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

Adriana Leonardi, Tamara Sajevic, Jože Pungerčar, and Igor Kržiš

Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova cesta 39, SI-1000 Ljubljana, Slovenia

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 A2 (sPLA2s), 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, sPLA2s, 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-IIle 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, V. ammodytes 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 A2 (sPLA2s), known as ammodytoxins (Atxs).9 A comparative analysis of the Vaa and Vam proteomes...

Received: February 18, 2019
Published: April 24, 2019

DOI: 10.1021/acs.jproteome.9b00120
J. Proteome Res. 2019, 18, 2287–2309
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 snacles (snake C-type lectin-like proteins) and sPLA2s. 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.15 The nucleotide sequences were determined by Microsynth AG (Switzerland) using the dideoxynucleotide 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% (v/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-ZnCl2, the gel was scanned by an ImageScanner 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 +0.6 Da, respectively; carbamidomethyl Cys (C) as the fixed modification and oxidized methionine (Mox) 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...
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 CaCl2, 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
plasmin 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
cid 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 snacels (13.8%), SVSPs
(11.8%), P-III class SVMPs (11.0%), sPLA2s (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 (CRISP), 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. Snacel
precursors were thus represented by nine, SVSPs by eight, P-
III SVMPs by six, common MPis, BPP, and NP peptide
precursors by five, and sPLA2s 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
adamentus.21 Protobothrops flavoviridis,22 Bothrops colombiensis,
and Echis ocellatus.23 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 were
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.25 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.
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 vitro.
MPs, subunit A of heterodimeric VaH4 and homodimeric VaH3, 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, VaF129 and Vaa-MPIII-1, 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, 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.
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 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-

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-Zn²⁺ method.

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 Vbh 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 intra-molecular 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 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
Table 1. Assignment of the Vaa Venom Proteins in 2-DE Spots to Protein Families by LC–ESI-MS/MS Analysis of Tryptic Peptides

| 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 |
| 3        | metalloproteinase [E. coloratus] | ADI47654 | 55138 | 48 | 1 | MP |
| 5        | MP (type III) [C. adamanteus] | AFJ49231 | 67329 | 106 | 2 | MP |
| 6        | Vaa-MPII-1, Vaa-MPII-2 [V. a. ammodytes] | KT148831, KT148832 | 53479 | 60 | 1 | MP |
| 7        | Vaa-MPIII-1 [V. a. ammodytes] | MG958500 | 68844 | 43 | 1 | MP |
| 8        | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 45 | 1 | MP |
| 9        | Vaa-SP-6 [V. a. ammodytes] | KT148826, KT148828 | 28168, 25253 | 85 | 2 | SP |
| 10       | LAAO B variant 1 [E. coloratus] | JAC96580 | 56738 | 262 | 5 | LAAO |
| 11       | MP (type III) [C. adamanteus] | AFJ49231 | 67329 | 237 | 4 | MP |
| 12       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 216 | 3 | MP |
| 13       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 90 | 2 | snaclec |
| 14       | Vaa-MPII-1, Vaa-MPII-2 [V. a. ammodytes] | KT148831, KT148832 | 53479 | 237 | 4 | MP |
| 15       | MP (type III) [C. adamanteus] | AFJ49231 | 67329 | 187 | 3 | MP |
| 16       | Vaa-SP-6 [V. a. ammodytes] | MG958495 | 28317 | 130 | 3 | SP |
| 17       | MP [E. coloratus] | ADI47654 | 55138 | 81 | 2 | MP |
| 18       | MP [E. coloratus] | ADI47654 | 55138 | 85 | 2 | MP |
| 19       | MP [E. coloratus] | ADI47654 | 55138 | 80 | 2 | MP |
| 20       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 191 | 3 | MP |
| 21       | VaH3 [V. a. ammodytes] | AGL45259 | 68546 | 142 | 2 | MP |
| 22       | MP [E. coloratus] | ADI47654 | 55138 | 57 | 1 | MP |
| 23       | Vaa-LAAO-II [V. a. ammodytes] | MG958502 | 57102 | 609 | 10 | LAAO |
| 24       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 297 | 5 | MP |
| 25       | MP (type III) [C. adamanteus] | AFJ49231 | 67329 | 66 | 1 | MP |
| 26       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 209 | 3 | MP |
| 27       | MP (type III) [C. adamanteus] | AFJ49231 | 67329 | 190 | 3 | MP |
| 28       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 161 | 3 | MP |
| 29       | VaH3 [V. a. ammodytes] | AGL45259 | 68546 | 148 | 2 | MP |
| 30       | MP (type III) [C. adamanteus] | AFJ49231 | 67329 | 187 | 3 | MP |
| 31       | VaH3 [V. a. ammodytes] | AGL45259 | 68546 | 142 | 2 | MP |
| 32       | VaH3 [V. a. ammodytes] | AGL45259 | 68546 | 148 | 2 | MP |
| 33       | VaH3 [V. a. ammodytes] | AGL45259 | 68546 | 148 | 2 | MP |
| 34       | Vaa-LAAO-II [V. a. ammodytes] | MG958502 | 57102 | 609 | 10 | LAAO |
| 35       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 297 | 5 | MP |
| 36       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 209 | 3 | MP |
| 37       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 190 | 3 | MP |
| 38       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 161 | 3 | MP |
| 39       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 124 | 2 | MP |
| 40       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 105 | 3 | MP |
| 41       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 49 | 1 | MP |
| 42       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 105 | 3 | MP |
| 43       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 49 | 1 | MP |
| 44       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 105 | 3 | MP |
| 45       | Vaa-MPIII-2 [V. a. ammodytes] | MG958498 | 69218 | 49 | 1 | MP |
| spot no. | protein family | protein | NCBI accession number | protein mass (Da) | Mascot score | matched peptides | protein family |
|---------|----------------|---------|-----------------------|------------------|--------------|------------------|----------------|
| 44      | [D. ruscii] glutaminyl-peptide cyclotransferase | AF84762 | 42116                 | 323              | 6            | QC               |
| 45      | [O. okinavensis] PLB | BAN82155 | 64133                 | 154              | 3            | PLB              |
| 47      | [V. a. ammodytes] Vaa-SP-3, Vaa-SP-4 | KTI48827 | 28587                 | 148              | 3            | SP               |
| 48      | [V. a. ammodytes] renin-like AP [E. ocellatus] | CAGS260 | 43872                 | 4                | 1            | AP               |
| 49      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 153              | 3            | SP               |
| 50      | [V. a. ammodytes] Vaa-SP-2, Vaa-SP-3, Vaa-SP-5 | KT148825 | 28885                 | 212              | 4            | SP               |
| 51      | [V. a. ammodytes] Vaa-SP-2, Vaa-SP-5 | KT148825 | 28885                 | 243              | 5            | SP               |
| 52      | [V. a. ammodytes] Vaa-SP-2, Vaa-SP-5 | KT148825 | 28885                 | 249              | 4            | SP               |
| 53      | [V. a. ammodytes] Vaa-SP-2, Vaa-SP-5 | KT148825 | 28885                 | 238              | 5            | SP               |
| 56      | [V. a. ammodytes] Vaa-SP-4 | KT148827 | 28587                 | 226              | 5            | SP               |
| 57      | [V. a. ammodytes] Vaa-SP-8 | MG955497 | 28795                 | 4                | 1            | SP               |
| 58      | [V. a. ammodytes] Vaa-SP-8 | MG955497 | 28795                 | 171              | 3            | SP               |
| 59      | [V. a. ammodytes] Vaa-SP-4 | MG955497 | 28795                 | 158              | 3            | SP               |
| 60      | [V. a. ammodytes] Vaa-SP-3, Vaa-SP-5 | KT148826, KT148828 | 28168                 | 107              | 2            | SP               |
| 61      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 290              | 5            | SP               |
| 62      | [V. a. meridionalis] ammodytin I2 (C) isoform | CAE47236 | 15391                 | 89               | 2            | PLA            |
| 63      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 244              | 4            | SP               |
| 64      | [V. a. ammodytes] Vaa-SP-8 | MG955495 | 28317                 | 304              | 6            | SP               |
| 65      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 193              | 3            | SP               |
| 66      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 163              | 3            | SP               |
| 67      | [V. a. ammodytes] Vaa-SP-8 | MG955495 | 28317                 | 151              | 3            | SP               |
| 68      | [V. a. ammodytes] Vaa-SP-3, Vaa-SP-5 | KT148826, KT148828 | 28168                 | 106              | 2            | SP               |
| 69      | [V. a. ammodytes] Vaa-SP-8 | MG955497 | 28795                 | 47               | 1            | SP               |
| 70      | [V. a. ammodytes] Vaa-SP-8 | MG955497 | 28795                 | 178              | 3            | SP               |
| 71      | [V. a. ammodytes] Vaa-SP-4 | KT148827 | 28587                 | 111              | 2            | SP               |
| 72      | [V. a. ammodytes] Vaa-SP-4 | KT148824 | 26910                 | 135              | 2            | SP               |
| 73      | [V. a. ammodytes] Vaa-SP-8 | MG955497 | 28795                 | 160              | 3            | SP               |
| 74      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 215              | 4            | SP               |
| 75      | [V. a. ammodytes] Vaa-SP-3 | KT148824 | 26910                 | 69               | 1            | SP               |
| 76      | [V. a. ammodytes] Vaa-SP-7 | KT148824 | 26910                 | 102              | 2            | SP               |
| 77      | [C. adamanteus] calmodulin | AFJ49577 | 16838                 | 144              | 3            | E-hand          |
| 78      | [V. a. ammodytes] Vaa-SP-1 | KT148824 | 26910                 | 83               | 1            | SP               |
| 79      | [V. a. ammodytes] Vaa-SP-3 | KT148824 | 26910                 | 129              | 3            | SP               |
| 80      | [V. a. ammodytes] Vaa-SP-3 | KT148824 | 26910                 | 263              | 4            | SP               |
| 81      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 204              | 5            | SP               |
| 82      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 206              | 4            | SP               |
| 83      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 227              | 4            | SP               |
| 84      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 132              | 2            | MP               |
| 85      | [V. a. ammodytes] Vaa-SP-6 | MG955495 | 28317                 | 183              | 3            | MP               |
Table 1. continued

