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Comprehensive Study of the Proteome and Transcriptome of the Venom of the Most Venomous European Viper: Discovery of a New Subclass of Ancestral Snake Venom Metalloproteinase Precursor-Derived Proteins

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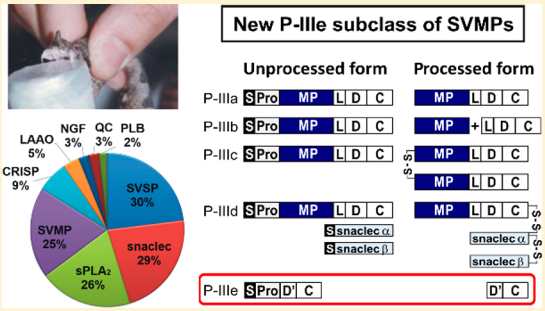
Supporting Information

ABSTRACT: The nose-horned viper, its nominotypical subspecies *Vipera ammodytes ammodytes* (*Vaa*), in particular, is, medically, one of the most relevant snakes in Europe. The local and systemic clinical manifestations of poisoning by the venom of this snake are the result of the pathophysiological effects inflicted by enzymatic and nonenzymatic venom components acting, most prominently, on the blood, cardiovascular, and nerve systems. This venom is a very complex mixture of pharmacologically active proteins and peptides. To help improve the current antivenom therapy toward higher specificity and efficiency and to assist drug discovery, we have constructed, by combining transcriptomic and proteomic analyses, the most comprehensive library yet of the *Vaa* venom proteins and peptides. Sequence analysis of the venom gland cDNA library has revealed the presence of messages encoding 12 types of polypeptide precursors. The most abundant are those for metalloproteinase inhibitors (MPis), bradykinin-potentiating peptides (BPPs), and natriuretic peptides (NPs) (all three on a single precursor), snake C-type lectin-like proteins (snaclecs), serine proteases (SVSPs), P-II and P-III metalloproteinases (SVMPs), secreted phospholipases A_2 (sPLA$_2$s), and disintegrins (Dis). These constitute >88% of the venom transcriptome. At the protein level, 57 venom proteins belonging to 16 different protein families have been identified and, with SVSPs, sPLA$_2$s, snaclecs, and SVMPs, comprise ∼80% of all venom proteins. Peptides detected in the venom include NPs, BPPs, and inhibitors of SVSPs and SVMPs. Of particular interest, a transcript coding for a protein similar to P-III SVMPs but lacking the MP domain was also found at the protein level in the venom. The existence of such proteins, also supported by finding similar venom gland transcripts in related snake species, has been demonstrated for the first time, justifying the proposal of a new P-IIIe subclass of ancestral SVMP precursor-derived proteins.



KEYWORDS: *Vipera ammodytes ammodytes, snake, Viperidae, venom composition, transcriptomics, proteomics, metalloproteinase, new subclass*

1. INTRODUCTION

Snake venoms are highly complex cocktails mainly comprising proteins and peptides involved in the immobilization and initial digestion of prey. They are also a rich source of bioactive compounds exploited by humans for the diagnosis and therapy of a variety of diseases.[1] Venom toxins have evolved from closely related body proteins that have been diversified functionally by gene duplication and adaptive evolution, generating multigene families specific for venom glands.[2] The ancestral genes were recruited from various types of tissue and usually code for the key secreted proteins involved in diverse biological processes.[3] Some time ago, it was reported by our laboratory that animal toxin multigene families have evolved under a strong positive selection that favors amino acid replacements serving to adapt the duplicated gene to a new function.[4] Venomous snakes are found in different snake families, especially those whose venom apparatus is highly developed, such as Elapidae and Viperidae. The latter, vipers, constitute a monophyletic lineage of venomous snakes comprising approximately 330 species distributed worldwide and currently divided into three subfamilies, Azemiopinae, Crotalinae, and Viperinae.[5]

The nose-horned viper, *Vipera ammodytes*, is the most venomous snake in Europe. It is found mainly in southern Europe and partly in western Asia. Spreading from the northwest to the southeast, at least four subspecies, *ammodytes* (*Vaa*), *meridionalis* (*Vam*), *montandoni*, and *transcaucasiana*, are usually recognized.[6] *V. ammodytes* venom induces mainly hemotoxic and neurotoxic effects, which, in rare cases, can lead to human death.[7,8] In contrast with that from other subspecies, *Vaa* venom contains highly neurotoxic monomeric secreted phospholipases A_2 (sPLA$_2$s), known as ammodytoxins (Atxs).[9] A comparative analysis of the *Vaa* and *Vam* proteomes

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revealed the presence of 38 venom components in the former.[10] Recently, we studied the proteome of the common European adder, subspecies *Vipera berus berus* (*Vbb*), and compared it with that of *Vaa*.[11] The *Vbb* proteome was shown to be much less complex than that of *Vaa*, in particular, possessing smaller amounts of snaclecs (snake C-type lectin-like proteins) and $sPLA_2$s. The *Vaa* venom is rich in compounds that interfere with hemostasis,[12,13] with some that are potentially anti-tumor-active.[14,15]

The main aim of the present comprehensive transcriptomic and proteomic study was to identify and build a complete library of *Vaa* venom proteins and peptides. The accumulated data will direct the production of a more specific and effective antivenom with which to treat venomous *Vaa* bites. Such antivenoms can be, namely, produced by injecting horses with a mixture of antigens stemming from the most critical toxic components of the venom only. It will also facilitate structure-based drug design, especially for the treatment of certain neurological, cardiovascular, and cancer disorders.

2. MATERIALS AND METHODS

2.1. Venom and Reagents

Vaa venom, collected in 2005 from snakes from different parts of Croatia, was a gift from the Institute of Immunology, Zagreb, Croatia. Fibrinogen was from Hypen BioMed (France). Acetonitrile (ACN; Merck, Germany), trifluoroacetic acid (TFA; from Sigma-Aldrich, USA), and formic acid (Fluka, Germany) were of HPLC gradient grade or higher. Deionized water was purified using a Direct-Q 5 system (Millipore, Billerica, MA).

2.2. Analysis and Sequencing of cDNA

cDNAs encoding venom proteins were obtained by random screening of a representative plasmid cDNA library. Sequences encoding the complete protein-coding regions of *Vaa* venom gland transcripts were determined by using internal sequencing primers deduced from previously sequenced regions. The library was recently prepared from venom glands isolated 2 days after milking from a single *Vaa* specimen captured in the wild in the area of northeastern Slovenia.[14] The nucleotide sequences were determined by Microsynth AG (Switzerland) using the dideoxy chain-termination method. They were subsequently analyzed by free, publicly available, bioinformatics services. They were submitted to GenBank under the accession numbers KU249650−KU249656, KT148817−KT148834, and MG958491−MG958504.

2.3. Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed under optimized conditions.[16] 500 μg of crude *Vaa* venom was dissolved in 450 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 30 mM Tris, 1% (v/v) ampholytes, 0.25% (*m*/*v*) ASB-14, 2.5% (*m*/*v*) CHAPS, 0.002% (*m*/*v*) bromophenol blue, and 12 μL/mL DeStreak reagent (GE Healthcare, Amersham Biosciences). A 24 cm immobilized pH gradient (IPG) strip (GE Healthcare, Amersham Biosciences), covering the pH range 3−11 NL, was rehydrated passively with the sample overnight. The first dimension separation (isoelectric focusing (IEF)) and the second dimension separation (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE)) were carried out using the reported experimental protocols.[16] Following reverse staining with imidazole-SDS-Zn^{2+}, the gel was scanned by an Image Scanner using LabScan 5 software (GE Healthcare, Amersham Biosciences). The image was analyzed by Image Master 2D Platinum 6.0 software (GE Healthcare, Amersham Biosciences). The protein spots detected were cut out automatically using an Ettan Spot Picker (GE Healthcare, Amersham Biosciences) and kept at −20 °C before analysis.

2.4. RP-HPLC Analysis

One g of crude *Vaa* venom was separated by gel filtration on Sephacryl S-200, as described.[17] The resulting fractions, B2, C1, C2, C3, and D, were separated successively by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C4 (Aquapore BU-300, 7 μm, 300 Å, 4.6 × 30 mm, PerkinElmer, USA) column and a Poroshell 120 EC-C18 column (4.6 × 150 mm, 2.7 μm, 120 Å, Agilent Technologies, USA) equilibrated with 0.1% (v/v) TFA in water. Column-retained molecules were eluted by applying a discontinuous gradient of 90% (v/v) ACN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min as follows: (i) in the case of an RP-C4 column: 0−20% for 5 min, 20−45% for 15 min, 45−60% for 5 min; (ii) in the case of an EC-C18 column: 0−20% for 10 min, 20−40% for 40 min. Proteins and peptides were detected by absorbance at 215 nm; peak samples were collected manually and dried in a SpeedVac (Savant, USA).

2.5. Protein Identification by Mass Spectrometry

Protein spots were destained and treated with trypsin in-gel, and the resulting peptides were analyzed using an ion trap mass spectrometer 1200 series HPLC-Chip-LC/MSD Trap XCT Ultra (Agilent Technologies, Waldbronn, Germany).[16] Spectral data were exported as Mascot generic format (mgf) files using in-house Agilent Technologies software, Data Analysis for 6300 series Ion Trap LC−MS version 3.4 (Build 175). A search against the nonredundant National Center for Biotechnology Information (NCBI) Snakes database (taxid 8750, December 2017, 159 187 entries) supplemented with our *Vaa* transcriptome data deposited in the GenBank NCBI database was performed using a licensed version 2 of the MASCOT program, applying the following restrictions: 2+ and 3+ peptide charge; two miscleavages allowed; peptide and fragment mass tolerance of ±1.2 and ±0.6 Da, respectively; carbamidomethyl Cys (C) as the fixed modification and oxidized methionine (M_{ox}) as variable; and an automatic decoy database search. The results were further validated using Scaffold software (version 2, Proteome Software, USA) with the following thresholds: protein confidence of 99% and one peptide per protein at 95% confidence. Proteins were identified at 0.1% Prophet false discovery rate (FDR), and peptides were identified at 5.27% Prophet FDR. Data are available via ProteomeXchange with the identifier PXD012752.

Low-molecular-mass peptides isolated by RP-HPLC were analyzed using a Q-TOF Premier mass spectrometer (Waters-Micromass, GB) as described.[18]

2.6. Polypeptide Sequencing by Edman Degradation

Isolated polypeptides were sequenced from the N-termini using Edman degradation performed automatically on a Procise 492A automated sequencing system (Applied Biosystems, USA).

2.7. Inhibition of Fibrinogenolytic Activity of Snake Venom Metalloproteinases by an Endogenous Tripeptide Inhibitor

The tripeptide inhibitor in the gel filtration fraction E (Figure 6A) was tested for its ability to inhibit the fibrinogenolytic

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activity of the *Vaa* snake venom metalloproteinases (SVMPs) present in the gel filtration fraction A. The vacuum-dried fraction RP-C18 containing the tripeptide was dissolved in 5 μL (0.5 μg) of fraction A supplemented with 2 mM serine protease inhibitor Pefabloc (Sigma-Aldrich, USA) and incubated for 30 min at 37 °C. Five μL (30 μg) of fibrinogen in 20 mM Tris, 50 mM NaCl, 2 mM $CaCl_2$, pH 7.0 was then added. The reaction was stopped after 15 min by adding 10 μL of reducing SDS-PAGE buffer. The reaction mixture was heated for 5 min at 95 °C and analyzed by 12.5% SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue R250.

3. RESULTS AND DISCUSSION

3.1. Transcriptomic Analysis

Of the 520 randomly selected cDNA clones, 254 (48.8%) coded for precursors of *Vaa* venom proteins and peptides (toxic and nontoxic), whereas the remaining recombinant plasmids harbored either nonvenomous or other sequences encoding unidentified proteins. For example, those encoding phospholipase B (PLB; GenBank accession number MG958504) and leucine aminopeptidase (incomplete cDNA) were excluded from the venom-related transcripts secreted by a pair of *Vaa* venom glands. A search for the presence of a potential signal peptide in the deduced amino acid sequences of precursors of these two proteins and of closely similar proteins in databases did not support the assumption that these proteins are actually secreted by venom glands. Interestingly, PLB was detected at the protein level in the *Vaa* venom (see below). This enzyme was also reported in venoms of other snakes, either viperids, such as *Pelias* species,[19] or elapids, such as *Pseudechis guttatus*.[20]

The venom-related transcriptome thus includes 254 partial and complete sequences of 45 different mRNA transcripts that are branched into 12 different groups (Table S-1). Despite the relatively small number of analyzed cDNAs, the comparison of these groups with those observed in the proteomic analysis (see below) suggests that this result is an indicative snapshot of the biosynthesis of venom proteins and of their relative distribution in *Vaa* venom glands. The most abundant were transcripts encoding common precursors of tripeptide inhibitors of MPs (MPis), bradykinin-potentiating peptides (BPPs), and natriuretic peptides (NPs) (25.6% of all venom transcripts), followed by those of snaclecs (13.8%), SVSPs (11.8%), P-III class SVMPs (11.0%), $sPLA_2$s (10.6%), P-II class SVMPs (9.4%), and disintegrins (Dis; 5.9%) (Figure 1). These seven major groups comprise >88% of all mRNAs isolated from the *Vaa* venom glands. Each of the remaining five groups—SP inhibitors (SPis), vascular endothelial growth factors (VEGFs), Cys-rich secretory proteins (CRISPs), L-amino acid oxidases (LAAOs), and venom nerve growth factors (VNGFs)—constitutes <5% of the transcriptome.

