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Proteomics and immunocharacterization of Asian mountain pit viper (*Ovophis monticola*) venom

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Abstract

The venomic profile of Asian mountain pit viper *Ovophis monticola* is clarified in the present study. Using mass spectrometry-based proteomics, 247 different proteins were identified in crude venom of *O. monticola* found in Thailand. The most abundant proteins were snake venom metalloproteases (SVMP) (36.8%), snake venom serine proteases (SVSP) (31.1%), and phospholipases A_2 (PLA_2) (12.1%). Less abundant proteins included L-amino acid oxidase (LAAO) (5.7%), venom nerve growth factor (3.6%), nucleic acid degrading enzymes (3.2%), C-type lectins (CTL) (1.6%), cysteine-rich secretory proteins (CRISP) (1.2%) and disintegrin (1.2%). The immunoreactivity of this viper's venom to a monovalent antivenom against green pit viper *Trimeresurus albolabris*, or to a polyvalent antivenom against hemotoxic venom was investigated by indirect ELISA and two-dimensional (2D) immunoblotting. Polyvalent antivenom showed substantially greater reactivity levels than monovalent antivenom. A titer for the monovalent antivenom was over $1:1.28x10^7$ dilution while that of polyvalent antivenom was $1:5.12x10^7$. Of a total of 89 spots comprising 173 proteins, 40 spots of predominantly SVMP, SVSP and PLA_2 were specific antigens for antivenoms. The 49 unrecognized spots containing 72 proteins were characterized as non-reactive proteins, and included certain types of CTLs and CRISPs. These neglected venom constituents could limit the effectiveness of antivenom-based therapy currently available for victims of pit viper envenomation.

Introduction

Envenomation from snakebites affects over 2.7 million people in tropical and subtropical countries each year, leading to more than 130,000 deaths among victims [1]. Severe injuries and complications from bites also can lead to permanent disabilities and long-term health

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problems in survivors. In addition, the majority of snakebite victims have been reported within the productive age of the workforce [2]. This creates socio-ecomomic loss as seen in developing countries of Asia and Africa [3].

Currently, there are at least six known species of the venomous *Ovophis* genus (Family Viperidae, Subfamily Crotalinae) existed according to the phylogenetic and morphological analyses. Five closely related species are distributed across several geographical areas of the Asian mainland [4]. *Ovophis tonkinensis* occurs in northern Vietnam and southern China; *O. zayuensis* in southern China (Yunnan), northeastern India and Myanmar; *O. makazayazaya* in southern China (Sichuan, Yunnan), Taiwan and northern Vietnam; *O. convictus* is restricted to western Malaysia; and *O. monticola* is found in Nepal, northeastern India [5], southern China, Myanmar, southern Laos, central Vietnam and northern Thailand [4, 6]. The other member of this genus, *O. okinavensis*, inhabits Ryukyu Island of Japan [7].

In Thailand, the Asian mountain pit viper *O. monticola* is found in high-altitude mountains, particularly in the northern province of Chiang Mai [6]. It has a stout body with a short snout. Its triangular head is covered by small, smooth scales rather than large shields. These vipers also exhibit sexual dimorphism in body size, with an average male length of 49 cm and female length of 110 cm. These montane, terrestrial, nocturnal vipers generally live under the forest litter and prey on small mammals [8].

The medical significance of pit viper envenomation primarily relates to the hematotoxic activity on human victims. Severe clinical manifestation includes local damage (*e.g.*, painful oedema, tissue necrosis) and systemic injuries, including haemorrhage, coagulopathy and thrombocytopenia, critically resulting in high mortality and morbidity [5, 9].With advanced proteomic technology, the heterogeneity of snake venoms has progressively been elucidated. For the *Ovophis* spp., venomic profiles of *O. convictus* from western Malaysia, *O. tonkinensis* from northern Vietnam and southern China and Japanese hime habu *O. okinavensis* from Okinawa, Japan were recently reported. The abundance of four major enzymes namely snake venom serine proteinase (SVSP), phospholipases A_2 (PLA_2), L-amino acid oxidases (LAAO) and snake venom metalloproteases (SVMP) were dominant within all venoms. Among these enzymatic proteins, SVSP was found in the greatest proportion, accounting for 35–53% of all constituents. The second most abundant enzyme was PLA_2 ranging from 19–26%. In addition, various non-enzymatic proteins and peptides including cysteine-rich secretory proteins (CRISP), venom nerve growth factor (VNGF), venom endothelial growth factor (VEGF), kunitz peptides (KUN) and C-type lectins/snaclecs (CTL) were recorded, in varying amounts [10]. However, variation in snake venom composition occurs not only among distinct species but also among different population of the same species, due to ecological niches as well as availability of preys [11].