| spot no. | protein                        | NCBI accession number | protein mass (Da) | Mascot score | matched peptides | protein family |
|----------|--------------------------------|-----------------------|-------------------|--------------|------------------|----------------|
| 84       | Vaa-SP-6 [V. a. ammodytes]     | MG958495              | 28317             | 113          | 2                | SP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 110          | 2                | SP             |
|          | nikobin [V. nikobiki]          | CBW30778              | 28216             | 168          | 3                | SP             |
| 85       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 235          | 4                | SP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 104          | 2                | SP             |
|          | VaaH4-A [V. a. ammodytes]      | AHB62069              | 68662             | 113          | 2                | MP             |
| 86       | VaaH4-A [V. a. ammodytes]      | AHB62069              | 68662             | 382          | 5                | SP             |
|          | VaaH4-A [V. a. ammodytes]      | AHB62069              | 68662             | 123          | 2                | SP             |
|          | VaaH4-A [V. a. ammodytes]      | AHB62069              | 68662             | 131          | 2                | MP             |
| 87       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 202          | 2                | SP             |
|          | nikobin [V. nikobiki]          | CBW30778              | 28216             | 112          | 3                | SP             |
| 88       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 325          | 5                | SP             |
|          | nikobin [V. nikobiki]          | CBW30778              | 28216             | 162          | 3                | SP             |
| 89       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 257          | 4                | SP             |
| 90       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             |              |                  |                |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 87           | 1                | SP             |
| 91       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             |              |                  |                |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 87           | 1                | SP             |
| 92       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 180          | 3                | SP             |
| 93       | VaaH4-A [V. a. ammodytes]      | AHB62069              | 68662             | 122          | 2                | MP             |
| 94       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             |              |                  |                |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 105          | 2                | MP             |
| 95       | VaaH4-A [V. a. ammodytes]      | AHB62069              | 68662             | 121          | 2                | MP             |
| 96       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 113          | 2                | MP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 113          | 2                | MP             |
| 97       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 105          | 2                | MP             |
| 98       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 68           | 2                | MP             |
| 99       | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 100      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 101      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 102      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 103      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 104      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 122          | 2                | MP             |
| 105      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 106      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 122          | 2                | MP             |
| 107      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 122          | 2                | MP             |
| 108      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 122          | 2                | MP             |
| 109      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 110      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
|          | Vaa-SF-1 [V. a. ammodytes]     | KT148824              | 26910             | 122          | 2                | MP             |
| 111      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 112      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 113      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |
| 114      | Vaa-SF-6 [V. a. ammodytes]     | MG958495              | 28317             | 122          | 2                | MP             |

