Exploring the venom proteome of the African puff adder, *Bitis arietans*, using a combinatorial peptide ligand library approach at different pHs

Elisa FASOLI[1], Libia SANZ[2], Simon WAGSTAFF[3], Robert A. HARRISON[3], Pier Giorgio RIGHETTI[2], Juan J. CALVETE[2*]

[1] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, 20131 Milan, Italy

[2] Instituto de Biomedicina de Valencia, Consejo Superior de Investigaciones Científicas (CSIC), Jaume Roig 11, 46010 Valencia, Spain.

[2]Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Pembroke

Running title: Venom proteome of *Bitis arietans*

[*] **Address correspondence to**: Juan J. Calvete, Instituto de Biomedicina de Valencia, Consejo Superior de Investigaciones Científicas (CSIC), Jaume Roig 11, 46010 Valencia, Spain. Phone: +34-96-339-1778; Fax: +34-96-369-0800; Email: jcalvete@ibv.csic.es

Keywords: *Bitis arietans*, African puff adder, snake venomics, venom proteome, venom gland transcriptomics, N-terminal sequencing, mass spectrometry, low-abundance proteins, combinatorial peptide ligand library, ProteoMiner™.

ABSTRACT

We report the 2DE-based proteomic characterization of the venom of the medically important African puff adder, *Bitis arietans*, after prefractionation by incubation with a solid-phase combinatorial hexapeptide ligand library at three different pH values. This approach yielded partially overlapping yet clearly distinct sets of proteins. The *B. arietans* venom proteome, merged from the four sets of proteins comprises at least 43 distinct proteins from 9 toxin families. In line with a previous reverse-phase HPLC-based venomic characterization on the same species, SVMPs, serine proteinases, C-type lectin-like proteins, and to a minor extent PLA_2, disintegrin bitistatin, and cystatin, comprise the major toxins in the venom of *B. arietans*. However, the 2D-CPLL approach employed here identified both a significantly higher (about double) number of proteins than a previous venomic approach, and many very minor components barely, or not at all, detectable in the 2DE separation of whole venom. 30 proteins from the CPLL-merged venom proteome match any of the 63 toxin clusters generated by sequencing one thousand randomly selected venom gland cDNA library clones of the same species. The low (47%) concordance between transcriptome and proteome may be interpreted in terms of intraspecific venom variation. Comparison of the reverse-phase HPLC separations of the venom proteins of *B. arietans* from Ghana and Nigeria supports this view.

INTRODUCTION

Unravelling the total protein composition of complex proteomes is a major challenge of current proteomic technologies. This goal is often complicated by the wide dynamic range of proteomes, which in the case of human serum, per instance, comprises at least nine orders of magnitude [1]. To minimize this problem and allow access to the ‘‘deep proteome’’, a number of multidimensional pre-fractionation and tools for immunodepleting the most abundant proteins have been described [2-4]. Further, the use of a combinatorial library of hexapeptide ligands (Equalizer beads or ProteoMiner™, Bio-Rad) has been shown to allow access to low-abundance proteins undetectable by classical analytical methods [5-8]. The use of large peptide libraries under overloading conditions to capture and “amplify” low abundance proteins from the whole proteome is based on a seminal paper by Thulasiraman et al. [9] and has been applied by PG Righetti´s group for exploring the "hidden proteome" of a number of biological samples, including human urine [10] and serum [11], human platelets [12], human erythrocytes [13], chicken egg white [14] and yolk [15], plant tissues [16], and for detecting rare protein impurities [17,18]. Equalizer beads reduce the dynamic range of proteomes by simultaneously diluting high-abundance proteins and concentrating low-abundance proteins.

Recent contributions of the literature of snake venom have underscored the need for multifaceted approaches for maximizing proteome coverage [19-21]. Very recently we have applied two solid-phase combinatorial hexapeptide ligand libraries to explore the occurrence of low-abundance proteins in the venom of *Crotalus atrox* [22]. *C. atrox* is the largest western rattlesnake and one of the more aggressive rattlesnake species found in North America. It is responsible for the majority of snakebite fatalities in northern Mexico and for the second greatest number in the USA after *C. adamanteus*

[23]. This study showed the occurrence in the venom of protein spots barely, or not at all, detectable in the whole venom, including a C-type lectin-like protein, several PLA_2 molecules, PIII-SVMP isoforms, glutaminyl cyclase isoforms, and a 2-cys peroxiredoxin [22]. Peroxiredoxin and glutaminyl cyclase may participate, respectively, in redox processes leading to the structural/functional diversification of toxins, and in the N-terminal pyrrolidone carboxylic acid formation required in the maturation of bioactive peptides such as bradykinin-potentiating peptides and endogenous inhibitors of metalloproteases.

We have initiated a snake venomics project whose long-term goal is the in-depth analysis of viperid venom proteomes [24-26]. Here, we have explored the venom proteome of the African puff adder, *Bitis arietans*. With an average size is about 1 m in length and very stout, this species is probably the most common and widespread snakes in Africa [20]. Its common occurrence in most sub-Saharan habitats except true deserts and rain forests, potent venom, and willingness to bite make *B. arietans* responsible for more fatalities than any other African snake [23, 27-29]. The venom of *B. arietans* is one of the most toxic of any viper (LD_{50} values in mice vary from 0.4–2.0 mg/kg i.v., 0.9–3.7 mg/kg i.p., to 4.4–7.7 mg/kg s.c. [30]), and typically interferes with the hemostatic and coagulation systems. *B. arietans* causes up to 32.000 deaths per year and with many more victims ending up with permanent physical disability, due to local necrosis, and with psychological sequelae, both of which greatly jeopardize the quality of their lives [31]. Defining the deep proteome of *B. arietans* venom may provide a comprehensible catalogue of secreted proteins, which may contribute to a deeper understanding of the biological effects of the venom, and may also serve as a starting point for studying structure-function correlations of individual toxins.

EXPERIMENTAL SECTION

Enrichment of Bitis arietans venom proteins by treatment with solid-phase combinatorial peptide libraries at different pHs

The lyophilized venom pooled from adult specimens of *Bitis arietans* (West Africa) was purchased from Latoxan (Valence, France). Samples of two hundred mgs of lyophilized venom were dissolved at room temperature in 15 mL of the following buffers: 25 mM acetate buffer pH 3.82 containing 50 mM potassium chloride, 25 mM phosphate, 50 mM potassium chloride, pH 7.15, 25 mM Tris-HCl, 50 mM potassium chloride, pH 9.97, each containing the complete protease inhibitor cocktail (Roche Diagnostics, Basel, CH). After centrifugation at 13.000 xg for 10 min (in an Eppendorf centrifuge) to pellet non-soluble materials, the three samples were then incubated with 200 µL of ProteoMiner™ beads (a mixture of 100 µL of EBeads + 100 µL of its carboxylated version, SE-Beads) pre-equilibrated with the same buffers and the proteins were allowed to adsorb to the beads by gentle shacking for 3 hrs at room temperature. The solid-phase combinatorial libraries consist of poly(hydroxymetacrilate) beads, each displaying the same hexapeptide. The ligand density is ca. 40-60 mmol/mL bead volume, and the complete library may contain a set of 64 millions different hexapeptides [5]. After washing to remove excess soluble proteins not bound to the beads, the flow-though fractions were pooled, and the captured proteins were separately eluted from each bead batch in three steps (400 µL + 400 µL + 200 µL) with 7 M urea, 2 M thiourea, 2% CHAPS and 20 mM cysteic acid, pH 3.3 [32]. The three eluates of each sample were pooled and used as such for two-dimensional electrophoretic analysis. Peptide libraries and all materials for electrophoresis, such as gel plaques and reagents, were from Bio-Rad Laboratories (Hercules, CA, USA).

