISTLNPLAK	2	759.94	69.35
			WSPSDSLYGSR	2	627.8	54.71
			FVLLDLIGAR	2	558.85	52.56
			NTYQIQGIDLFVLLDLIGAR	1	2263.29	0.53
Renin-like aspartic protease	Echis ocellatus	109287598	GFLSQDIVR	1	1034.57	0.49
Phospholipase B	Crotalus adamanteus	338855308	VVPESLFAWER	1	1332.72	74
			HGLEFSYEMAPR	1	1436.66	97
			NGYWPSYNIPIFDK	1	1600.77	67
			HQGLPESYNDFVMTMKPVL	1	2222.07	48

Peptides scores of the different search engines are: Peaks—regular, Mascot—**bold**, and ProLuCID—*italics* letters.

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cryptic snake *Drysdalia coronoides* [21]. While the three dimensional structure of this enzyme have not yet been resolved, it is known to form both monomers and dimers. The peptides of the PLB encountered in the venom of *S. c. edwardsii* show identity to the PLB detected in the transcriptome of the venom gland of the related rattlesnake *C. adamanteus*. The entire protein sequence of the PLB in the venom of *C. adamanteus* shows 553 amino acids, including a 27 residue signal peptide and a 526 amino acid phospholipase B domain [52]. Elucidation of the structure and function(s) of this protein from *S. c. edwardsii* venom may reveal diverse phospholipase subtypes in this venom and help explain the lack of PLA₂ diversity. It is interesting to compare the sequences identified in the venom of *S. c. edwardsii* in the present study with the sequences detected by Edman degradation and *de novo* sequencing of MS/MS spectra in the same venom in the study by Sanz and coworkers. While we were able to match nearly all of the sequences determined by N-terminal sequencing (S3 Table) in the study by Sanz *et al.* [7] to protein snake venom families of *S. c. edwardsii* identified in the present work, peptide sequences inferred by *de novo* sequencing (S3 Table) were more difficult to match. Interestingly, many sequences in the paper by Sanz *et al.* refer to SVMP's (S2 Table), which we were not able identify in the venom of *S. c. edwardsii*, in spite of the fact that both studies utilized venom from the same source population. This might point to venom heterogeneity among this population of snakes occurring in a rather limited area (~1600 hectares).

The relatively high abundance of metalloproteinases in the venom of *S. edwardsii*, which have the ability to cleave extracellular matrix and other structural proteins, indicate that the envenomation of prey is primarily related to hemorrhagic/tissue damaging events rather than myotoxic effects. This conclusion is also supported by the observation that specific small peptide myotoxins, such as myotoxin a from *C. viridis viridis* venom [53], and prominent PLA₂ myotoxins [54], appear to be absent from the venom. Human envenomations by *Sistrurus catenatus* are uncommon; for example, only 9/650 reported snakebites resulted from *S. catenatus* [55]. Similarly, case reports are rare, but the clinical presentation is considered to be similar to *Crotalus* sp. bites, requiring antivenom treatment but typically with less severe outcome [56, 57]. Bites by *S. c. edwardsii* are even less frequent, but because this species has a toxic venom (mouse LD₅₀ = 0.60 µg/g; [58]) which contains abundant serine proteases, coagulopathies including hypofibrinogenemia and thrombocytopenia are to be expected.