Journal of Proteome Research
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               | PL2            |
| 122      | ammodytin I2 [V. a. ammodytes] | P34180           | 15309             | 187          | 3               | PL2            |
| 123      | Vaa-CRISP-1 [V. a. ammodytes] | KT148819          | 25459             | 220          | 4               | CRISP          |
| 125      | Vaa-CRISP-1 [V. a. ammodytes] | KT148819          | 25459             | 96           | 2               | CRISP          |
| 126      | GSH peroxidase 3 [P. microsquama] | XP_015679695     | 27808             | 89           | 2               | GSH peroxidase |
| 135      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 108          | 2               | PL2            |
| 139      | S'-nucleotidase [G. brevicauda] | BAG82602         | 64433             | 88           | 2               | S'-nucleotidase |
| 140      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 138          | 2               | PL2            |
| 147      | calmodulin [C. adamanteus] | AFJ49577         | 16838             | 174          | 3               | EF-hand        |
| 148      | venom NGF [V. urinii] | AEHS95882        | 27284             | 250          | 4               | NFG            |
| 149      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 107          | 2               | PL2            |
| 150      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 141          | 2               | PL2            |
| 151      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 132          | 2               | PL2            |
| 152      | Vaa-smacle-9 [V. a. ammodytes] | MG058494        | 18081             | 263          | 5               | snaclec        |
| 153      | Vaa-smacle-7 [V. a. ammodytes] | KU249653        | 15269             | 48           | 1               | snaclec        |
| 154      | Vaa-smacle-9 [V. a. ammodytes] | MG058494        | 18081             | 143          | 3               | snaclec        |
| 155      | factor X activator light chain 2 [M. lebetina] | AAT91068        | 18093             | 107          | 2               | snaclec        |
| 156      | actin, cytoplasmic 1 [C. adamanteus] | AFJ49302        | 41736             | 48           | 1               | actin          |
| 157      | venom NGF [V. urinii] | AEHS95882        | 27284             | 145          | 2               | NFG            |
| 158      | Vaa-smacle-1 [V. a. ammodytes] | KT148820        | 15708             | 71           | 1               | snaclec        |
| 159      | Vaa-smacle-7 [V. a. ammodytes] | KU249653        | 15269             | 157          | 3               | snaclec        |
| 160      | Vaa-smacle-9 [V. a. ammodytes] | MG058494        | 18081             | 207          | 4               | snaclec        |
| 161      | actin, cytoplasmic 1 [C. adamanteus] | AFJ49302        | 41736             | 48           | 1               | actin          |
| 162      | venom NGF [V. urinii] | AEHS95882        | 27284             | 145          | 2               | NFG            |
| 163      | Vaa-smacle-1 [V. a. ammodytes] | KT148820        | 15708             | 90           | 2               | snaclec        |
| 164      | Vaa-smacle-9 [V. a. ammodytes] | MG058494        | 18081             | 159          | 3               | snaclec        |
| 165      | Vaa-smacle-2 [V. a. ammodytes] | KT148821        | 15200             | 45           | 1               | snaclec        |
| 166      | Vaa-smacle-9 [V. a. ammodytes] | MG058494        | 18081             | 144          | 3               | snaclec        |
| 167      | Vaa-smacle-1 [V. a. ammodytes] | KT148820        | 15708             | 115          | 2               | snaclec        |
| 168      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 195          | 4               | PL2            |
| 169      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236       | 15391             | 436          | 6               | PL2            |
| 170      | Vaa-smacle-1 [V. a. ammodytes] | KT148820        | 15708             | 193          | 3               | snaclec        |
| 171      | Vaa-smacle-2 [V. a. ammodytes] | KT148821        | 15200             | 189          | 3               | snaclec        |

Mascot score: 2296
DOI: 10.1021/acs.jproteome.9b00120
J. Proteome Res. 2019, 18, 2287–2309
Table 1. continued

| spot no. | protein                            | NCBI accession number | protein mass (Da) | Mascot score | matched peptides | protein family |
|----------|------------------------------------|-----------------------|-------------------|--------------|-----------------|----------------|
| 170      | ammodytin I2 (C) isoform [V. a. ammodytes] | KT148822 | 15519        | 97           | 2               | snaclec        |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 13785        | 52           | 1               | snaclec        |
| 171      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 394          | 5               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 293          | 5               | PL A           |
| 172      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 449          | 6               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 286          | 5               | PL A           |
| 173      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 290          | 5               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 164          | 3               | snaclec        |
| 174      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 337          | 5               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 112          | 2               | PL A           |
| 176      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 325          | 5               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 171          | 3               | snaclec        |
|          | Vaa-snaclec-2 [V. a. ammodytes]    | KT148823 | 15519        | 98           | 2               | snaclec        |
|          | Vaa-snaclec-1 [V. a. ammodytes]    | KT148823 | 15519        | 53           | 1               | snaclec        |
| 177      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 136          | 2               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 13785        | 3               | PL A           |
| 178      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 233          | 4               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 47           | 1               | snaclec        |
| 179      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 238          | 4               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 233          | 4               | PL A           |
| 180      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 136          | 2               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 233          | 4               | PL A           |
| 181      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 238          | 4               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 233          | 4               | PL A           |
| 182      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 136          | 2               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 233          | 4               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 233          | 4               | PL A           |
| 183      | ammodytin I2 (C) isoform [V. a. meridionalis] | CAE47236 | 15391        | 136          | 2               | PL A           |
|          | Vaa-snaclec-3 [V. a. ammodytes]    | KT148823 | 15519        | 233          | 4               | PL A           |
| spot no. | protein | NCBI accession number | protein mass (Da) | Mascot score | matched peptides | protein family |
|---------|---------|-----------------------|-------------------|--------------|-----------------|----------------|
| 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] | KT249653 | 15269 | 41 | 1 | snaclec |
| 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 I1 [V. a. ammodytes] | K34180 | 15309 | 286 | 5 | PLA2 |
|         | ammodytin I2 [C] isoform [V. a. meridionalis] | CAE47236 | 15391 | 282 | 5 | PLA2 |
| 187     | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 371 | 5 | PLA2 |
|         | 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] | KT148820 | 15708 | 205 | 3 | snaclec |
|         | snaclec VP12 subunit A [D. palaestinae] | P0DJL4 | 12125 | 45 | 1 | snaclec |
|         | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 261 | 4 | PLA2 |
|         | ammodytin I2 [C] isoform [V. a. meridionalis] | CAE47236 | 15391 | 186 | 3 | PLA2 |
|         | ammodytin B [V. a. ammodytes] | K14107 | 15498 | 102 | 2 | PLA2 |
| 189     | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 338 | 6 | PLA2 |
|         | ammodytin I2 [C] isoform [V. a. meridionalis] | CAE47236 | 15391 | 318 | 5 | PLA2 |
|         | 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] | KT249653 | 15269 | 48 | 1 | snaclec |
| 190     | ammodytin I2 [C] isoform [V. a. meridionalis] | CAE47236 | 15391 | 332 | 5 | PLA2 |
|         | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 212 | 3 | PLA2 |
|         | ammodytin I1 [E] isoform [V. aspis aspis] | CAE47133 | 15428 | 86 | 2 | PLA2 |
|         | Vaa-snaclec-1 [V. a. ammodytes] | KT148820 | 15708 | 185 | 3 | snaclec |
|         | Vaa-snaclec-7 [V. a. ammodytes] | KT148820 | 15200 | 108 | 2 | snaclec |
|         | Vaa-snaclec-3 [V. a. ammodytes] | KT148822 | 15519 | 92 | 2 | snaclec |
| 192     | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 284 | 4 | PLA2 |
|         | ammodytin I2 [C] isoform [V. a. meridionalis] | CAE47236 | 15391 | 176 | 3 | PLA2 |
|         | vammin [V. a. ammodytes] | ACN22045 | 16307 | 37 | 1 | VEGF |
| 193     | C-type lectin-like protein 3B [M. lebetina] | AJ070723 | 17043 | 234 | 3 | snaclec |
| 194     | C-type lectin-like protein 3B [M. lebetina] | AJ070723 | 17043 | 159 | 3 | snaclec |
|         | VaaDis-2 [V. a. ammodytes] | KU249655 | 12146 | 117 | 2 | Dis |
| 195     | C-type lectin-like protein 3B [M. lebetina] | AJ070723 | 17043 | 297 | 5 | snaclec |
|         | Vaa-snaclec-8 [V. a. ammodytes] | KU249654 | 15102 | 279 | 5 | snaclec |
| 196     | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 215 | 4 | PLA2 |
|         | 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 | PLA2 |
|         | 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] | KT249653 | 15269 | 106 | 2 | snaclec |
|         | Vaa-Sp-6 [V. a. ammodytes] | MG958495 | 28317 | 105 | 2 | SP |
| 199     | ammodytin I2 [V. a. ammodytes] | K34180 | 15309 | 99 | 2 | PLA2 |
| 200     | ammodytin I2 [C] isoform [V. a. meridionalis] | CAE47236 | 15391 | 372 | 6 | PLA2 |
|         | ammodytin I2 [V. a. ammodytes] | K34180 | 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] | KT148820 | 15708 | 51 | 1 | snaclec |
|         | Vaa-snaclec-3 [V. a. ammodytes] | KT148822 | 15519 | 48 | 1 | snaclec |
| 201     | Vaa-snaclec-8 [V. a. ammodytes] | KT148820 | 15708 | 282 | 5 | snaclec |
|         | C-type lectin-like protein 3B [M. lebetina] | AJ070723 | 197043 | 318 | 5 | snaclec |
|         | VaaDis-1 [V. a. ammodytes] | KT148829, KT148830 | 13983 | 140 | 2 | Dis |
High-molecular-mass proteins (the protein family pro-SVMPs, LAAOs, snaclecs, CRISPs, and Dis.35 Figure 5 shows the relative distribution of spots among the most frequently represented Vaa venom high-molecular-mass proteins (~10–60 kDa) according to the molecular masses of 31.3 and 34.3 kDa were purified from Vaa venom.33 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 and identified as VaSP1 and VaSP2, respectively.33,34 VaSP2 is able to degrade prothrombin, FX, and plasminogen.35,36 In addition, VaSP2 contains a high-hematotoxic potential.12,37,38 Members of SVSP, sPLA2, and SVMP protein families were found in most of the spots.