Interestingly, the five most abundant venom-related mRNA transcripts were also the most heterogeneous. Snaclec precursors were thus represented by nine, SVSPs by eight, P-III SVMPs by six, common MPi, BPP, and NP peptide precursors by five, and $sPLA_2$s by three different mRNAs.

P-II and P-III class SVMPs together form the largest group of *Vaa* venom-gland-encoded enzymes, comprising more than one-fifth (20.4%) of the transcriptome. A large proportion of the SVMP transcripts, ranging from 24 to 58%, has also been observed in the venom gland transcriptome of most other Viperidae species reported so far, for example, *Crotalus*

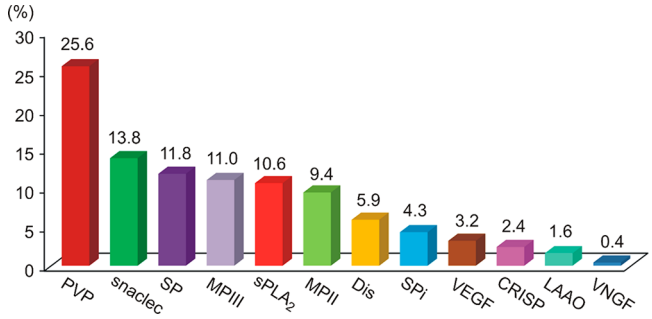

Figure 1. Relative distribution of protein groups in the transcriptome of *Vaa* venom glands. Percentages were calculated according to the total number, that is, 254, of venom-related transcripts. Abbreviations: PVP, the common precursor of venom peptides (NPs, BPPs, and MPis); snaclec, snake C-type lectin-like protein; SP, serine protease; MP, metalloproteinase (class II or III); $sPLA_2$, secreted phospholipase A_2; Dis, disintegrin; SPi, serine protease inhibitor (Kunitz-type); VEGF, vascular endothelial growth factor; CRISP, Cys-rich secretory protein; LAAO, L-amino acid oxidase; VNGF, venom nerve growth factor.

adamanteus,[21] *Protobothrops flavoviridis*,[22] *Bothrops colombiensis*,[23] and *Echis ocellatus*.[24] All of these evolved from an ancient ADAM (a disintegrin and a metalloproteinase) gene that was recruited into the venom gland of snakes and are responsible for the wide spectrum of severe local and cardiovascular pathologies observed in victims of viper envenomation.[25]

3.1.1. mRNA Transcripts Encoding Precursors of MPi, BPPs, and NPs. The largest portion of *Vaa* mRNA transcripts, that is, about one-quarter (see above), contained information for the precursors of biologically active peptides—MPis, BPPs, and NPs—similar to those previously found in viperid snakes.[26] Six different mRNAs were recognized that can be divided into two groups, whose leading representatives we termed Vaa-MPi-1 and Vaa-MPi-2 (Figure 2). Vaa-MPi-1 encodes a precursor protein of 180 amino acid residues, and Vaa-MPi-2 encodes a precursor of 244 residues. These two share 64% amino acid identity and possess the identical sequence of a putative signal peptide of 23 residues (Figure 2A). Interestingly, the same signal peptide sequence has also been observed in three MPi polypeptide precursors from the Viperinae snake *E. ocellatus*.[27] Highly similar nucleotide and deduced amino acid sequences were also found in a genome database with whole genome shotgun data of *Vbb* (Viperinae) and *Protobothrops mucrosquamatus* (Crotalinae), enabling the presumed (first) intron position within the Vaa-MPi-2 nucleotide sequence to be deduced.

The sequences of Vaa-MPi-1 and Vaa-MPi-1′ differ in only one amino acid residue (resulting from only one nucleotide residue) at the C-terminal end, probably representing two allelic forms. In contrast, the shorter transcripts Vaa-MPi-5 and Vaa-MPi-3, with deletions of 43 and 36 amino acid residues respectively (Figure 2B,C), could be the result of alternative splicing. In transcript Vaa-MPi-4, displaying a deletion of 106 nucleotides relative to Vaa-MPi-2, an open-reading frameshift occurs that results in a premature ending of the polypeptide chain, thus lacking the C-terminal NP sequence (Figure 2C). This may also be due to alternative splicing. Another possibility is that Vaa-MPi-3, Vaa-MPi-4, and Vaa-MPi-5 mRNAs were transcribed from recently duplicated copies of the Vaa-MPi-2 gene.

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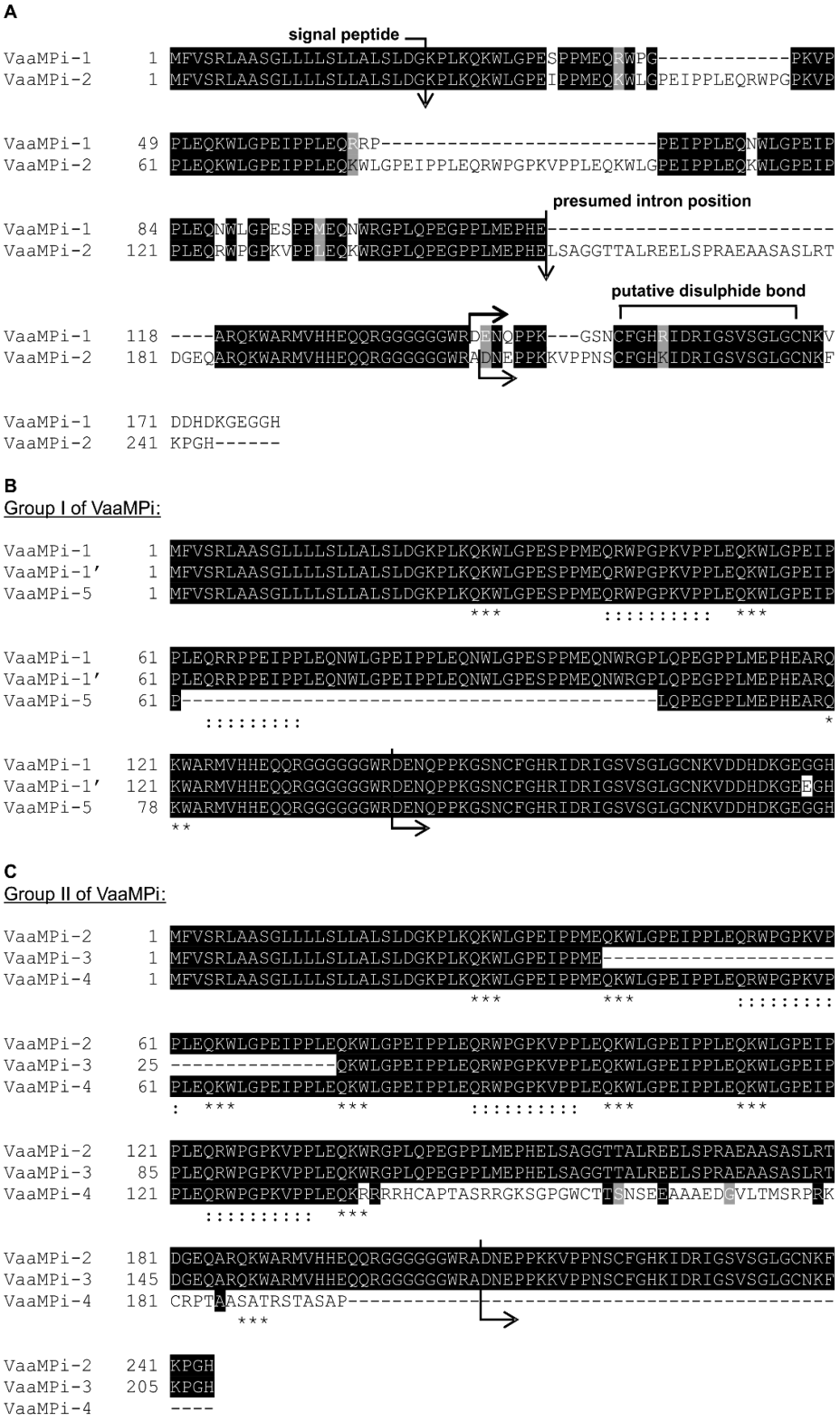

Figure 2. Alignment of translated *Vaa* MPi transcripts. (A) Comparison of two representative Vaa-MPi precursors. (B) Alignment of the deduced protein sequences similar to that of the Vaa-MPi-1 precursor. (C) Alignment of the deduced protein sequences similar to that of the Vaa-MPi-2 precursor. Identical and similar amino acid residues are highlighted in black and gray, respectively. Gaps introduced to optimize the alignment are indicated by dashes. Peptides present in the *Vaa* venom are denoted as follows: MPis by asterisks, BPPs by colons, and the N-terminal sequences of NPs by arrows pointing to the right.

3.1.2. Transcripts Coding for a New Ancestral SVMP Precursor-Derived Protein. Twenty-eight transcripts coding for precursors of P-III class SVMP proteins were grouped into six groups (encoded by full-length transcripts) corresponding to six different pre-pro-proteins. Two of them correspond to two previously identified and characterized *Vaa* hemorrhagic

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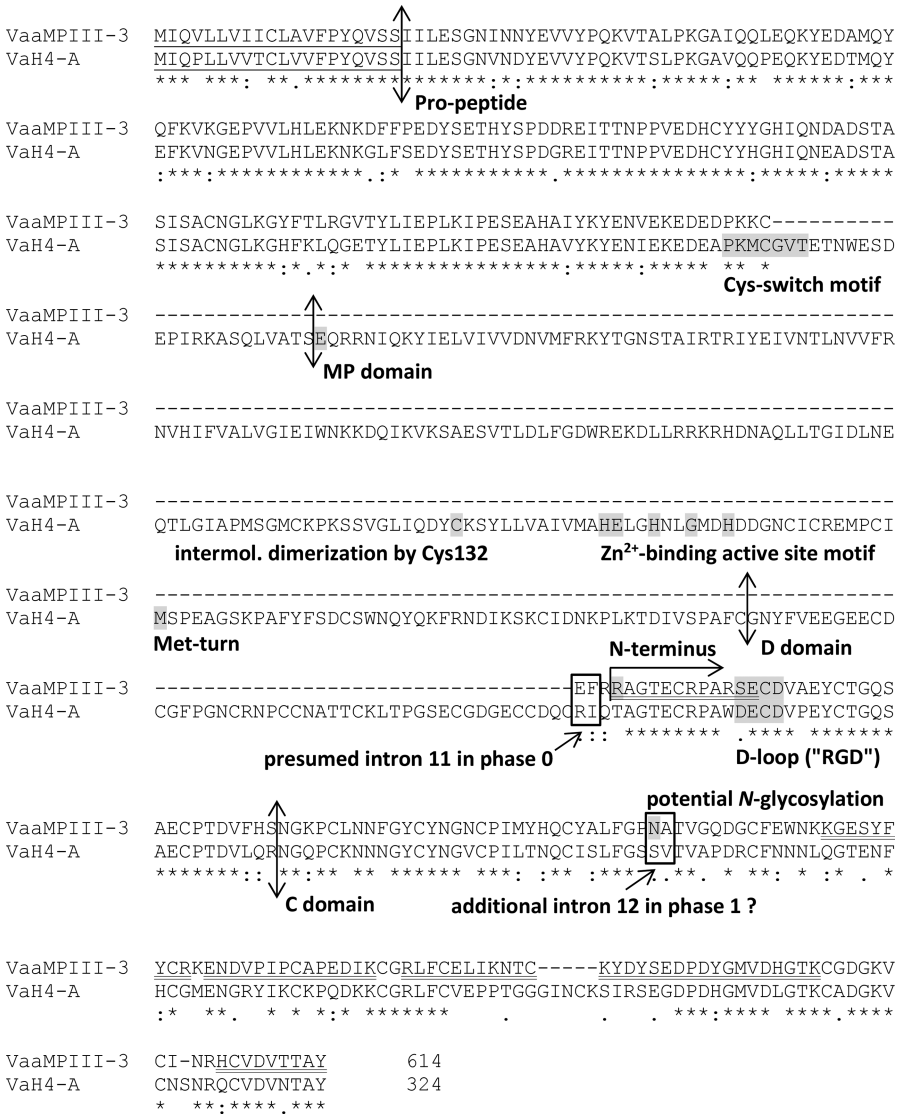

Figure 3. Alignment of the deduced precursor sequence of Vaa-MPIII-3 lacking the MP domain with that of the VaH4-A subunit of heterodimeric hemorrhagin VaH4 from the same venom. Important residues and motifs are highlighted in gray. Identical, conserved, and semiconserved amino acid residues are designated by asterisks, colons, and dots, respectively. Putative signal peptides are underlined once, and the N-terminus of the mature Vaa-MPIII-3 is shown by an arrow pointing right. The deletion of 284 amino acid residues in Vaa-MPIII-3, including the last part of the pro-peptide with the inhibitory Cys-switch motif, the entire MP domain, and the first part of the D domain, is indicated by dashes. The phase 0 intron is located between two codons, whereas the phase 1 intron separates codons between the first and the second nucleotides. Part of the Vaa-MPIII-3 sequence (64%), covered by Edman and MS sequencing, is underlined by a double line. In contrast with Vaa-MPIII-3, the sequence of VaH4-A includes a canonical zinc-binding active site motif, followed by a methionine turn characteristic of the metzincin superfamily of catalytically active MPs.

