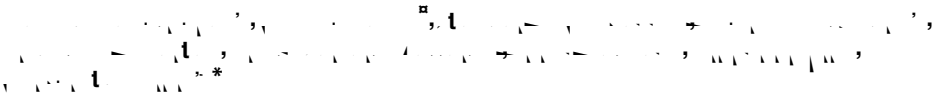
RESEARCH ARTICLE

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

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 *C b ,* , 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 *E a ,* 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 *T a a ,* , and a closely related marine species *H a ,* 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 *S , , a a , a* (Desert Massasauga Rattlesnake), a subspecies of *S , , a a ,* , 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 *S , ,* 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. . a* 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 A_2, bradykinin-potentiating protein, and L-amino acid oxidase, previously described in the transcriptome of *S. . a* . In addition, our analysis revealed the presence of snake venom protein families not detected in the venom gland transcriptome or previous studies, including glutaminylhe 7

Tryptic digestion of the venom

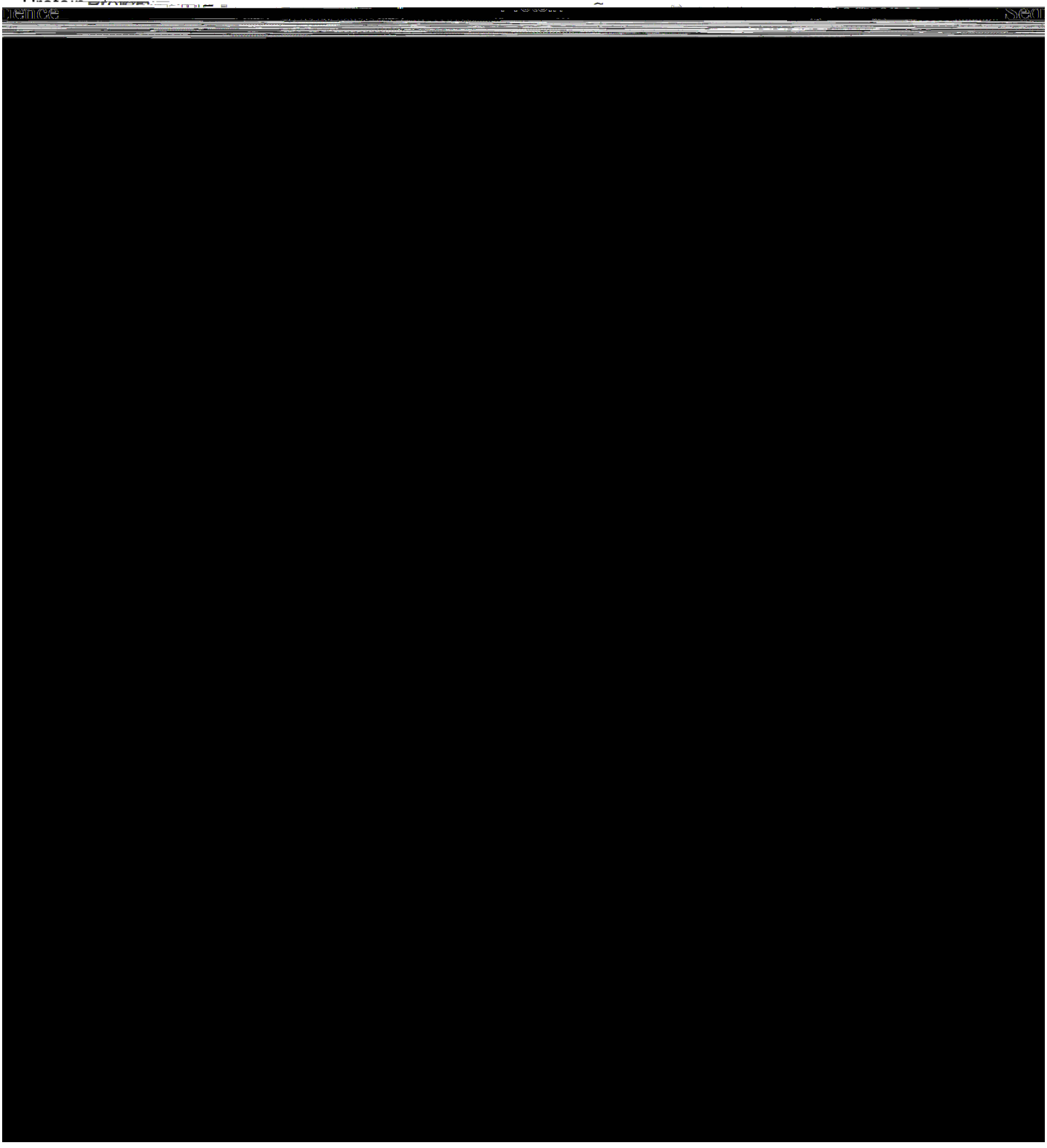
Lyophilized crude venom (600 µ

Data analysis

Database searches of the mass spectra acquired on the MALDI mass spectrometers were searched against all entries of NCBInr (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.