In contrast, in a proteomic study of Vaa venom from Bulgaria only 139 protein spots were reported on a 2-DE gel.39 Of these, only 38 venom component 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.40 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 proteomics.

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 fibrinogenase without the unconventional catalytic triad with Vaa-SP-7, 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 well 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 fibrinogenase without the unconventional catalytic triad with Vaa-SP-7, 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 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 fibrinogenase without the unconventional catalytic triad with Vaa-SP-7, 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...
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-SHP-1 neutral proteins. As is usual for viperid SVSPs, 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 pl’s 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 disulphides as in Trimeresurus stejnegeri venom plasminogen activator, TSV-PA, 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, so these proteins are not expected to be enzymes as well. Vaa-SPs, other than Vaa-SPH-1, are proteases that hydrolyse 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 VLF, and 72 and 88% with VLBF) and from Daboia russelli siamensis venom (71 and 74% with RVAF) (Figure S-2). Vaa-SP-3 and Vaa-SPH-5 are, however, more similar to plasminogen activators (e.g., ≥73% identity with TSV-PA, Haly-PA, and LV-PA).42-44 The very high sequence identity (95.7%) of Vaa-SP-7 to VLCLLP from M. lebetina, an angiotensin-cleaving enzyme and weak fibrinogenase with chymotrypsin-like activity, 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 (ESAJX2).

3.2.1.2. Phospholipases. Some years ago, we determined both protein and cDNA sequences of 5 Vaa venom sPLA₂s (presynaptically neurotoxic ammodytoxins (Atxs) A, B, and C, nontoxic ammodytins (Atns) I1 and I2) and one enzymatically inactive myotoxic sPLA₂, homologue, AtnL.56 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, another AtnI2 (C) isoform 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.25 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 Ophiophagus hannah, in a cobrarah 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, VaFl, ammodytase), homo- or heterodimeric P-IIIc (VaH3, VaH4, ammodytagn) and the oligomeric P-IIIId subclass of SVMPs (VAFXA-I and VAFXA-II). Additionally, new Vaa SVMPs, corresponding to transcripts Vaa-MPIII-I, Vaa-MPIII-2, and Vaa-MPIII-4 (Figure S-3), but not to Vaa-MPIII-S, 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-IIIId 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-IId 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
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 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 P-IIIe. The function of such D domain protein is probably glycosylated, as is the QC from Vaa-MPIII-3.

Some SVMPs (P-IIIb subclass) undergo autolysis at the D domain of Le-3, from M. lebetina, which also undergoes processing to the MP- and DC domains in the venom,63 and to MPII precursors from Echis snake venoms (Figure S-4). A specific DC domain peptide arising from Vaa-MPII-1 was detected in spot 206, together with two peptides from Vaa-Dis-2, suggesting that these two DCs 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. I-Amino Acid Oxidases. SV LAAsOs are dimeric FAD- or FMN-binding enzymes giving venoms a characteristic yellowish color.64 In the 2-DE gel, we identified Vaa venom LAAsOs 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 LAAsO precursor Vaa-LAAO-II (a 504 amino acid pre-pro-protein) that shares 92% amino acid identity with the mature form of Vaa-LAAO-I65 (Figure S-6). Whereas Vaa-LAAO-I was the major LAAsO 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 LAAsO isoform was identified in 2-DE spots 10 and 22 (Figure 4, Table 1, Table S-2), which is like Ehis coloratus (JAC96580). LAAsOs 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, LAAsOs comprise one-third of the venom protein content. The pathophysiological effects of LAAsOs, 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. russelli (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.7,16,69 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 S-5′-nucleotidase are such enzymes in Vaa venom.
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 vipers11,19 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 blomhoffii 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). 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 vipers11,19 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 blomhoffii 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 Ca2+/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

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

| 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; Mox, oxidized Met. |
In venoms, snacles 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 α/β heterodimers, (αβ)₂, (αβ)₄, and (α₁β₁)(α₂β₂). Snaclec structures of ∼50 ((αβ)₂) and ∼25 kDa (αβ), 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 (αβ)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 αβ₁-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-Illd SVMP that activates coagulation FX.⁵⁸ In procoagulant P-Illd 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 Vaa6, are encoded per se, by short-coding mRNAs that do not include a message for the MP domain.⁶⁰ Heterodimeric Dis are combinations of two diverse α subunits or one α and one β subunit, whereas just α subunits constitute homodimeric Dis. Vaa6 forms homodimers. Because sequences of Vaa-Dis-2 and Vaa6 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.⁸⁵ 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 α₁β₃. 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 α₁β₃, 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 αβ₁, 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 VDG-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 and β integrins located on inflammatory and vascular endothelial cells, thus interfering with cell adhesion, proliferation, migration, and invasion.⁹⁰⁻⁹¹ For example, lebein-2 from Macroviperera lebetina and VLOSB from Macroviperera obtusa block the binding to β₃ 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 VLOSB, 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.¹³

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 (plm PF00188), whose members occur in all life kingdoms and are involved in diverse patho/physiological processes.⁹⁴⁻⁹⁶ 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 nikolskia (B7FD10), 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 Protothrops flavovirdis,⁹⁷ a Ca²⁺-channel blocker (Figure S-9). Furthermore, Vaa-CRISP-1 exhibits high amino
acids (nature similar (~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 (AAV64646, 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, FIRDTPAPE (F), and PERRPPEIPP (F), and thereby 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. 