MPs, subunit A of heterodimeric VaH4 and homodimeric VaH3,[14,28] whose cDNAs were isolated by initial random screening, followed by PCR. The remainder encode new, previously unknown *Vaa* P-III SMVP proteins of a high degree of amino acid sequence identity, which were named Vaa-MPIII-2, Vaa-MPIII-3, Vaa-MPIII-4, and Vaa-MPIII-5. Unlike the others, Vaa-MPIII-3 exhibits a large deletion of 284 amino acid residues in the middle part (Figure S-1). In the present transcriptomic analysis, no transcripts corresponding to the two previously identified P-III class SVMPs, VaF1[29] and Vaa-MPIII-1,[11] were found, but those two were then obtained by PCR amplification.

Interestingly, the protein-coding sequences of VaH3 and Vaa-MPIII-5 cDNAs of 1851 nt share a high level of nucleotide identity (95.2%). They differ only in their 579 nt pre-pro-regions (28 nt differences leading to 14 aa replacements), whereas their mature protein-coding regions of 1272 nt show 100% nucleotide and amino acid identity (Figure S-1). These figures may reflect a recent duplication event in the evolution of their genes, opening up the possibility of a fine-tuning of their processing. A similar observation was also noted in the case of two snaclec precursors in which Vaa-snaclec-5 and Vaa-snaclec-6 differ only in the signal peptide region, their mature protein regions being identical (see Figure S-7).

Four transcripts, of a total of 28 encoding P-III class SVMPs, coded for a precursor protein of 324 amino acids, designated as Vaa-MPIII-3. The Vaa-MPIII-3 mRNA encodes a mature protein without the MP domain, possessing only the C-terminal part of the Dis-like (D) domain, with a D-loop (an XXCD, i.e., an RGD-like motif), termed here the D′ domain,

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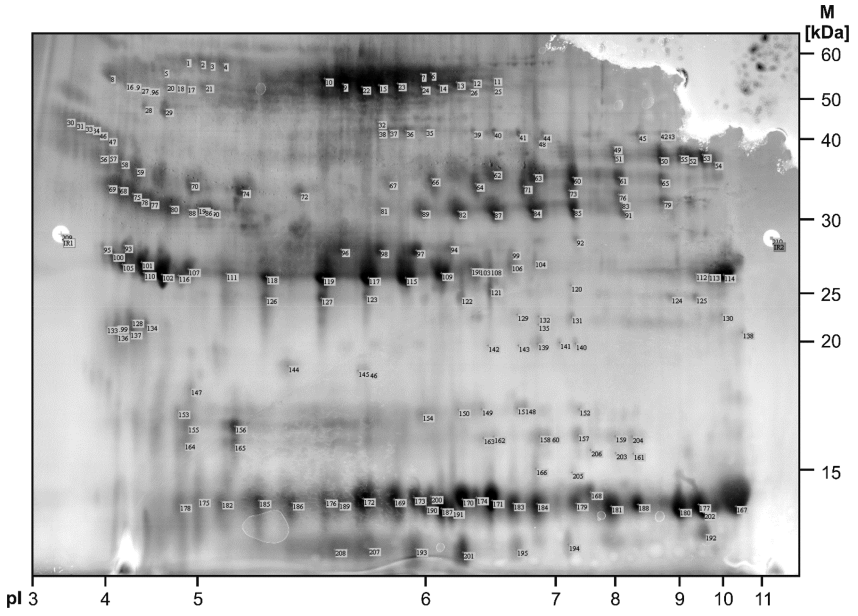

Figure 4. Two-dimensional gel electrophoresis of the *Vaa* venom. 500 μg of crude *Vaa* venom was separated with IEF on a 24 cm IPG strip, pH 3–11 NL, in the first dimension. Proteins were then reduced and alkylated and separated on a 10% SDS-PAGE gel in the perpendicular dimension, according to their molecular masses, using the Tris/Taurin buffer system. The gel was stained using the imidazole-SDS-Zn^{2+} method.

and the Cys-rich domain (C domain). The deduced pre-pro-protein sequence of Vaa-MPIII-3 is shown compared with that of VaH4-A[14] in Figure 3. The existence of Vaa-MPIII-3 mature protein in *Vaa* venom was confirmed by its isolation from the venom and sequencing by Edman degradation and MS (see below, Figure 6B, Table 2). The N-terminal amino acid sequence of this protein was determined to be RAGTECRPA-RSE. The Vaa-MPIII-3 mature protein of 151 amino acid residues is thus presumably preceded by a signal peptide of 20 residues and a pro-peptide of 153 residues.

Loss of the MP domain has already been observed in one of the P-III class SVMP precursors from *Echis carinatus sochureki* venom (GenBank No. GU012129).[30] However, in contrast with the case presented here, the deleted region was much shorter (196 vs 284 amino acid residues), resulting in a longer DC domain of the deduced mature protein. The existence of such a protein in *Echis* was, however, not confirmed on the protein level. These results showed that the evolutionary history of viperid SVMPs was punctuated repeatedly by domain loss, resulting in frequent alterations to the molecular scaffold. Furthermore, catalytically inactive P-II class SVMPs, with substitutions in the canonical zinc-binding motif, have also been isolated from *Bothriechis lateralis* venom and characterized, indicating that enzymatically inactive SVMP homologues deserve further investigation of their toxicity role in snake venoms.[31]

A BLASTp search through the nonredundant NCBI database, using the 324 residue pre-pro-Vaa-MPIII-3 as a query sequence, revealed Eoc89, a translated cDNA sequence of GenBank No. AM039699 from *Echis ocellatus* venom glands,[32] to be one of the most closely similar structures, with ~82% amino acid identity in the overlapping N- and C-terminal parts. The first draft of the genomic organization of a PIII-SVMP gene, that of the Eoc89-like protein, has been reported.[33] Its gene consists of 12 exons separated by 11 introns. Notably, the presumed position of intron 11 in the Vaa-MPIII-3 sequence is very close to the N-terminus of the mature protein (Figure 3), indicating that this intron could play a significant role in the evolution of the Vaa-MPIII-3 gene, in which the catalytic MP domain and subsequent first part of the D domain had been lost. The nucleotide sequence of Vaa-MPIII-3 was also used for a similarity search through the whole genome shotgun data. It appears that, at least in some of the similar gene sequences, such as those in the *Vbb* genome, the C-terminal domain sequence may be interrupted by additional introns, but this remains to be confirmed.

The Vaa-MPIII-3 transcript from *Vaa*, encoding the N-terminal signal peptide and pro-peptide, lacks the central MP domain and possesses, at its C-terminal end, a truncated D (D′) and the complete C domain, thus encoding a new type of P-III class SVMP-like proteins. We therefore suggest a new P-IIIe subclass of ancestral SVMP precursor-derived proteins. Precursors, such as those for Vaa-MPIII-3 and the P-III class SVMP from *Echis carinatus sochureki* venom (GenBank No. GU012129), encode mature proteins consisting of a partial (D′) or complete Dis-like domain (D), followed by a Cys-rich domain (i.e., D′C and DC proteins). The gene structure and evolution of Vaa-MPIII-3, its precise precursor processing, and actual function in the snake venom have yet to be elucidated. According to our proteome results (Figure 6B), the mature Vaa-MPIII-3 protein, presumably possessing eight intramolecular disulfide bonds and a free Cys residue, exists in *Vaa* venom as a glycosylated monomer.

3.2. High-Molecular-Mass Proteome Profiling of the *Vaa* Venom

In the present study, the optimized 2-DE conditions[16] allowed resolution of crude *Vaa* venom into 208 distinct spots in the molecular mass range of 10 to 60 kDa (Figure 4). Each spot was subjected to in-gel digestion and LC–ESI–MS/MS analysis. The MS spectra were searched against the non-redundant protein NCBI database of snake species, supplemented with the transcriptomic data obtained from our *Vaa* venom gland cDNA library analysis. Proteins were identified unambiguously in 176 spots (Table 1, Table S-2). Members of different protein families were detected in certain spots. Of the

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Table 1. Assignment of the *Vaa* Venom Proteins in 2-DE Spots to Protein Families by LC−ESI-MS/MS Analysis of Tryptic Peptides[a]

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
2	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168, 25253	89	2	SP
	metalloproteinase [*E. coloratus*]	ADI47654	55138	48	1	MP
3	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168, 25253	85	2	SP
5	MP (type III) [*C. adamanteus*]	AFJ49231	67329	106	2	MP
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479	60	1	MP
	Vaa-MPIII-1 [*V. a. ammodytes*]	KT148834	68388	53	1	MP
	Vaa-MPIII-4 [*V. a. ammodytes*]	MG958500	68844	43	1	MP
6	MP [*E. coloratus*]	ADI47654	55138	91	2	MP
	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	45	1	MP
7	MP [*E. coloratus*]	ADI47654	55138	139	3	MP
	VaH3 [*V. a. ammodytes*]	AGL45259	68546	104	2	MP
9	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	626	9	LAAO
10	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	647	12	LAAO
	LAAO B variant 1 [*E. coloratus*]	JAC96580	56738	262	5	LAAO
	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	237	4	MP
	VaH3 [*V. a. ammodytes*]	AGL45259	68546	216	3	MP
	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	17711	90	2	snaclec
11	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	130	3	SP
	MP [*E. coloratus*]	ADI47654	55138	81	2	MP
12	MP [*E. coloratus*]	ADI47654	55138	85	2	MP
13	MP [*E. coloratus*]	ADI47654	55138	80	2	MP
14	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	191	3	MP
	VaH3 [*V. a. ammodytes*]	AGL45259	68546	142	2	MP
	MP [*E. coloratus*]	ADI47654	55138	57	1	MP
15	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	609	10	LAAO
	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	297	5	MP
17	MP (type III) [*C. adamanteus*]	AFJ49231	67329	66	1	MP
18	MP (type III) [*C. adamanteus*]	AFJ49231	67329	209	3	MP
19	MP (type III) [*C. adamanteus*]	AFJ49231	67329	190	3	MP
20	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479	213	3	MP
	MP (type III) [*C. adamanteus*]	AFJ49231	67329	187	3	MP
21	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	113	2	MP
22	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	638	12	LAAO
	LAAO B variant 1 [*E. coloratus*]	JAC96580	56738	244	4	LAAO
	Vaa-LAAO-I [*V. a. ammodytes*]	P0DI84	54748	154	3	MP
	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	204	4	MP
	VaH3 [*V. a. ammodytes*]	AGL45259	68546	148	2	MP
23	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	676	12	LAAO
	VaH3 [*V. a. ammodytes*]	AGL45259	68546	210	3	MP
	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	161	3	MP
24	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	303	5	LAAO
	VaH3 [*V. a. ammodytes*]	AGL45259	68546	190	3	MP
	Vaa-MPIII-2 [*V. a. ammodytes*]	MG958498	69218	124	2	MP
	MP [*E. coloratus*]	ADI47654	55138	93	2	MP
	VaF1 [*V. a. ammodytes*]	AJC52543	68745	88	2	MP
29	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	283	5	MP
32	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	144	3	LAAO
	MP [*E. coloratus*]	ADI47654	55138	95	2	MP
	VaF1 [*V. a. ammodytes*]	AJC52543	68546	86	2	MP
33	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	42	1	SP
34	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	126	2	SP
35	MP [*E. coloratus*]	ADI47654	55138	105	2	MP
36	MP [*E. coloratus*]	ADI47654	55138	49	1	MP
38	Vaa-LAAO-II [*V. a. ammodytes*]	MG958502	57102	159	3	LAAO
39	glutaminyl-peptide cyclotransferase [*D. russelii*]	AFE84762	42116	432	8	QC
40	glutaminyl-peptide cyclotransferase [*D. russelii*]	AFE84762	42116	392	7	QC
41	glutaminyl-peptide cyclotransferase [*D. russelii*]	AFE84762	42116	432	8	QC
42	VaH3 [*V. a. ammodytes*]	AGL45259	68546	176	3	MP
	PLB [*O. okinavensis*]	BAN82155	64133	123	2	PLB