Two-dimensional electrophoresis

The pH values of the three eluates were adjusted to neutrality and their protein concentration adjusted to 2 mg/mL. Disulphide bond reduction was performed at room temperature for 60 min by addition of TCEP [Tris(2-carboxyethyl)phosphine hydrochloride] to a final concentration of 5 mM. For alkylating sulphydryl groups De-Streak [Bis-(2-hydroxyethyl)disulphide, $(HOCH_2CH_2)_2S_2$)] was added to the solution at a final concentration of 150 mM (by dilution from the 8.175 M stock solution, Sigma-Aldrich), followed by addition of Ampholine (from the 40% stock solution to a final concentration of 0.5%) and a trace amount of bromophenol blue.18-cm long IPG strips (Bio-Rad), pH 3-10 NL, were rehydrated with 150 µL of protein solution for 5 h at room temperature. Isoelectric focusing (IEF) was carried out using a linear voltage gradient from 100 to 1000 V for 5 h, 1000 V for 4 h, followed by an exponential gradient up to 10000 V, until each strip was electrophoresed for 25 kV/h. For the second dimension, the IPGs strips were equilibrated at room temperature for 25 min in a solution containing 6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl (pH 8.8) under gentle shaking. The IPG strips were then laid on an 8-18% acrylamide gradient SDS-PAGE with 0.5% agarose in the cathode buffer (192 mM glycine, 0.1% SDS and Tris-HCl to pH 8.3). The electrophoretic run was performed by setting a current of 5 mA/gel for 1h, followed by 10 mA/gel for 1h and 15 mA/gel until the dye front reached the bottom of the gel. Gels were stained with Coomassie Blue and destained in 7% acetic acid till clear background, followed by a rinse in distilled water. The 2-DE gels were scanned with a Versa-Doc image system (Bio-Rad) by fixing the acquisition time at 10 sec; the relative gel images were captured via the PDQuest software (Bio-Rad). After

filtering the gel images for removing the background, spots were automatically detected, manually edited and then counted.

In-gel enzymatic digestion and mass fingerprinting

Protein bands of interest were excised from Coomassie Brilliant Blue-stained 2DE gels and subjected to automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing grade porcine pancreas trypsin (Promega) using a ProGest digestor (Genomic Solutions) following the manufacturer's instructions. 0.65 μL of the tryptic peptide mixtures (from a total volume of ~ 20 μL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 70% acetonitrile containing 0.1% TFA, air-dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (SwissProt accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450-3300 Da).

Collision-induced dissociation tandem mass spectrometry (CID- MS/MS)

For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization (ESI) mass spectrometric analysis using a QTrap 2000 mass spectrometer (Applied Biosystems) [33] equipped with a nanospray source (Protana, Denmark). Doubly- or triply-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q_0 trapping. Enhanced Resolution was performed at 250 amu/s

across the entire mass range. Settings for MS/MS experiments were as follows: Q1- unit resolution; Q1-to-Q2 collision energy - 30-40 eV; Q3 entry barrier - 8 V; LIT (linear ion trap) Q3 fill time - 250 ms; and Q3 scan rate - 1000 amu/s. CID spectra were interpreted manually or using a licensed version of the MASCOT program (http://www.matrixscience.com) against a private database containing 1083 viperid protein sequences deposited in the SwissProt/TrEMBL database (UniProtKB/Swiss-Prot Release 56.7 of 20-Jan-2009; http://us.expasy.org/sprot/) complemented by previously assigned peptide ion sequences from snake venomics projects carried out in our laboratory [34-43] and contigs assembled from EST sequencing of a *B. arietans* venom gland cDNA library (see below). MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program [44] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de.

cDNA library construction and sequencing

A venom gland cDNA library was constructed from ten wild-caught specimens of *B. arietans* (Nigeria), maintained in the herpetarium of the Liverpool School of Tropical Medicine, using identical protocols described for the construction of the venom gland cDNA library from *E. ocellatus* [45]. Briefly, one thousand randomly selected cDNA library clones were sequenced in the forward direction (Cogenics, UK) after which low quality, contaminating vector sequences and poly A+ tracts were removed using Trace2dbEST within the PartiGene pipeline [46] using the same high stringency clustering protocols used previously [45], then assembled into contigs before BLAST annotation against Uniprot and TrEMBL databases.

RESULTS AND DISCUSION

The Bitis arietans venom proteome merged by CPLL capture at different pHs

Recently we have employed solid-phase combinatorial peptide ligand libraries (CPLL) to explore the venom proteome of the western diamondback rattlesnake, *Crotalus atrox* [22]. Treatment of the venom with ProteoMiner™ beads under overloading conditions resulted in the capture and amplification of several low abundance proteins, complementing data gained using our snake venomics protocol [24-26] towards a more complete description of the venom proteome. Indications in the literature of differences in the protein patterns recovered when the same library was eluted at slightly acidic or slightly alkaline pH conditions [47], prompted us to explore the venom proteome of *B. arietans* employing a two-dimensional solid-phase combinatorial hexapeptide ligand library approach. Indeed, incubation of crude venom samples with the ProteoMiner™ beads in different pH conditions resulted in the recovery of partially overlapping yet clearly distinct sets of proteins (Fig.1, Table 1). Noteworthy, no overall tendency for preferential capturing of acidic versus alkaline proteins when the venom was incubated at pH 3.82 or pH 9.97, respectively, was observed. On the other hand, 16 proteins (including all 5' nucleotidase and cystatin isoforms, a Kunitz-type inhibitor, and the disintegrin bitistatin) were uniquely identified in the pooled flow-through (FT) fraction; 10 unique proteins were identified in the pH 3.82 fraction. This fraction appeared to to be particularly good at capturing C-type lectin-like and snake venom metalloproteinases (SVMPs). 1 C-type lectin-like molecule (spot 65 in Fig.1C) was exclusively recovered in the set of proteins adsorbed at pH 7.15; and 7 unique proteins were found in the pH 9.97 fraction, including the single PLA_2

protein (spot 78, Fig.1D) identified in the venom proteome. All other proteins were shared between two, or more, fractions (Table 1).

Differentially captured proteins included both high and low abundance species. Many of the latter proteins were barely, if not at all, detectable in the 2DE separation of whole venom when the same amount of total proteins were loaded in the IPG strips (compare panels A with B-E of Fig.1). Per instance, this is particularly evident in the case of the Two-Kunitz inhibitor identified in the heterogeneous series of spots labelled 50 and 51 in Fig. 1C, the PI-SVMPs in spots 47 and 69 (Fig.1D), the serine proteinase 28 (Fig.1B) or the L-amino acid oxidase isoform in spot 9 (Fig.1B).

The *B. arietans* venom proteome, merged from the four sets of proteins recovered from the CPLL treatment at different pHs, comprises at least 43 distinct proteins (Table 1) from 9 toxin families (Fig.2). However, the finding of multiple spots sharing peptide mass fingerprint, indicated the existence extensive diversity of isoforms among all the major protein families (Table 1). In addition, the characterization of a number of serine proteinase, snake venom metalloproteinases (SVMPs) and L-amino acid oxidase (LAO) fragments absent in the starting material indicated that proteolysis occurred during handling of the samples. Proteolysis during ProteoMiner™ treatment of *C. atrox* venom has been previously reported [22].