Proteome of the venom of *S. c. edwardsii*

The sequence coverages accomplished by mass spectrometry of the different venom proteins range from 16.8% (BPP) to 82% (PLA₂). This distribution is reflected in the corresponding transcriptome analysis of the venom of *S. c. edwardsii*, in which the BPP represents the lowest abundance protein (0.28%) and the PLA₂ the highest abundance protein (28.06%) of the single protein identifications. The SVMPs of the venom of *S. c. edwardsii* reveal moderate sequence coverage of up to 56% that might be related to the fact that the prodomain of the corresponding isoforms is included in the sequence of the mature proteins. However, the prodomain of SVMPs might be proteolytically cleaved *before* secretion into the venom gland lumen [16]. In this case, the sequence of the mature SVMPs would lack the prodomain, hence, the coverage would significantly increase. The differentiation of multiple isoforms of proteins by mass spectrometry is particularly demanding due to pronounced sequence homology, as is the case with SVSPs from the venom of *S. c. edwardsii*. However, we were able to distinguish ten out of eleven predicted isoforms of SVSPs (Fig 2) based on the identification of unique peptides of the corresponding proteins. The complementary use of ESI and MALDI ionization techniques leads to increased sequence coverage of the proteins investigated, compared to the sole application of one of these techniques [59, 60]. Indeed, inspection of the peptides detected revealed the identification of different sets of peptides of the corresponding proteins depending on the ionization technique applied. In some cases, such as the C-type lectin isoforms and the vascular endothelial growth factor, peptides were predominantly identified by MALDI, while other proteins like CRISP and the L-amino acid oxidase were detected by the identification of tryptic peptides ionized primarily by ESI. Different studies have shown that in MALDI experiments, peptides containing arginine residues generated through tryptic digestion are preferentially ionized compared to peptides that carry a lysine residue [61–63]. This has been related to the increased gas phase basicity of arginine compared to lysine. During the course of the manual MS/MS spectra analysis we also noted that rather large peptides (>2500 Da) revealed in many cases improved sequence fragmentation when detected by MALDI-TOF/TOF, compared to the same peptide analyzed by ESI in the linear trap. The use of ETD (electron transfer dissociation) [64] as a fragmentation technique of the tryptic peptides had only a minor impact on the improvement of the sequence coverage of the proteins investigated. However, triply charged precursor ions fragmented by ETD yielded (in some cases) more complete series of product ions and therefore more extensive sequence information when compared with the corresponding CID spectra. It is also important to note that the database search of the MS/MS spectra revealed substantially more positive results when semi-tryptic sequences were considered compared to the fully tryptic approach (dual tryptic termini). However, database searches including no specification of the enzyme did not improve protein identifications. This could possibly be explained as a significant increase in the search space, which ultimately reduces the search engine's sensitivity [65].

Conclusions

The combined approach of electrospray and MALDI mass spectrometry increased the sequence coverage of the predicted protein families (metalloproteinases, serine-proteinases, CRISP, C-type lectin, L-amino acid oxidase, vascular endothelial growth factor, bradykinin-potentiating protein and phospholipase A₂) and their corresponding isoforms when compared to one ionization technique alone. Additionally, this approach also revealed the presence of snake venom protein families (glutaminyl cyclase, renin-like aspartic protease and ecto 5'-nucleotidase) previously not encountered in the transcriptome of the venom gland of *S.c edwardsii*. These results support the use of a dual technical approach toward determining the proteome of

venoms, which have both abundant and rare protein components, in order to obtain a more complete analysis. Our results revealed increased diversity of venom constituents in this venom and provide support for future studies of structure-function relationships of several venom protein family isoforms.

Supporting Information

S1 Table. Sequences of venom proteins from *S. c. edwardsii* identified by tandem mass spectrometry. Search engine color code for the identified peptides: Peaks white, ProLucid grey, and Mascot light blue.

(DOC)

S2 Table. Comparison of the sequences determined by *de novo* sequencing of MS/MS spectra of the venom of *S. edwardsii* (Sanz *et al.*) with the sequences detected in the present study.

(DOC)

S3 Table. Comparison of the sequences determined by Edmann sequencing of the venom of *S. edwardsii* (Sanz *et al.*) with the sequences detected in the present study.

(DOC)

S4 Table. Sequence coverages of some of the predicted venom gland proteins of *S. c. edwardsii* as revealed by the combined approach of MALDI and ESI tandem mass spectrometry. Protein sequences including specific domains are indicated by colored bars; below these, corresponding peptides identified by ESI (black lines) and MALDI (red lines) are indicated (Part 1).

(DOCX)

S5 Table. Sequence coverages of some of the predicted venom gland proteins of *S. c. edwardsii* as revealed by the combined approach of MALDI and ESI tandem mass spectrometry. Protein sequences including specific domains are indicated by colored bars; below these, corresponding peptides identified by ESI (black lines) and MALDI (red lines) are indicated (Part 2).

(DOCX)

Author Contributions

Conceived and designed the experiments: AC RMK JP. Performed the experiments: AC ATF QL MAR. Analyzed the data: AC PCC RV. Contributed reagents/materials/analysis tools: AC JP PCC RMK. Wrote the paper: AC SPM.

