Original Article

Check for updates

Qualitative Analysis of Proteins in Two Snake Venoms, Gloydius Blomhoffii and Agkistrodon Acutus

Su-Jeong Ha[1†], Yeo-Ok Choi[2†], Eun-Bin Kwag[1], Soo-Dam Kim[1], Hwa-seung Yoo[4], In-Cheol Kang[3,5]*, So-Jung Park[1]*

[1]East West Cancer Center, Daejeon Korean Medicine Hospital, Daejeon University, Daejeon, Republic of Korea
[2]Bio Research Institute of Biotechnology, Goyang, Republic of Korea
[3]Department of Biological Science and BioChip Research Center, Hoseo University, Asan, Republic of Korea
[4]East West Cancer Center, Seoul Korean Medicine Hospital, Daejeon University, Seoul, Republic of Korea
[5]InnoPharmaScreen Inc., Incheon, Republic of Korea

*Corresponding Author
So-Jung Park
East West Cancer Center, Daejeon Korean Medicine Hospital, Daejeon University, Daejeon 35235, Republic of Korea
Tel: +82-42-470-9136
E-mail: vivies@hanmail.net

In-Cheol Kang
Department of Biological Science and BioChip Research Center, Hoseo University, Asan 336795, Republic of Korea
Tel: +82-41-540-5974
E-mail: ickang@hoseo.edu

[†]These authors contributed equally to this work.

Objectives: Snake venom is a complex mixture of various pharmacologically active substances, such as small proteins, peptides, and organic and mineral components. This paper aims to identify and analyse the proteins in common venomous snakes, such as *Gloydius blomhoffii* (*G. blomhoffii*) and *Agkistrodon acutus* (*A. acutus*), in Korea.
Methods: We used mass spectrometry, electrophoresis, N-terminal sequencing and in-gel digestion to analyse the proteins in these two snake venoms.
Results: We identified eight proteins in *G. blomhoffii* venom and four proteins in *A. acutus* venom. The proteins detected in *G. blomhoffii* and *A. acutus* venoms were phospholipase A_2, snake venom metalloproteinase and cysteine-rich secretory protein. Snake C-type lectin (snaclec) was unique to *A. acutus* venom.
Conclusion: These data will contribute to the current knowledge of proteins present in the venoms of viper snakes and provide useful information for investigating their therapeutic potential.

Keywords: gloydius blomhoffii, agkistrodon acutus, proteomics, venomics, venom proteome

INTRODUCTION

Snake venom has been considered nature's most attractive toxic substance. It contains various pharmacologically active substances, such as small proteins, peptides and organic and mineral components [1, 2]. Although the high toxicity of snake venom can cause death and significant morbidity [3], the biological and toxicological mechanisms enhance its potential value as a therapeutic agent [4, 5].

Snake venom is also a mixture of many different toxic substances, most venomous being cardiotoxins and neurotoxins [6]. Generally, cardiotoxins and neurotoxins are three-finger protein structured substances. The members of these toxin groups are small-molecular-weight proteins (6-7.5 kDa) with 60 to 75 amino acids [7]. The primary structure is similar in these two toxins groups; however, they show different biological activities. The target of neurotoxins is the nicotinic acetylcholine receptor at the post-synaptic level of the neuromuscular junction [8, 9]. Neurotoxins block the receptor and thus, prevent binding with the acetylcholine receptor [10, 11]. Cardiotoxins cause depolarisation and contraction of muscle cells and loss of excitability and depolarisation of nerve cells [12]. They can also prevent aggregation caused by the lytic effect on platelets [13]. Few cell types, such as erythrocytes, epithelial cells and foetal lung cells, can undergo lysis on exposure to higher concentrations of cardiotoxin [14-16].

The present study investigated the protein compositions of two Viperidae snakes, *Gloydius blomhoffii* (*G. blomhoffii*) and *Agkistrodon acutus* (*A. acutus*) venoms. We selected these two species due to their medical significance in Southeast Asia. *G. blomhoffii* is commonly known as salmosa (Korea) or mamushi (Japan).

Many studies have examined the proteins and peptides in the venom of these species. One study on *G. blomhoffii* venom showed that it contained two neurotoxins, α-toxin and β-toxin. α-toxin is a post-synaptic inhibitor, and β-toxin is a pre-synaptic inhibitor [17]. *G. blomhoffii* venom was also reported to contain an anticoagulant, mamushi L-amino-acid oxidase (M-LAO) [18], and peptide albumin [19]. Other studies on *A. acutus* reported that the venom of *A. acutus* contained activating or inhibiting factors of the plasmatic coagulation system, thrombin-like enzymes for fibrinogen conversion and platelet aggregation inhibitors, or even activators [1, 20].

This study used mass spectrometry, electrophoresis, N-terminal sequencing and in-gel digestion to analyse the proteins in two snake venoms. We applied N-terminal sequencing and in-gel digestion methods to investigate proteins with molecular weights below 20 kDa because the most toxic and medically valuable substances in snake venom, such as cardiotoxins and neurotoxins, are generally low-molecular-weight proteins [7, 8]. The results of this study may promote basic research on snake venoms for the development of new therapeutic agents.

MATERIALS AND METHODS

1. Materials

The venoms of *G. blomhoffii* and *A. acutus* used in this study were milked from adult snakes. Manual milking was performed by placing the snake's fangs on a parafilm-covered sterile container. The venom samples were immediately frozen at –80℃, lyophilised via a freeze-dryer and stored at –20℃ until used. Crude venoms were purchased from Geolim Pharmaceutical Company (Hampyeong, Korea). The yields of *G. blomhoffii* and *A. acutus* venoms were approximately 25% and 42.5%, respectively.