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Table 1. continued

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
44	glutaminyl-peptide cyclotransferase [*D. russelii*]	AFE84762	42116	323	6	QC
	PLB [*O. okinavensis*]	BAN82155	64133	154	3	PLB
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	105	2	SP
45	PLB [*O. okinavensis*]	BAN82155	64133	227	4	PLB
47	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	148	3	SP
	renin-like AP [*E. ocellatus*]	CAJ55260	43872	44	1	AP
48	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	153	3	SP
	PLB [*O. okinavensis*]	BAN82155	64133	40	1	PLB
49	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	369	7	SP
50	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	212	4	SP
51	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	243	5	SP
52	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	249	4	SP
53	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	238	5	SP
54	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	227	4	SP
55	Vaa-SP-2 [*V. a. ammodytes*]	KT148825	28885	349	7	SP
56	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	106	2	SP
	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	103	2	SP
57	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	226	5	SP
	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	110	2	SP
58	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	102	2	SP
	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	45	1	SP
59	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	171	3	SP
	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	158	3	SP
	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	107	2	SP
60	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	290	5	SP
	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	144	2	SP
61	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	285	5	SP
	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	150	2	SP
62	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	89	2	PLA_2
63	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	244	4	SP
64	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	316	6	SP
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	116	2	SP
65	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	193	3	SP
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	163	3	SP
66	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	304	6	SP
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	151	3	SP
67	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	203	3	SP
68	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	106	2	SP
	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	48	1	SP
69	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	47	1	SP
70	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	178	3	SP
	Vaa-SP-4 [*V. a. ammodytes*]	KT148827	28587	111	2	SP
71	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	135	2	SP
72	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	185	3	SP
	Vaa-SP-8 [*V. a. ammodytes*]	MG958497	28795	160	3	SP
73	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	263	4	SP
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	215	4	SP
74	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	69	1	SP
75	Vaa-SP-3 [*V. a. ammodytes*]	KT148826	28168	102	2	SP
76	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	381	7	SP
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28216	182	4	SP
	calmodulin [*C. adamanteus*]	AFJ49577	16838	144	3	EF-hand
77	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	83	1	SP
78	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	129	3	SP
79	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	283	5	SP
80	Vaa-SP-3 [*V. a. ammodytes*]	KT148826	28168	204	5	SP
81	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	206	4	SP
82	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	227	4	SP
	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	132	2	MP
83	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	183	3	MP

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Table 1. continued

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	113	2	SP
	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	110	2	SP
84	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	235	4	SP
	nikobin [*V. nikolskii*]	CBW30778	28216	168	3	SP
	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	104	2	SP
	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	113	2	MP
85	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	282	5	SP
	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	123	2	SP
	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	131	2	MP
86	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	180	3	MP
	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	88	2	SP
87	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	202	3	SP
	nikobin [*V. nikolskii*]	CBW30778	28216	112	2	SP
88	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	158	3	MP
89	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	325	5	SP
	nikobin [*V. nikolskii*]	CBW30778	28216	162	3	SP
90	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	44	1	SP
91	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	257	4	SP
	Vaa-SPH-1 [*V. a. ammodytes*]	KT148824	26910	87	1	SP
92	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	180	3	SP
93	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	122	2	MP
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	64	1	MP
94	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	176	3	snaclec
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	105	2	MP
95	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	121	2	MP
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	88	2	MP
96	Vaa-snaclec-8 [*V. a. ammodytes*]	KT148834	15102	113	2	snaclec
	VaH4-A [*V. a. ammodytes*]	AHB62069	68662	105	2	MP
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	93	2	snaclec
97	Vaa-SP-3, Vaa-SP-5 [*V. a. ammodytes*]	KT148826, KT148828	28168	49	1	SP
98	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	107	2	snaclec
	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	88	2	snaclec
99	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	58	1	snaclec
100	Vaa-MPII-1, Vaa-MPII-2, Vaa-MPII-3 [*V. a. ammodytes*]	KT148831, T148832, KT148833	53479, 53471, 53068	78	1	MP
101	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	292	5	MP
102	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	423	7	MP
103	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	53479, 53471	170	3	MP
104	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	206	4	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	177	3	snaclec
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	54586	148	3	MP
	lebetase Le3 [*M. lebetina*]	CAA66471, Q98995	53480	58	1	MP
105	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	54586	267	4	MP
106	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	198	4	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	183	3	snaclec
	lebetase Le3 [*M. lebetina*]	CAA66471	53480	107	2	MP
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	54586	53	1	MP
107	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	54586	351	6	MP
	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	100	2	CRISP
108	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	175	3	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	88	2	snaclec
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	196	3	PLA_2
	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	54586	97	2	MP
109	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	218	4	PLA_2
	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	111	2	CRISP
110	Vaa-MPII-1, Vaa-MPII-2 [*V. a. ammodytes*]	KT148831, KT148832	54586	277	4	MP
111	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	102	1	CRISP
112	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	70	1	CRISP
113	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	115	2	CRISP
114	CRISP B [*E. coloratus*]	JAC96631	26686	50	1	CRISP

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Table 1. continued

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
115	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	106	2	CRISP
117	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	213	3	CRISP
118	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	324	6	CRISP
119	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	349	6	CRISP
121	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	306	5	PLA_2
122	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	187	3	PLA_2
	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	146	2	snaclec
123	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	220	4	CRISP
	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	128	2	snaclec
125	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	96	2	CRISP
	GSH peroxidase 3 [*P. mucrosquamatus*]	XP_015679695	27808	89	2	GSH peroxidase
126	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	125	2	CRISP
127	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	283	5	CRISP
130	GSH peroxidase 3 [*P. mucrosquamatus*]	XP_015679695	27808	44	1	GSH peroxidase
135	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	108	2	PLA_2
	glutaminyl-peptide cyclotransferase [*C. atrox*]	AFE84758	42299	100	2	QC
139	5′-nucleotidase [*G. brevicaudus*]	BAG82602	64433	88	2	5′-nucleotidase
140	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	138	2	PLA_2
147	calmodulin [*C. adamanteus*]	AFJ49577	16838	174	3	EF-hand
148	venom NGF [*V. ursinii*]	AEH59582	27284	250	4	NGF
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	107	2	PLA_2
149	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	141	2	PLA_2
150	venom NGF [*V. ursinii*]	AEH59582	27284	200	3	NGF
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	132	2	PLA_2
	VaaDis-2 [*V. a. ammodytes*]	KU249655	12146	121	2	Dis
151	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	96	2	PLA_2
152	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	263	5	snaclec
153	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	48	1	snaclec
154	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	143	3	snaclec
	factor X activator light chain 2 [*M. lebetina*]	AAT91068	18093	107	2	snaclec
	Vaa-snaclec-5, Vaa-snaclec-6 [*V. a. ammodytes*]	KU249651, KU249652	18546	104	2	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	43	1	snaclec
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	89	2	PLA_2
	calmodulin [*C. adamanteus*]	AFJ49577	16838	45	1	EF-hand
155	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	98	2	snaclec
156	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	247	4	snaclec
157	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	236	4	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	71	1	snaclec
158	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	157	3	snaclec
159	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	257	5	snaclec
160	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	207	4	snaclec
161	actin, cytoplasmic 1 [*C. adamanteus*]	AFJ49302	41736	48	1	actin
162	venom NGF [*V. ursinii*]	AEH59582	27284	145	2	NGF
163	venom NGF [*V. ursinii*]	AEH59582	27284	131	2	NGF
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	90	2	snaclec
164	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	159	3	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	45	1	snaclec
165	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	144	3	snaclec
166	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	115	2	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	106	2	snaclec
167	ammodytin L [*V. a. ammodytes*]	P17935	15636	114	2	PLA_2
168	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	195	4	PLA_2
	ammodytin L [*V. a. ammodytes*]	P17935	15636	98	2	PLA_2
	ammodytoxin B [*V. a. ammodytes*]	P11407	15498	42	1	PLA_2
169	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	436	6	PLA_2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	330	5	PLA_2
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	193	3	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	189	3	snaclec

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Table 1. continued

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	97	2	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	52	1	snaclec
170	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	394	5	PLA_2
	ammodytoxin C [*V. a. ammodytes*]	P11407	15498	73	1	PLA_2
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	293	5	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	106	2	snaclec
171	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	449	6	PLA_2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	256	4	PLA_2
172	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	444	7	PLA_2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	274	4	PLA_2
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	286	5	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	275	5	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	106	2	snaclec
173	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	290	5	PLA_2
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	164	3	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	51	1	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	53	1	snaclec
174	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	337	5	PLA_2
	ammodytoxin C [*V. a. ammodytes*]	P11407	15498	112	2	PLA_2
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	157	3	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	144	3	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	95	2	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	92	2	snaclec
	snaclec VP12 subunit A [*D. palaestinae*]	P0DJL4	12125	89	2	snaclec
175	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	325	5	PLA_2
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	171	3	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	98	2	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	53	1	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	47	1	snaclec
176	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	297	5	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	242	4	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	157	3	snaclec
	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	61	1	snaclec
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	263	4	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	238	4	PLA_2
177	ammodytoxin B [*V. a. ammodytes*]	P14424	15529	233	4	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	136	2	PLA_2
178	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	211	3	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	155	3	snaclec
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	233	4	PLA_2
	ammodytin I1 [*V. a. ammodytes*]	Q910A1	15434	162	3	PLA_2
179	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	381	6	snaclec
	snaclec VP12 subunit A [*D. palaestinae*]	P0DJL4	12125	92	2	snaclec
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	282	5	PLA_2
	ammodytoxin B [*V. a. ammodytes*]	P11407	15498	146	2	PLA_2
180	ammodytoxin C [*V. a. ammodytes*]	P11407	15498	297	5	PLA_2
181	ammodytoxin B [*V. a. ammodytes*]	P11407	15498	218	4	PLA_2
182	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	273	5	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	172	3	snaclec
	snaclec VP12 subunit B [*D. palaestinae*]	P0DJL5	15157	105	2	snaclec
	Vaa-snaclec-9 [*V. a. ammodytes*]	MG958494	18081	105	2	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	56	1	snaclec
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	150	3	PLA_2
183	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	199	3	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	173	3	PLA_2
	ammodytoxin C [*V. a. ammodytes*]	P11407	15498	109	2	PLA_2
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	111	2	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	109	2	snaclec
184	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	255	5	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	223	3	snaclec