The present study aims to investigate the protein constituents of venom from the Asian mountain pit viper *O. monticola* found in Thailand. In addition, since there is no homospecific antivenom to *Ovophis* spp. venoms currently available, the therapeutic regime for bite victims depends largely on two types of antivenom: pit viper monovalent antivenom, raised against white-lipped green pit viper (*Trimeresurus albolabris*) venom; and polyvalent antivenom, produced against hematotoxic venom of *Calloselasma rhodostoma* (Malayan pit viper), *Daboia siamensis* (Russell's viper) and *T. albolabris*. Cross reactivity of *O. monticola* venom to these readily available antivenoms was therefore evaluated. Compositional profiles of immunoreactive versus non-reactive proteins in *O. monticola* venom were also clarified. Knowledge gained from this study not only extends the *Ovophis* spp. venomic database, but also can lead to better management and therapeutic approaches for mountain pit viper envenomation.

Materials and methods

Snakes, venom and antivenoms

All *O. monticola* pit vipers (Fig 1) were captured in the wild and transferred to Snake Farm, Queen Saovabha Memorial Institute (QSMI) before being quarantined. All procedures were performed following the safety protocol for working with venomous snakes (No. SN 001/2016). Routine snake care and the venom collection was conducted according to the specific protocol. All protocols were approved by the Ethic Committee of the Queen Saovabha Memorial Institute Animal Care and Use (No. QSMI-ACUC-02-2018) in accordance with the guideline of the National Research Council of Thailand. Information about individual snakes used in this study is shown in Table 1.

Monovalent antivenom against the green pit viper *T. albolabris* venom (batch no. TA00219; expiry date 08/10/2024) and hematotoxic polyvalent antivenoms (against the venom of *C. rhodostoma*, *D. siamensis* and *T. albolabris*) (batch no. HP 00118; expiry date 16/ 01/2023) produced by QSMI available as a freeze-dried $F(ab')_2$ form, isolated from horse immunoglobulins were used within their shelf-life. Following reconstitution, each milliliter of monovalent antivenom neutralized 0.7 mg of *T. albolabris* venom; one milliliter of hematotoxic polyvalent antivenom neutralized 0.7 mg of *T. albolabris* venom, 1.6 mg of *C. rhodostoma* venom and 0.6 mg of *D. siamensis* venom [12].

O. monticola *venom preparation and one-dimensional sodium dodecyl sulfate polyacrylamide* gel electrophoresis (SDS-PAGE)

Crude venom of *O. monticola* was mixed with lysis buffer (containing of 1% Triton X-100 (Merck, Germany), 1% sodium dodecyl sulfate (SDS) (Merck, Germany), and 1% NaCl

Fig 1. A wild juvenile Asian mountain pit viper (*O. monticola*) found in Northern Thailand. The venom of *O. monticola* was extracted and kept in individual 1.5 ml microcentrifuge tubes. After weighing, the fresh (liquid) venom was immediately frozen at -20˚C and lyophilized. The lyophilized venom was then pooled and stored at -20˚C until use.

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Table 1. Biological and geographical data for all snakes used in the study.

Species[a]	Voucher no.[b]	Sex[c]	Snout-Vent Length (cm)	Total Length (cm)	Locality[d]
Ovophis monticola	QSMI 1441	F	32.0	36.5	Omkoi-Chiang Mai
Ovophis monticola	QSMI1443	F	32.0	36.5	Doi Pui-Chiang Mai
Ovophis monticola	QSMI 1449	M	34.0	40.0	Omkoi-Chiang Mai
Ovophis monticola	QSMI 1469	M	31.0	38.0	Omkoi-Chiang Mai
Ovophis monticola	QSMI 1559	M	35.5	43.0	Omkoi-Chiang Mai

[a] The identification of *Ovophis monticola* was made by specialized veterinarians according to the identification key [8]. Key characters are body coloration and pattern: predominantly tan or reddish-grey with irregular short, black-edged crossbars or blotches along the vertebral ridge, including smaller irregular dark blotches on both sides of the body along the edges of the dorsal scales (Fig 1).

