Comparative Profiling of Three Atheris Snake Venoms: A. squamigera, A. nitschei and A. chlorechis

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Comparative profiling of three *Atheris* snake venoms: *A. squamigera, A. nitschei* and *A. chlorechis*

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Abstract

A proteomic and transcriptomic comparative analysis of the venoms of three *Atheris* species (*A. squamigera, A. nitschei* and *A. chlorechis*) was carried out by size exclusion liquid chromatography, gel electrophoresis, mass spectrometry, and mRNA sequencing. The improved proteomic profiling utilised in this work was combined with transcript studies, advancing our insights into venom composition, protein distribution and inter-species variation among the three bush vipers. Crude venoms of all three samples contained at least 10-20 protein components, ranging in size from <= 3KDa to >98KDa. Both approaches yielded converging overall information, pointing to phospholipases, disintegrins, serine proteases and metalloproteases as the major toxin classes, which are likely to explain the local and systemic symptoms observed in envenomation by *Atheris* genus. Being considered as the main factors involved in the distinct venom-induced pathologies, these identified snake venom proteins are of particular interest in terms of understanding their physiological and biological function as well as for their contribution in potential medical treatments.

Key words:

Atheris, proteomics, snake venom, toxins

List of Abbreviation

A. chlorechis	*Atheris chlorechis*
A. nitschei	*Atheris Nitschei*
A. squamigera	*Atheris squamigera*
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
CDD	Conserved domain database
cDNA	Complementary DNA
CHCA	Alpha-cyano-5-hydroxycinnamic acid
MALDI-TOF	Matrix-assisted laser desorption ionization, time-of-flight
mRNA	Messenger RNA
MS	Mass spectrometry
NCBI	National Centre of Biotechnology Information
NUP	Nested universal primer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pHpG	Poly-histidine and poly-glycine
RACE	Rapid Amplifiction of cDNA Ends
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SMART	Switching Mechanism At 5' end of RNA Transcript
SVMP	Snake venom metalloproteinases
SVSP	Snake venom serine proteinases

Introduction

Viper snakebites account for the majority of deadly envenomation accidents all over the world, caused by the action of venom toxins [1]. Viper venoms are usually composed of toxins belonging to several protein families: snake venom serine proteinases (SVSP), phospholipases, snake venom metalloproteinases (SVMP), L-amino acid oxidases as well as non-enzymatic proteins such as disintegrins and protease inhibitors [2-3]. For species-specific snake venoms, their intraspecific variability in protein composition, distinct distribution of proteomic components and the presence of isoforms are common problems that one has to face when the development of a specific antidote is needed [4]. However, this issue has encouraged the isolation and characterisation of snake venom components, in particular proteins, at a large scale to improve the quality of therapeutic strategies. Moreover, snake venom proteins are known to be of considerable physiological, biological and medical value, making them an important resource of bioactive molecules with great potential for new drug development [5].

The genus *Atheris* (bush vipers) belongs to the family Viperidae and occurs in the African rainforests of the tropical subsaharan regions [6]. A brief description of the three species studied here is listed in Table 1[6]. These snakes have been held responsible for serious bites in quite a few of cases [7-10]. Envenomation upon bites of *Atheris* are clinically-characterised by coagulation disturbance (prolonged coagulation time and non-clotting of blood), bleeding and haemorrhaging, local pain, and oedema [8,11]. Some victims developed acute renal failure [9] and even hypertension [10]. Symptomatic replacement therapy is commonly applied due to the absence of an *Atheris* specific antivenom [7-8,10]. However, treatment of *A. chlorechis* envenomation using a non-specific antiserum (FAV AFRIQUE) described by Top [9], appeared to be efficient in neutralising the toxins, although knowledge about the antidotal effect was incomplete at that time. Interestingly, an antivenom against

another viper genus, *Echis*, has been reported to be effective in a case of human envenomation by *A. squamigera* [12]. The venom compositions of species from the genus *Atheris* have not been fully elucidated yet. Biochemical and biological investigations of venom from *A. squamigera* revealed the existence of some components causing haemocoagulation disorders [8]. These studies emphasised the need for further investigation on *Atheris* venom toxins, in particular the identification of venom components, the elucidation of mechanisms leading to venom toxicity as well as the quantitative and qualitative characterization in terms of the venom protein pattern.