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Table 1. continued

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
	ammodytoxin C [*V. a. ammodytes*]	P11407	15498	157	2	PLA_2
185	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	181	3	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	120	2	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	108	2	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	41	1	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	41	1	snaclec
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	298	5	PLA_2
186	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	400	6	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	97	2	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	44	1	snaclec
	ammodytin I_2 [*V. a. ammodytes*]	P34180	15309	286	5	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	282	5	PLA_2
187	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	371	5	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	246	4	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	184	3	snaclec
188	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	418	7	snaclec
	snaclec VP12 subunit A [*D. palaestinae*]	P0DJL4	12125	45	1	snaclec
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	261	4	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	186	3	PLA_2
	ammodytoxin B [*V. a. ammodytes*]	P11407	15498	102	2	PLA_2
189	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	338	6	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	318	5	PLA_2
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	205	3	snaclec
	Vaa-snaclec-4 [*V. a. ammodytes*]	KT148823	13785	143	3	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	142	2	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	87	2	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	48	1	snaclec
190	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	332	5	PLA_2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	212	3	PLA_2
	ammodytin I1 (E) isoform [*V. aspis aspis*]	CAE47133	15428	86	2	PLA_2
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	185	3	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	108	2	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	92	2	snaclec
192	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	284	4	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	176	3	PLA_2
	vammin [*V. a. ammodytes*]	ACN22045	16307	37	1	VEGF
193	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	234	3	snaclec
194	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	159	3	snaclec
	VaaDis-2 [*V. a. ammodytes*]	KU249655	12146	117	2	Dis
195	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	297	5	snaclec
	Vaa-snaclec-8 [*V. a. ammodytes*]	KU249654	15102	279	5	snaclec
196	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	215	4	PLA_2
	MP (type III) [*C. adamanteus*]	AFJ49231	67329	190	3	MP
197	Vaa-SP-3 [*V. a. ammodytes*]	KT148826	28168	126	3	SP
198	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	196	4	PLA_2
	Vaa-CRISP-1 [*V. a. ammodytes*]	KT148819	25459	173	3	CRISP
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	112	2	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	106	2	snaclec
	Vaa-SP-6 [*V. a. ammodytes*]	MG958495	28317	105	2	SP
199	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	99	2	PLA_2
200	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	372	6	PLA_2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	212	3	snaclec
	Vaa-snaclec-1 [*V. a. ammodytes*]	KT148820	15708	201	3	snaclec
	Vaa-snaclec-2 [*V. a. ammodytes*]	KT148821	15200	78	2	snaclec
	snaclec VP12 subunit A [*D. palaestinae*]	P0DJL4	12125	90	2	snaclec
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	51	1	snaclec
	Vaa-snaclec-3 [*V. a. ammodytes*]	KT148822	15519	48	1	snaclec
201	Vaa-snaclec-8 [*V. a. ammodytes*]	KU249654	15102	282	5	snaclec
	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	318	5	snaclec
	VaaDis-1 [*V. a. ammodytes*]	KT148829, KT148830	13983	140	2	Dis

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Table 1. continued

spot no.	protein	NCBI accession number	protein mass (Da)	Mascot score	matched peptides	protein family
202	ammodytoxin B [*V. a. ammodytes*]	P14424	15529	287	5	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	151	2	PLA_2
203	ammodytoxin B [*V. a. ammodytes*]	P14424	15529	126	2	PLA2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	108	2	PLA_2
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	99	2	PLA_2
	VaaDis-2 [*V. a. ammodytes*]	KU249655	12146	117	2	Dis
204	venom NGF [*V. ursinii*]	AEH59582	27284	262	4	NGF
	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	125	2	PLA_2
	ammodytoxin B [*V. a. ammodytes*]	P14424	15529	112	2	PLA_2
	ammodytin I2 [*V. a. ammodytes*]	P34180	15309	107	2	PLA_2
205	ammodytin I2 (C) isoform [*V. a. meridionalis*]	CAE47236	15391	222	3	PLA_2
	Vaa-snaclec-7 [*V. a. ammodytes*]	KU249653	15269	183	3	snaclec
206	VaaDis-2 [*V. a. ammodytes*]	KU249655	12146	138	2	Dis
	venom NGF [*V. ursinii*]	AEH59582	27284	123	2	NGF
	Vaa-MPII-1 [*V. a. ammodytes*]	KT148831	54586	68	1	MP/Dis
207	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	308	5	snaclec
	Vaa-snaclec-8 [*V. a. ammodytes*]	KU249654	15102	248	4	snaclec
208	C-type lectin-like protein 3B [*M. lebetina*]	AJO70723	17043	292	5	snaclec
	Vaa-snaclec-8 [*V. a. ammodytes*]	KU249654	15102	281	5	snaclec

[a]Cysteine residues were carbamidomethylated before MS/MS analysis. Abbreviations: AP, aspartic protease; CRISP, cysteine-rich secretory protein; Dis, disintegrin; GSH, glutathione; LAAO, L-amino acid oxidase; MP, metalloproteinase; NGF, nerve growth factor; QC, glutaminyl cyclase; PL, phospholipase; SP, serine protease.

32 spots in which proteins could not be recognized, some gave poor spectrometric data, probably due to insufficient protein levels, whereas others may just represent staining artifacts.[34] The identified proteins were assigned to 16 protein families, 7 of which are typical for viperid snake venoms—SVSPs, $sPLA_2$s, SVMPs, LAAOs, snaclecs, CRISPs, and Dis.[35] Figure 5 shows the protein family profile of the most abundant *Vaa* venom high-molecular-mass proteins (~10−60 kDa) according to the number of spots in which members of a particular family have been found. In accordance with the high hematotoxic potential of *Vaa* venom,[12] members of SVSP, snaclec, $sPLA_2$, and SVMP protein families were found in most of the spots.

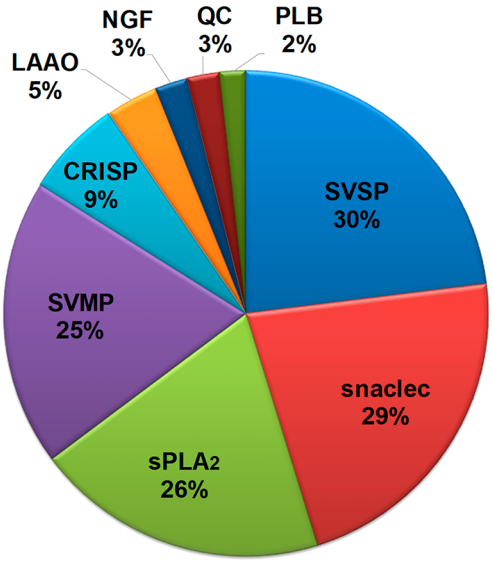

Figure 5. High-molecular-mass proteome profile of the *Vaa* venom. The profile is showing the relative distribution of spots among the most frequently represented *Vaa* protein families, that is, SVSP, snake venom serine protease; snaclec, snake C-type lectin-like protein; $sPLA_2$, secreted phospholipase A_2; SVMP, snake venom metalloproteinase; CRISP, Cys-rich secretory protein; LAAO, L-amino acid oxidase; NGF, nerve growth factor; QC, glutaminyl cyclase; and PLB, phospholipase B.

In contrast, in a proteomic study of *Vaa* venom from Bulgaria only 139 protein spots were reported on a 2-DE gel.[10] Of these, only 38 venom components were identified, being assigned to 8 protein families. This may well be the consequence of a lower sequence identity of the *Vaa* venom proteins to those in protein data banks. Although the nonredundant NCBI database currently contains >150 000 protein sequences from different snake species, identification of proteins by MS is limited due to the high level of interspecies sequence variation within a particular protein family, as well as to the presence of diverse post-translational modifications.[36] For this reason, a combination of transcriptomics and proteomics was used here to obtain a more complete proteome profile of the venom. Transcriptomic data contributed to the high rate of protein identification as well as to the high sequence coverage of the identified proteins. Practically all of the proteins predicted from the transcriptome analysis were then confirmed by the venomics.

3.2.1. Protein Families in the *Vaa* Venom. All of the protein components described in the *Vaa* venom are discussed below, grouped in protein families, from the most abundant to those present only in minute amounts. We begin with enzymes and conclude with nonenzymatic venom proteins.

3.2.1.1. Serine Proteases. Despite being abundantly present in *Vaa* venom, representatives of the SVSP family have been poorly investigated. Two kallikrein-like enzymes with apparent molecular masses of 31.3 and 34.3 kDa were purified from *Vaa* venom in 1976.[37] Only recently, we reported on the 31.5 kDa fibrin(ogen)ase with the unconventional catalytic triad, VaSP1 that can also degrade prothrombin, FX, and plasminogen.[38] In the current study, cDNA sequences of 8 SVSPs (Figure S-2) were determined. With the exception of Vaa-SP-7, their presence or that of their structurally close relatives was

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confirmed in almost one-third (i.e., 53) of all of the protein-containing 2-DE spots, mostly in the molecular mass range 30 to 45 kDa (Figure 4, Table 1, Table S-2). The cDNA transcript of Vaa-SP-5 is incomplete, lacking ~30 amino acids at the C-terminus. However, the known part of the molecule shows 96% sequence identity with Vaa-SP-3 (Figure S-2). Of the 12 spots in which we identified peptides common to both proteins, in only three were the Vaa-SP-3-specific peptides identified.

Vaa-SP-2 is a basic protein while Vaa-SP-3, Vaa-SP-4, Vaa-SP-5, and Vaa-SP-8 are acidic and Vaa-SP-6 and Vaa-SPH-1 neutral proteins. As is usual for viperid SVSPs,[39] the Vaa-SPs also possess various numbers of consensus *N*-glycosylation sites in their sequences, and thus have the potential of becoming *N*-glycosylated. Four such sites have been found in Vaa-SP-2 and Vaa-SP-6, three in Vaa-SP-4 and Vaa-SP-8, and two in each of Vaa-SP-3, Vaa-SP-5, and Vaa-SPH-1. *N*-glycosylation could explain the multiple pIs and much higher apparent molecular masses of these proteins than would be expected from their primary structures. Glycosylation is known to affect the stability of SVSPs, their activity and their responses to protein inhibitors.[40,41]

In spite of the wide range of substrate specificity, viperid SVSPs exhibit extensive sequence similarity.[39] Common structural characteristics of SVSPs—a C-terminal extension and 12 Cys residues that are assumed to form disulfides as in *Trimeresurus stejnegeri* venom plasminogen activator, TSV-PA,[42] are preserved in all full-length Vaa-SP transcripts (Figure S-2). The canonical active site catalytic triad, His−Asp−Ser, is preserved in all Vaa-SPs (Figure S-2) except Vaa-SPH-1, in which it is replaced by Arg−Asp−Asn. As expected, Vaa-SPH-1 is devoid of proteolytic activity.[13] It is, however, a strong inhibitor of coagulation, acting as an antagonist of FIXa. As deduced from their respective cDNAs, the same active site replacements as in Vaa-SPH-1 are also present in SVSP homologues from *Macrovipera lebetina* and *Bitis gabonica*,[43,44] so these proteins are not expected to be enzymes as well. Vaa-SPs, other than Vaa-SPH-1, are proteases that hydrolyze fibrinogen and activate FIX and FX, but not prothrombin.[12] The highest sequence identity was observed between Vaa-SP-2/Vaa-SP-4 and fibrogenases from *Macrovipera lebetina* (75% both with VLAF, and 72 and 88% with VLBF)[45] and from *Daboia russeli siamensis* venom (71 and 74% with RVAF)[46] (Figure S-2). Vaa-SP-3 and Vaa-SP-5 are, however, more similar to plasminogen activators (e.g., ≥ 73% identity with TSV-PA, Haly-PA, and LV-PA).[42,47,48] The very high sequence identity (95.7%) of Vaa-SP-7 to VLCTLP from *M. lebetina*, an angiotensin-cleaving enzyme and weak fibrinogenase with chymotrypsin-like activity,[49] was found. Similarly, Vaa-SP-6 differs in only four amino acid residues from nikobin (97.7% identity), an SVSP from *V. berus nikolskii* with an as yet unknown function (E5AJX2).

3.2.1.2. Phospholipases. Some years ago, we determined both protein and cDNA sequences of 5 *Vaa* venom $sPLA_2$s (presynaptically neurotoxic ammodytoxins (Atxs) A, B, and C, nontoxic ammodytins (Atns) I1 and I2) and one enzymatically inactive myotoxic $sPLA_2$ homologue, AtnL.[9,50] These were also found in the present proteomic analysis (Table 1, Table S-2). However, we could not discriminate between the Atx isoforms A and C in protein spots, since the peptides analyzed did not allow differentiation between these two proteins, whose sequences differ only in two amino acid residues at the C-terminal end.

In several 2-DE spots, instead of the expected AtnI2 sequence,[51] another AtnI2 (C) isoform[52] was detected (Figure S-5) that is usually the component of venoms of two other, eastern European *V. ammodytes* subspecies, *Vam* and *V. a. montadoni*. Because the *Vaa* venom analyzed was in fact a mixture of *Vaa* venom samples from different regions in Croatia, such a finding appears to be the consequence of a gene flow present in some of the viper specimens used for milking. Furthermore, besides AtnI1, found previously in *Vaa*, its (E) isoform was also detected in one of the 2-DE spots. This isoform has been identified so far only in another viper species, *V. aspis aspis* in southern France.[52] Indeed, peptides corresponding to the (E) isoform of AtnI1 were also found in a venom pool obtained from 8 *Vaa* specimens captured in the northwestern region of Bulgaria.[10] Again, this may also reflect a gene flow, in this case even between different viperid species.