[b] Voucher no. was attached to each preserved snake after it died.

[c] Sex: F: Female; M: Male.

[d] District or subdistrict-province in Thailand where snakes were captured.

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(Merck, Germany). The venom was estimated for protein concentration by Quick Start™ Bradford Protein Assay (Bio-Rad, USA). A 30 μg sample of *O. monticola* venom was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, USA) and stained by Coomassie R-250 solution (Bio-Rad, USA) as previously described [13]. The whole lane of venom was excised into 10 pieces and further subjected to in-gel digestion.

Two-dimensional polyacrylamide gel electrophoresis (2DE)

A 100 μg protein was mixed with IPG sample buffer containing 8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 15 mM dithiothreitol (DTT), and 0.5% IPG sample buffer [14]. Afterwards, the protein solution was rehydrated overnight into a non-linear immobilized pH gradient (IPG) strip (pH 3–10; Amersham Bioscience, USA). Isoelectric focusing (pI) was done using an Ettan IPGphorII instrument (Amersham Bioscience, USA) with the following settings: 30 V for 14 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3500 V for 1 h, and 8000 V for 18 h. The IPG strips were equilibrated with DTT for 15 min and with iodoacetamine for 15 min. After incubation, the strips were placed onto a 12% SDS-PAGE gel. All three 2DE gels were stained with silver stain and the immunoreactive spots in these gels were excised and pooled for mass spectrometric analysis. Other two 2DE gels were used for immunoblotting.

In-gel digestion

A mixture of 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate was used for de-staining the blue color from gel slides [13]. Venom proteins were reduced by 4mM DTT and incubated at 60˚C for 15 min. The reduced proteins were further alkylated by 250 mM iodoacetamine (IAA) (Sigma-Aldrich, USA) and incubated at room temperature for 30 min in dark. The gel pieces were dehydrated by removing all solution and adding 100% ACN (Thermo Scientific, USA). For tryptic digestion, trypsin (Sigma-Aldrich, USA, T6567) in 50 mM ammonium bicarbonate (Sigma-Aldrich, USA) was added to rehydrate the gels, which were then incubated overnight at 37 ˚C. Peptide extraction was performed by adding 100% ACN and incubating for 15 min. The resulting solution was transferred into a new microcentrifuge tube and dried using a centrifugal concentrator (TOMY, Japan). The peptide mixtures were stored at -20˚C prior to mass spectrometric analysis.

Mass spectrometric analysis

Venom peptides were dissolved in 0.1% formic acid (Sigma-Aldrich, USA) and subjected to an Ultimate® 3000 Nano-LC systems (Thermo Scientific, USA). The peptides were eluted and infused to a microTOF-Q II (Bruker, Germany). The acquisition was operated by HyStar™ version 3.2 (Bruker, Germany), and the resulting data were processed and converted to mascot generics format (.mgf) files using Compass DataAnalysis™ software version 3.4 (Bruker, Germany). A database search was performed using Mascot Daemon software (Matrix Science, USA) against the NCBI snake database with the following parameters: one missed cleavage site, variable modifications of carbamidomethyl (C) and oxidation (M), 0.8 Da for MS peptide tolerance and 0.8 Da for MS/MS tolerance. The significance threshold was set at 95%. Three biological replications were performed for protein identification.