References

1. Lukas RJ, Morimoto H, Hanley MR, Bennett EL (1981) Radiolabeled alpha-bungarotoxin derivatives: kinetic interaction with nicotinic acetylcholine receptors. *Biochemistry* 20: 7373–7378. PMID: [7326232](#)
2. OmPraba G, Chapeaurouge A, Doley R, Devi KR, Padmanaban P, et al. (2010) Identification of a novel family of snake venom proteins Veficolins from *Cerberus rynchops* using a venom gland transcriptomics and proteomics approach. *J Proteome Res* 9: 1882–1893. doi: [10.1021/pr901044x](#) PMID: [20158271](#)
3. Wagstaff SC, Sanz L, Juarez P, Harrison RA, Calvete JJ (2009) Combined snake venomomics and venom gland transcriptomic analysis of the ocellated carpet viper, *Echis ocellatus*. *J Proteomics* 71: 609–623. doi: [10.1016/j.jprot.2008.10.003](#) PMID: [19026773](#)
4. Calvete JJ, Ghezellou P, Paiva O, Matainaho T, Ghassempour A, et al. (2012) Snake venomomics of two poorly known *Hydrophiinae*: Comparative proteomics of the venoms of terrestrial *Toxicocalamus*

- longissimus* and marine *Hydrophis cyanocinctus*. J Proteomics 75: 4091–4101. doi: [10.1016/j.jprot.2012.05.026](https://doi.org/10.1016/j.jprot.2012.05.026) PMID: [22643073](https://pubmed.ncbi.nlm.nih.gov/22643073/)
5. Holycross AT, Mackessy SP (2002) Variation in the diet of *Sistrurus catenatus* (Massasauga), with emphasis on *Sistrurus catenatus edwardsii* (Desert Massasauga). J Herpetol 36: 454–464.
 6. Hobert JP, Montgomery CE, Mackessy SP (2004) Natural history of the Massasauga *Sistrurus catenatus edwardsii*, in Southeastern Colorado. Southwest Nat 49: 321–326.
 7. Sanz L, Gibbs HL, Mackessy SP, Calvete JJ (2006) Venom proteomes of closely related *Sistrurus* rattlesnakes with divergent diets. J Proteome Res 5: 2098–2112. PMID: [16944921](https://pubmed.ncbi.nlm.nih.gov/16944921/)
 8. Pahari S, Mackessy SP, Kini RM (2007) The venom gland transcriptome of the Desert Massasauga rattlesnake (*Sistrurus catenatus edwardsii*): towards an understanding of venom composition among advanced snakes (Superfamily *Colubroidea*). BMC Mol Biol 8: 115–132. PMID: [18096037](https://pubmed.ncbi.nlm.nih.gov/18096037/)
 9. Mackessy SP (1988) Venom ontogeny in the pacific rattlesnakes *Crotalus-viridis-helleri* and *Crotalus-iridis-oreganus*. Copeia: 92–101.
 10. Xu T, Venable JD, Park SK, Cociorva D, Lu B, et al. (2006) ProLuCID, a fast and sensitive tandem mass spectra-based protein identification program. Mol Cell Proteomics 5: S174.
 11. Carvalho PC, Fischer JSG, Xu T, Cociorva D, Balbuena TS, et al. (2012) Search engine processor: Filtering and organizing peptide spectrum matches. Proteomics 12: 944–949. doi: [10.1002/pmic.201100529](https://doi.org/10.1002/pmic.201100529) PMID: [22311825](https://pubmed.ncbi.nlm.nih.gov/22311825/)
 12. Gutierrez JM, Rucavado A, Escalante T, Diaz C (2005) Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. Toxicol 45: 997–1011. PMID: [15922771](https://pubmed.ncbi.nlm.nih.gov/15922771/)
 13. Gutierrez JM, Rucavado A (2000) Snake venom metalloproteinases: Their role in the pathogenesis of local tissue damage. Biochimie 82: 841–850. PMID: [11086214](https://pubmed.ncbi.nlm.nih.gov/11086214/)
 14. Teixeira CF, Fernandes CM, Zuiliani JP, Zamuner SF (2005) Inflammatory effects of snake venom metalloproteinases. Mem Inst Oswaldo Cruz 100: 181–184. PMID: [15962120](https://pubmed.ncbi.nlm.nih.gov/15962120/)