The lack of information may derive from some difficulties and the limits of analytical techniques, which require alternative approaches to separate complex protein mixture. However, recent developments in proteomics provide helpful tools to overcome this issue and could thus help to improve a comprehensive analysis of crude snake venom and identification of the containing proteins from such complex mixture [13-14]. Therefore, proteomic studies of snake venoms could give new insight into the global venom proteome, focusing on the protein expression pattern and functional characterisation. To explain the molecular basis of envenomation pathophysiologies, our comparative, proteomic analyses allowed us to hypothesise about the roles of different proteins in toxicological processes, thereby providing a way to understand their clinical significance. In this work, the characterisation and comparative study of the venom proteomes of three *Atheris* bush vipers by use of SDS-PAGE, chromatographic fractionation and mass spectrometry, is described. In addition, the venom gland complementary DNA libraries were constructed and molecular cloning of the main venom transcripts was performed. We previously reported the cloning and characterisation of three novel cDNAs encoding disintegrin precursors [15] and three novel cDNAs encoding phospholipase A_2 precursors [16] from the venoms of the three *Atheris* vipers from the present study using the same molecular cloning strategy. The existing set of

venom transcripts derived from the three *Atheris* species were used and combined with the proteomic data from the present study aiming to elucidate a general, diagnostic profile of different *Atheris* venoms.

Materials and methods

Preparation of venoms

100 mg of pooled venom samples from three bush viper species (*Atheris squamigera, Atheris nitschei* and *Atheris chlorechis*) were purchased from Latoxan, Rosans, France. All venoms were stored lyophilised at -20ºC until used.

Size-exclusion liquid chromatography and identification

To obtain a general elution profile, venoms were analysed via size exclusion chromatography (SEC) using an ÄKTAprime™ plus system (GE Healthcare) and a Superdex 75 10/300 GL column. Each crude venom (10-12mg) was resuspended in 1 ml of buffer (0.05%/30%/70% (v/v/v) TFA/acetonitrile/H_2O) and 400 µl were injected into the column equilibrated with the same buffer. The separation was carried out under isocratic conditions at a flow rate of 0.5 ml/min over 60 min and absorbance was detected at 280 nm.

Identification of proteins or peptides present in the collected fractions was performed using a Voyager DE Matrix-assisted laser desorption/ionization-Time of Flight (MALDI-TOF) mass spectrometer under the control of the Applied Biosystems Voyager System ™ (Applied Biosystems, Framingham, MA). One microliter of each fraction was applied on a 100 well sample-loading plate (Applied Biosystems, Framingham, MA) and pre-coated with alpha-cyano-5-hydroxycinnamic acid (CHCA, 10mg/ml in 70% acetonitrile/0.01% trifluoroacetic acid). The spectrometer operated in positive reflector and in linear modes, spectra were acquired in the mass range of 500 to 20,000 Da.

SDS-PAGE and protein identification

Venom samples were diluted to a final concentration of 2.0mg/ml crude venom in phosphate buffered saline (PBS). For quantification of total protein present in the crude venom samples the Bicinchoninic acid protein assay (BCA Protein Assay kit, Thermo Scientific Pierce, UK) was used. NuPage 4-12% pre-cast acrylamide gels (Invitrogen, UK) were loaded with 32 µg of each venom sample, run in 1x SDS (sodium dodecyl sulfate) running buffer for 35 min at 200V, and proteins were stained with Coomassie Brilliant Blue (Invitrogen, UK). The relative amounts of stained protein bands were measured by densitometry using the Gel-Pro Analyzer 4.0 software (Media cybernetics, MA, USA). In addition, all SEC fractions were assessed for their protein concentration using the BCA Protein Assay kit (Thermo Scientific Pierce, UK) and subsequently loaded on a NuPage 4-12% pre-cast acrylamide gel for SDS-PAGE.