Indirect enzyme-linked immunosorbent assay (ELISA)

Immunoreactivity of protein antigens in *O. monticola* venom to monovalent and polyvalent antivenom was assessed by indirect enzyme-linked immunosorbent assay (ELISA) modified from Gawtham and colleagues [15]. Each well of a 96-well Maxisorp Nunc immune plate (Thermo Fisher Scientific, Denmark) was coated with 5 ng of *O. monticola* venom in 0.05 M carbonate/bicarbonate buffer pH 9.6 (50 μl/well) and kept at 4˚C overnight. Plates were washed three times with phosphate-buffered saline (PBS) pH 7.2, blocked by adding 200 μL of PBS containing 2% (*w*/*v*) bovine serum albumin (BSA) (Capricorn Scientific GmBH, Ebsdorfergrund, Germany) and incubated for 1.5 h at 37˚C. The plates were then washed three times with PBS-0.05% Tween (PBST). They were incubated again for 1 h at 37˚C with 50 μL of the serial dilution of either monovalent or polyvalent antivenom ($1:10^5$–$1:5x10^7$ in 0.2% BSA-PBS). After washing the plate three times with PBST, 50 μL of horseradish peroxidase-conjugated goat anti-horse-IgG (Abcam, Cambridge, UK) in PBST (1:1000) was added into each well and further incubated for another hour at 37˚C. Plates were then washed three times with PBST. Fifty microliters of substrate solution (SureBlue TMB microwell peroxidase, Seracare Life Sciences, Milford, MA) was subsequently added to each well, and the plate was kept in the dark for 10 min at room temperature for the reaction to occur. The absorbance at 630 nm was read using a microplate reader (TECAN InfinitePro 200, Switzerland).

Immunoblot analysis

The separated polypeptide spots from 2DE gels were transferred to nitrocellulose membrane for 90 min at 18 V on a Trans-blot semi-dry Transfer CellTM (Biorad) in semi-dry transfer buffer (48 mM Tris and 2.93 g glycine) pH 9.2 containing 20% methanol. The membranes were blocked using 5% (w/v) non-fat milk in PBS for 2 h at room temperature. The membranes were rinsed twice with PBS-T buffer pH 7.4 (8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM NaCl, 2.7 mM KCl and 0.5% v/v Tween) for 30 s each. The blotted membranes were incubated with either monovalent or polyvalent antivenom (1:1000 in 0.2% BSA-PBS). After washing the membrane three times with PBS-T, 50 μL of horseradish peroxidase-conjugated goat anti-horse-IgG (Abcam, Cambridge, UK) in PBS-T (1:2000) was added, and the mixture was incubated for 1 h at ambient temperature under constant agitation. Membranes were washed three times with PBS-T buffer and one time with PBS. Immunogen spots were visualized by detection of peroxidase activity using Ultra TMB-Blotting Solution (ThermoFisher Scientific, UK).

Statistical analysis

Quantitative data are presented as mean ± SEM. Statistical significance between groups was analyzed using standard t-tests or two-way ANOVA followed by the Bonferroni test. Significant p-values are indicated within the figure panels. Error bars indicate SEM.

Results

Proteomic analysis of *O. monticola* venom

Detectable proteins in venom of *O. monticola* were between 10–95 kDa (Fig 2A). Intense protein bands at 10, 15, 50 and 72 kDa and faint bands at 26, 28, 30, 34 and 95 kDa were recorded. There were 247 proteins found in *O. monticola* venom (a list of all proteins is shown in S1 Table). A classification of constituent proteins based on their biological properties is presented in Fig 2B. The most abundant proteins were snake venom metalloproteases (SVMP) (36.8%), snake venom serine proteases (SVSP) (31.1%), and phospholipases A_2 (PLA_2) (12.1%). Less abundant groups included L-amino acid oxidase (LAAO) (5.7%), venom nerve growth factor (3.6%), nucleic acid degrading enzymes (3.2%) C-type lectins (CTL) (1.6%), cysteine-rich secretory proteins (CRISP) (1.2%) and disintegrin (1.2%). Toxin biosynthesis and other proteins comprised 0.4%. The top 15 unique proteins identified in *O. monticola* venom are shown in Table 2.