2. Quadrupole time-of-flight (Q-TOF) mass spectrometry

Dried samples were weighed (10 mg) and diluted using water. Then, the extract was centrifuged, and the supernatant was collected for further analysis. Liquid chromatography (LC) separation was performed using an Acquity I-Class UPLC system (Waters, Manchester, UK) with an Acquity UPLC protein BEH C4 column (1.7 um, 2.1 mm × 100 mm). The column was maintained at a temperature of 40℃. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The injection volume was 2 uL, and the flow rate was 0.4 mL/min. Mass spectrometry (MS) measurements were performed on a SYNAPT G2-Si system (Waters, Manchester, UK). Data were acquired in the MS^E mode. The ionisation mode was electrospray ion (ESI)-positive. The source temperature was set at 120℃, and the reservation temperature was set at 300℃. The lock mass compound used leucine enkephaline (556.2771 in positive, 554.2615 in negative) for the external standard. The operation parameters were as follows: ESI positive and negative capillary voltages were set at 3 kV and 2.5 kV, respectively, and cone voltage was set at 30 V. The collision energies were set as 6 eV (trap) for the low-energy scan and 20- ~ 45-eV ramp (trap) for the high-energy scan. The scanned mass ranged from 100 to 1,500 m/z. The liquid chromatography-mass spectrometry (LC-MS) data acquisition was controlled using a MassLynx 4.1 system (Waters, Manchester, UK). The acquisition data processing was performed using UNIFI1.8. Software.

3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of venoms

The lyophilised venoms (*G. blomhoffii* and *A. acutus* venom) were dissolved in phosphate-buffered saline (PBS), and their protein concentrations were determined using the Bradford method. Different amounts (100, 50, 25 and 12.5 μg) of both venom proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4%-12% gel (bis-tris protein gel, Invitrogen). The gels were stained with Coomassie blue staining solution (50% MeOH, 10% acetic acid, 1% Coomassie blue) for 30 min, after which they were destained with destaining solution (20% MeOH, 10% acetic acid) and then dried with drying solution (30% MeOH, 5% glycerol). The molecular weights of the protein bands were estimated with reference to molecular weight markers. Next, the gels were soaked in transfer buffer (NuPAGE™ Transfer Buffer, Invitrogen), after which the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific binding sites were blocked using 5% non-fat dry milk. The membranes

were stained with Ponceau S solution (Sigma, P7170-1L) for 5 min, destained with 50% MeOH and dried at 4℃.

4. N-terminal sequencing

After isolating the bands in the PVDF membranes corresponding to 20 kDa or less, N-terminal amino sequencing was conducted using the Edman degradation method with a Procise 491 HT protein sequencer instrument (Applied Biosystems, USA). We searched for amino acid sequence similarities in the non-redundant protein sequences database using Blastp (protein-protein BLAST), found at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

5. In-gel digestion

Stained protein bands on 1D-gel were excised using a scalpel to 1 mm with bands less than 20 kDa in total and transferred to new Eppendorf Lobind tubes. The gel slices were subjected to digestion using the in-gel digestion protocol. The gels were destained with 50% (v/v) acetonitrile/50 mM Bacto agar and squeezed 3-4 times with 100% (v/v) acetonitrile. After drying the organic solvent in a speed vacuum concentrator (Savant, Holbrook, NY), the gels were incubated with 5 mM DTT/50 mM Bacto agar at 56℃ for 2 h for protein reduction, followed by 10 mM iodoacetamide (Sigma, T6508)/50 mM Bacto agar in-dark reaction for 30 min for cysteine alkylation. The gels were then rinsed a few times with distilled water and squeezed using 100% (v/v) acetonitrile. The gels were vortexed and completely dried in the speed vacuum concentrator. Trypsin (1 µg)
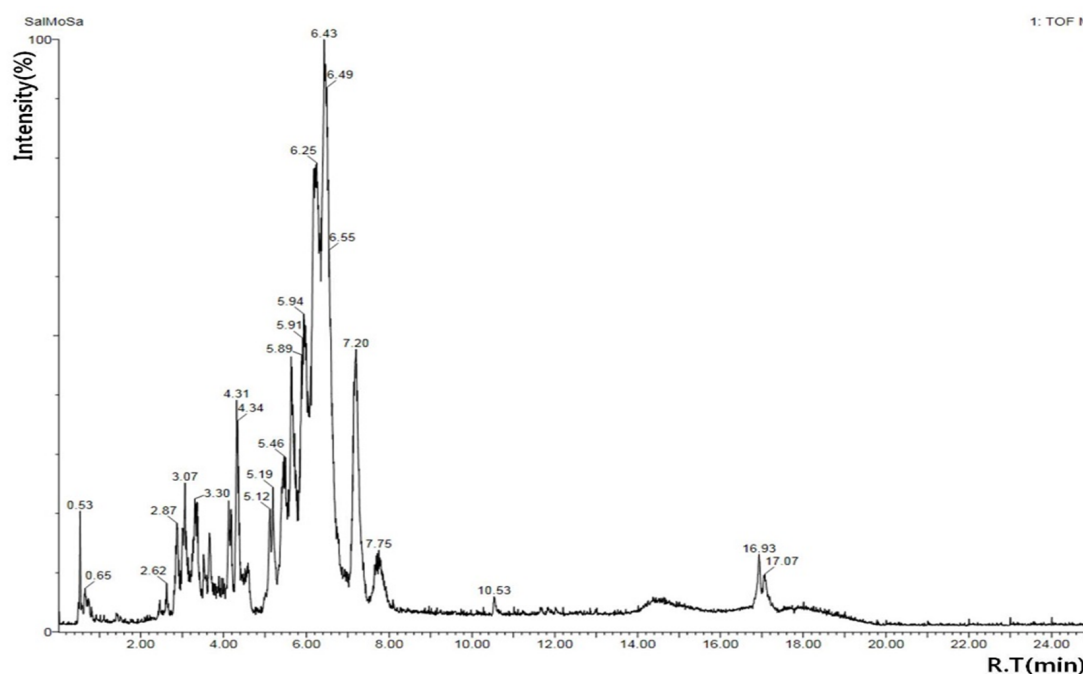

Figure 1. UPLC/Q-TOF MS chromatogram of *G. blomhoffii* venom.