No direct toxic activity of VNGFs has been demonstrated so far, 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. 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. 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 vanmin, was identified in 2-DE spots 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. Vanmin affects vasconstriction by inducing hypotension and vascular permeability by a specific interaction with Tyr kinase receptor VEGFR-2 and the activation of the nitric oxide pathway. 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 venom of Viperidae and Elapidae. They exhibit a structural fold similar to that in bovine pancreatic trypsin inhibitor. Vaa venom contains potent inhibitors of trypsin and chymotrypsin (Table 3). Like orthologues from D. russelli and Pseudonaja textilis venom, 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. In such a way, Vaa chymotrypsin inhibitor forms a complex with neurotoxic sPLA2, AtxA, thus augmenting its toxicity.

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 cardiac-renal homeostasis. Many snake venoms harbor such peptides, thereby participating in prey immobilization by inducing severe hypotension. The latter is one of the common symptoms following Vaa envenomation in humans, so it was no surprise that NPs were found in Vaa venom (Table 3). Group I Vaa-MPis (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-MPis (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
disulfide bond and a 17 amino-acid-residue ring of highly conserved primary structure. Besides lowering the blood pressure, Vaa NPs can inhibit platelet aggregation by analogy to the homologous lebetin-2 from Macrovipera lebetina and with PNP, the NP from Pseudocerastes persicus venom.

3.3.1.3. Bradykinin-Potentiating Peptides. SV BPPs are Pro-rich peptides of 5 to 14 amino acid residues that induce systemic hypotension. 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. 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. 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 (pERWPGRPKVPP). 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.

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, and the presence of tripeptide inhibitors. 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 pyroglutamform of the inhibitor, pEKW, is the major constituent of the Vaa venom gel filtration fraction E. 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), snacles, SVSPs, P-II and P-III SVMPs, sPLA2s, 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, sPLA2s, snacles, 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-snacles 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 MPMPI transcripts with the most similar P-II SVMPs. Figure S-4. Amino acid sequence alignment of Vaa-MPII precursors with the most similar P-III SVMPs. Figure S-5. Amino acid sequence alignment of precursors of sPLA2S 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 snacles 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)

AUTHOR INFORMATION

Corresponding Author

*E-mail: igor.krizaj@ijs.si. Tel: +386 1 477 3626. Fax: +386 1 477 3984.

ORCID

Adriana Leonardi: 0000-0001-9854-9955
Jože Pungerčar: 0000-0001-7228-336X
Igor Krizaj: 0000-0003-0203-0708
Notes
The authors declare no competing financial interest. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD012752 (DOI: 10.6019/PXD012752).

ACKNOWLEDGMENTS
This work was supported by the Slovenian Research Agency grant (P1-0207). We are grateful to Dr. Roger H. Pain for critical reading of the manuscript. We also thank Jernej Pušnik, M.Sc., for his contribution to the analysis of the Vaa venom gland cDNA library.