Immunoreactivity of protein antigens in *O. monticola* venom to monovalent and polyvalent antivenoms by indirect ELISA

Since there is no homospecific antivenom to *Ovophis* spp. venoms currently available, all pit viper envenoming victims are recommended to receive either monovalent antivenom (raised against *T. albolabris* venom) or hematotoxic polyvalent antivenom (produced against venoms of *C. rhodostoma*, *D. siamensis* and *T. albolabris*) to alleviate symptoms [16]. Indirect ELISA was used to determine the cross-reactivity of these antivenoms to *O. monticola* venom. Hematotoxic polyvalent antivenom exhibited a significantly greater level of immunoreactivity than the monovalent antivenom by 30–50% (up to the dilution 1: $1.6x10^6$), $P<0.001$ (Fig 3). A titer for the monovalent antivenom was over $1:1.28x10^7$ dilution, while that of hematotoxic polyvalent antivenom was $1:5.12x10^7$ (Fig 3).

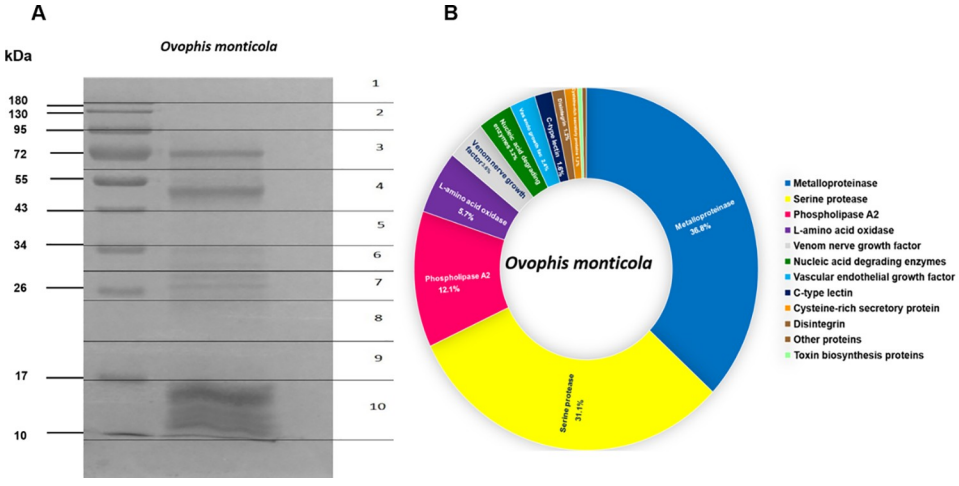

Fig 2. Venomics of Asian mountain pit viper *O. monticola* from Thailand. (A) Coomassie blue-stained 12% SDS-PAGE of *O. monticola* venom (30 μg) under reducing conditions. (B) Proteome classification of *O. monticola* venom; percentages indicate relative abundance (% of total venom proteins) of protein family in snake venom.

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Table 2. Fifteen most abundant unique proteins identified in *Ovophis monticola* venom.

No.	Accession no.	Protein	Score	MW[a] (Da)	No. of peptide	% Sequence coverage	pI[b]	emPAI[c]
1	sp|P0C2D5.2|OXLAPROFL	L-amino-acid oxidase	534	3601	2	80	4.44	14.55
2	sp|O93517.1|VM3S4GLOBR	Zinc metalloproteinase/disintegrin	1293	11254	5	48.6	4.42	5.18
3	sp|Q9PRP4.1|VSPFLACMR	Thrombin-like enzyme LMR-47	481	3168	2	100	4.31	3.82
4	sp|P0C590.1|VSP2GLOUS	Thrombin-like enzyme calobin-2	481	2159	2	100	4.65	3.81
5	sp|C0HLA2.1|VSP3LACMR	Thrombin-like enzyme LmrSP-3	335	2942	1	50	4.1	2.22
6	sp|P81478.1|PA2A2TRIGA	Acidic phospholipase A2 2	621	13784	4	33.6	4.95	1.73
7	sp|C0HLA3.1|VSP4LACMR	Snake venom serine protease LmrSP-4	1090	5841	2	62.3	4.28	1.60
8	sp|Q90W54.1|OXLA_GLOBL	L-amino-acid oxidase	2334	57056	19	32.7	6.52	1.37
9	BAA01566.1	Phospholipase A2	621	15697	3	40.6	4.99	1.35
10	pdb|1WVR|A	Chain A, Triflin	1099	24782	3	13.6	7.03	1.15
11	sp|Q7ZT99.1|CRVPCROAT	Cysteine-rich venom protein catrin	1099	26629	3	19.2	8.42	1.04
12	sp|Q7ZTA0.1|CRVPAGKPI	Cysteine-rich venom protein piscivorin	1099	26664	3	26.3	7.83	1.04
13	sp|E5L0E5.1|VSPPAAGKPL	Venom plasminogen activator	558	28060	5	12.8	5.78	1.02
14	AAM80563.1	Acidic phospholipase A2	372	15403	3	18.1	5.65	0.95
15	sp|P82896.1|PA2A5TRIST	Acidic phospholipase A2 5	427	13870	2	32.8	4.72	0.93