Protein bands of interest were excised according to their density and distribution and digested overnight using trypsin (Sigma, UK). The resultant tryptic peptides were subjected to an LCQ Fleet Ion Trap MS (Thermo Fisher, UK) with a Phenomonex Jupiter C18 column (5 µm, 150 × 2 mm) using a gradient of 0–60% acetonitrile/0.1 M acetic acid at a flow rate of 0.2 µl/min. Eluted peptides were analysed in a data-dependent mode to select multicharged peptides for further MS/MS fragmentation of the ten most abundant ions.

Construction of venom-derived cDNA libraries

Complementary DNA libraries were constructed by reverse transcription of polyadenylated mRNA extracted from 5 mg of lyophilized venom using Dynabeads® M DIRECT™ Kit (Dynal Biotech, UK). A SMART™ Rapid Amplification of cDNA ends (RACE) cDNA Amplification kit (BD Clontech, UK), both 5'-and 3' ends cDNA ends were synthesized using SMART (Switching Mechanism At 5' end of RNA Transcription) cDNA synthesis technology. For the reactions, a nested universal primer (NUP) and degenerated sense

primers were applied. Two types of primers were applied: (1) homologous primers designed on the basis of conserved 3'-untranslated region of analogue snake toxins identified from evolutionary related species of the same subfamily Viperinae like Bitis and Echis[15-16]; (2) degenerated primers for reverse transcription based on the identified protein sequences obtained by MS/MS fragmentation. The obtained cDNA fragments with the expected size were purified and used for transformation of *E. coli* cells using the pGEM-T vector system for subsequent blue-white screening according to manufacturer's manual (Promega, UK). After transformation, a PCR of the plasmid DNA obtained from positive *E. coli* cells was performed, and the PCR fragments were purified with a DNA Extraction kit (RBD Bioscience) for subsequent sequencing using a 3730 DNA sequencer (Applied Biosystems).

Bioinformatic analysis

The BLAST tool of the National Centre for Biotechnology Information (NCBI) was employed for protein identification of all obtained sequences and to exclude possible contaminations. Multiple sequence alignments of the DNA and amino acid sequence were performed using the Vector NTI software.

Result

The venom compounds of three *Atheris* species

Each of the three *Atheris* venom samples exhibited distinct chromatographic profiles using SEC (Figure 1). The observed m/z ratios from MALDI-TOF analysis of the collected SEC fractions are shown in Table 2.

The most abundant components eluted at a retention time between 20 and 35min corresponding to protein sizes of 43-0.2 kDa. The collected fractions contained polypeptides of about 7-14 kDa. Finally, short peptides ranging from 2-4.5 kDa, although not visable after SDS-PAGE, were detectable through MALDI-TOF MS analyses.

SDS-PAGE was run to compare the protein pattern of the three crude venoms. Comparable to SEC, different patterns in terms of band intensity and distribution were observed (Figure 2). A number of protein bands occurred in all three samples, ranging in size from approximately 3-65 kDa, but also a weak band at a size of ca. 100 kDa was present in the samples of *A. squamigera* and *A. nitschei*. The selected protein bands were cut and prepared for in-gel digestion. Based on the identified peptide sequences obtained from subsequent tandem mass spectrometry analyses, the presences of the three protein families disintegrin, phospholipase A_2 (7-14 kDa) and SVMP (ca. 40-55 kDa) in all of the three *Atheris* venoms samples confirmed in this study(Table 3). Moreover, identified peptides suggesting the presence of SVSP in the venom of *A. squamigera* and *A. nitschei*, whereas this protein family was absent in *A. chorechis*.

Some low molecular weight peptides (< 3,000 Da) were found in both *A. squamigera* and *A. chlorechis* venoms, but not in the venom of *A. nitschei*. However, a cluster of small peptides was detected from all venoms (Table 2). The primary structures identified by MS/MS sequencing did not show any sequence homology with previously published viper peptide toxins, such as myotoxins and neurotoxins.