REFERENCES
(1) Waheed, H.; Moin, S. F.; Choudhary, M. I. Snake venom: from deadly toxins to life-saving therapeutics. Curr. Med. Chem. 2017, 24, 1874–1891.
(2) Fry, B. G. From genome to "venome": Molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res. 2005, 15, 403–420.
(3) Fry, B. G.; Scheib, H.; Junqueira de Azevedo, I. de L. M.; Silva, D. A.; Caswell, N. R. Novel transcripts in the maxillary venom glands of advanced snakes. Toxicon 2012, 59, 696–708.
(4) Kordis, D.; Gubensk, F. Adaptive evolution of animal toxin multigene families. Gene 2000, 261, 43–52.
(5) Alencar, L. R. V.; Quental, T. B.; Graziottin, F. G.; Alfaro, M. L.; Martins, M.; Venzon, M.; Zaher, H. Diversification in vipers: Phylogenetic relationships, time of divergence and shifts in speciation rates. Mol. Phylogenet. Evol. 2016, 103, 50–62.
(6) Urenbacher, S.; Schweiger, S.; Tomović, L.; Crnobrnja-Isailovic, J.; Fumagalli, L.; Mayer, W. Molecular phylogeography of the nose-horned viper (Vipera ammodytes, Linnaeus (1758)): Evidence for high genetic diversity and multiple refuge in the Balkan peninsula. Mol. Phylogenet. Evol. 2008, 46, 1116–1128.
(7) Lukšić, B.; Bradarić, N.; Prgomet, S. Venomous snakebites in southern Croatia. Coll. Antropol. 2006, 30, 191–197.
(8) Karabuva, S.; Vrklić, I.; Brzić, I.; Ivić, I.; Lukšić, B. Venomous snakebites in children in southern Croatia. Toxicon 2010, 56, 112, 8–15.
(9) Kriz, I. Ammodytoxin: A window into understanding pharmacological toxicity of secreted phospholipases A2 and more. Toxicon 2011, 58, 219–229.
(10) Georgieva, D.; Risch, M.; Kardas, A.; Buck, F.; Von Bergen, M.; Betzel, C. Comparative analysis of the venom proteomes of Vipera ammodytes ammodytes and Vipera ammodytes meridionalis. J. Proteome Res. 2008, 7, 866–886.
(11) Latinovic, Z.; Leonardi, A.; Šribar, J.; Savjevic, T.; Žužek, M. C.; Frangē, R.; Halassy, B.; Trampuš-Bakija, A.; Pungerčar, J.; Kriz, I. Venomics of Vipera berus berus to explain differences in pathology elicited by Vipera ammodytes ammodytes envenomation: Therapeutic implications. J. Proteomics 2016, 146, 34–47.
(12) Savjevic, T.; Leonardi, A.; Kriz, I. An overview of hemostatically active components of Vipera ammodytes ammodytes venom. Toxins Rev. 2014, 33, 33–36.
(13) Latinovic, Z.; Leonardi, A.; Kovačič, L.; Koh, C. Y.; Šribar, J.; Bakija, A. T.; Venkateswarlu, D.; Kini, R. M.; Kriz, I. The first intrinsic tenase complex inhibitor with serine protease structure offers a new perspective in anticoagulant therapy. Thromb. Haemostasis 2018, 118, 1713–1728.
(14) Leonardi, A.; Savjevic, T.; Kovačič, L.; Pungerčar, J.; Lang Balija, M.; Halassy, B.; Trampuš-Bakija, A.; Kriz, I. Hemorrhagin VaH4, a covalent heterodimeric P-III metalloproteinase from Vipera ammodytes ammodytes with a potential antitumour activity. Toxicon 2014, 77, 141–155.
(15) Latinović, Z.; Leonardi, A.; Petan, T.; Žalaipah, M.; Kriz, I. Disintegrins from the venom of Vipera ammodytes ammodytes efficiently inhibit migration of breast cancer cells. Acta Chim. Slov. 2017, 64, 555–559.
(16) Leonardi, A.; Bias, D.; Kordiš, D.; Stöcklin, R.; Favreau, P.; Kriz, I. Conus consors snail venom proteomics proposes functions, pathways, and novel families involved in its venomic system. J. Proteome Res. 2012, 11, 5046–5058.
(17) Leonardi, A.; Gubensk, F.; Kriz, I. Purification and characterisation of two hemorrhagic metalloproteinases from the venom of the long-nosed viper, Vipera ammodytes ammodytes. Toxicon 2002, 40, 55–62.
(18) Sosić, I.; Gobec, M.; Brus, B.; Knež, D.; Živec, M.; Konc, J.; Lešnik, S.; Ogrižek, M.; Obreza, A.; Žigon, D.; et al. Nonpeptidic selective inhibitors of the chymotrypsin-like (pS i) subunit of the immunoproteasome. Angew. Chem., Int. Ed. 2016, 55, 5745–5748.
(19) Kováč, S. I.; Zignanshin, R. H.; Starkov, V. G.; Tselin, V. I.; Utkin, Y. N. Quantitative proteomic analysis of venoms from Russian vipers of Pelias group: Phospholipases A2 are the main venom components. Toxins 2016, 8, 105.
(20) Viala, V. L.; Hildebrand, D.; Trusch, M.; Arni, R. K.; Pimenta, D. C.; Schlüter, H.; Betzel, C.; Spencer, P. J. Pseudechis catenatus venom proteome: Insights into evolution and toxin clustering. J. Proteomics 2014, 110, 32–44.
(21) Rokyta, D. R.; Lemmon, A. R.; Margres, M. J.; Aronow, K. The venom-land grafitomique of the eastern diamondback rattlesnake (Crotalus adamanteus). BMC Genomics 2012, 13, 312.
(22) Aird, S. D.; Watanabe, Y.; Villar-Briones, A.; Roy, M. C.; Terada, K.; Mikheyev, A. S. Quantitative high-throughput profiling of snake venom gland transcriptomes and proteomes (Oovphis okavainensis and Protobothrops flavoviridis). BMC Genomics 2013, 14, 790.
(23) Suntravat, M.; Uzcategui, N. L.; Athaisit, C.; Helme, T. J.; Lucena, S. E.; Sánchez, E. E.; Acosta, A. R. Gene expression profiling of the venom gland from the Venezuelan mapanare (Bothrops colombiensis) using expressed sequence tags (ESTs). BMC Mol. Biol. 2016, 17, 7. 
(24) Wagstaff, S. C.; Sanz, L.; Juárez, P.; Harrison, R. A.; Calvete, J. J. Combined snake venoms and venom gland transcriptomic analysis of the occluded carpet viper, Echis ocellatus. J. Proteomics 2009, 71, 609–623.
(25) Caswell, N. R. On the ancestral recruitment of metalloproteinases into the venom of snakes. Toxicon 2012, 60, 449–454.
(26) Higuchi, S.; Murayama, N.; Saguchi, K.; Ohi, H.; Fujita, Y.; Camargo, A. C. M.; Ogawa, T.; Deshimaru, M.; Ohno, M. Bradykinin-potentiating peptides and C-type natriuretic peptides from snake venom. Immunopharmacology 1999, 44, 129–135.
(27) Wagstaff, S. C.; Favreau, P.; Cheneval, O.; Laing, G. D.; Wilkinson, M. C.; Miller, R. L.; Stöcklin, R.; Harrison, R. A. Molecular characterisation of endogenous snake venom metalloproteinase inhibitors. Biochem. Biophys. Res. Commun. 2008, 365, 650–656.
(28) Savjevic, T.; Leonardi, A.; Kovačič, L.; Lang Balija, M.; Kurtović, T.; Pungerčar, J.; Halassy, B.; Trampuš-Bakija, A.; Kriz, I. VaH3, one of the principal hemorrhagins in Vipera ammodytes ammodytes venom, is a homodimeric P-IIIc metalloproteinase inhibitor. Biochim. Biophys. Res. Commun. 2008, 565, 650–656.
(29) Leonardi, A.; Savjevic, T.; Latinovic, Z.; Pungerčar, J.; Lang Balija, M.; Trampuš Bakija, A.; Vidmar, R.; Halassy, B.; Kriz, I. Structural and biochemical characterisation of VaF1, a P-IIIa fibrinogenolytic metalloproteinase from Vipera ammodytes ammodytes venom. Biochimie 2015, 109, 78–87.
(30) Caswell, N. R.; Wagstaff, S. C.; Harrison, R. A.; Renjifo, C.; Wüster, W. Domain loss facilitates accelerated evolution and neofunctionalization of duplicate snake venom metalloproteinase toxin genes. Mol. Biol. Evol. 2011, 28, 2637–2649.
(31) Camacho, E.; Sanz, L.; Escalante, T.; Pérez, A.; Villalta, F.; Lomonte, B.; Neves-Ferreira, A. G. C.; Feoli, A.; Calvete, J. J.; Gutierrez, J. M.; et al. Novel catalytically-inactive PII metalloproteinases from a viperid snake venom with substitutions in the canonical zinc-binding motif. Toxins 2016, 8, 292.
alternative-splicing or genome alteration.

lebetina snake venom gland serine proteinase homologs - result of

Thromb. Haemostasis fibrinogenases from W. The crystal structure of the novel snake venom plasminogen activator proteinase from Russell's vipers.

analysis of serine proteases from Russell's vipers.

focusing and immobilized pH gradient gels with imidazole and zinc salts: Its application to the identification of isolectric focusing separated isoforms by in-gel proteolysis. Electro- phoresis 2001, 22, 1677−1685.

Calvete, J. J.; Sanz, L.; Angulo, Y.; Lumonte, B.; Gutierrez, J. M. Venoms, venomics, antivenomics. FEBS Lett. 2009, 583, 1736−1743. 

Fox, J. W.; Serrano, S. M. T. Exploring snake venom proteomes: Multifaceted analyses for complex toxin mixtures. Proteomics 2008, 8, 909−920.

Bailey, G. S.; Shipolini, R. A. Purification and properties of a kininogenin from the venom of Vipera ammodytes ammodytes. Biochem. J. 1976, 153, 409−414.

Kurtović, T.; Brgles, M.; Leonard, A.; Lang Balija, M.; Sajevic, T.; Allmaier, G.; Marchetti-Deschmann, M.; Halassy, B. VaSP1, catalytically active serine proteinase from Vipera ammodytes ammodytes venom with unconventional active site triad. Toxicon 2014, 77, 93−104.

Vaiyapuri, S.; Thiyagarajan, N.; Hutchinson, E. G.; Gibbins, J. M. Sequence and phylogenetic analysis of viper venom serine proteases. Bioinformation 2012, 8, 763−772.

Samel, M.; Subbi, J.; Siigur, J.; Siigur, E. Biochemical characterization of fibrinogenolytic serine proteinases from Vipera lebetina snake venom. Toxicon 2002, 40, 51−54.

Zhu, Z. Z.; Liang, Z.; Zhang, T.; Zhu, Z. Z.; Xu, W.; Teng, M.; Niu, L. Crystal structures and amidolytic activities of two glycosylated snake venom serine proteinases. J. Biol. Chem. 2008, 283, 10524−10532.