[a]MW: Molecular weight (Dalton).

[b]pI: isoelectric point.

[c]emPAI: exponentially modified protein abundance index.

The information of identified proteins including NCBI accession number (Accession no.), protein name (Protein), protein score (Score), molecular weight of protein in Dalton unit (Da), Number of identified peptides (No. of peptide), % sequence coverage of the identified peptides (%Sequence coverage), isoelectric point of protein (pI) and exponentially modified protein abundance index for semi-quantification (emPAI) are demonstrated.

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O. monticola venom protein analysis by two-dimensional electrophoresis (2DE)

In order to explore the protein antigens present in *O. monticola* venom, crude venom was subjected to 2DE gel electrophoresis. There were 89 spots detected, with pI values ranging from 3 to 10 and MW from 10 to 95 kDa. Within the particular MW regions of 10–15, 30–34, 50 and 72–90 lies the greatest abundance of protein spots (Fig 4A). Using MALDI-TOF/TOF-MS/MS, all protein spots in 2DE gels were identified, and are listed in S2 Table. There were 461 different sequences, which correspond to 173 peptide accession identities.

Immunoreactive proteins in *O. monticola* venom by immunoblot analysis

The immunoblot analysis was performed with either monovalent or polyvalent antivenom to characterize specific protein antigens within the *O. monticola* venom. Twenty-six immunoreactive spots were detected with monovalent antivenom, with pI values ranging from 3 to 6, and MW ranging from 17 to 95 kDa. Most of these spots were observed at MW 50 to 95 kDa and pI between 3 to 5 (Fig 4B). When probed with hematotoxic polyvalent antivenom, 40 immunoreactive spots were recorded with a broader range of pI values from 3 to 8, and MW ranging from 17 to 95 kDa. A high number of the spots were detected within a MW range of 40–55 kDa (Fig 4C). Comparing the immunoreactive spots obtained from polyvalent antivenom with all protein spots visualized by silver staining (Fig 4A), 49 spots (numbered 24–25, 31–32, 38–40, 42–45, 48–51, 54–73 and 76–89) were not immunologically recognized. These non-reactive spots were grouped according to their MW and pI values into three clusters. Cluster 1 appeared in the MW range from 26