A qualitative and quantitative distribution of the different venom protein families identified in the three *Atheris* venoms is shown in Table 3, pointing to PLA_2, disintegrins, serine proteases and metalloproteases as the major toxin compounds.

General description of venom transcripts

The species-specific cDNA libraries have been constructed from the venoms of *A. squamigera*, *A. nitschei* and *A. chlorechis* with a molecular cloning strategy developed in our laboratory. To gather the maximum amount of information about the putative secreted proteins, selected sequences presented in Table 3 were used to design degenerated primers for the cloning of the venom-derived cDNA libraries. These fragments played an extensive role

in the genomic investigation for a homologue primer design as well. As a result, three novel Group II PLA_2 precursor transcripts were consistently cloned and named PLA_2-A.C., PLA_2-A.N. and PLA_2-A.S, respectively (Genbank accession numbers KP119682 to KP119684).

In this research, the signal peptide and part of the propeptide sequence of SVMP has been cloned from the venom-derived cDNA library of *Atheris squamigera* (Figure 3). According to the CDD (conserved domain database) analysis, the propeptide can be classified as a member of the Pep_M12B_propeptide group SVMP [17]. Although the whole sequence of SVMP was not cloned successfully with our standard approach, a platelet aggregation inhibiting activity of the corresponding snake venom fraction could approve the presence of this protein family.

Discussion

The three species of *Atheris* (*A. squamigera, A. nitschei* and *A. chlorechis*) were studied using SDS-PAGE of crude venoms, chromatographic profiling (SEC) and an established molecular cloning strategy. Combining all methodologic approaches, at least 10-20 protein components, ranging in size from ⩽3 kDa to > 98 kDa, were apparent in the crude venoms. Several components are common to all venoms although with quantitative differences, such as disintegrins, phospholipases and SVMPs, whereas some identified components were species-specific. These differences among species could be the result of diverse factors such as geographic region, ecological factors, prey patterns, evolutionary traits, ontogenetic or individual variability, sexual recombination and other genetic factors as well as age and the storage conditions of a venom sample [3, 24].

A summary of venom gland transcripts (cDNA) found in the Atheris genus, including those previously reported and the present study is presented in Table 4. The nucleotide and deduced amino acid sequences of all three PLA2 have been reported previously [16]. We have also reported the first genomic sequences of three cDNA encoding disintegrin

precursors form Atheris venoms [15], named DS-A.C., DS-A.N. and DS-A.S. (EMBL Nucleotide Sequence Database: accession numbers HF543862-HF543864). At the molecular level, the proteomic and transcriptomic datasets exhibit high compositional concordance. These data emphasize the value of combining two approaches to acquire a more complete understanding of the toxicological profile of the snake venom.

It is noteworthy that the venom of *A. squamigera* contains a peptide with a unique structure in having high repetition of the single amino acids, histidine and glycine (pHpG) probably leads to specific biological and pathological effects [18]. Part of the pHpG show significant sequence identity with the short enzyme inhibitor from *Echis ocellatus* venom that specifically targets the zinc-metalloproteinase [19]. Chemically synthesised replicates of pHpG peptides will be carried out for a spectrum of bioactive assays in the future work.

As an example of their vast potential and as an invaluable source of biological compounds, the venoms of vipers are of great significance. In snakebites, the tissues or vessels of prey are pierced by sharp fangs and an often massive volume of venom will be injected [20]. With the assistance of enzymes/toxins in their venoms, snakes can easily kill prey by chemical poisoning as well as by the normal mechanical methods [21]. The venom components usually have high proteolytic activities for pre-digestion of prey that can also provide assistance to the normal mechanical method. Thus the enzymatic activities of venoms, especially those of some proteinases, are closely correlated with prey patterns [22-23]. The *Atheris* genus contains species that, in common with other species of Viperidae snakes, usually feed upon amphibians, lizards and even larger prey-birds and snakes (Table 1) [6], suggesting the importance of the enhanced pre-digestive activities of their venoms, although *Atheris* species are not well-characterized for their venom toxins. However, evidence from the present study confirms that *Atheris* venoms contain powerful components, such as SVSP, phospholipases,

SVMP and the tissue-destroying disintegrins, making the snakes potentially lethal for humans while promoting initial digestion of the natural preys of bush vipers.