 15. Lu Q, Navdaev A, Clemetson JM, Clemetson KJ (2005) Snake venom C-type lectins interacting with platelet receptors. Structure-function relationships and effects on haemostasis. Toxicol 45: 1089–1098. PMID: [15876445](https://pubmed.ncbi.nlm.nih.gov/15876445/)
 16. Fox JW, Serrano SM (2008) Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. Fcbs J 275: 3016–3030. doi: [10.1111/j.1742-4658.2008.06466.x](https://doi.org/10.1111/j.1742-4658.2008.06466.x) PMID: [18479462](https://pubmed.ncbi.nlm.nih.gov/18479462/)
 17. Fox JW, Serrano SMT (2009) Timeline of key events in snake venom metalloproteinase research. J Proteomics 72: 200–209. doi: [10.1016/j.jprot.2009.01.015](https://doi.org/10.1016/j.jprot.2009.01.015) PMID: [19344655](https://pubmed.ncbi.nlm.nih.gov/19344655/)
 18. Takeda S, Takeya H, Iwanaga S (2012) Snake venom metalloproteinases: structure, function and relevance to the mammalian ADAM/ADAMTS family proteins. Biochim Biophys Acta 1824: 164–176. doi: [10.1016/j.bbapap.2011.04.009](https://doi.org/10.1016/j.bbapap.2011.04.009) PMID: [21530690](https://pubmed.ncbi.nlm.nih.gov/21530690/)
 19. Cominetti MR, Ribeiro JU, Fox JW, Selistre-de-Araujo HS (2003) BaG, a new dimeric metalloproteinase/disintegrin from the *Bothrops alternatus* snake venom that interacts with alpha5beta1 integrin. Arch Biochem Biophys 416: 171–179. PMID: [12893294](https://pubmed.ncbi.nlm.nih.gov/12893294/)
 20. Valente RH, Guimaraes PR, Junqueira M, Neves-Ferreira AGC, Soares MR, et al. (2009) *Bothrops insularis* venomomics: A proteomic analysis supported by transcriptomic-generated sequence data. J Proteomics 72: 241–255. doi: [10.1016/j.jprot.2009.01.001](https://doi.org/10.1016/j.jprot.2009.01.001) PMID: [19211044](https://pubmed.ncbi.nlm.nih.gov/19211044/)
 21. Chatrath ST, Chapeaurouge A, Lin Q, Lim TK, Dunstan N, et al. (2011) Identification of novel proteins from the venom of a cryptic snake *Drysdalia coronoides* by a combined transcriptomics and proteomics approach. J Proteome Res 10: 739–750. doi: [10.1021/pr1008916](https://doi.org/10.1021/pr1008916) PMID: [21133350](https://pubmed.ncbi.nlm.nih.gov/21133350/)
 22. Rokyta DR, Lemmon AR, Margres MJ, Aronow K (2012) The venom-gland transcriptome of the eastern diamondback rattlesnake (*Crotalus adamanteus*). BMC Genomics 13.
 23. Juarez P, Comas I, Gonzalez-Candelas F, Calvete JJ (2008) Evolution of snake venom disintegrins by positive Darwinian selection. Mol Bio Evol: 2391–2407.
 24. Markland FS (1998) Snake venoms and the hemostatic system. Toxicol 36: 1749–1800. PMID: [9839663](https://pubmed.ncbi.nlm.nih.gov/9839663/)
 25. Pirkle H (1998) Thrombin-like enzymes from snake venoms: an updated inventory. Thromb Haemost 79: 675–683. PMID: [9531061](https://pubmed.ncbi.nlm.nih.gov/9531061/)
 26. Serrano SM, Maroun RC (2005) Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. Toxicol 45: 1115–1132. PMID: [15922778](https://pubmed.ncbi.nlm.nih.gov/15922778/)
 27. Lee JW, Seu JH, Rhee IK, Jin I, Kawamura Y, et al. (1999) Purification and characterization of brevinase, a heterogeneous two-chain fibrinolytic enzyme from the venom of Korean snake, *Agkistrodon blomhoffii brevicaudus*. Biochem Biophys Res Commun 260: 665–670. PMID: [10403823](https://pubmed.ncbi.nlm.nih.gov/10403823/)