Table 1. Peaks that were identified for *G. blomhoffii* venom by using Q-TOF MS

No.	Name	Type	RT (min)	m/z	Mass (kDa)
1	Salmosa venom 423	Analyte	3.07	1,064.1456	7.442
2	Salmosa venom 463	Analyte	3.34	1,079.8689	7.552
3	Salmosa venom 602	Analyte	4.31	1,159.3231	15.071
4	Salmosa venom 864	Analyte	6.22	242.2824	1.676
5	Salmosa venom 1000	Analyte	7.20	1,081.6899	6.189

in 100 μL with 50 mM Bacto agar was added to each tube, and the tubes were incubated at 37℃ for a maximum of 16 h for full digestion. The peptides were collected in extraction steps using 100 μL of 50 mM bicarbonate, 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid in acetonitrile, 100% (v/v), sequentially. The resulting peptide extracts were pooled and dried in the speed vacuum concentrator. The peptides were extracted using 100 μL of 50 mM bicarbonate and 0.1% trifluoroacetic acid, 0.1% trifluoroacetic acid in acetonitrile and 100% acetonitrile, sequentially. After freeze-drying the extracted peptides, the digested peptides were placed in an oasis solid-phase extraction (SPE) (water) column and desalted under vacuum according to the method provided with the oasis SPE column.

6. Protein analyses using Nano UPLC-high-definition mass spectrometry (HDMSE)

The tryptic peptide mixtures were separated on an Acquity™ HSS T3 1.8-um Trizaic™ nano-Tile column (85 um × 100 mm) using a nano-ACQUITY Ultra Performance™ chromatography system (Waters Corporation) with a Synapt G2-Si HDMSE system (Waters Corp., Milford, MA, USA). The mass spectrometer was operated in the positive ESI resolution mode with a resolution of > 250,000 FWHM. During data acquisition, the collision energies were changed to the low and the elevated (20-38 eV) collision energies on the Triwave collision cell with a scan time of 1.2 s per function over 100-2,500 m/z. MS spectral data were collected in triplicate. Tryptic peptides (4 μL) were loaded onto the enrichment column with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in
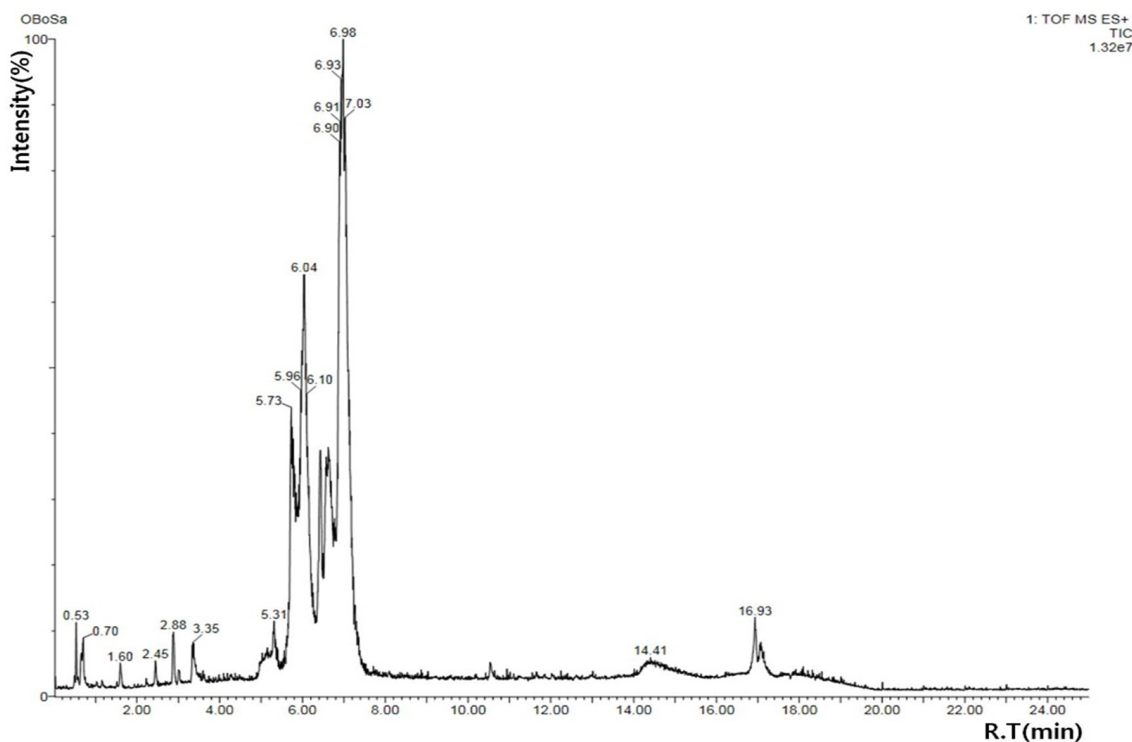

Figure 2. UPLC/Q-TOF MS chromatogram for *A. acutus* venom.

Table 2. Peaks that were identified for *A. acutus* venom by using Q-TOF MS

No.	Name	Type	RT (min)	m/z	Mass (kDa)
1	Obosa venom 838	Analyte	6.04	1,038.7026	22.829
2	Obosa venom 925	Analyte	6.62	1,099.4354	22.964
3	Obosa venom 969	Analyte	6.98	1,149.1576	22.963

acetonitrile). Step gradient was applied at a flow rate of 400 nL/min; the gradient consisted of 97% mobile phase A initially, followed by sharp decreases to 90% mobile phase A for 3 min, 65% mobile phase A for 40 min and 20% mobile phase A for 47 min; an increase to 97% mobile phase A for 50 min and a final sharp increase to 97% mobile phase A for the last 10 min. [Glu1]-fibrinopeptide (0.5 μM/min) was used to calibrate the time-of-flight analyser in the range of m/z from 100 to 1,500, and [Glu1]-fibrinopeptide (m/z = 785.8426) was used for the lock mass correction.

7. Protein identification and quantitative analyses

The continuum LC-MSE data were processed and searched using the PLGS (Protein Lynx Global Server) version 3.0 (Waters Corporation). Data acquired using alternating low and elevated energy modes in the LC-MSE were automatically smoothed, with the background-subtracted, centred, de-isotoped and charge-state reduced. Then, the alignment of the precursor and the fragmentation data were combined with a retention time tolerance of ± 0.05 min using PLGS software.