Our bioinformatic analysis of the deduced *Vaa* PLB precursor sequence (MG958504) did not reveal the presence of an obvious signal peptide that would allow its secretion to the lumen of viper venom glands. However, in 2-DE spots 42, 44, 45, 48 (Figure 4), peptides homologous to stretches of PLB were identified (Table 1, Table S-2). In fact, PLB activity was reported in snake venoms a long time ago.[53,54] Only recently, however, the first protein sequences of SV PLBs have been obtained—in two pit vipers, *Protobothrops flavoviridis* and *Ovophis okinavensis*,[22] and in a colubrid *Spilotes sulphureus* snake.[55]

3.2.1.3. Metalloproteinases. *Vaa* venom is rich in SVMPs, found here in 44 spots on the 2-DE gel (25%) (Figure 4, Table 1, Table S-2). *Vaa* SVMPs are approximately equally represented by P-II and P-III class SVMPs. The latter are well characterized and exhibit a wide array of biological activities, most affecting the hemostatic system. They are, for example, hemorrhagic and fibrino(geno)lytic, activating or degrading blood coagulation factors (FX, FIX, prothrombin) and inhibiting platelet aggregation.[12,14,17,28,29,56−58] These *Vaa* venom components belong to monomeric P-IIIa (VaH1, VaH2, VaF1, ammodytase), homo- or heterodimeric P-IIIc (VaH3, VaH4, ammodytagin) and the oligomeric P-IIId subclass of SVMPs (VAFXA-I and VAFXA-II). Additionally, new *Vaa* SVMPs, corresponding to transcripts Vaa-MPIII-1, Vaa-MPIII-2, and Vaa-MPIII-4 (Figure S-3), but not to Vaa-MPIII-5, were detected at the protein level. Vaa-MPIII-1 and Vaa-MPIII-4 were identified in only one low intensity spot (spot 5), so it is not surprising that they have not yet been isolated and characterized. Their primary sequences show their high similarity to hemorrhagins from other viperid venoms (Figure S-3). They lack Cys176, Cys132 or both, that is, the residues involved in dimerization, and therefore belong to the P-IIIa subclass of SVMPs. Judged from their position on the 2-DE gel, they are acidic proteins with an apparent molecular mass of ~56 kDa. The discrepancy between their apparent and theoretical (~46 kDa) molecular masses probably reflects *N*-glycosylation, since five potential *N*-glycosylation sites are present in Vaa-MPIII-1 and one in Vaa-MPIII-4. Furthermore, all other characterized *Vaa* P-III SVMPs are glycoproteins. Vaa-MPIII-2 (spots 6, 10, 14, 15, 22−24) is most probably a P-IIId subclass SVMP, since it exhibits a high degree of sequence identity with the partial sequence of the heavy chain of FX activator from *Vaa*, VAFXA-I,[58] as well as with those of P-IIId SVMPs from other snake venoms expressing the same activity, VLFXA (Q7T046) and RVV-X (Q7LZ61) (Figure S-3). Peptides arising from the MP domain of VaH4-A were also

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identified in the 2-DE spots, with molecular masses of ∼30 kDa, indicating that this SVMP is processed, increasing the structural and functional complexity of the venom. Namely, some SVMPs (P-IIIb subclass) undergo autolysis at the D domain, releasing the C-terminal DC part (DC domains), retaining their platelet binding capability, and acting as platelet aggregation inhibitors.[59] However, the presence of the DC domain of VaH4-A in the venom was not confirmed.

Vaa-MPIII-3, encoded by the unique mRNA that lacks a part coding for the MP domain, was also identified at the protein level (Figure 6B, Table 2). It is presumably a glycoprotein with

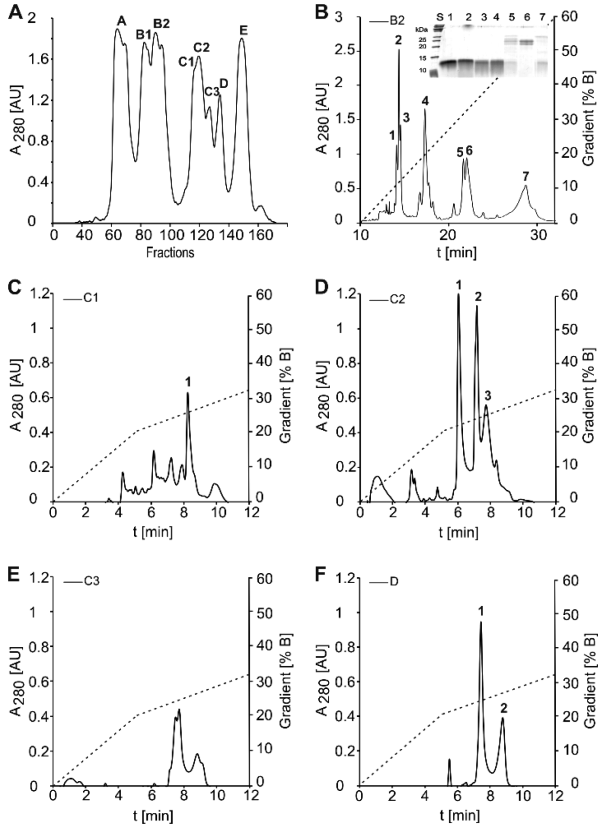

Figure 6. Low-molecular-mass proteome profiling of the *Vaa* venom. (A) Filtration of the crude *Vaa* venom on Sephacryl S200 gel resulted in eight fractions. (B) Gel filtration fraction B2 was further separated on the RP-HPLC C18 column. Figure inset shows the protein composition of peaks 1−7, as analyzed by 12.5% SDS-PAGE under nonreducing conditions. (C−F) Gel filtration fractions C1, C2, C3, and D were analyzed using the RP-HPLC C4 column. Proteins and peptides found in fractions were structurally characterized by N-terminal sequencing or MS/MS (Tables 2 and 3).

an apparent molecular mass of 21 kDa. The confirmation of the existence of such an SVMP-related protein in the venom as well led us to propose the introduction of a novel P-III subclass SVMP, a subclass P-IIIe. The function of such D′C domain proteins in the venom may be similar to that of the DC domain products of the post-translational processing of P-IIIb SVMPs, that is inhibition of platelet aggregation.[60−62] The potential platelet-binding capability of a D′C domain protein is probably related to an RGD-like motif in its D-loop.

All three Vaa-MPIIs are proteolytically processed to the P-I SVMP and Dis, as their MP domains have been found in numerous spots (93−95 and 98−110). These spots are distributed over a broad range of pIs in the region of molecular masses 26−28 kDa. Their masses are higher than those predicted theoretically. It is most probable that Vaa-MPIIs are *N*-glycosylated, as indicated by the existence of a potential *N*-glycosylation site at position 283 in the sequence of all three Vaa-MPIIs (Figure S-4) and an additional one at position 375 in the case of Vaa-MPII-3. All three Vaa-MPIIs show the highest sequence identity (89%) with fibrinogenolytic P-II SVMP, Le-3, from *M. lebetina*, which also undergoes processing to the MP- and Dis domain in the venom,[63] and to MPII precursors from *Echis* snake venoms (Figure S-4). A specific Dis domain peptide arising from Vaa-MPII-1 was detected in spot 206, together with two peptides from Vaa-Dis-2, suggesting that these two Dis form a disulfide linked dimer that was not completely reduced before the second-dimension SDS-PAGE. Because monomeric Dis have molecular masses <10 kDa, they should migrate with the electrophoretic front on a 2-DE gel, so they could not be spotted in this way. We analyzed them using a combination of liquid chromatography techniques (Figure 6; Table 2; Disintegrins section).

3.2.1.4. L-Amino Acid Oxidases. SV LAAOs are dimeric FAD- or FMN-binding enzymes giving venoms a characteristic yellowish color.[64] In the 2-DE gel, we identified *Vaa* venom LAAOs in six ∼55 kDa (9, 10, 15, 22−24) and two ∼43 kDa spots (32 and 38) (Figure 4, Table 1, Table S-2). Our cDNA library analysis revealed the presence of an LAAO precursor Vaa-LAAO-II (a 504 amino acid pre-pro-protein) that shares 92% amino acid identity with the mature form of Vaa-LAAO-I[65] (Figure S-6). Whereas Vaa-LAAO-I was the major LAAO isoform in the Bulgarian *Vaa* venom, Vaa-LAAO-II was in the majority in the Croatian venom that we analyzed. In addition to Vaa-LAAO-II, another LAAO isoform was identified in 2-DE spots 10 and 22 (Figure 4, Table 1, Table S-2), which is like *Ehis coloratus* (JAC96580). LAAOs are present in viperid venoms in different quantities, being a minor component, as in *Vaa* and *Vam* venoms,[10] or a major one, as in *Crotalus rhodostoma* venom.[66] In the latter case, LAAOs comprise one-third of the venom protein content. The pathophysiological effects of LAAOs, involving induction or inhibition of platelet aggregation, induction of apoptosis, hemolysis, hemorrhage, and edema, depend mainly on their production of hydrogen peroxide.[64,67]

3.2.1.5. Glutaminyl Cyclases. Glutaminyl cyclases (QCs) were detected in five 2-DE spots (39−41, 44, 135) (Figure 4, Table 1, Table S-2) with peptide sequences matching QCs from *D. russelii* (AFE84762) and *Crotalus atrox* (AFE84758) venoms. The *Vaa* venom QC is a ∼40 kDa protein that is most probably glycosylated, as is the QC from *C. atrox*. The primary structures of SVQCs, including two *N*-glycosylation sites, are highly conserved.[68] The ∼22 kDa protein with QC sequence in 2-DE spot 135 is probably a product of the proteolytic degradation of full-length QC. Although present in venoms in minute amounts, QCs have been found to be important in the post-translational modification of some venom proteins and peptides, for example, SVMPs and their tripeptide inhibitors, BPPs, and three-finger toxins.[27,68,69] They catalyze the formation of the N-terminal pyroglutamate residue in proteins and peptides, protecting them from degradation by exopeptidases.

3.2.1.6. Low-Abundance Enzymes. Some enzymes are present rarely and in low amounts in snake venoms.[70] Glutathione (GSH) peroxidase, aspartic protease, and 5′-nucleotidase are such enzymes in *Vaa* venom.

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Table 2. Low-Molecular-Mass Proteins Identified in the *Vaa* Venom[a]

HPLC fraction	protein	NCBI Acc. No.	N-terminal sequence	MS/MS peptide sequences	observed m/z	z	protein Mascot score	M + H$^+$ (Da)	protein family
1	Vaa-Dis-1 Vaa-Dis-1' Vaa-Dis-2 VA6 [*Vaa*]	KT148829 KT148830 KU249655 P0C6A5	NSANP						Dis
2	Vaa-Dis-2	KU249655	NSANPXXDPVTXKPRRGEHX	NSANPCCDPVTCKPR	888.54	2	209		
				FLNAGTICQYAR	707.79	2			
				NCKFLNAGTICQYAR	606.14	3		13861	
				GDDMNDYCTGISSDCPR	982.43	2		13844	
								13878	Dis
	VA6 [*Vaa*]	P0C6A5	NSANPXXDPVTXKPRRGEHX	NSANPCCDPVTCKPR	888.54	2	102	13828	
				GDDMNDYCTGISSDCPR	982.43	2		13954	
	Vaa-MPII-2	KT148832	NSGNPXXDPVTXKPRLGEHX	AVGDDMDDYCTGISSDCPR	1067.52	2	68	13973	
	Vaa-MPII-3	KT148833							
3	Vaa-Dis-1 Vaa-Dis-1' Vaa-Dis-2	KT148829 KT148830 KU249655	NSANP						
	VA6 [*Vaa*]	P0C6A5							Dis
	Vaa-MPII-1	KT148831	NSGNP						
	Vaa-MPII-2	KT148832							
	Vaa-MPII-3	KT148833							
4	Vaa-Dis-2	KU249655	NSANPXXDPVTXKPRRGEHX	NSANPCCDPVTCKPR	888.53	2	192		
				FLNAGTICQYAR	707.88	2			
				NCKFLNAGTICQYAR	606.22	3			
				GDDMoxNDYCTGISSDCPR	990.05	2		14027	
	VA6 [*Vaa*]	P0C6A5	NSANPXXDPVTXKPRRGEHX	NSANPCCDPVTCKPR	888.53	2	86	14044	Dis
				GDDM$_{ox}$NDYCTGISSDCPR	990.05	2		14420	
	Vaa-Dis-1 Vaa-Dis-1'	KT148829 KT148830	NSANPXXDPITXKPRKGEHX	NSANPCCDPITCKPR	597.66	3	105		
				FLNPGTICK	525.52	2			
5	Vaa-Dis-1, Vaa-Dis-1'	KT148829 KT148830	NSANPXXDPITX					14501	Dis
								14485	
								14536	
6	Vaa-MPIII-3	MG958499	RAGTEXRPARSE	ENDVPIPCAPEDIK	799.47	2	315		MP
				GESYFYCR	541.46	2			
				HCVDVTTAY	533.47	2			
				KGESYFYCR	605.53	2			
				LFCELIK	461.89	2			
				NTCKYDYSEDPDYGMVDHGTK	832.48	3			
				YDYSEDPDYGMVDHGTK	996.54	2			
7	vammin [*Vaa*]	ACN22045	blocked	CTPVGKHTVDIQIMR	877.45	2	139		VEGF
				EVRPFLEVHER	464.25	3			
				WVRPFLEVHER	489.26	3			

[a]Fraction B2 after gel filtration of crude *Vaa* venom (Figure 6A) was separated by RP-HPLC (Figure 6B), and the fractions were subjected to Edman sequencing. Major HPLC peaks were analyzed by nonreducing SDS-PAGE, proteins were in-gel digested with trypsin, and the resulting peptides were analyzed by tandem MS. Cys residues were carbamidomethylated before MS analysis but not before Edman sequencing. X denotes an unidentified amino acid residue, which is Cys in homologous sequences. Masses of molecular ions were determined by ESI-TOF analysis. Dis, disintegrin; MP, metalloproteinase; VEGF, vascular endothelial growth factor; M_{ox}, oxidized Met.