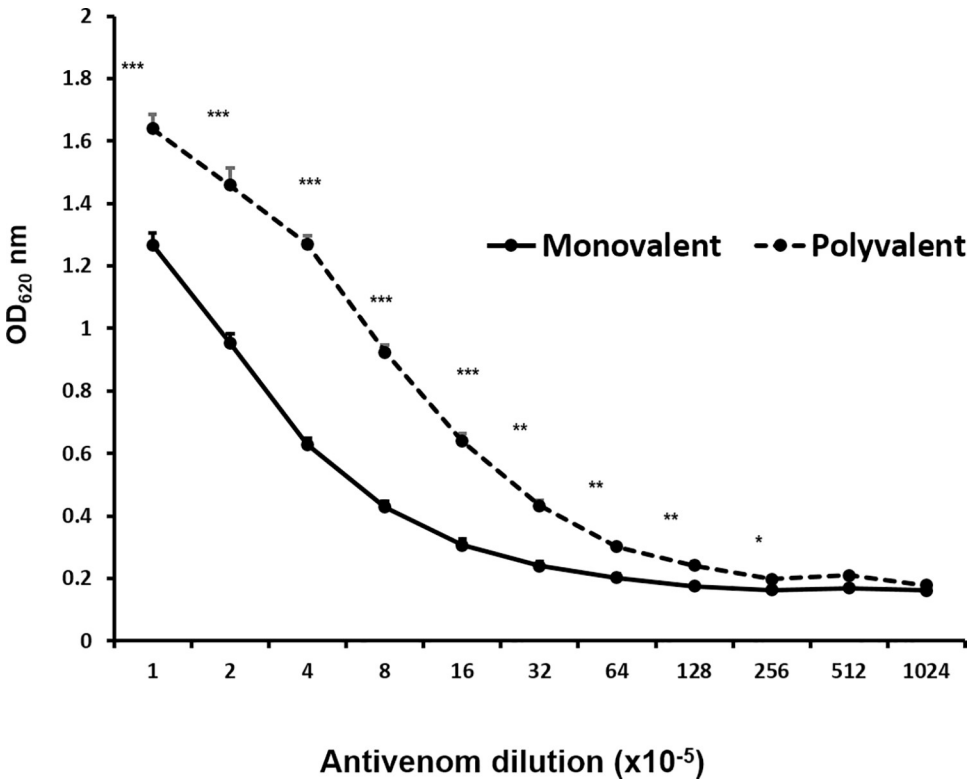

Fig 3. Immunoreactivity of *O. monticola* venom to antivenoms. Cross-reactivity of crude *O. monticola* venom to monovalent antivenom raised against green pit viper venom and polyvalent antivenom against snake hemotoxins. Data represent the mean ± SEM from two independent experiments; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

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to 43 kDa, with high pI values (7–8); cluster 2 included those with MW ranging from 26 to 43 and with low pI values (3–5); and cluster 3 contained those with low MW from 10 to 26 kDa and with low pI values (3–5).

Identification of immunoreactive and non-reactive peptides in *O. monticola* venom by LC-MS/MS

LC-MS/MS analysis revealed a total of 202 distinct sequences in *O. monticola* venom identified within 101 protein types that were immunologically reactive with a polyvenom. All

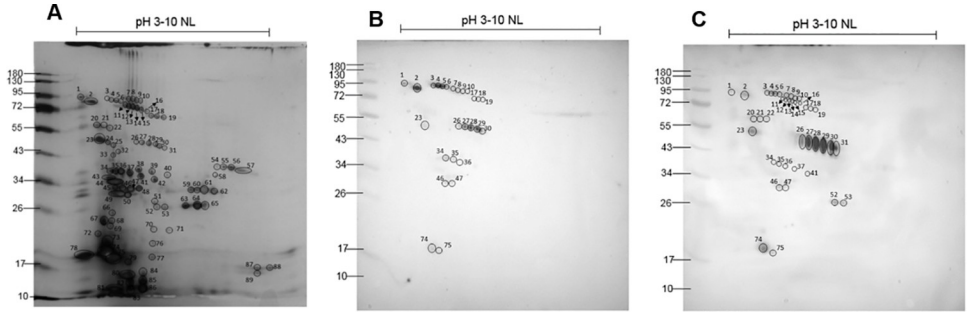

Fig 4. The 2DE separations and immunoblot analysis of *O. monticola* venom. (A) 2DE gels stained with silver stain; (B) 2D immunoblot of *O. monticola* proteins probed with monovalent antivenom and (C) polyvalent antivenom. Matched spots selected for subsequent LC-MS/MS analysis are marked and numbered.

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immunoreactive proteins recognized by monovalent and polyvalent antivenom are listed in Table 3. However, from all 173 protein types appearing by silver staining, 72 proteins were left unrecognized by both antivenoms, and are shown in Table 4. Interestingly, the majority of these neglected peptides are well-known members of CTLs (e.g., C-type lectin, snaclec and galactose-binding lectin) and different CRISPs including okinavin, catrin and piscivorin.

Discussion

The protein constituents within the venom of Asian mountain pit viper *O. monticola* from northern Thailand were investigated in the present study. SDS-PAGE revealed a protein band pattern ranging between MW 10–90 kDa with intense bands representing low MW proteins (10–15 kDa), those of 50 and 72 kDa, and faint bands between 30–50 kDa. This corresponded well to the spot pattern obtained from 2DE, where the clouds of protein spots were observed within MW regions of 10–15, 30–34, 50 and 72–90 kDa. The overall MW range of proteins in *O. monticola* venom was comparable to those from three other *Ovophis* species, namely *O. convictus*, *O. tonkinensis* and *O. okinavensis*. The pattern of dominant bands was most similar to the venom of O. *tonkinensis* found in China [10].