RESULTS

1. Q-TOF mass spectrometry

Mass spectrometry for *G. blomhoffii* venom revealed five peaks: one each for Salmosa venom 423 (molecular weight: 7,442 g/mol), Salmosa venom 463 (molecular weight: 7,552 g/mol), Salmosa venom 602 (molecular weight: 15,071 g/mol), Salmosa venom 864 (molecular weight: 1,676 g/mol) and Salmosa venom 1,000 (molecular weight: 6,189 g/mol) (Fig. 1, Table 1). Mass spectrometry for *A. acutus* venom re-

vealed three peaks: one each for Obosa venom 838 (molecular weight: 22,829.46 g/mol), Obosa venom 925 (molecular weight: 22,964.00 g/mol) and Obosa venom 969 (molecular weight: 22,963.15 g/mol) (Fig. 2, Table 2).

2. SDS-PAGE analyses of venom proteins

The overall protein compositions of *G. blomhoffii* and *A. acutus* venoms were assessed using gradient SDS-PAGE (Fig. 3). This result showed protein profiles with varying molecular weight distributions. Differences in fraction numbers, electrophoretic mobility and densities of venom proteins were observed between the two snakes. The separations revealed that the two venoms were composed of heterogeneous proteins varying in band intensity and migration. SDS-PAGE results show bands at molecular weights below 20 kDa.

3. N-terminal sequencing

The N-terminal sequences of *G. blomhoffii* and *A. acutus* venom were obtained using Edman degradation. The amino acids identified from the low-molecular-weight proteins of *G. blomhoffii* and *A. acutus* venoms are listed in Tables 3, 4. The N-terminal sequence of the *G. blomhoffii* venom did not correspond to any of the proteins in the databases. This finding may reflect the almost complete absence of *G. blomhoffii* protein entries in the databases and/or the lack in the *G. blomhoffii* proteins of a significant set of tryptic peptides with identical masses in homologue proteins from other snake species represented in the databanks. Amino acid sequence similarity for *A. acutus* venom was searched in the database using Blastp (protein-protein BLAST) (Table 5). Results showed that acutolysin A had a sequence of proteins similar to the *A. acutus* venom.
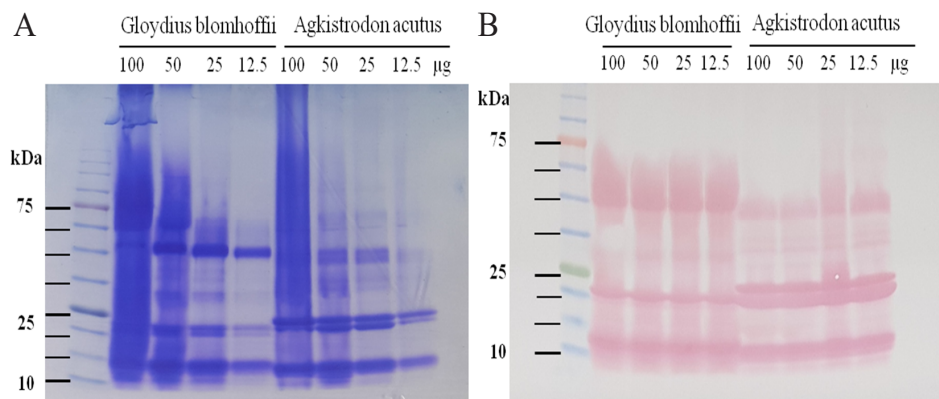

Figure 3. (A) Staining with Coomassie blue gel: Electrophoretic profiles of *G. blomhoffii* and *A. acutus* venoms are presented in a 4%-12% gradient SDS-PAGE gel. Picture showing individual venoms prepared and run in various concentrations. Proteins were visualised using a staining solution of 50% MeOH, 10% acetic acid and 1% Coomassie blue. (B) Ponceau S membrane staining: gels with the proteins were transferred to polyvinylidene difluoride (PVDF) membranes and visualised using Ponceau S solution.

4. Analyses of proteins using Nano UPLC- HDMSE

The venom proteins of *G. blomhoffii* and *A. acutus* were separated and identified using Nano UPLC-HDMSE. Five bands of *G. blomhoffii* venom (Fig. 4A) and six bands of *A. acutus* venom (Fig. 4B) were analysed; the continuum LC-MSE data were processed and searched using the PLGS, and the re-

sults are shown in Table 6.

<div style="background:#ed1c24;color:white;padding:2px 6px;text-align:center;font-weight:bold;">DISCUSSION</div>

The present study used the N-terminal sequencing analysis to detect only one protein (acutolysin A) in *A. acutus* venom. We identified no proteins with sequences similar to the results

Table 3. Amino acids identified from *G. blomhoffii* venom using N-terminal sequencing

No.	Amino acid
1	The result is uncertain
2	Pro
3	Ala
4	Gln
5	The result is uncertain
6	Not detected
7	Thr
8	Ile
9	Not detected
10	Leu

The standard three letter code was used for the amino acid residues.

Table 4. Amino acids identified from *A. acutus* venom by using N-terminal sequencing

No.	Amino acid
1	The result is uncertain
2	Pro
3	Glu, Gly, Tyr
4	Phe
5	Gln
6	Not detected
7	Tyr
8	Met
9	Not detected
10	Ile

The standard three letter code was used for the amino acid residues.