SVMPs, serine proteinases, and C-type lectin-like (CTL) proteins, and to a minor extent PLA_2, disintegrin bitistatin, and cystatin, comprise the major toxins in the venom of *B. arietans* (Fig.1A). This view is in general agreement with the outcome of a previous reverse-phase HPLC-based proteomic characterization of the same venom [48]. Its toxin composition may account for the severe local and systemic symptoms, which bites from *B. arietans* can produce in humans including cytotoxic and necrotic effects, coagulopathies and hemorrhages evident as ecchymosis, incoagulable blood,

and swelling. [27,28]. However, the 2D-CPLL approach employed here identified both, i) a significantly higher (about double) number of proteins than the previous venomic approach [46], and ii) minor novel components (eg., a 5'-nucleotidase found in spots 104 and 105, Fig.1E and Table 1).

Correlating the B. arietans venom proteome with the venom gland transcriptome

The CPLL-merged venom proteome includes 30 proteins encoded by contigs sequenced in a venom gland cDNA library of the same species (Table 2). The pseudo expression levels of these toxins is displayed in Figure 2. The venom proteome comprises also about 14 other proteins (including 1 serine proteinase, 7 CTLs, 2 SVMPs, and 4 unknown proteins) for which the MS/MS-derived peptide sequences did not match to any of the 63 toxin clusters generated by sequencing one thousand randomly selected cDNA library clones. In other words, the venom proteome gathered by the 2D-CPLL approach and transcriptome share only 47% of components. This figure is significantly lower than the 80% concordance between transcriptome and proteome reported by us for *Echis ocellatus* [42].

Understandably, the transcriptome covers more than the proteome, especially in cases e.g. the SVMPs, when there are many isoforms. This is to be expected since separation of proteins with closely related physiochemical properties can be difficult and 1000 EST sequences gives quite a considerable depth of random sequencing compared to proteomic sampling. However, qualitative discrepancies in the compositional agreement of venom acquired using proteomic and transcriptomic approaches have been reported for other species, i.e. *Lachesis muta* [39] and *Bitis gabonica gabonica* [49]. Intraspecific venom variation represents a well documented phenomenon since more than 70 years [50,51], and is particularly notorious among

species, which like *B. arietans*, have a wide distribution range [52]. Intraspecific compositional variation between venoms among specimens inhabiting different geographic regions may be due to evolutionary environmental pressure acting on isolated populations. The occurrence of mRNAs that are abundant and readily identifiable in the transcriptome, yet apparently absent from the venom proteome may also be explained by a number of factors. Firstly, these messengers may exhibit transient, individual or a temporal expression patterns over the life time of the snake. Alternatively, mRNAs whose predicted polypeptides were not found to be secreted in venom may represent very low-abundance toxins that play a hitherto unrecognised physiological function in the venom gland, or may simply represent a hidden repertoire of non-translated orphan molecules which may eventually become functional for the adaptation of snakes to changing ecological niches and prey habits. At this respect, it should be mentioned that the 4 unidentified CTL clusters are relatively small with a mean size of 2.3 ESTs; the majority of the 8 unidentified clusters of serine proteinases are small (with the exception of one cluster containing 6 ESTs) with a mean size of 2.1 ESTs; and the unidentified cluster of PLA_2 is also small, with a mean size of only 1.5 ESTs. SVMPs are relatively poorly covered, with only 7 out of a 22 isoforms detected in the proteome compared to the transcriptome (Table 2). The unidentified clusters are typically small, however, with a mean size of only 1.6 ESTs. The notable exception is SVMP (BAR00015) which in the transcriptome is represented with 90 EST copies (by far the most abundant venom component) but was not detected in the proteome. One possible explanation is that BAR00015 is unstable in venom and has been broken down, or represents a case of intraspecific variability. Comparison of the reverse-phase HPLC separations of the venom proteomes of *B. arietans* from Ghana and Nigeria displayed in Fig.3 illustrates this point. On the other hand, the discrepancy between the venom

proteome and venom gland transcriptome concerning the absence of venom gland transcripts matching proteins found in the venom (Table 1) indicates that EST clusters assembled from sequences of 1000 random colonies carrying cDNAs generated from the venom gland a small number of geographical restricted (Nigerian) specimens do not provide a complete representation of the proteome of a widely distributed species such as *B. arietans*. The ocurrence of intra-species venom variation might be a matter of concern from the perspective of the possible consequences on the clinical manifestations of envenoming and patient response to antivenom.

Concluding remarks

Our findings underscore the usefulness of combinatorial peptide libraries as powerful tools for mining below the tip of the iceberg**.** The realization that incubation at different pHs results in the capture of distinct sets of venom proteins offers new analytic possibilities. The strength of the 2D-CPLL approach resides in its unique capability to capture and amplify the concentration of minor proteins, many of which may pass unnoticed in a standard proteomic protocol. The Achilles heel of this technique is its intrinsic lack of quantitative character. On the other hand, our reverse-phase HPLC-based snake venomic protocol was developed to address the need of quantifying the relative amounts of venom toxins, and is appropriate for characterizing proteins representing more than 0.05% of the total venom proteins [24-26]. Abundant venom proteins may perform generic killing and digestive functions that are not prey specific, low abundance proteins may be more plastic either in evolutionary or ecological timescales. Mutations provide the ground on which natural selection operates to create functional innovations. A subset of low abundance proteins may serve to "customize" an individual venom to feeding on particular prey or may represent orphan molecules

evolving under neutral selection "in search for a function". Hence, whereas abundant proteins are the primary targets for antivenom therapy, minor components may represent scaffolds for biotechnological developments. Proteomic techniques offer their full analytical possibilities in the context of a suitable genomic or transcriptomic databases. Hence, CPLL, venomic, and transcriptomic approaches complement each other towards a complete visualization of venom proteomes.

ACKNOWLEDGEMENTS

This study has been financed by grant BFU2007-61563 from the Ministerio de Educación y Ciencia, Madrid. EF gratefully acknowledge EMBO for a short-term fellowship in JJC´s laboratory. PGR was supported by Fondazione Cariplo (Milano), by PRIN 2009 (MURST, Rome), and by an ERC Advanced Grant from the EC.

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LEGENDS TO FIGURES

Fig.1. ***Exploring the venom proteome of C. atrox using combinatorial peptide libraries.***

Colloidal Coomassie blue-stained 2DE maps of crude *C. atrox* venom (control sample) (**A**), the venom proteins adsorbed to the ProteoMiner™ beads at pHs, 3.82 (**B**), 7.15 (**C**), and 9.95 (**D**), and the proteins in the flow-through (non-adsorbed) fraction (**E**). Table 1 lists the sets of proteins identified in the different fraction.