Moreover, the clinical symptoms observed following envenomation by *Atheris* vipers, could obviously occur due to several families of proteins identified in this work and previous studies from our lab [15-16]. Local haemorrhage, pain and oedema are mainly caused by enzymatic components, and severe coagulopathy could result from the actions of disintegrins that mainly contribute to the platelet aggregative inhibition. Although there is little information on the clinical manifestations of *Atheris* viper bites [12], its critical features would be closely correlated with the protein composition. It was therefore important to perform further studies on the structural, biochemical and pharmacological characteristics of the venom proteins in order to give a better insight into their functional relationships and their roles in discrete envenomation.

The comparative biochemical studies on three venoms studied in the current work (*A. squamigera*, *A. nitschei* and *A. chlorechis*) indicate that intriguing variations exist in composition and concentration of venom protein molecules that play distinct roles. The identification of variable venom compositions may contribute to overcome the obstacles for the development of effective clinical therapies, because the antidote available for one species could be either partially effective or ineffective against the toxicity of another species [25-26]. In the case of *Atheris* envenomation, antivenom administration is even more complicated because specific antidotes are lacking and thus only general substances or antivenoms for other species can be applied. Consistently, optimisation of therapeutic envenomation treatment, a more comprehensive knowledge about the snake venom heterogeneity and detailed individual, species-specific molecular characterisations are of needed.

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figure Legends

Figure 1. Venom profile of three *Atheris* species(A) *Atheris squamigera*, (B) *Atheris nitschei*, (C) *Atheris* chlorechis. The electrophoresis analysis of fractions collected from the size-exclusion liquid chromatography is displayed on top of each spectrum. Molecular masses of chromatographic fractions are listed in Table 2.

Figure 2. The electrophoretic patterns of venom proteinsobtained using polyacrylamide gels (4-12%). A.C.-*Atheris chlorechis*, A.N.-*Atheris nitschei*, A.S.-*Atheris squamigera*, MM-Molecular Weight Markers with sizes were indicated on the right. Identified proteins bands were numbered from 1 to 16. Molecular masses, sequence tags and database hits are listed in Table 3.

Figure3. (A) Nucleotide sequence and the deduced amino acid sequence of the open-reading frame (ORF) of the clones encoding partial of the metalloproteinase precursor (Metallopretease-1). The putative signal sequence is double underlined. Only part of the sequence identified belonging to the Pep_M12B_propeptide family has been cloned. (B) Amino acid sequence multiple alignments of Metalloprotease-1 precursor found in this work and other M12B metalloproteases from the Genbank database. The accession number of each protein is marked at the front of array. Identical bases are shaded in black and consensus bases are shaded gray. The amino acid is numbering at the top.

Table Legends

Table 1. Description of the three *Atheris* snake species.

Table 2. Corresponding identification of chromatoghraphic eluted portions by Matrix-assisted laser desorption/ionization-Time of Flight (MALDI-TOF) mass spectrometry: (a) *Atheris squamigera*, (b) *Atheris nitschei*, (c) *Atheris chlorechis*.

T**able 3.** Summary of the relative quantities of *Atheris* snake venom proteins identified by structural class through the combined data derived from electrophoresis, in-gel digestion and mass spectrometric strategies. Average abundance was calculated according to the electrophoretic pattern obtained for each snake venom as shown in Figure 3.3.4.