Table 5. N-terminal sequencing of the venom from the *A. acutus* snake

| No.	Protein name	Sequence	Uniprot
0	A.acutus sample-1	P E F Q _ Y M	P60244
1	Chain A, ACUTOLYSIN A FROM SNAKE VENOM OF *A. ACUTUS* AT PH 7.5	_ _ F Q R Y M	
2	Chain A, ACUTOLYSIN A FROM SNAKE VENOM OF *A. ACUTUS* AT PH 5.0	_ _ F Q R Y M	
0	A.acutus sample-2	P G F Q _ Y M	P60244
1	Chain A, ACUTOLYSIN A FROM SNAKE VENOM OF *A. ACUTUS* AT PH 7.5	_ _ F Q R Y M	
2	Chain A, ACUTOLYSIN A FROM SNAKE VENOM OF *A. ACUTUS* AT PH 5.0	_ _ F Q R Y M	
0	A.acutus sample-3	P Y F Q _ Y M	P60244
1	Chain A, ACUTOLYSIN A FROM SNAKE VENOM OF *A. ACUTUS* AT PH 7.5	_ _ F Q R Y M	
2	Chain A, ACUTOLYSIN A FROM SNAKE VENOM OF *A. ACUTUS* AT PH 5.0	_ _ F Q R Y M	

A

B
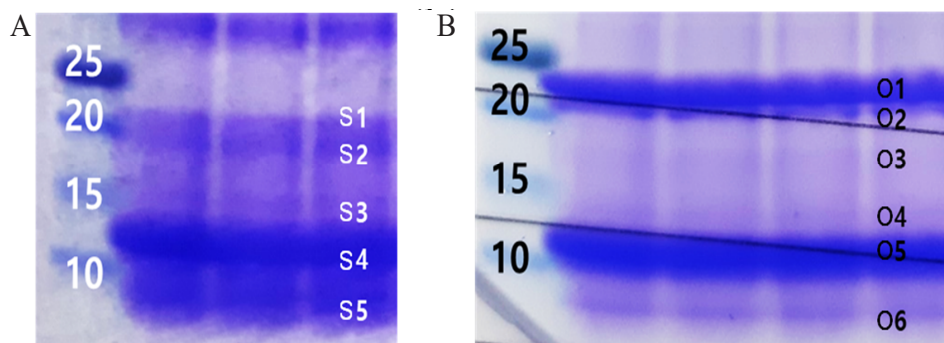
Figure 4. In-gel digestion of different protein bands with molecular weights less than 20 kDa on 1D-gel in (A) *G. blomhoffii* and (B) *A. acutus* venoms for protein identification. After SDS-PAGE using 4%-12% gradient gel, the gel slices were subjected to digestion using the in-gel digestion protocol (described in Materials and Methods).

Table 6. Proteins in *G. blomhoffii* (S1-S5) and *A. acutus* (O1-O6) venoms detected from in-solution digests by using UPLC-HDMSE

Band	Accession	Description	Protein family	Score	Avg mass (Da)	Matched products	Matched peptides	Digest peps	SeqCover (%)
S1	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	518.431	13,973.892	20	3	2,205	22.58
	P22796	Hemorrhagic factor II (EC 3.4.24.-)	SVMP	767.0865	22,595.849	38	5	3,801	21
	P79845	Cysteine-rich venom protein precursor	CRiSP	1,053.453	20,377.963	30	2	3,444	14.21
	P34179	Adamalysin II (EC 3.4.24.46)	SVMP	201.4728	23,075.626	13	1	3,864	4.93
S2	P22796	Hemorrhagic factor II (EC 3.4.24.-)	SVMP	1,678.013	22,595.849	62	8	3,801	34
	P79845	Cysteine-rich venom protein precursor	CRiSP	1,051.569	20,377.963	20	4	3,444	27.87
	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	2,028.666	13,973.892	42	4	2,205	20.97
S3	P04417	Phospholipase A2_ basic (EC 3.1.1.4) (PA2-I)	PLA$_2$	2,161.394	13,982.312	28	3	2,163	15.57
	P00623	Phospholipase A2 alpha (EC 3.1.1.4)	PLA$_2$	2,064.735	13,679.499	37	4	2,163	37.7
	P14421	Phospholipase A2_ neutral (EC 3.1.1.4)	PLA$_2$	4,071.618	13,868.646	32	5	2,163	36.89
	P00624	Phospholipase A2 (EC 3.1.1.4)	PLA$_2$	1,582.225	13,595.372	25	3	2,163	34.43
	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	2,517.537	13,973.892	54	5	2,205	20.97
	P79845	Cysteine-rich venom protein precursor	CRiSP	437.537	20,377.963	11	1	3,444	7.1
S4	P04417	Phospholipase A2_ basic (EC 3.1.1.4) (PA2-I)	PLA$_2$	1,915.819	13,982.312	32	3	2,163	21.31
	P00623	Phospholipase A2 alpha (EC 3.1.1.4)	PLA$_2$	2,064.735	13,679.499	37	4	2,163	37.7
	P14421	Phospholipase A2_ neutral (EC 3.1.1.4)	PLA$_2$	4,071.618	13,868.646	32	5	2,163	36.89
	P00624	Phospholipase A2 (EC 3.1.1.4)	PLA$_2$	1,582.225	13,595.372	25	3	2,163	34.43
	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	3,290.186	13,973.892	63	5	2,205	33.87
S5	P00623	Phospholipase A2 alpha (EC 3.1.1.4)	PLA$_2$	747.4556	13,679.499	12	2	2,163	26.23
	P00624	Phospholipase A2 (EC 3.1.1.4)	PLA$_2$	478.1932	13,595.372	25	4	2,163	18.85
	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	731.7947	13,973.892	18	1	2,205	8.87
O1	Q9PW35	Acutolysin A precursor (EC 3.4.24.-)	SVMP	672.6088	46,564.95	35	4	8,274	9.44
O2	Q9PW35	Acutolysin A precursor (EC 3.4.24.-)	SVMP	5,820.132	46,564.95	259	22	8,274	26.15
O3	Q9PW35	Acutolysin A precursor (EC 3.4.24.-)	SVMP	515.6565	46,564.95	49	6	8,274	15.74
	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	533.1411	13,973.892	15	2	2,205	8.87
	P79845	Cysteine-rich venom protein precursor	CRiSP	641.5217	20,377.963	16	2	3,444	7.1
O4	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	735.4298	13,973.892	19	2	2,205	8.87
O5	P81114	Alboaggregin A subunit 4.	CTL/ snaclec	1,126.348	14,543.723	24	2	6	26.02
	Q9PW35	Acutolysin A precursor (EC 3.4.24.-)	SVMP	1,579.929	47,192.186	57	6	35	19.85
	P14418	Phospholipase A2_ acidic (EC 3.1.1.4)	PLA$_2$	1,291.795	14,772.193	30	2	14	17.74
O6	Q9PW35	Acutolysin A precursor (EC 3.4.24.-)	SVMP	1,962.108	47,192.186	110	8	35	23.97