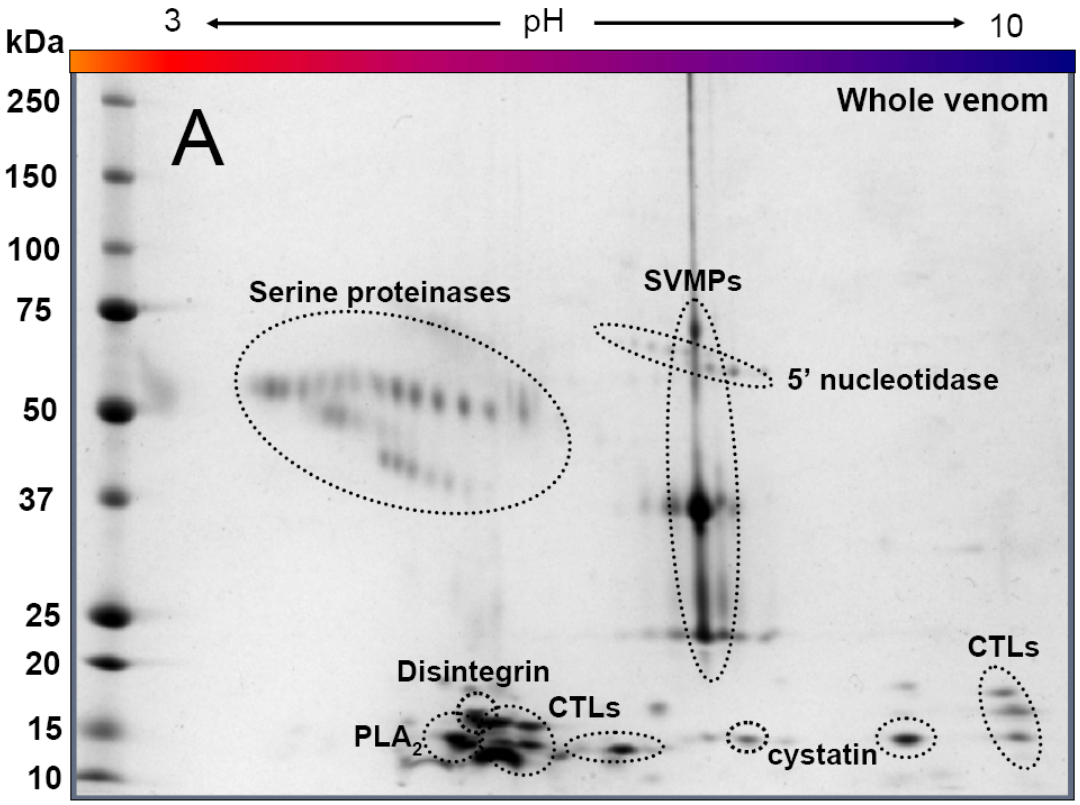
Fig.2. ***Comparison of the expression levels of toxin isoforms identified in the set of venom proteins adsorbed to the ProteoMiner™ beads at pHs***.

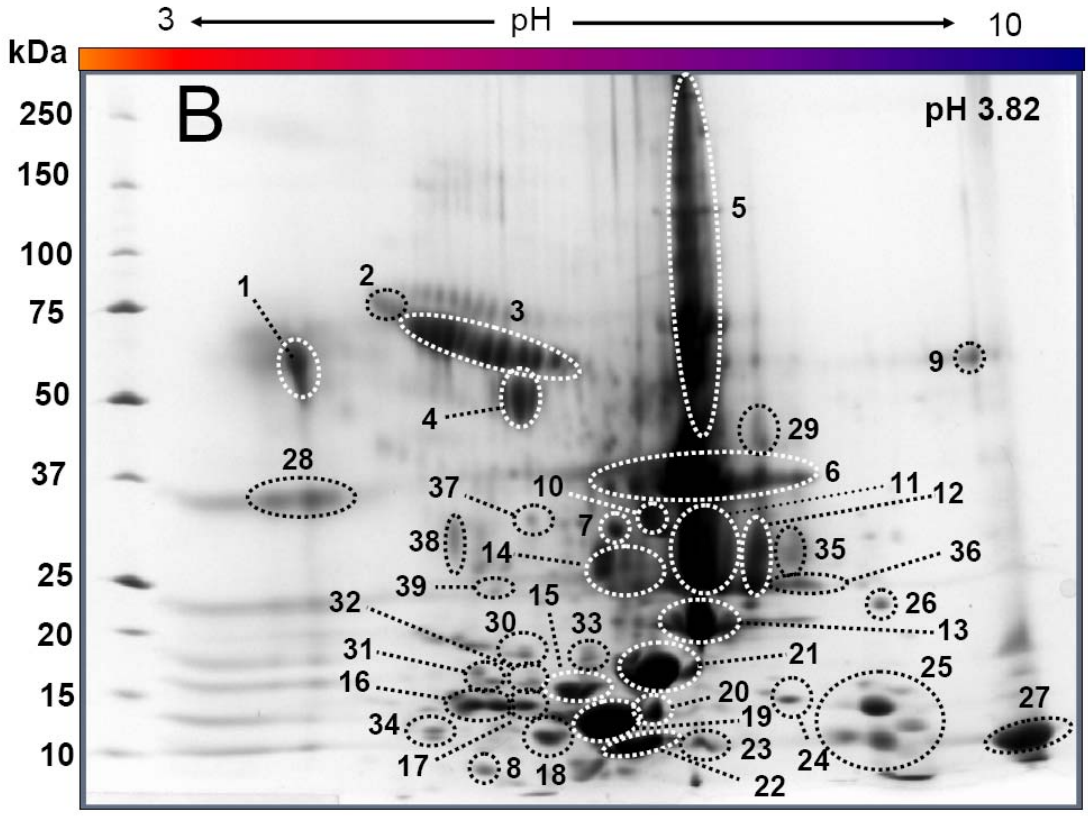
Distribution of proteins across the 4 fractions captured at different pHs by the CLLP approach against their relative representation in terms of number of ESTs in each contig of the *B. arietans* venom gland transcriptome. Asteriks indicate protein species uniquely recovered in the corresponding CLLP fraction. SVMP, snake venom metalloproteinase; Dis, disintegrin; CTL, C-type lectin-like; LAO, L-amino acid oxidase; N´tidase, 5´-nucleotidase; PLA2, phospholipase A2; Kunitz-type, Kunitz-type serine proteinase inhibitor; Cystatin, cysteine proteinase inhibitor.

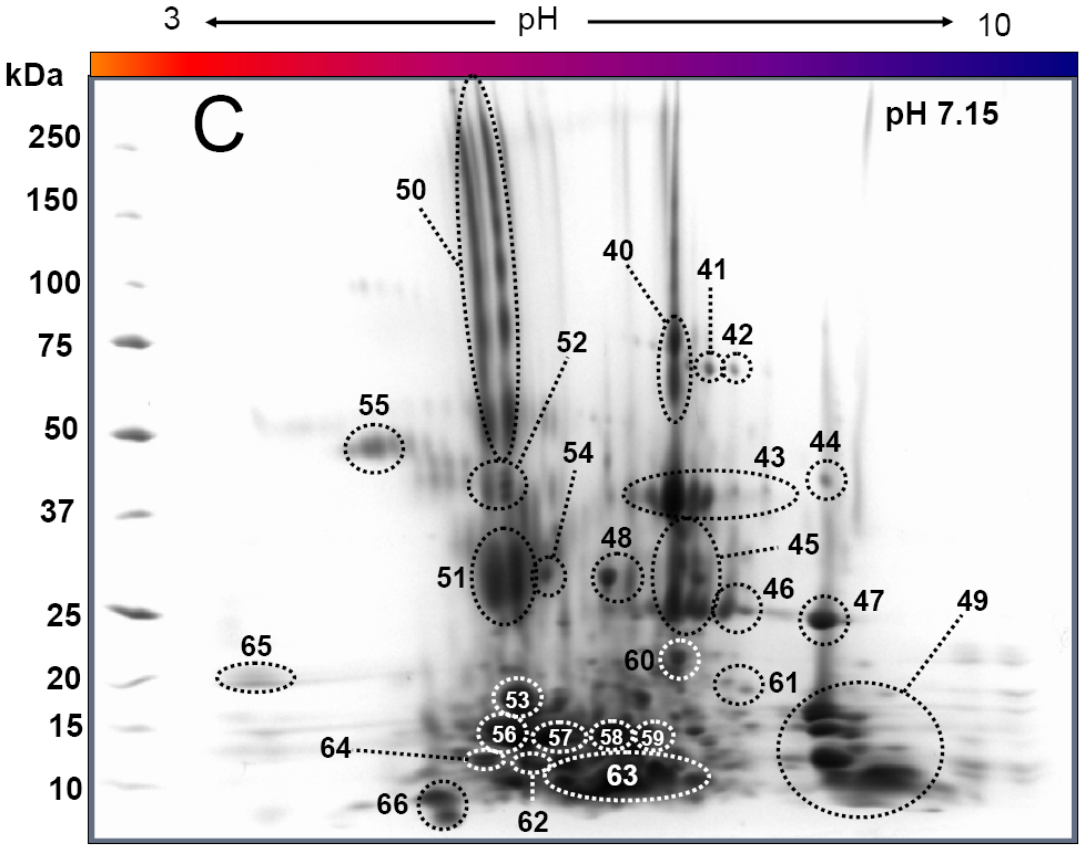
Fig.3. Intraspecific geographical venom variation