GSH peroxidase is an antioxidant enzyme that catalyzes the reduction of hydrogen peroxide to water by reduced glutathione. It was found in *Vaa* venom by 2-DE in two basic pI spots, 125 and 130 (Figure 4, Table 1, Table S-2). As a minor component, GSH peroxidase has been reported in venoms of only a few other snakes.[70,71] Its possible role in the venom is to protect lipids and proteins against oxidative damage by hydrogen peroxide.

Renin-like aspartic protease was found in only one 2-DE spot (spot 47) (Figure 4, Table 1, Table S-2). Thus far, such a protease has been identified as a minor venom component of various Russian vipers[11,19] and the Indian saw-scaled viper, *Echis c. carinatus*.[72] The latter protease was recently purified from the venom, and its renin-like activity was confirmed.[73] Renin is a mammalian aspartic protease catalyzing the first step of the renin−angiotensin pathway in which angiotensinogen is processed to angiotensin I. This is then cleaved by angiotensin-converting enzyme to angiotensin II, a vasoconstrictor. By exerting renin-like activity, SV aspartic proteases can induce hypertensive effects, local or systemic, as was reported in the case of *Vbb* envenomation.[74,75] In accord with the negligible quantity of the enzyme in the *Vaa* venom, no such effects have so far been reported following a *Vaa* venomous bite.

The *Vaa* venom 5′-nucleotidase was identified by two peptides identical to peptides from a 55 kDa 5′-nucleotidase (BAG82602) from *Gloydius blomhoffi brevicaudus* (Table S-2). However, spot 139 (Figure 4) harboring these two peptides was located at ∼20 kDa on the 2-DE gel, which suggests that the *Vaa* enzyme had undergone proteolytic cleavage. 5′-Nucleotidases are ubiquitous in SVs, although usually, as in the case of *Vaa* venom, in very small quantities.[76] They cleave 5′-nucleotides to liberate adenosine, which then induces various pharmacological effects, such as vasodilation or inhibition of platelet aggregation, in this way potentiating the overall venom toxicity.

3.2.1.7. Snaclecs. Snaclecs are the largest nonenzymatic group of proteins in the *Vaa* venom. They are found in almost one-third (51 spots) of all identified 2-DE spots (Figure 4, Table 1, Table S-2). The snaclec family of venom proteins comprises C-type lectin-like proteins, which do not bind sugars due to lack of the Ca^{2+}/sugar-binding loop in their domains homologous to the carbohydrate recognition domain (CRD) but are still able to bind various physiologically important proteins and receptors.[77,78] Snaclecs bind to receptors on platelets, inducing either inhibition or activation of their aggregation.[78] By provoking thrombocytopenia, they contribute to the venom toxicity that was also observed in *Vaa* envenomed patients.[7,79,80] Some of these patients that suffered severe coagulopathy developed acute thrombocytopenia without significant changes in blood coagulation kinetics or fibrinogen level, which supports a nonenzymatic mechanism of platelet-related snaclecs' toxicity.[81−83] Snaclecs also potentiate the hemorrhagic activity of SVMPs.[84]

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In venoms, snaclecs are present as heterodimers of α (14 to 15 kDa) and β (13 to 14 kDa) subunits cross-linked by a disulfide bond or as oligomers of the same or different $\alpha\beta$ heterodimers, $(\alpha\beta)_2$, $(\alpha\beta)_4$, and $(\alpha_1\beta_1)(\alpha_2\beta_2)$.[77] Snaclec structures of ~50 ($(\alpha\beta)_2$) and ~25 kDa ($\alpha\beta$), have been discovered in *Vaa* venom.[12] Five of the nine *Vaa* snaclec monomers characterized in this study have sequences similar to those of α subunits (Vaa-snaclec-1, -3, -5, -6, and -9), and the other four have sequences similar to β subunits (Vaa-snaclec-2, -4, -7, and -8) of snaclecs from other snake venoms (Figure S-7). As previously noted, Vaa-snaclec-5 and Vaa-snaclec-6 have identical mature amino acid sequences. The $(\alpha\beta)_2$ snaclec is composed of Vaa-snaclec-3 and Vaa-snaclec-2.[12] The greatest amount of amino acid sequence identity (mostly >90%) of *Vaa* snaclecs was found with various snaclecs from the *M. lebetina* venom, all of still unknown activity. Vaa-snaclec-1 and Vaa-snaclec-4 share high sequence similarity with the subunits A and B (83 and 98%) of snaclec VP12 from *Daboia palestinae*, which inhibits integrin $\alpha_2\beta_1$-dependent melanoma metastasis.[85] Vaa-snaclec-8, however, shows high sequence identity to that of the partial sequence of a light chain 1 (the snaclec subunit) of VAFXA-II from *Vaa*, the P-IIId SVMP that activates coagulation FX.[58] In procoagulant P-IIId SVMPs, as in VAFXA-II, dimeric snaclecs are present as subunits linked to the C domain by a disulfide bond. The snaclec subunit serves to bind the substrate, FX, at its Gla (γ-carboxyglutamate residues containing) domain, to present it properly to the catalytic site at the MP domain for effective proteolytic activation.[77]

3.2.1.8. Disintegrins. Disintegrins comprise another family of nonenzymatic dimeric toxins present in the *Vaa* venom (Tables 1 and 2; Table S-2).[12,15,86] They are common constituents of Viperinae venoms that act as integrin antagonists.[86−88] β-Subunits of dimeric Dis are derived from P-II SVMP precursors (e.g., Vaa-MPII-1, Vaa-MPII-2, and Vaa-MPII-3) in the process of post-translational proteolytic processing. α-Subunits, for example, Vaa-Dis-1, Vaa-Dis-2, and VA6, are encoded per se, by short-coding mRNAs that do not include a message for the MP domain.[86] Heterodimeric Dis are combinations of two diverse α subunits or one α and one β subunit, whereas just α subunits constitute homodimeric Dis. VA6 forms homodimers. Because sequences of Vaa-Dis-2 and VA6 differ in only four amino acid residues, three of which are similar, Vaa-Dis-2 probably also forms homodimers (Figure S-8). Such a conclusion is also supported by the molecular ion mass of 14 027 ± 1 Da, determined for a native protein in the HPLC fraction 2 (Figure 6B, Table 2), agreeing with the predicted masses of VA6 and Vaa-Dis-2 homodimers. However, of the other Dis molecular masses listed in Table 2, only two could be obtained by combining the theoretical masses of the known *Vaa* Dis monomers: 13 844 and 13 828 Da may be the masses of heterodimeric Dis that comprise Vaa-Dis-2 or VA6 as the α subunit and Vaa-MPII-1-Dis or Vaa-MPII-3-Dis as the β subunit. Many as-yet unknown Dis isoforms are therefore expected in *Vaa* venom. The feature common to α and β Dis subunits is that both possess 10 strictly conserved Cys residues that form the intra- and interchain disulfide bonds that define the conformation of the integrin-binding loop.[86] The specific recognition of integrins by Dis is defined primarily by the sequence of the integrin-binding motif at the tip of the integrin-binding loop (e.g., RGD, KGD, MGD, VGD, WGD, MLD) but also involves the amino acid residues flanking the tripeptide motif, where Dis display the highest level of sequence variability (Figure S-8). At least four different integrin-binding motifs, RGD, KGD, VGD, and MLD, are present in the *Vaa* Dis subunits (Figure S-8). The first two are found typically in Dis, where they inhibit platelet aggregation by binding to the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$. This interaction, already demonstrated in the case of VA6,[86] is additionally supported by the strong inhibition of ADP-induced platelet aggregation by crude *Vaa* venom as well as by the gel filtration fraction B2 that contains Dis.[12] Moreover, Dis, derived from Vaa-MPII-1 with the KGD motif, could represent a selective inhibitor of the integrin $\alpha_{IIb}\beta_3$, as shown for KGD-Dis barbourin from *Sistrurus barbouri*.[89]

Dis target integrin receptors of extracellular matrix proteins on various types of cell, in this way affecting adhesion between cells and the extracellular matrix, of the highest importance for normal tissue homeostasis. Misregulation of this process can result in the initiation and progression of a variety of diseases, such as cardiovascular, autoimmune, and cancer.[88] The receptor for fibronectin, integrin $\alpha_5\beta_1$, which is involved in angiogenesis, is targeted by different viperid RGD- and VGD-Dis, including VA6.[86] The same specificity is expected from Vaa-Dis-2 with the RGD motif and from VGD-Dis that stem from Vaa-MPII-2 and Vaa-MPII-3 (Figure S-8). Furthermore, MLD-containing Dis have been shown to bind various α_4 and β_1 integrins located on inflammatory and vascular endothelial cells, thus interfering with cell adhesion, proliferation, migration, and invasion.[90,91] For example, lebein-2 from *Macrovipera lebetina* and VLO5B from *Macrovipera lebetina obtusa* block the binding to β_1 integrins of laminin and the vascular cell adhesion molecule 1.[92] The MLD motif is also present in Vaa-Dis-1. Furthermore, its primary structure differs in only a few amino acids from those of lebein-2 and VLO5B, so the same activity can also be assumed for this molecule. As expected, a mixture of *Vaa* Dis significantly slowed down the migration of cancer cells.[15]

3.2.1.9. Cys-Rich Secretory Proteins. Vaa-CRISP-1 homologues were identified in 15 spots on 2-DE (Figure 4; Table 1, Table S-2) as having an apparent molecular mass of ~26 kDa. Some of these spots (114−119) were among the most intense in the 2-DE gel. Although acidic Vaa-CRISP isoforms prevail, basic CRISPs were found in spots 112 to 114. Vaa-CRISP-1 is, like other SV CRISPs, a single-chain protein containing 16 strictly conserved Cys residues that form eight disulfide bonds. Ten of the Cys residues are clustered at the C-terminal end of the molecule (Figure S-1), which is structurally similar to the K^+ channel blockers.[93] SV CRISPs constitute a subfamily of the large CAP protein superfamily (pfam PF00188), whose members occur in all life kingdoms and are involved in diverse patho/physiological processes.[94] Despite the wide distribution of CRISPs in snake venoms, the biological functions of only a few have been established. Most of these inhibit the contraction of smooth muscles by blocking ion-gated, voltage-gated, or cyclic nucleotide-gated ion channels.[95,96] Vaa-CRISP-1 exhibits the highest sequence identity (~96%) with two CRISPs from Viperinae snake venoms, *Vbb* (CAP74089) and *V. berus nikolskii* (B7FDI0), neither of whose activity is known.[97] Slightly less than identical to Vaa-CRISP-1 are ES-CRISP (~85% identity) from *Echis carinatus sochureki*,[98] having antiangiogenic activity, and triflin (~80%) from *Protobothrops flavoviridis*,[99] a Ca^{2+}-channel blocker (Figure S-9). Furthermore, Vaa-CRISP-1 exhibits high amino

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Table 3. Peptides from the *Vaa* Venom[a]