Abbreviations: CTL, C-type lectin; CRiSP, cysteine-rich secretory protein; PLA$_2$, phospholipase A$_2$; SVMP, snake venom metalloproteinase.

of N-terminal sequencing for the *G. blomhoffii* venom. Using in-gel digestion with the UPLC-HDMSE approach, we detected a total of eight proteins belonging to three snake-venom protein families in *G. blomhoffii* venom and four proteins belonging to four snake-venom protein families in *A. acutus* venom. The *G. blomhoffii* venom protein families consisted of phospholipase A$_2$ (PLA$_2$), snake venom metalloproteinase (SVMP) and cysteine-rich secretory protein (CRiSP) families. The *A. acutus* venom families consisted of PLA$_2$, SVMP, CRiSP and C-type lectin/snaclec (CTL/ snaclec) families.

Generally, viper venoms induce haemorrhagic, hypotensive and inflammatory effects due to high concentrations of metal-

loproteases, serine proteases and C-type lectins [21]. The results of the present study do not deviate much from this general fact. The toxin families detected in the two snakes are discussed below.

1. SVMP family

The SVMP proteins are important compounds in most viper venoms. The SVMPs are known to show a wide range of physiological activities (haemorrhagic, fibrinolytic and apoptotic activities), inhibition of platelet aggregation, prothrombin and blood coagulation factor X activation and deactivation of blood serine proteinase inhibitors [22]. SVMPs are responsible for the classic Viperid envenoming effect, the ability to cause haemorrhage at the bite site [23, 24]. Interestingly, SVMPs can be a source of therapeutic agents due to their ability to cause haemorrhage, coagulopathy and inflammatory responses. Based on their structures, these proteins can be classified into three classes: P-I, P-II and P-III. The P-I class (with only a metalloproteinase domain) is the smallest at 20-30 kDa. The proteins in the P-II class have molecular weights between 30 and 60 kDa and contain a metalloproteinase domain, followed by a disintegrin domain. The proteins in the P-III class are the largest at 60 to 100 kDa, consisting of a metalloproteinase domain and disintegrin-like and cysteine-rich domains [23, 25]. Generally, the proteins in the P-III class are considered the most potent of SVMPs because they may play a role in targeting the protein to a particular site in cells (such as platelets and endothelial cells), integrins and the extracellular matrix [26-30]. However, all SVMP proteins detected in this study belong to the P-I class. We report two SMVPs (haemorrhagic factor II, adamalysin II) for the *G. blomhoffii* venom and one SMVP (acutolysin A precursor) for the *A. acutus* venom (Table 6).

2. CRiSP family

CRiSPs are relatively low-molecular-weight glycoproteins (20-30 kDa) with a highly conserved specific pattern of 16 cysteine residues and high similarity of amino acid sequences [31-33]. Some biological functions, such as gamete fusion, sperm maturation, sperm chemoattraction, anti-microbial activity, matrix degradation, Ca^{2+} and K^+ channel blocking, stress resistance regulation and protease-like activity, have been investigated [34]. CRiSPs are found in most snake families, including vipers, and the CRiSPs in snake venoms have Ca^{2+} channel blocker-like

properties and cyclic nucleotide-gated channel blocker activities [35, 36]. This study revealed one CRiSP protein in the *G. blomhoffii* venom and one in the *A. acutus* venom (Table 6).

3. PLA$_2$ family

Widely distributed in nature, PLA$_2$ has been identified in venoms from all snake families, including Colubridae, Elapidae, Viperidae and Hydrophilae [37]. PLA$_2$ activates the hydrolysis of glycerophospholipids at the sn-2 position of the glycerol backbone to dissociate fatty acids and corresponding 1-acyl lysophospholipids [38, 39]. PLA$_2$s are enzymes of high medical interest because they are involved in several inflammatory human diseases and present important effects, including neurotoxic, myotoxic, cardiotoxic, cytotoxic, haemolytic, hypotensive, platelet aggregation, anticoagulant, pro-inflammatory, oedematogenic and bactericidal activities [40, 41]. Lipolytic PLA$_2$ enzymes have been explored as novel anticancer agents targeting altered lipid biosynthesis and deregulated lipogenesis, which are the typical features of cancer [42-44]. Acidic and basic PLA$_2$s also possess antitumour and antiangiogenic properties [44-47]. Some PLA$_2$s isolated from viper snake venoms are capable of antitumoural activity, suggesting that these molecules may be a new class of anticancer agents [48]. Here, we report relatively high amounts (62.5%) of PLA$_2$ enzymes in *G. blomhoffii* venom and PLA$_2$ in *A. acutus* venom (Table 6). We identified the presence of acidic, basic and neutral PLA$_2$ isoenzymes in *G. blomhoffii* venom and one acidic form in *A. acutus* venom.

4. CTL/snaclec family

The term "snaclec" avoids confusion between classic C-type lectins and C-type lectin proteins. Classic C-type lectins bind to calcium and sugar residue, but the C-type lectin-like proteins in snake venom do not contain calcium and sugar-binding loops, so they do not exhibit lectin activity [49, 50]. Snaclecs display numerous biological activities, including anticoagulation, procoagulation and platelet modulation [51]. Some CTL/snaclecs show potential prospects in cancer therapy [52]. Snaclecs are considered abundant components of snake venom, particularly in viper venom [53]. We report one CTL/snaclec, alboaggregin, in *A. acutus* venom (Table 6).

The present study has some limitations. The initial focus of this study was to identify the low-molecular-weight proteins in

the venoms of two viper snakes, *G. blomhoffii* and *A. acutus*. The Q-TOF results for *G. blomhoffi* venom showed peaks with values of 1,676 kDa and 1,5071 kDa. However, we focused on confirming the presence of proteins below 20 kDa and conducted N-terminal sequencing and in-gel digestion for bands corresponding to 20 kDa or less. The results showed proteins with a higher molecular weight. We assume that this result came from a protein with high molecular weight being cleaved during electrophoresis. Furthermore, the proteins found by using N-terminal sequencing analyses may not be truly present because those results were based on the similarity of the N-terminal sequence. Western blots may help the definitive identification of proteins in these snake venoms.