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 A_2 [12–14], as well as by the detri-



Protein sequences including specific domains are indicted by colored bars; below these, corresponding peptides identified by ESI (black lines) and MALDI (red lines) are indicated.

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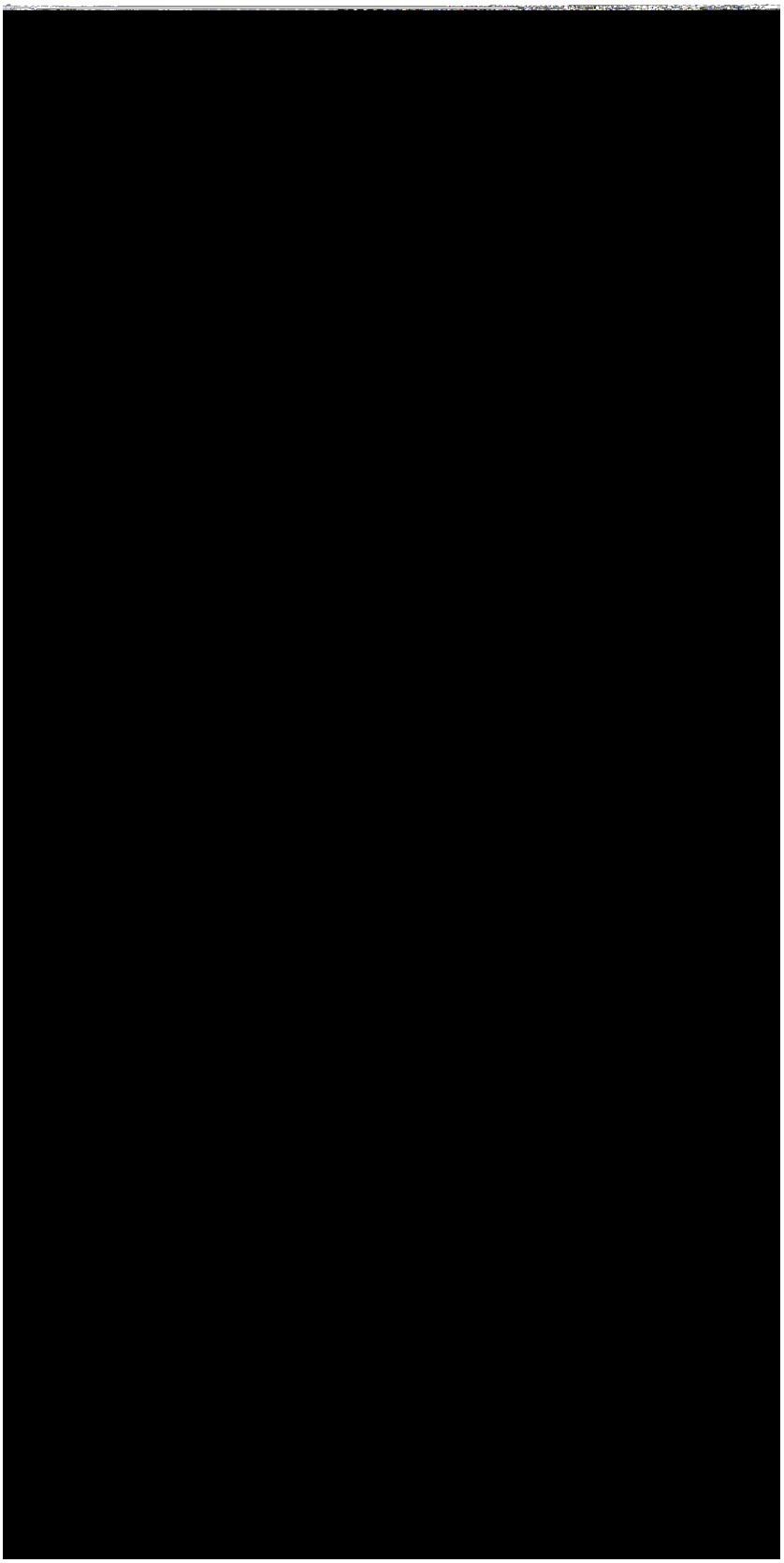

(Continued)