28. Kini RM (1997) In: Kini RM (ed). John Wiley & Sons: Chichester, England p 1–28
29. Kini RM (2006) Anticoagulant proteins from snake venoms: structure, function and mechanism. *Biochem J* 397: 377–387. PMID: [16831131](#)
30. Yamazaki Y, Morita T (2004) Structure and function of snake venom cysteine-rich secretory proteins. *Toxicon* 44: 227–231. PMID: [15302528](#)
31. Shikamoto Y, Suto K, Yamazaki Y, Morita T, Mizuno H (2005) Crystal structure of a CRISP family Ca²⁺-channel blocker derived from snake venom. *J Mol Biol* 350: 735–743. PMID: [15953617](#)
32. Yamazaki Y, Koike H, Sugiyama Y, Motoyoshi K, Wada T, et al. (2002) Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. *Eur J Biochem* 269: 2708–2715. PMID: [12047379](#)
33. Yamazaki Y, Hyodo F, Morita T (2003) Wide distribution of cysteine-rich secretory proteins in snake venoms: isolation and cloning of novel snake venom cysteine-rich secretory proteins. *Arch Biochem Biophys* 412: 133–141. PMID: [12646276](#)
34. Clemetson KJ, Morita T, Kini RM (2009) Classification and nomenclature of snake venom C-type lectins and related proteins. *Toxicon* 54: 83. doi: [10.1016/j.toxicon.2009.04.001](#) PMID: [19362105](#)
35. Hirabayashi J, Kusunoki T, Kasai K (1991) Complete primary structure of a galactose-specific lectin from the venom of the rattlesnake *Crotalus atrox*. Homologies with Ca²⁺-dependent-type lectins. *J Biol Chem* 266: 2320–2326. PMID: [1989986](#)
36. Du XY, Clemetson KJ (2002) Snake venom L-amino acid oxidases. *Toxicon* 40: 659–665. PMID: [12175601](#)
37. Villard E, Soubrier F (1996) Molecular biology and genetics of the angiotensin-I-converting enzyme: potential implications in cardiovascular diseases. *Cardiovasc Res* 32: 999–1007. PMID: [9015402](#)
38. Linz W, Wiemer G, Gohlke P, Unger T, Schölkens BA (1995) Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol Rev* 47: 25–49. PMID: [7784479](#)
39. Yamazaki Y, Takani K, Atoda H, Morita T (2003) Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2). *J Biol Chem* 278: 51985–51988. PMID: [14600159](#)
40. Yamazaki Y, Matsunaga Y, Nakano Y, Morita T (2005) Identification of vascular endothelial growth factor receptor-binding protein in the venom of eastern cottonmouth. A new role of snake venom myotoxic Lys49-phospholipase A2. *J Biol Chem* 280: 29989–29992. PMID: [16014630](#)
41. Huang KF, Liu YL, Cheng WJ, Ko TP, Wang AH (2005) Crystal structures of human glutaminyl cyclase, an enzyme responsible for protein N-terminal pyroglutamate formation. *Proc Natl Acad Sci U S A* 102: 13117–13122. PMID: [16135565](#)
42. Pawlak J, Mackessy SP, Sixberry NM, Stura EA, Le Du MH, et al. (2009) Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB J* 23: 534–545. doi: [10.1096/fj.08-113555](#) PMID: [18952712](#)
43. Hinke S, Pospisilik JA, Demuth HU, Mannhart S, Kühn-Wache K, et al. (2000) Dipeptidyl peptidase IV (DPIV/CD26) degradation of glucagon. Characterization of glucagon degradation products and DPIV-resistant analogs. *J Biol Chem* 275: 3827–3834. PMID: [10660533](#)
44. Pawlak J, Kini RM (2006) Snake venom glutaminyl cyclase. *Toxicon* 48: 278–286. PMID: [16863655](#)
45. Georgieva D, Ohler M, Seifert J, von Bergen M, Arni RK, et al. (2010) Snake venomomics of *Crotalus durissus terrificus*—correlation with pharmacological activities. *J Proteome Res* 9: 2302–2316. doi: [10.1021/pr901042p](#) PMID: [20205475](#)
46. Calvete JJ, Fasoli E, Sanz L, Boschetti E, Righetti PG (2009) Exploring the Venom Proteome of the Western Diamondback Rattlesnake, *Crotalus atrox*, via Snake Venomomics and Combinatorial Peptide Ligand Library Approaches. *J Proteome Res* 8: 3055–3067. doi: [10.1021/pr900249q](#) PMID: [19371136](#)
47. Fox JW, Ma L, Nelson K, Sherman NE, Serrano SMT (2006) Comparison of indirect and direct approaches using ion-trap and Fourier transform ion cyclotron resonance mass spectrometry for exploring viperid venom proteomes. *Toxicon* 47: 700–714. PMID: [16574175](#)
48. Kini RM, Evans HJ (1990) Effects of snake-venom proteins on blood-platelets. *Toxicon* 28: 1387–1422. PMID: [2089737](#)
49. Ouyang C, Huang TF (1983) Inhibition of platelet-aggregation by 5'-nucleotidase purified from *Trimeresurus gramineus* snake-venom. *Toxicon* 21: 491–501. PMID: [6312633](#)
50. Hart ML, Kohler D, Eckle T, Kloor D, Stahl GL, et al. (2008) Direct treatment of mouse or human blood with soluble 5'-nucleotidase inhibits platelet aggregation. *Arterioscler Thromb Vasc Biol* 28: 1477–1483. doi: [10.1161/ATVBAHA.108.169219](#) PMID: [18511695](#)