CONCLUSION

In conclusion, by combining mass spectrometry, electrophoresis, N-terminal sequencing and in-gel digestion, we identified eight proteins from *G. blomhoffii* venom and four proteins from *A. acutus* venom. The proteins detected in *G. blomhoffii* and *A. acutus* were PLA$_2$, SVMP and CRiSP. CTL/snaclec was unique to the *A. acutus* venom. These data will contribute to the current knowledge of proteins in viper-snake venom and may be beneficial in obtaining a clinical prognosis for patients envenomed by these snakes.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

DATA AVAILABILITY

The data used to support the findings of this study are included in the article.

ORCID

Su-Jeong Ha, https://orcid.org/0000-0002-8089-5515
Yeo-Ok Choi, https://orcid.org/0000-0002-2317-8771
Eun-Bin Kwag, https://orcid.org/0000-0002-7068-4888
Soo-Dam Kim, https://orcid.org/0000-0002-4872-2462
Hwa-seung Yoo, https://orcid.org/0000-0003-3738-3239
In-Cheol Kang, https://orcid.org/0000-0002-4685-2917
So-Jung Park, https://orcid.org/0000-0002-8829-1716

REFERENCES

1. Markland FS. Snake venoms and the hemostatic system. Toxicon. 1998;36(12):1749-800.
2. Fry BG. Structure-function properties of venom components from Australian elapids. Toxicon. 1999;37(1):11-32.
3. Williams D, Gutiérrez JM, Harrison R, Warrell DA, White J, Winkel KD, et al. The global snake bite initiative: an antidote for snake bite. Lancet. 2010;375(9708):89-91.
4. Kasai K, Ishikawa T, Nakamura T, Miura T. Antibacterial properties of L-amino acid oxidase: mechanisms of action and perspectives for therapeutic applications. Appl Microbiol Biotechnol. 2015;99(19):7847-57.
5. Chellapandi P. Structural, functional and therapeutic aspects of snake venom metalloproteinases. Mini Rev Org Chem. 2014; 11(1):28-44.
6. Chong HP, Tan KY, Tan CH. Cytotoxicity of snake venoms and cytotoxins from two Southeast Asian cobras (*Naja sumatrana*, *Naja kaouthia*): exploration of anticancer potential, selectivity, and cell death mechanism. Front Mol Biosci. 2020;7:583587.
7. Dufton MJ, Hider RC. Conformational properties of the neurotoxins and cytotoxins isolated from Elapid snake venoms. CRC Crit Rev Biochem. 1983;14(2):113-71.
8. Basus VJ, Song G, Hawrot E. NMR solution structure of an alpha-bungarotoxin/nicotinic receptor peptide complex. Biochemistry. 1993;32(46):12290-8.
9. Conti-Tronconi BM, Diethelm BM, Wu XD, Tang F, Bertazzon T, Schröder B, et al. Alpha-bungarotoxin and the competing antibody WF6 interact with different amino acids within the same cholinergic subsite. Biochemistry. 1991;30(10):2575-84.
10. Tzartos SJ, Remoundos MS. Fine localization of the major alpha-bungarotoxin binding site to residues alpha 189-195 of the Torpedo acetylcholine receptor. Residues 189, 190, and 195 are indispensable for binding. J Biol Chem. 1990;265(35):21462-7.
11. Radding W, Corfield PW, Levinson LS, Hashim GA, Low BW. Alpha-toxin binding to acetylcholine receptor alpha 179-191 peptides: intrinsic fluorescence studies. FEBS Lett. 1988;231(1):

212-6.

12. Grognet JM, Ménez A, Drake A, Hayashi K, Morrison IE, Hider RC. Circular dichroic spectra of elapid cardiotoxins. Eur J Biochem. 1988;172(2):383-8.

13. Kini RM, Evans HJ. Mechanism of platelet effects of cardiotoxins from Naja nigricollis crawshawii (spitting cobra) snake venom. Thromb Res. 1988;52(3):185-95.

14. Hinman C, Lepisto E, Stevens R, Montgomery I, Rauch H, Hudson R. Effects of cardiotoxin D from Naja naja siamensis snake venom upon murine splenic lymphocytes. Toxicon. 1987;25(9):1011-4.

15. Takechi M, Tanaka Y, Hayashi K. Binding of cardiotoxin analogue III from Formosan cobra venom to FL cells. FEBS Lett. 1986;205(1):143-6.

16. Gatineau E, Takechi M, Bouet F, Mansuelle P, Rochat H, Harvey AL, et al. Delineation of the functional site of a snake venom cardiotoxin: preparation, structure, and function of monoacetylated derivatives. Biochemistry. 1990;29(27):6480-9.

17. Igari R, Iseki K, Abe S, Syoji M, Sato M, Shimomura K, et al. [Binocular diplopia and ptosis due to snakebite (Agkistrodon blomhoffi "mamushi")--a case report]. Brain Nerve. 2010;62(3):273-7. Japanese.

18. Sakurai Y, Shima M, Matsumoto T, Takatsuka H, Nishiya K, Kasuda S, et al. Anticoagulant activity of M-LAO, L-amino acid oxidase purified from Agkistrodon halys blomhoffii, through selective inhibition of factor IX. Biochim Biophys Acta. 2003;1649(1):51-7.

19. Yamazaki Y, Koike H, Sugiyama Y, Motoyoshi K, Wada T, Hishinuma S, et al. Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. Eur J Biochem. 2002;269(11):2708-15.

20. Sajevic T, Leonardi A, Križaj I. Haemostatically active proteins in snake venoms. Toxicon. 2011;57(5):627-45.

21. Vaiyapuri S, Wagstaff SC, Watson KA, Harrison RA, Gibbins JM, Hutchinson EG. Purification and functional characterisation of rhiminopeptidase A, a novel aminopeptidase from the venom of Bitis gabonica rhinoceros. PLoS Negl Trop Dis. 2010;4(8):e796.