Serine proteinase	Viridovipera stejnegeri	82242793	IIGGDECNIDEHR	2	764.36	56.68
C-type lectin	Sistrurus miliarius	21530567	GLQQGTNYHK	2	382.53	72.72
			FCSEQAEGGHLVSIESSEEAA	1	2239	0.54
			WSDGSSVSYENWIEAESK	1	2073.83	
			DCPSGWSSYDQHCYR	1	1917.74	
C-type lectin	Sistrurus miliarius	21530570	YDVWIGLR	2	511.28	60.77
			WSDGSSVNYENLIK	2	806.4	75.31
			DFDCPSDWYAYDQYCYR	1	2323.83	
C-type lectin	Sistrurus miliarius	21530564	FTSMWIGLK	1	1082.57	
			LASIHSSEEEAFVGK	1	1603.81	0.52
			TWDDAESFCYTQHR	1	1815.69	
C-type lectin	Sistrurus miliarius	21530573	QNQYYVWIGLR	1	1439.75	
			ETEFLQWYNTDCEEK	1	1991.88	
C-type lectin	Crotalus adamanteus	338855278	YEDWAEESYCVYFK	1	1888.79	
C-type lectin	Crotalus durissus terrificus	82129809	WSDGSSVNYENLLK	2	806.40	75.31
			QNKYYVWIGLR	1	1439.75	
			ETEFLQWYNTDCEEK	1	1991.86	
L-amino acid oxidase	Bothrops neuwiedi pauloensis	195927838	GNPLEECFR	2	561.26	59.52
			NGLSATSNPK	2	495.25	45.52
L-amino acid oxidase	Demansia vestigiata	118151720	YPVKPSEK	2	474.27	42.82
L-amino acid oxidase	Viridovipera stejnegeri	34014953	LSAAYVLAGAGHEVTVLEASER	1	2244.2	0.56
L-amino-acid oxidase	Naja kaouthiapauloe67minoj1aoutf3SELEIA813.2869511.28 02Naj4 kTPYQFQgHFSEALTiariWSDGSSK					1

[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.* . *a* 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 *S* , , , *a* , , *C* , *a* , *a a-* , *a* , , , and *C* , *a* , , were also noted (Table 1). This brings the number of C-type

Scdad 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. . a* to a total of nine isoforms and might indicate a prominent role of this protein family to the envenomation of prey by *S. . a* .

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. . a* 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. . a* 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 *B.* and *La* , BPPs play a minor role in envenomation by *S a* .

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. . a* we identified the predicted VEGF-F together with two peptides that showed homology to VEGF-Fs from the viper *B. a* (Central America) and the elapid *C.* (eastern small-eyed snake; coastal eastern states of Australia). Again, there appears to be greater diversity in the proteome of *S. . a* 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. . a* . 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 [transcrip4rl[(lrort)-8.6proteispe

Ecto-5'-nucleotidase	Gloydius blomhoffi	211926756	SSGNPILLNK	2	521.80	73.51
			ETPVLSNPGPYLEFR	1	1718.83	
			LTAVLPFGGTFDLLQIK	1	1834.08	0.58
Glutaminyl 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	
			HGLEFSYEMAPR	1	1436.66	
			NGYWPSYNIPFDK	1	1600.77	
			HQGLPESYNFDFVTMKPVL	1	2222.07	

Peptides scores of the different search engines are: Peaks—regular, Mascot—

cryptic snake *D* [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.* show identity to the PLB detected in the transcriptome of the venom gland of the related rattlesnake *C.* . The entire protein sequence of the PLB in the venom of *C.* 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.* venom may reveal diverse phospholipase subtypes in this venom and help explain the lack of PLA_2 diversity. It is interesting to compare the sequences identified in the venom of *S.* in the present study with the sequences detected by Edman degradation and sequencing of MS/MS spectra in the same venom in the study by Sanz . [7] to protein snake venom families of *S.* identified in the present work, peptide sequences inferred by sequencing (S3 Table) were more difficult to match. Interestingly, many sequences in the paper by Sanz . refer to SVMP's (S2 Table), which we were not able identify in the venom of *S.* , 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.* , 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.* venom [53], and prominent PLA_2 myotoxins [54], appear to be absent from the venom. Human envenomations by *S* - are uncommon; for example, only 9/650 reported snakebites resulted from *S.* [55]. Similarly, case reports are rare, but the clinical presentation is considered to be similar to *C* sp. bites, requiring antivenom treatment but typically with less severe outcome [56, 57]. Bites by *S.* are even less frequent, but because this species has a toxic venom (mouse LD_{50} = 0.60 μg/g; [58]) which contains abundant serine proteases, coagulopathies including hypofibrinogenemia and thrombocytopenia are to be expected.

Proteome of the v-50om-2185.7of

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