Parry, M. A.; Jacob, U.; Huber, R.; Wisner, A.; Bon, C.; Bode, W. The crystal structure of the novel snake venom plasminogen activator TSV-PA: a prototype structure for snake venom serine proteases. Structure 1998, 6, 1195−1206.

Siigur, E.; Aaspöll, A.; Siigur, J. Sequence diversity of Vipera lebetina snake venom gland serine proteinase homologs - result of alternative-splicing or genome alteration. Gene 2001, 263, 199−203.

Calvete, J. J.; Marcinikiewicz, C.; Sanz, L. Snake venomics of Idesia platyclada and Daboia russelli russelli: target recognizing model for ADAM/reprolysin family proteins. Biochem. Biophys. Res. Commun. 2009, 386, 156−164.

Menezes, M. C.; De Oliveira, A. K.; Melo, R. L.; Lopes-Ferreira, M.; Rioli, V.; Balan, A.; Paes Leme, A. F.; Serrano, S. M. T. Disintegrin-like/cysteine-rich domains of the represyn HF3: Site-directed mutagenesis reveals essential role of specific residues. Biochimie 2011, 93, 345−351.

Aaspöll, A.; Siigur, J. C. Phylogenetic analysis of serine proteases from Russell's vipers. Arch. Biochem. Biophys. 2003, 420, 395−401.

Zhu, Z. Z.; Gao, Y.; Zhu, Z. Z.; Yu, Y.; Zhang, X.; Zang, J.; Teng, M.; Niu, L. Structural basis of the autolysis of AaHIV suggests a novel target recognizing model for ADAM/reprolysin family proteins. Biochem. Biophys. Res. Commun. 2009, 386, 156−164.

Guo, C.; Liu, S.; Yao, Y.; Zhang, Q.; Sun, M.-Z. Past decade study of snake venom L-amino acid oxidase. Toxicon 2012, 60, 302−311.

Georgieva, D.; Kardas, A.; Buck, F.; Perbandt, M.; Betzel, C. Isolation, crystallization and preliminary X-ray diffraction analysis of L-amino acid oxidase from Vipera ammodytes ammodytes venom. Acta Crystallogr., F. Struct. Biol. Cryst. Commun. 2008, 64, 918−921.

Ponnudurai, G.; Chung, M. C.; Tan, N. H. Purification and properties of the L-amino acid oxidase from Malayan pit vipers (Calloselasma rhodostoma) venom. Arch. Biochem. Biophys. 1994, 313, 373−378.

Izidoro, L. F. M.; Sobrinho, J. C.; Mendes, M. M.; Costa, T. R.; Grabner, A. N.; Rodrigues, V. M.; da Silva, S. L.; Zanchi, F. B.; Zuliani, J. P.; Fernandes, C. F. C.; et al. Snake venom L-amino acid oxidases: trends in pharmacology and biochemistry. BioMed Res. Int. 2014, 2014, 1−19.
1246. Experiences with 147 cases. G.; Koutsojannis, C. M. Snake venom poisoning in Greece. Toxicon 2010, 56, 278–286.

73) Wilkinson, M. C.; Nightingale, D. J. H.; Harrison, R. A.; Wagstaff, S. C. Isolation and characterization of renin-like aspartic proteases from Echis ocellatus venom. Toxicon 2017, 137, 92–94.

74) Garkowski, A.; Czpurna, P.; Zajkowska, J.; Grzybucka, S.; Golbik, P.; Letmanowski, M.; Zajkowska, J. Vipera berus bites in Eastern Poland - a retrospective analysis of 15 case studies. Ann. Agric. Environ. Med. 2012, 19, 793–797.

75) Malina, T.; Krecak, L.; Warrell, D. A. Neurotoxicity and hypertension following European adder (Vipera berus berus) bites in Hungary: Case report and review. QJM 2008, 101, 801–806.

76) Dhananjaya, B. L.; D’Souza, C. J. M. The pharmacological role of phosphatases (acid and alkaline phosphomonoesterases) in snake venoms related to release of purines – a multitoxin. Basic Clin. Pharmacol. Toxicol. 2011, 108, 79–83.

77) Morita, T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. Toxicon 2005, 45, 1099–1114.

78) Clemetson, K. J. Snakecide (snake C-type lectins) that inhibit or activate platelets by binding to receptors. Toxicon 2010, 56, 1236–1246.

79) Frangides, C. Y.; Koulouras, V.; Kouni, S. N.; Tzortzatos, G. V.; Nikolau, A.; Pneumaticos, J.; Piriakaros, C.; Niarchos, C.; Kounis, N. G.; Koutsojannis, C. M. Snake venom poisoning in Greece. Experience with 147 cases. Eur. J. Intern. Med. 2006, 17, 24–27.

80) Al, B.; Orak, M.; Aldemir, M.; Glioglu, C. Snakebites in adults from the Diyarbakır region in southeast Turkey. Ulus. Travma Acil Cerrahi Derg. 2010, 16, 210–214.

81) Marinov, I.; Atanasov, V. N.; Stankova, E.; Duhalov, D.; Petrova, S.; Hubenova, A. Severe coagulopathy induced by Vipera ammodytes ammodytes snakebite in Bulgaria: A case report. Toxicon 2010, 56, 1066–1069.

82) Kurtović, T.; Brvar, M.; Grenc, D.; Lang Balija, M.; Križaj, L.; Halassy, B. A single dose of Viperafav(tm) may be inadequate for Vipera ammodytes snake bite: A case report and pharmacokinetic evaluation. Toxins 2016, 8, 244.

83) Brvar, M.; Kurtović, T.; Grenc, D.; Lang Balija, M.; Križaj, L.; Halassy, B. Vipera ammodytes bites treated with antivenom ViperaTab: a case series with pharmacokinetic evaluation. Clin. Toxicol. 2017, 55, 241–248.

84) Lucavdaco, A.; Soto, M.; Escalante, T.; Loria, G. D.; Arni, R.; Gutierrez, J. M. Thrombocytopenia and platelet hypopaggregation induced by Bothrops asper snake venom. Toxins involved and their contribution to metalloproteinase-induced pulmonary hemorrhage. Thromb. Haemostasis 2005, 94, 123–131.

85) Staniszworska, I.; Walsh, E. M.; Rothman, V. L.; Gaathan, A.; Tuszynski, G. P.; Calvete, J. J.; Lazarovici, P.; Marcinkiewicz, C. Effect of VP12 and viperinstitin on inhibition of collagen-receptor-dependent melanoma metastasis. Cancer Biol. Ther. 2009, 8, 1507–1516.

86) Calvete, J. J.; Moreno-Murciano, M. P.; Theakston, R. D. G.; Kiselew, D. G.; Marcinkiewicz, C. Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering. Biochem. J. 2003, 372, 725–734.

87) Calvete, J. J. Brief History and Molecular Determinants of Snake Venom Disintegrin Evolution. In Toxins and Hemostasis; Springer Netherlands: Dordrecht, The Netherlands, 2010; pp 285–300.