HPLC fraction	protein	NCBI accession number	N-terminal sequence	MS/MS peptide sequence	M + H$^+$ (Da)	peptide family
C1-1	Vaa-MPi-2, Vaa-MPi-3	KT148818, MG958491	DNEPPKKVPPNSXFG		3859	NP
C2-1	Vaa-MPi-1, Vaa-MPi-1′, Vaa-MPi-5	KU24965, KT148817, MG958493	DENQPPK			NP
	Vaa-MPi-2, Vaa-MPi-3	KT148818, MG958491	DNEPPKK			
	Vaa-MPi-1, Vaa-MPi-1′	KU24965, KT148817	blocked	pERRPPEIPP	1073	BPP
C2-2	trypsin inhibitor [*Vaa*]	P00991, AMH40741	blocked		6842, 7402	SPi
	Vaa-MPi-2, Vaa-MPi-3	KT148818, MG958491	DNEPPKKVPP			NP
	Vaa-MPi-1, Vaa-MPi-1′	KU24965, KT148817	blocked	pERRPPEIPP	1073	BPP
C2-3	trypsin inhibitor [*Vaa*]	P00991, AMH40741	blocked		6842, 7402	SPi
	Vaa-MPi-1, Vaa-MPi-1′, Vaa-MPi-5	KU24965, KT148817, MG958493	DENQPPKGSN			NP
	Vaa-MPi-1, Vaa-MPi-1′	KU24965, KT148817		ERRPPEIPP	1090	BPP
D-1	Vaa-MPi-1, Vaa-MPi-1′	KU24965, KT148817	ERRPPE	ERRPPEIPP	1090	BPP
	Vaa-MPi-1, Vaa-MPi-1′, Vaa-MPi-2, Vaa-MPi-3, Vaa-MPi-4, Vaa-MPi-5	KU24965, KT148817, KT148818, MG958491, MG958492, MG958493	blocked	pERWPGPKVPP	1145	
D-2	Vaa-MPi-1, Vaa-MPi-1′, Vaa-MPi-2, Vaa-MPi-3, Vaa-MPi-4, Vaa-MPi-5	KU24965, KT148817, KT148818, MG958491, MG958492, MG958493	blocked	pERWPGPKVPP	1145	BPP

[a]Fractions C1, C2, C3, and D obtained by gel filtration of crude *Vaa* venom (Figure 6A) were separated by RP-HPLC (Figure 6C−F), and designated major peaks were analyzed by Edman and ESI−MS/MS sequencing. Molecular ion masses were determined by ESI-TOF analysis. BPP, bradykinin-potentiating peptide; NP, natriuretic peptide; SPi, Kunitz-type serine protease inhibitor; pE, pyroglutamic acid.

acid similarity (~50%) to human CRISP-2 (NP_003287) and CRISP-3 (P54108).

3.2.1.10. Venom Nerve Growth Factor. Vaa-VNGF was identified in six 2-DE spots (148, 150, 162, 163, 204, and 206) (Figure 4, Table 1, Table S-2). It exhibits 97 to 98% sequence identity with VNGFs from *V. ursini* (AEH59582) and *M. lebetina* (AAV64846, P25428) venom (Figure S-10). Although the only isolated Vaa-VNGF cDNA (MG958503) codes for the C-terminally truncated protein, we were able to identify the missing sequence in two peptides, FIRIDTA**CVCVISR** and IDTA**CVCVISR**, in 2-DE spots, confirming that the full-length protein is expressed in the venom. VNGF, found in the venom of all venomous snake families, stimulates the growth of sensory and sympathetic nerves.[100,101] No direct toxic activity of VNGFs has been demonstrated so far,[2] but it has been suggested that they potentiate the action of certain other toxic components in venoms by binding to specific membrane receptors in a victim, increasing the vasopermeability or affecting its immune system.[100,102] VNGF from *Naja kaouthia* inhibits the proteolytic activity of SVMPs, to a degree comparable to that of inhibition of human MPs with the human β-NGF.[103] This suggests a further role of VNGFs in the regulation of the proteolytic activity of SVMPs.

3.2.1.11. Vascular Endothelial Growth Factor. Vaa-VEGF, or vammin, was identified in 2-DE spot 192 (Figure 4, Table 1, Table S-2) and in RP-C18 fraction 7 (Figure 6B, Table 2). It is a 25 kDa homodimer, a subtype of the VEGF-F molecule.[104] Vammin affects vasoconstriction by inducing hypotension and vascular permeability by a specific interaction with Tyr kinase receptor VEGFR-2 and the activation of the nitric oxide pathway.[105,106] In this way, it assists spreading of the venom from the bite site.

3.3. Low-Molecular-Mass Proteome Profiling of the *Vaa* Venom

The crude *Vaa* venom was first separated by gel filtration (Figure 6A). Fractions containing low-molecular-mass proteins and peptides (B2, C1, C2, C3, and D) were here analyzed by RP-HPLC (Figure 6B−F). The following low-molecular-mass proteins—Dis, a new P-IIIe SVMP subclass protein (Vaa-MPIII-3), and VEGF (Table 2), together with peptides—Kunitz-type SPis, NPs, and BPPs (Table 3)—were identified in the HPLC fractions by Edman sequencing and MS/MS analysis.

3.3.1. Peptide Families in the *Vaa* Venom. In the *Vaa* venom, peptides were discovered that can be classified into four groups according to their structure or biological activity. They are discussed below.

3.3.1.1. Kunitz-Type Serine Protease Inhibitors. Kunitz-type SPis are ~60 amino acid long polypeptides found in the venoms of Viperidae and Elapidae. They exhibit a structural fold similar to that in bovine pancreatic trypsin inhibitor.[107] *Vaa* venom contains potent inhibitors of trypsin and chymotrypsin (Table 3).[108,109] Like orthologues from *D. r. russelii* and *Pseudonaja t. textilis* venoms,[110,111] trypsin inhibitor also inhibits plasmin and plasma kallikrein, thus affecting fibrinolysis and blood coagulation. SPis can form complexes with other venom components to enhance or moderate their pathophysiological activities.[107] In such a way, *Vaa* chymotrypsin inhibitor forms a complex with neurotoxic sPLA$_2$, AtxA, thus augmenting its toxicity.[112]

3.3.1.2. Natriuretic Peptides. NPs are hormones that exert diuretic, natriuretic, and vasorelaxant activities by interacting with specific receptors, thus playing an important role in cardio-renal homeostasis.[113] Many snake venoms harbor such peptides, thereby participating in prey immobilization by inducing severe hypotension.[114] The latter is one of the common symptoms following *Vaa* envenomation in humans,[7,8,79] so it was no surprise that NPs were found in *Vaa* venom (Table 3). Group I Vaa-MPi precursors (Figure 2B) code for two 40 amino-acid-residue-long NP sequences that differ in only one amino acid residue at the C-terminus (Gly or Glu at position 38), whereas group II Vaa-MPi precursors (Figure 2C) encode a single 36 amino-acid-residue-long NP sequence. *Vaa* NPs exhibit substantial sequence identity with NPs from other snake venoms and with human NPs (Figure S-11), in which two strictly conserved Cys residues form a

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disulfide bond and a 17 amino-acid-residue ring of highly conserved primary structure.[115] Besides lowering the blood pressure, *Vaa* NPs can inhibit platelet aggregation by analogy to the homologous lebetin-2 from *Macrovipera lebetina*[116] and with PNP, the NP from *Pseudocerastes persicus* venom.[117]

3.3.1.3. Bradykinin-Potentiating Peptides. SV BPPs are Pro-rich peptides of 5 to 14 amino acid residues that induce systemic hypotension.[114] Their modular structure includes a pyroglutamic acid (pGlu or pE) at the N-terminus, the PXP motif (X is usually R, H, or G) in the middle, and the IPP sequence at the C-terminus.[118] Of the possible BPP sequences found in six Vaa-MPi precursors (Figure 2), only two, QRRPPEIPP and QRWPGPKVPP, were also detected in the venom (Table 3). Both have two Pro residues at the C-terminus, suggestive of strong bradykinin-potentiating activity.[119,120] All Vaa-MPi transcripts encode the decapeptidic BPP in different numbers of copies. In the venom, only one form of BPP was found, having a pE at its N-terminus (pERWPGPKVPP). The message for a shorter BPP was found, however, in only two transcripts, Vaa-MPi-1 and Vaa-MPi-1′, presumably representing allelic forms. This BPP was expressed in both the N-terminally blocked (pGlu at the N-terminus) and the free forms (Glu at the N-terminus). Although the latter is expected to be more susceptible to hydrolysis by aminopeptidases, its physiological effect may be greater than that of the former, as demonstrated in the case of BPP from *Gloydius halys* venom.[119]

3.3.1.4. Snake Venom Metalloproteinase Inhibitors. The catalytic activity of SVMPs in the venom is reduced by low pH, high concentrations of citrate ions,[121] and the presence of tripeptide inhibitors.[27,122−124] The latter are reversible, low-affinity inhibitors, highly concentrated in the venom gland. The sequence of a tripeptide inhibitor (QKW) is encoded frequently by the *Vaa* MPi transcripts (Figure 2). The pyroglutamic form of the inhibitor, pEKW, is the major constituent of the *Vaa* venom gel filtration fraction E.[12] It effectively inhibits the fibrinogenolytic activity of *Vaa* SVMPs (Figure S-12). Although the transcripts Vaa-MPi-1 and Vaa-MPi-2 code for a similar inhibitory tripeptide QNW, this, expectedly as pENW, has not been detected in the venom so far.

4. CONCLUSIONS

This work is the most comprehensive transcriptomic and proteomic survey of the *Vaa* venom to date. 45 different venom-related mRNA transcripts encoding peptide and protein precursors of 12 diverse types are characterized. More than 88% of the venom transcriptome comprises messages for MPis, BPPs, and NPs (all three on the same precursor), snaclecs, SVSPs, P-II and P-III SVMPs, $sPLA_2$s, and Dis. In the venom, representatives of 16 protein families, altogether 57 different proteins, were identified. Four of them—actin, calmodulin, PLB, and glutathione peroxidase—are likely to be contaminants that entered the venom from damaged cells lining the venom gland. Peptides identified in the venom were NPs, BPPs, inhibitors of SVSPs, and inhibitors of SVMPs. The most abundant and diversified venom proteins were SVSPs, $sPLA_2$s, snaclecs, and SVMPs, which account for 80% of all of the venom proteins and are responsible for the main toxic effects of the venom, including hemorrhage, coagulopathy, inhibition of platelet aggregation, and neurological disturbance. The production of antivenoms directed against their most toxic representatives is the way to a more effective and safer treatment of envenomed patients. Some newly discovered *Vaa* venom components open up novel lines of pharmacological research, for example, Vaa-LAAOs as potential antimicrobial, antitumor, and antiprotozoal agents, Vaa-snaclecs as inhibitors of melanoma metastasis, angiogenesis, and ion-channel activity, and Vaa-Dis as anticancer or antiplatelet agents. Venom peptides are also exciting; according to their structure, both Vaa-BPPs are expected to be endowed with a strong bradykinin-potentiating activity. Finally, our transcriptomic and proteomic analyses resulted in the discovery of an original SV protein, Vaa-MPIII-3. Its transcript is similar to that of P-III SVMPs but lacks the entire MP domain. The mature protein consists of just two domains, (truncated) D and C, thus defining a new subclass of SVMPs, the subclass P-IIIe. Such venom proteins presumably bind platelets and interfere with the hemostasis of the prey.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.9b00120.

Table S-1. Analysis of *Vaa* venom gland transcriptome by nucleotide sequencing of randomly selected cDNAs encoding venom-related peptide and protein precursors. Figure S-1. Alignment of translated *Vaa* MPIII transcripts. Figure S-2. Amino acid sequence alignment of Vaa-SP precursors with the most similar SVSPs. Figure S-3. Amino acid sequence alignment of *Vaa* MPIII transcripts with the most similar P-III SVMPs. Figure S-4. Amino acid sequence alignment of Vaa-MPII precursors with the most similar P-II SVMPs. Figure S-5. Amino acid sequence alignment of precursors of $sPLA_2$s found in *Vaa* venom. Figure S-6. Amino acid sequence alignment of the Vaa-LAAO-II (MG958502) precursor with the most similar SV LAAOs. Figure S-7. Alignment of precursor amino acid sequences of *Vaa* snaclecs with those of the most similar proteins from other snake venoms. Figure S-8. Amino acid sequence alignment of novel mature *Vaa* Dis with the most similar SV Dis. Figure S-9. Amino acid sequence alignment of mature Vaa-CRISP-1 with the most similar CRISPs from snake venoms. Figure S-10. Amino acid sequence alignment of the Vaa-VNGF precursor with those of the most similar SV VNGFs. Figure S-11. Amino acid sequence alignment of natriuretic peptides (NPs) from the *Vaa* venom with the most similar SV and human peptides. Figure S-12. Inhibition of fibrinogenolytic activity of *Vaa* SVMPs by a tripeptide inhibitor (PDF)

Table S-2. Report of MS data (XLSX)

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Notes

The authors declare no competing financial interest.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[125] partner repository with the data set identifier PXD012752 (DOI: 10.6019/PXD012752).

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