51. Wagstaff SC, Harrison RA (2006) Venom gland EST analysis of the saw-scaled viper, *Echis ocellatus*, reveals novel alpha9beta1 integrin-binding motifs in venom metalloproteinases and a new group of putative toxins, renin-like aspartic proteases. *Gene* 377: 21–32. PMID: [16713134](#)
52. Rokyta DR, Wray KP, Lemmon AR, Lemmon EM, Caudle SB (2011) A high-throughput venom-gland transcriptome for the Eastern Diamondback Rattlesnake (*Crotalus adamanteus*) and evidence for pervasive positive selection across toxin classes. *Toxicon* 57: 657–671. doi: [10.1016/j.toxicon.2011.01.008](#) PMID: [21255598](#)
53. Fox JW, Elzinga M, Tu AT (1979) Amino-acid sequence and disulfide bond assignment of myotoxin-a isolated from the venom of prairie rattlesnake (*Crotalus-viridis-viridis*). *Biochemistry* 18: 678–684. PMID: [570412](#)
54. Gutierrez JM, Ownby CL, Odell GV (1984) Skeletal-muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops-asper* (Fer-de-lance). *Toxicon* 22: 719–731. PMID: [6523503](#)
55. Russell FE (1980) Snake Venom Poisoning Scholium International, Inc, Great Neck, New York 562 pp.
56. Sing K, Erickson T, Aks S, Rothenberg H, Lipscomb J (1994) Eastern Massasauga rattlesnake envenomations in an urban wilderness. *J Wild Med* 5: 77–87.
57. Barceloux DG (2008) Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals. John Wiley & Sons, Hoboken, New Jersey 1200 pp.
58. Gibbs HL, Mackessy SP (2009) Functional basis of a molecular adaptation: Prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* 53: 672–679. doi: [10.1016/j.toxicon.2009.01.034](#) PMID: [19673082](#)
59. Stapels MD, Barofsky DF (2004) Complementary use of MALDI and ESI for the HPLC-MS/MS analysis of DNA-binding proteins. *Anal Chem* 76: 5423–5430. PMID: [15362902](#)
60. Yang Y, Zhang S, Howe K, Wilson DB, Moser F, et al. (2007) A comparison of nLC-ESI-MS/MS and nLC-MALDI-MS/MS for GeLC-based protein identification and iTRAQ-based shotgun quantitative proteomics. *J Biomol Tech* 4: 226–237. PMID: [17916795](#)
61. Brancia FL, Oliver SG, Gaskell SJ (2000) Improved matrix-assisted laser desorption/ionization mass spectrometric analysis of tryptic hydrolysates of proteins following guanidination of lysine containing peptides. *Rapid Commun Mass Spectrom* 14: 2070–2073. PMID: [11085420](#)
62. Beardsley RL, Karty JA, Reilly JP (2000) Enhancing the intensities of lysine-terminated tryptic peptide ions in matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* 14: 2147–2153. PMID: [11114023](#)
63. Carabetta VJ, Li T, Shakya A, Greco TM, Cristea IM (2010) Integrating Lys-N Proteolysis and N-Terminal Guanidination for Improved Fragmentation and Relative Quantification of Singly-Charged Ions. *J Am Soc Mass Spectrom* 21: 1050–1060. doi: [10.1016/j.jasms.2010.02.004](#) PMID: [20207164](#)
64. Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc Natl Acad Sci USA* 101: 9528–9533. PMID: [15210983](#)
65. Borges D, Perez-Riverol Y, Nogueira FCS, Domont GB, Noda J, et al. (2013) Effectively addressing complex proteomic search spaces with peptide spectrum matching. *Bioinformatics* 29: 1343–1344. doi: [10.1093/bioinformatics/btt106](#) PMID: [23446294](#)