88) Marcinkiewicz, C. Applications of snake venom components to modulate integrin activities in cell–matrix interactions. Int. J. Biochem. Cell Biol. 2013, 45, 1974–1986.

89) Scarborough, R. M.; Naughton, M. A.; Teng, W.; Rose, J. W.; Phillips, D. R.; Nannini, L.; Artisen, A.; Campbell, A. M.; Charo, I. F. Design of potent and specific integrin antagonists. Peptide antagonists with high specificity for glycoprotein IIb-IIIa. J. Biol. Chem. 1993, 268, 1066–1073.

90) Walsh, E. M.; Marcinkiewicz, C. Non-RGD-containing snake venom disintegrins, functional and structural relations. Toxicon 2011, 58, 355–362.

91) Arruda Macêdo, J.; Fox, J.; Souza Castro, M. Disintegrins from snake venoms and their applications in cancer research and therapy. Curr. Protein Pept. Sci. 2015, 16, 532–548.

92) Eble, J. A.; Bruckner, P.; Mayer, U. Vipera lebetina venom contains two disintegrins inhibiting laminin-bonding beta1 integrins. J. Biol. Chem. 2003, 278, 26488–26496.

93) Gao, M.; Teng, M.; Niu, L.; Liu, Q.; Huang, Q.; Hao, Q. Crystal structure of the cysteine-rich secretory protein stecrisp reveals that the cysteine-rich domain has a K' channel inhibitor-like fold. J. Biol. Chem. 2005, 280, 12405–12412.

94) Abraham, A.; Chandler, D. E. Tracing the evolutionary history of the CAP superfamily of proteins using amino acid sequence homology and conservation of splice sites. J. Mol. Evol. 2017, 85, 137–157.

95) Yamazaki, Y.; Morita, T. Structure and function of snake venom cysteine-rich secretory proteins. Toxicon 2004, 44, 227–231.

96) Matsuanga, Y.; Yamazaki, Y.; Hyodo, F.; Sugiyama, Y.; Nozaki, M.; Morita, T. Structural divergence of cysteine-rich secretory proteins in snake venoms. J. Biochem. 2009, 145, 365–375.

97) Ramazanova, A. S.; Starkov, V. G.; Ouspov, A. V.; Zagazin, R. H.; Filkin, S. Y.; Tsetlin, V. I.; Utkin, Y. N. Cysteine-rich venom proteins from the snakes of Viperinae subfamily - Molecular cloning and phylogenetic relationship. Toxicon 2009, 53, 162–168.

98) Lecht, S.; Chiaverelli, R. A.; Gerstenhaber, J.; Calvete, J. J.; Lazarovici, P.; Casevell, N. R.; Harrison, R.; Lelkes, P. I.; Marcinkiewicz, C. Anti-angiogenic activities of snake venom CRISP isolated from Echis carinatus sochureki. Biochim. Biophys. Acta, Gen. Subj. 2015, 1850, 1169–1179.

99) Yamazaki, Y.; Koike, H.; Sugiyama, Y.; Motoyoshi, K.; Wada, T.; Hishinuma, S.; Mit, M.; Morita, T. Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. Eur. J. Biochem. 2002, 269, 2708–2715.

100) Koistia, T.; Meier, J. Nerve growth factors from snake venoms: chemical properties, mode of action and biological significance. Toxicon 1996, 34, 787–806.

101) Trummal, K.; Töningmädgi, K.; Paalme, V.; Järvelä, L.; Siigur, J.; Siigur, E. Molecular diversity of snake venom nerve growth factors. Toxicon 2011, 58, 363–368.

102) Sunagar, K.; Fry, B. G.; Jackson, T. N. W.; Casevell, N. R.; Undheim, E. A. B.; Vidal, N.; Ali, S. A.; King, G. F.; Vasudevan, K.; Vasconcelos, V.; et al. Molecular evolution of vertebrate neurotrophins: co-option of the highly conserved nerve growth factor gene into the advanced snake venom arsenal. PLoS One 2013, 8, 81827.

103) Wijeyewickrema, L. C.; Gardiner, E. E.; Gladigau, E. L.; Berndt, M. C.; Andrews, R. K. Nerve growth factor inhibits metalloproteinase-disintegrins and blocks ecdysionegging of platelet glycoprotein VI. J. Biol. Chem. 2010, 285, 11793–11799.

104) Yamazaki, Y.; Morita, T. Molecular and functional diversity of vascular endothelial growth factors. Mol. Diversity 2006, 10, 515–527.

105) Yamazaki, Y.; Takani, K.; Atoda, H.; Morita, T. Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2). J. Biol. Chem. 2003, 278, 51985–51988.
Identification of proteins interacting with ammodytoxins in Vipera Lang Balija, M.; Allmaier, G.; Marchetti-Deschmann, M.; Halassy, B. Anal. Bioanal. Chem. ammodytes ammodytes venom by immuno-affinity chromatography. Studies. Vipera ammodytes inhibitors from Pathophysiol. Haemostasis Thromb. aggregation inhibitors. 434 Heart Assoc. 2015 agents. Curr. Med. Chem. peptides and low mass proteins: Molecular tools and therapeutic Soares, A. M.; Vale, N.; de C. Gomes, P. A.; et al. Snake venom regulation of cardiovascular physiology and metabolic events. Journal of Proteome Research Article Natriuretic peptide drug leads from snake venom. 154 Pseudonaja textilis textilis from Winzor, D. J.; Watters, D. J.; Lavin, M. F.; Gaffney, P. J. Textilinins inhibitors that reduce bleeding in an animal model. Fibrinolysis 2000, 11, 385–393. Characterization of a Kunitz-type protease inhibitor peptide (Rusvukiunin) purified from Daboia russelli russelli venom. Int. J. Biol. Macromol. 2014, 67, 154–162. Identification of proteins interacting with ammodytoxins in Vipera ammodytes ammodytes venom by immuno-affinity chromatography. Anal. Bioanal. Chem. 2014, 406, 293–304. Natriuretic peptides in the regulation of cardiovascular physiologic physiology and metabolic effects. J. Am. Heart Assoc. 2015, 4, 002423. El Ayeb, M. Lebetin peptides: potent platelet aggregation inhibitors. Pathophysiol. Haemostasis Thromb. 2002, 31, 207–210. Identification of proteins interacting with ammodytoxins in Vipera ammodytes ammodytes venom by immuno-affinity chromatography. Anal. Bioanal. Chem. 2014, 406, 293–304. Natriuretic peptides in the regulation of cardiovascular physiology and metabolic effects. J. Am. Heart Assoc. 2015, 4, 002423. El Ayeb, M. Lebetin peptides: potent platelet aggregation inhibitors. Pathophysiol. Haemostasis Thromb. 2002, 31, 207–210. Identification of proteins interacting with ammodytoxins in Vipera ammodytes ammodytes venom by immuno-affinity chromatography. Anal. Bioanal. Chem. 2014, 406, 293–304. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45. The modular nature of bradykinin-potentiating peptides isolated from snake venoms. J. Venomous Anim. Toxins Incl. Trop. Dis. 2017, 23, 45.