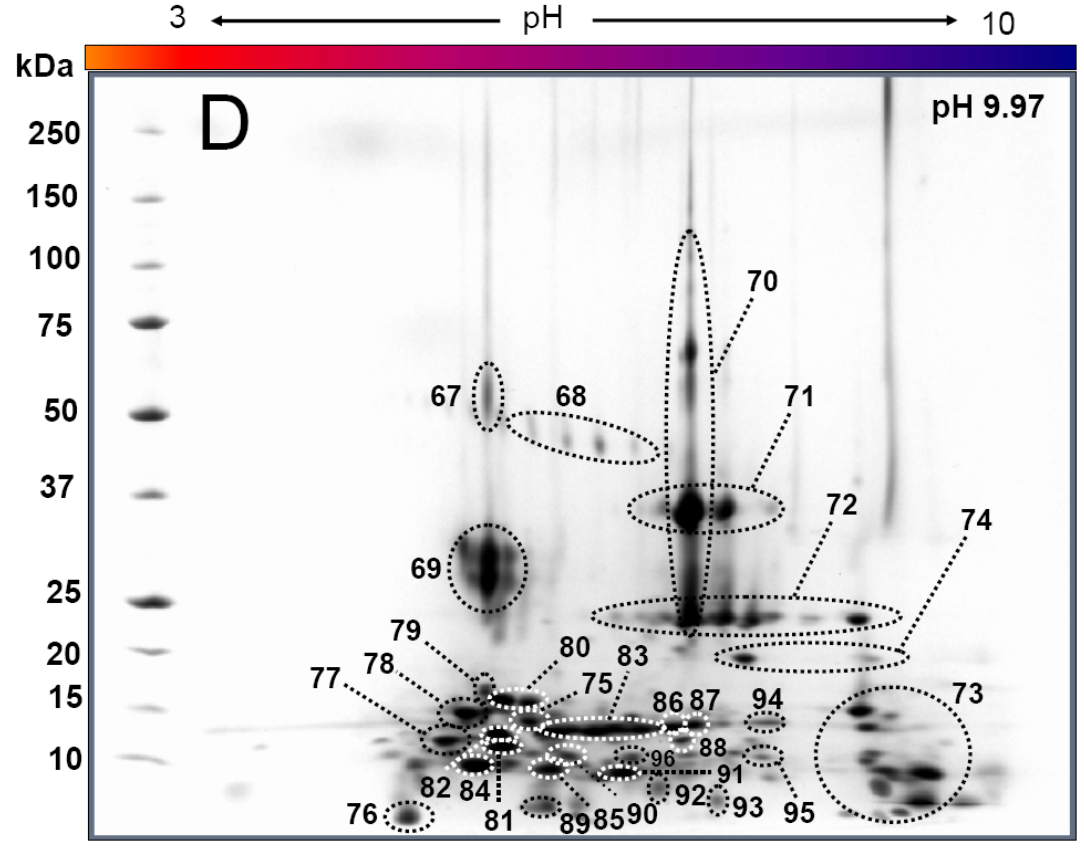
Comparison of the reverse-phase HPLC separations of the venom proteomes of Bitis arietans from West Africa (panel A) and Nigeria (panel B). Distinct proteins in each venom are labelled with asterisks and their protein families are specified.

Figure 1


3
pH
10
kDa
250
150
100
75
50
37
25
20
15
10
A
Whole venom
Serine proteinases
SVMPs
5' nucleotidase
Disintegrin
CTLs
PLA2
cystatin
CTLs


3
pH
10
kDa
250
150
100
75
50
37
25
20
15
10
B
pH 3.82
1
2
3
4
5
6
7
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39


3
pH
10
kDa
250
150
100
75
50
37
25
20
15
10
C
pH 7.15
50
40
41
42
52
55
54
43
44
48
45
51
46
47
49
65
60
61
53
56
57
58
59
64
63
66
62


3
pH
10
kDa
250
150
100
75
50
37
25
20
15
10
D
pH 9.97
70
67
68
71
72
74
69
79
80
83
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82
84
92
93
95
76
81
89
85
90

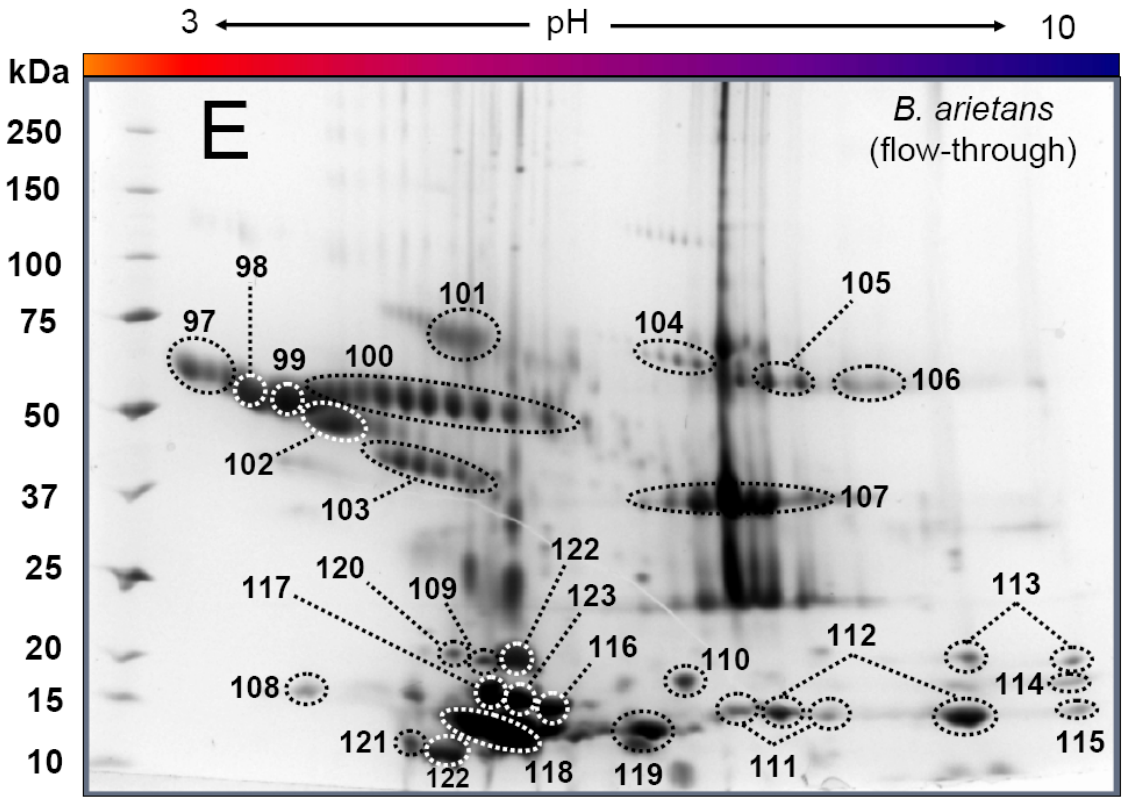

3
pH
10
kDa
250
150
100
75
50
37
25
20
15
10
E
B. arietans
(flow-through)
98
97
99
100
101
102
103
104
105
106
107
122
120
117
109
123
116
108
110
112
113
114
115
121
122
118
119
111