22. Markland FS Jr, Swenson S. Snake venom metalloproteinases. Toxicon. 2013;62:3-18.

23. Fox JW, Serrano SM. Timeline of key events in snake venom metalloproteinase research. J Proteomics. 2009;72(2):200-9.

24. Fox JW, Serrano SM. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon. 2005;45(8):969-85.

25. Gutiérrez J, Rucavado A. Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage. Biochimie. 2000;82(9-10):841-50.

26. Estêvão-Costa MI, Diniz CR, Magalhães A, Markland FS, San-

chez EF. Action of metalloproteinases mutalysin I and II on several components of the hemostatic and fibrinolytic systems. Thromb Res. 2000;99(4):363-76.

27. Kamiguti AS, Markland FS, Zhou Q, Laing GD, Theakston RD, Zuzel M. Proteolytic cleavage of the beta1 subunit of platelet alpha2beta1 integrin by the metalloproteinase jararhagin compromises collagen-stimulated phosphorylation of pp72. J Biol Chem. 1997;272(51):32599-605.

28. Smith J, Dangelmaier C, Selak M. Identification of 50 kDa snake venom proteins which specifically inhibit platelet adhesion to collagen. FEBS Lett. 1991;283(2):307-10.

29. Sanchez EF, Bush LR, Swenson S, Markland FS. Chimeric fibrolase: covalent attachment of an RGD-like peptide to create a potentially more effective thrombolytic agent. Thromb Res. 1997;87(3):289-302.

30. Liu CZ, Huang TF. Crovidisin, a collagen-binding protein isolated from snake venom of Crotalus viridis, prevents platelet-collagen interaction. Arch Biochem Biophys. 1997;337(2):291-9.

31. Swenson S, Bush LR, Markland FS. Chimeric derivative of fibrolase, a fibrinolytic enzyme from southern copperhead venom, possesses inhibitory activity on platelet aggregation. Arch Biochem Biophys. 2000;384(2):227-37.

32. Yamazaki Y, Morita T. Structure and function of snake venom cysteine-rich secretory proteins. Toxicon. 2004;44(3):227-31.

33. Yamazaki Y, Morita T. Snake venom components affecting blood coagulation and the vascular system: structural similarities and marked diversity. Curr Pharm Des. 2007;13(28):2872-86.

34. Sunagar K, Johnson WE, O'Brien SJ, Vasconcelos V, Antunes A. Evolution of CRISPs associated with toxicoferan-reptilian venom and mammalian reproduction. Mol Biol Evol. 2012;29(7):1807-22.

35. Matsunaga 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(3):365-75.

36. Brown RL, Haley TL, West KA, Crabb JW. Pseudechetoxin: a peptide blocker of cyclic nucleotide-gated ion channels. Proc Natl Acad Sci U S A. 1999;96(2):754-9.

37. Tasoulis T, Isbister G. A review and database of snake venom proteomes. Toxins (Basel). 2017;9(9):290.

38. Koh DC, Armugam A, Jeyaseelan K. Snake venom components and their applications in biomedicine. Cell Mol Life Sci. 2006;63(24):3030-41.

39. Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. Toxicon. 2003;42(8):827-40.

40. Gutiérrez JM, Lomonte B. Phospholipases A2: unveiling the secrets of a functionally versatile group of snake venom toxins. Toxicon. 2013;62:27-39.

41. Cummings BS. Phospholipase A2 as targets for anti-cancer drugs. Biochem Pharmacol. 2007;74(7):949-59.

42. Mashima T, Seimiya H, Tsuruo T. De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy. Br J Cancer. 2009;100(9):1369-72.

43. Ferguson EL, Richardson SC, Duncan R. Studies on the mechanism of action of dextrin-phospholipase A2 and its suitability for use in combination therapy. Mol Pharm. 2010;7(2):510-21.

44. Roberto PG, Kashima S, Marcussi S, Pereira JO, Astolfi-Filho S, Nomizo A, et al. Cloning and identification of a complete cDNA coding for a bactericidal and antitumoral acidic phospholipase A2 from Bothrops jararacussu venom. Protein J. 2004;23(4):273-85.

45. Araya C, Lomonte B. Antitumor effects of cationic synthetic peptides derived from Lys49 phospholipase A2 homologues of snake venoms. Cell Biol Int. 2007;31(3):263-8.

46. Maity G, Mandal S, Chatterjee A, Bhattacharyya D. Purification and characterization of a low molecular weight multifunctional cytotoxic phospholipase A2 from Russell's viper venom. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;845(2):232-43.

47. Khunsap S, Pakmanee N, Khow O, Chanhome L, Sitprija V, Suntravat M, et al. Purification of a phospholipase A(2) from Daboia russelii siamensis venom with anticancer effects. J Venom Res. 2011;2:42-51.

48. Rodrigues RS, da Silva JF, Boldrini França J, Fonseca FP, Otaviano AR, Henrique Silva F, et al. Structural and functional properties of Bp-LAAO, a new L-amino acid oxidase isolated from Bothrops pauloensis snake venom. Biochimie. 2009;91(4):490-501.

49. Kessentini-Zouari R, Jebali J, Taboubi S, Srairi-Abid N, Morjen M, Kallech-Ziri O, et al. CC-PLA2-1 and CC-PLA2-2, two Cerastes cerastes venom-derived phospholipases A2, inhibit angiogenesis both in vitro and in vivo. Lab Invest. 2010;90(4):510-9.

50. Morita T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. Toxicon. 2005;45(8):1099-114.

51. Clemetson KJ. Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors. Toxicon. 2010;56(7):1236-46.

52. Calderon LA, Sobrinho JC, Zaqueo KD, de Moura AA, Grabner AN, Mazzi MV, et al. Antitumoral activity of snake venom proteins: new trends in cancer therapy. Biomed Res Int. 2014;2014:203639.

53. Kunalan S, Othman I, Syed Hassan S, Hodgson W. Proteomic characterization of two medically important Malaysian snake venoms, *Calloselasma rhodostoma* (Malayan Pit Viper) and *Ophiophagus hannah* (King Cobra). Toxins (Basel). 2018;10(11):434.