Table 4. A catalog of three *Atheris* venom gland cDNA for mainly secretary proteins

Table 1

Species	Common name *	Subspecies	General prey base	Geographic range
A.squamigera	Variable bush viper	0	Rodents, lizards and amphibians and snakes	Central Africa, from Nigeria to Uganda and Angola
A.nitschei	Great Lakes bush viper	1(A.n.nitschei ,A.n.rungweensis)	Rodents, lizard (especiallychameleons) and amphibians	From East Africa, Uganda to the Central Lake Tanganyika
A.chlorechis	Western bush viper	0	Rodents, lizards and amphibians (tree frogs)	Western Africa, including Guinea-Bissau, Guinea, Sierra Leone, et al.

*Only the most frequently-used common names are shown for each species.

Table2

(A)

Fraction Time (min)	M/Z ratio of components
17-18	6805/7141/13840
19-20	4945/7142/13840
21-24	4943/7119
29-30	1355/1703/2842/3170/3597
31-32	1355/1703/2344/2788/2844
33-34	1345/2100/2277/2758/2798
35-36	1523/2136/2369
37-40	1480/2135

(B)

Fraction Time (min)	M/Z ratio of components
21-22	7071/13975
23-24	4721/7084/13965
25-26	4727/7071
29-30	3311/4727
35-36	2787/3312/4718
37-38	1263/2609/2788/3311
47-48	1028/1263/2607
49-50	1027/1262

(C)

Fraction Time (min)	M/Z ratio of components
17-18	13961
19-20	6985/7012/13964
21-22	6989/7011/13961
35-36	3515/6985
37-38	2357/3519
39-40	2357/2961/3518
47-48	1061/2435
49-50	2456

Table 3

Band NO.	Molecular Mass (KDa)	MS/MS Sequencing	Identification	Snake species
1	<3-5	---	Short peptides	***A.squamigera***
2	~6-7	FINSGTICK	Disintegrin	
3	~14	NLFQFR	PLA2	
4	~20	---	Unknown	
5	~25-45	LFDYSVCR	SVSP	
6	~47-60	LPCAPEDIK	SVMP	
7	~6-7	NSPHPCCDPVTCK	Disintegrin	***A.nitschi***
8	~12-14	NPYK	Dimeric Disintegrin	
9	~14	NLFQFGSMIK	PLA2	
10	~25-45	IFDYSICR	SVSP	
11	~45-55	VNGEPVVLHLEK	SVMP	
12	<3-5	---	Short peptides	***A.chorechis***
13	~6-7	FMNSGTICK	Disintegrin	
14	~12-14	---	Unkown(Big possible be dimeric Disintegrin)	
15	~14	PQDDTNR	PLA2	
16	~30-45	LHPWVEGESGECCDK	SVMP	

Table 4

Snake species	Sequence name	Protein family	Description	Genbank[1]	MW[2]	Novel cDNA[3]	Function	Reference
A.squamigera	pHpG peptides	Poly G peptides	Full-length	Unpublished	2338	Y	Induced haemorrhage	Favreau et al, 2007; Wagstff et al, 2008
	DS-A.S.	Disintegrin	Full-length	HF543864	7042	Y	Platelet inhibitor	Wang et al, 2013
	PLA2-A.S.	Phospholipase A_2	Full-length	KP119684	13840	Y	Edema inducer	Wang et al, 2016
	Metallopretease-1	Metallopretease	Partial	Unpublished	NA[4]	Y	Haemorrhagic	This Work (Figure 4.)
A.nitschi	DS-A.N.	Disintegrin	Full-length	HF543863	7071	Y	Platelet inhibitor	Wang et al, 2013
	PLA2-A.N.	Phospholipase A_2	Full-length	KP119683	13975	Y	Edema inducer	Wang et al, 2016
A.chorechis	DS-A.C.	Disintegrin	Full-length	HF543862	7012	Y	Platelet inhibitor	Wang et al, 2013
	PLA2-A.C.	Phospholipase A_2	Full-length	KP119682	13964	Y	Edema inducer	Wang et al, 2016

1Genbank, NR database accession number

2MW, molecular mass of the mature protein

3Novel cDNA, Y=yes

[4]NA, Not Available