Figure 2

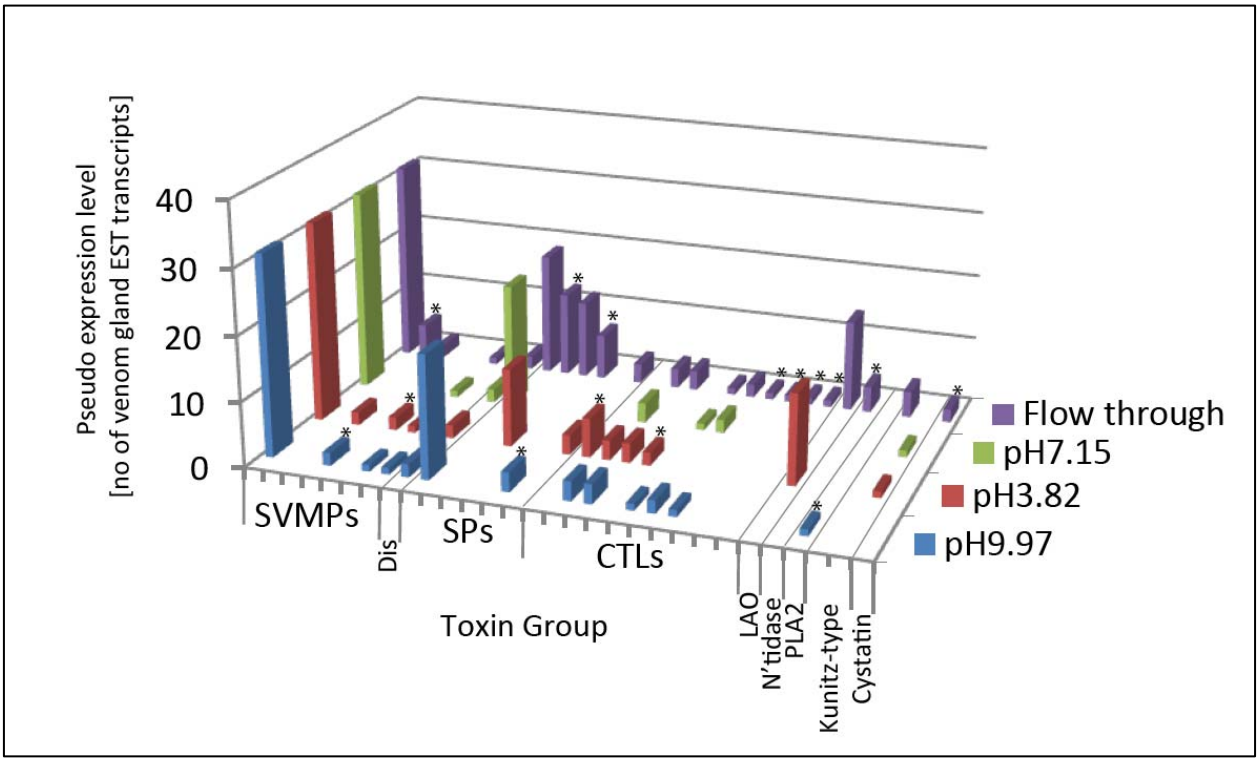
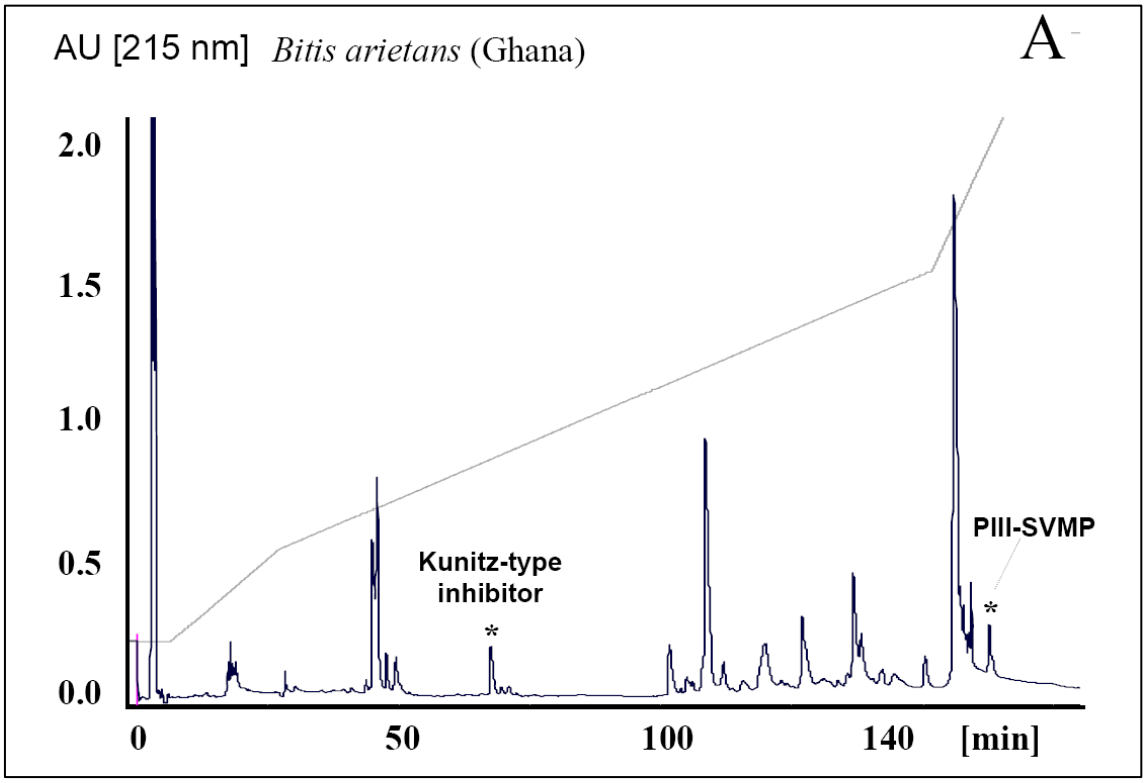

Pseudo expression level
[no of venom gland EST transcripts]
40
30
20
10
0
SVMPs
Dis
SPs
CTLs
LAO
N'tidase
PLA2
Kunitz-type
Cystatin
Toxin Group
Flow through
pH7.15
pH3.82
pH9.97

Figure 3


AU [215 nm] *Bitis arietans* (Ghana)
A
2.0
1.5
1.0
0.5
0.0
Kunitz-type inhibitor
PIII-SVMP
0
50
100
140
[min]

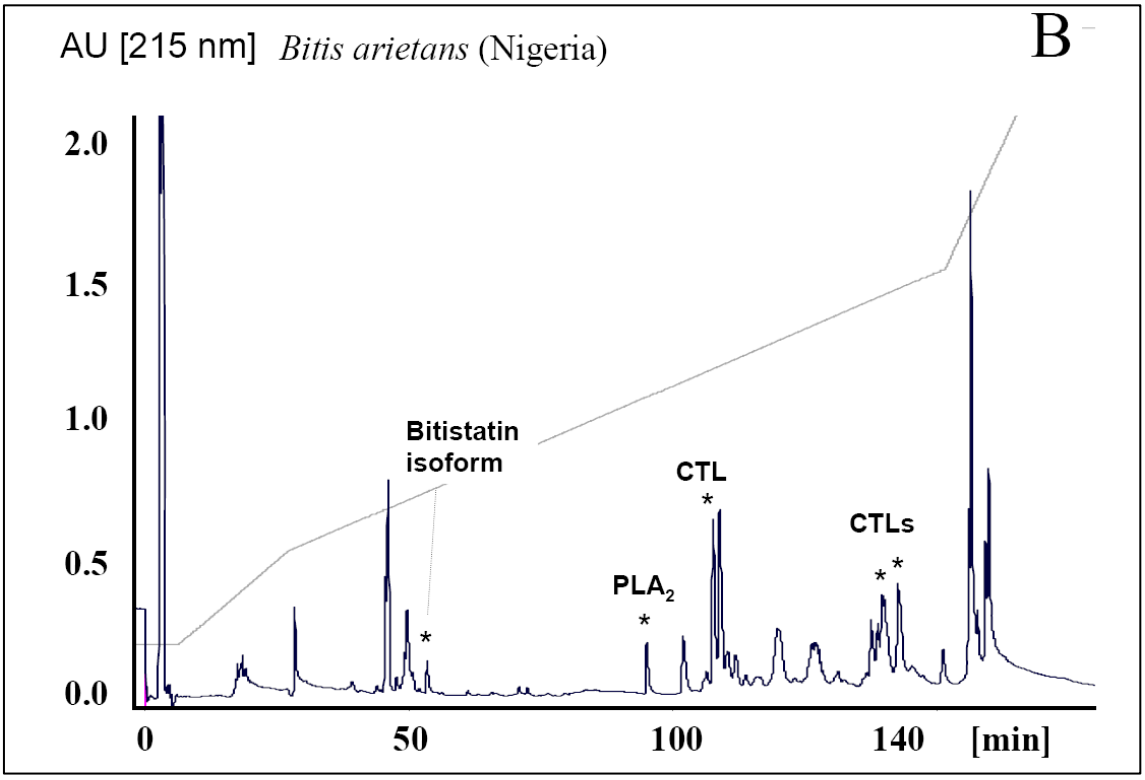

AU [215 nm] *Bitis arietans* (Nigeria)
B
2.0
1.5
1.0
0.5
0.0
Bitistatin isoform
CTL
CTLs
PLA2
0
50
100
140
[min]

TABLE 1

Assignment of the 2DE-separated proteins of *Bitis arietans* venom fractions, obtained after treatment with the combinatorial peptide libraries and eluted at different pHs, to protein families by collision-induced fragmentation by nanoESI-MS/MS of selected doubly and tryply-charged tryptic peptide ions gathered from in-gel digested protein bands labelled as in panels A-D of Fig.1. X, Ile or Leu. C, carbamidomethylated cysteine residue. Numbers correspond to contig identifiers in the transcriptomic database prefixed by a three letter species identifier (e.g. 00127 corresponds to BAR00127).

2DE spot number				peptide ion		MS/MS-derived sequence	Protein
pH 3.82	pH 7.15	pH 9.97	FT	m/z	z		
1			97	728.3	2	SLPSSPPNVGVCR	Serine proteinase 00127
				756.8	2	VIGGDECNINEHR	
				571.3	2	VFDYGDWIK	Serine proteinase 00031
			98	578.7	2	VFDYADWIK	Serine proteinase 00152
				734.7	2	VIGGAECNINEHR	
				919.3	2	IAPLSLPSSPPTVGSVCR	
				607.8	2	VFDYTDWIR	Serine proteinase 00034
	55	68	99,100	756.8	2	VIGGDECNINEHR	Serine proteinase 00034
				494.7	2	ATYWYER	
				607.8	2	VFDYTDWIR	
				647.6	3	IAPFSLPSRPPTVGSVCR	
				636.3	2	VFGDYTDWXR	Serine proteinase
		74		530.3	2	LFDYSVCR	Serine proteinase [~ Q6T6S7] 00263
				729.4	2	SLPSNPPSVGSVCR	
				618.1	3	IAPLSLPSNPPSVGSVCR	
		86		785.4	2	LSLPSNPPSVGSVCR	Serine proteinase (fragment) 00263
		87		764.4	2	ATVAQDACFQFNR	SVMP (fragment) 00101

		88		616.4	2	(157.1)DYFWXGFK	C-type lectin-like
2				633.3	2	VFNXVEXYR	PIII-SVMP
				579.3	2	(239.3)SYYDDVK	
				495.8	2	GSSDGYCR	
3			101	825.8	2	XYEXVNXXNEXYR	PIII-SVMP
				730.3	2	(216.3)CDXAEYCTGR	
				485.8	2	YYFYCR	PIII-SVMP 00077
				684.3	2	(158.2)ECPXDHFHR	PIII-SVMP
			102	807.9	2	STYWYELLPAQSR	Serine proteinase 00091
				607.9	2	VFDYTDWIR	
				756.8	2	VIGGDECNINEHR	
				737.3	3	GDSGGPLICNGQIHGIVEWSR	
			103	537.9	2	LYDYSVCR	Serine proteinase 00031
				614.9	2	GEFHGIVAWGR	
				571.6	2	VFDYGDWIK	
			104, 105	860.6	2	ETPVLSNPGPYLEFR	Ecto-5′-nucleotidase 00177
				791.9	2	NVNFPILSANIRPK	
			106	611.8	2	SAGQLYQASLGK	L-amino acid oxidase 00017
				647.3	2	EGWYANLGPMR	
				742.9	2	EADYEEFLEIAR	
				672.9	3	LNEFVQETENGWYFIK	
4				868.8	2	NGHPCQNNLGYCFR	PIII-SVMP 00027
				464.9	2	NLGYCFR	
		67,69		764.3	2	ATVAQDACFQFNR	PI-SVMP 00101
				566.3	2	LANDYGYCR	
				534.2	2	QCISLFGSR	
				847.8	2	LSCEASYLFSDCSR	PIII-SVMP 00377
5,6,35,36	40-49	70-73	107	857.9	2	VHQYFNTLNEMYR	PIII-SVMP 00042 (and fragments)
				644.9	2	YINVIVVADQR	
				753.3	2	SASDTLHSFAEWR	
				466.4	2	HSFAEWR	
				796.9	2	SAAVVMNYQPEIDR	PIII-SVMP 00577

				557.3	2	CSWNDYNR
				788.3	3	DFECPVEWCPYDQHCYR
			108	735.3	2	FCYLPAVEGPCR
			110	539.9	2	AESFFYCR
				778.6	3	SECDVAEYCTGQSADCPIDR
			111	468.9	2	VTYVNWR
				731.3	3	DFQCPSEWSAYGQHCYR
			112	602.3	3	DVTDPDVQEAAAFAVEK
				897.9	3	GYQEIQNCNLPPENQQEEITCK
				665.3	2	VVEAQSQVVDGVK
				587.3	2	YYLTMELLK
				398.6	2	IPGGLSPR
			113	467.9	2	YFWYTR
				524.9	2	FDYXAYTR
			114	510.7	2	NPFICEIK
		90,95		645.4	2	YINVIVVADQR
				826.6	2	XYEXVNXXNEXYR
				580.3	2	(256.1)WSNGAQXK
		96		753.3	2	SASDTLHSFAEWR
				557.3	2	CSWNDYNR
		91		826.6	2	XYEXVNXXNEXYR
				557.3	2	CSWNDYNR
	58			597.3	3	AVAAIMAHEMGHNLGIR
7-9,24				746.9	2	ADDKNPLEECFR
				742.6	2	EADYEEFLEIAR
10-15				753.4	2	SASDTLHSFAEWR
				466.4	2	HSFAEWR
				587.6	3	AVAAIMAHEMGHNLGIR
				557.3	2	CSWNDYNR
				698.1	2	(253.1)AFXCADAGCYCQTR

PIII-SVMP 00155
C-type lectin-like [~ Q6T7B6]
Kunitz-type inhibitor 00023
SVMP 00076
C-type lectin-like 00287
Cystatin 00172
C-type lectin-like 00475
C-type lectin-like 00298
SVMP 00042 fragment
SVMP fragment
C-type lectin-like
SVMP fragment 0042
PIII-SVMP 00155
SVMP fragment
PIII-SVMP 00155
SVMP fragment 00577
L-amino acid oxidase 00017 (+ fragments)
SVMP 0042 (fragment)
SVMP 00577 (fragment)
PIII-SVMP 00155
C-type lectin-like

16	56,57,59-63			496.8	2	YDAWIGLR	C-type lectin-like α-chain [JC5058] 00292
				727.8	2	DEGCLPDWSSYK	C-type lectin-like
				509.9	2	YDPWSSHK	C-type lectin-like
				522.9	2	XSSPATGSGXR	unknown
	64	77		674.3	2	NWQWSDGSIVR	C-type lectin-like 00086
17	54	75	118,122	688.9	2	CFGLKQYTGFR	C-type lectin-like 00292
				496.9	2	YDAWIGLR	
				647.9	3	DCLPDWSLYEGHCYR	
			119	888.8	2	WANEESNCFHEQPK	unknown
		76		728.9	2	(247.2)TNGNXDPWCK	unknown
	65			832.9	2	(260.1)XEFAEYXSDYR	C-type lectin-like [~ Q6T7B7]
				639.4	2	STCPFNWXPR	
				854.6	2	QXFCAEXVSYTGYR	
		78		722.3	2	GWGGNGKPIDATDR	D49-PLA$_2$ 00538
				763.8	3	CCFVHDCCYGNLPDCSPK	
				561.2	3	CGWGGNGKPIDATDR	
				539.3	2	ENEAIVCDK	
	66			796.9	2	SAAVVMNYQPEIDR	SVMP fragment 00577
18				557.3	2	CSWNDYNR	C-type lectin-like 00155
				796.4	2	SAAVVMNYQPEIDR	SVMP 00577
19				504.4	2	(243.1)GHNLGIR	SVMP
				796.4	2	SAAVVMNYQPEIDR	SVMP 00577
20				645.6	3	FISTHNPQCIHNQPSR	PII-SVMP [Q4JCR9] 00042
				548.8	2	(230.1)WNDYNR	C-type lectin-like
21-30				753.4	2	SAAVVMNYQPEIDR	SVMP fragment 00577
				504.4	2	(243.1)GHNLGIR	
31				557.3	2	CSWNDYNR	C-type lectin-like 00155
		82-85		753.6	2	SASDTLHSFAEWR	SVMP fragments 00042
				557.3	2	CSWNDYNR	C-type lectin-like 00155

32		80,81	115,116,123	546.6	2	VYDAWIGLR	C-type lectin-like α-chain [Q7LZK5] 00301
				620.3	2	FVYDAWIGLR	
				698.3	2	CFGLDVHTEYR	
				918.8	3	TQQCSPQWTDGSSVVYENVDEPTK	
				603.3	2	TWTDLPCGEK	
				517.3	2	VGTWEDAEK	
				603.7	2	TWTDLPCGEK	
				831.9	2	FCVENSGHLASIDSK	
				712.4	2	DPGCLPDWSSYK	
		89,92		728.4	2	DEGCLPDWSSYK	C-type lectin-like β-chain [Q7LZK8] 00062
				655.1	3	ALSDEPICFVAESFHNK	
		93		840.3	2	(279.1)EXXNYVNVFYK	Unknown
		94		580.2	2	(199.1)GWSNQEXK	Unknown
	52,53	79	109, 117	788.3	3	DFECPTEWCPYDQHCYR	C-type lectin-like [~ Q6T7B6] 00473
				860.3	2	SSPDYVWXGXWNQR	
				876.3	2	WTDGSSVXYQNVVER	C-type lectin-like
33				568.9	2	HDDIFAYEK	L-amino acid oxidase fragment 00017
34				454.6	2	EESAFVAR	C-type lectin-like 00113
37				746.9	2	ADDKNPLEECFR	L-amino acid oxidase 00017 (fragment)
				742.6	2	EADYEEFLEIAR	
				857.9	2	VHQYFNTLNEMYR	PIII-SVMP 00042
38	50,51			823.6	2	VFAYGGCQVAANNFK	Two-Kunitz inhibitor 00446
				527.6	2	GPCDEYTGR	
				696.9	2	FYXDPASNECK	Two-Kunitz inhibitor [~ AAR19275]
39				557.3	2	CSWNDYNR	C-type lectin-like 00155
				496.9	2	YDAWIGLR	C-type lectin-like α-chain [Q7LZK5] 000292
				727.9	2	DEGCLPDWSSYK	C-type lectin-like 00012
			120	728.4	2	DEGCLPDWSSYK	C-type lectin-like β-chain [Q7LZK8] 00062
			121	674.6	2	NWQWSDGSIVR	C-type lectin-like 00086
			122	665.7	2	GDWNDDYCTGK	Disintegrin bitistatin 00042

702.5	3	LTPGSQCNYGECCDQCR
501.5	2	SSDCPWNH

Table 2

Comparison of number of proteins from the different toxin families identified in the transcriptome and the proteome. Full details of the *Bitis arietans* venom gland transcriptome will be reported elsewhere. GO, gene ontology. Toxin class abbreviations as in Fig.2.

Toxin class	**GO description**	**Go term**	**Transcriptome**	**Proteome**
SVMP	Zn^{2+}metalloendopepti-dase activity	GO:0004222	22	8
SP	Serine-type endopeptidase activity	GO:0004252	13	6
CTL	Activators and inhibitors of blood coagulation	GO:0005529	14	10
Disintegrin	Inhibitors of integrin receptors	GO:0007229	2	1
PLA_2	Hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides	GO:0004623	2	1
LAO	Oxidative deamination of hydrophobic and aromatic L-amino acids	GO:0001716	2	1
5’ nucleotidase	Catabolism of nucleotides	GO:0008253	2	1
Kunitz-type protease inhibitor	Serine protease inhibitor activity	N/A	3	1
Cystatin	Cysteine protease inhibitor activity	GO:0004869	3	1