The proteomic profile showed that enzymatic components which are SVMP (36.8%), SVSP (31.1%), PLA_2 (12.1%) and LAAO (5.7%) mainly make up the venom of Asian mountain pit viper *O. monticola*. The overall composition of these major enzymes was comparable to those recently reported from venoms of *O. convictus*, *O. tonkinensis and O. okinavensis*, only content proportion seemed to be different. Among these three *Ovophis* spp., the most abundant proteins were of SVSP (35–53%), followed by PLA_2 (19–25%) and LAAO (5–17%). SVMP (11–19%) was detected at a lower percentage than in our venom [10]. Such venom variation in venom composition could would not only be attributed by speciation but also other factors including prey diversity reflecting different ecological habitats [11], snake sex, [17] and age [18, 19]. In addition, the variation in quantity of identified toxin types within snake venom might be resulted from the different quantitative approaches and accompanying calculation methods as well as proteomic database availability [20]. The 2DE indicated that *O. monticola* venom contained more acidic than basic protein spots. This finding was confirmed by our list of all identified peptides obtained from LC-MS/MS showing that the majority possessed pI values lower than 7. Our spot pattern also confirmed previous 2DE analysis of *Trimeresurus sumatranus* (another Viperid) venom, in which more proteins were identified in the acidic range than in Elapid venom [21]. The overall acidic properties of 4 main protein groups of vipers greatly contribute to the hemorrhagic and coagulopathic effect on victims [22, 23].

Our results revealed SVMPs as representing more than one-third of the entire *O. monticola* venom. The greatest amount found in *O. monticola* was sp|O93517.1|VM3S4_GLOBR Zinc metalloproteinase/disintegrin or disintegrin-like salmosin-4, first identified within Korean *Agkistrodon halys brevicaudus* snake venom [24]. SVMPs potentially inhibit platelet aggregation and integrin-dependent cell adhesion via interrupting glycoprotein IIb-IIIa/fibrinogen interaction and fibrinogenolysis [25, 26]. Additionally, SVMPs interact with the various types of cellular matrix and exerts the most haemorrhagic effect on hosts [27]. SVSPs were found to be second-most abundant in *O. monticola* venom. The majority are thrombin-like enzymes including sp|Q9PRP4.1|VSPF_LACMR thrombin-like enzyme LMR-47 and sp|Q9PRP4.1|VSPFLACMR thrombin-like enzyme calobin-2. Known as fibrinogen-clotting enzymes, they are common, and found in large amounts in the venoms of the genera *Agkistrodon*, *Bothrops*, *Lachesis* and *Trimeresurus* [28]. Thrombin-like enzymes demonstrate strong hydrolytic activity, primarily against triad residues of His57, Asp102 and Ser195 of fibrinogen [29]. Resembling thrombin, they act on blood plasma by forming friable and translucent clots which later

Table 3. List of identified proteins in *Ovophis monticola* venom immunologically reactive with monovalent and polyvalent antivenoms.

Spot no.	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Monovalent	Polyvalent
1	XP_029142019.1	Zinc metalloproteinase-disintegrin-like atrolysin-A, partial [*Protobothrops mucrosquamatus*]	60272	√	√
	JAS04843.1	Metalloproteinase type III 2b [*Crotalus horridus*]	68297		
	JAS04684.1	Metalloproteinase type III 1b [*Crotalus adamanteus*]	67284		
	AAA03326.1	Hemorrhagic toxin a (partial)[*Crotalus atrox*]	46848		
	GBP06242.1	Disintegrin and metalloproteinase domain-containing protein 12 [*Eumeta japonica*]	199170		
2	XP_029142019.1	Zinc metalloproteinase-disintegrin-like atrolysin-A, partial [*Protobothrops mucrosquamatus*]	60272	√	√
	JAS04843.1	Metalloproteinase type III 2b [*Crotalus horridus*]	68297		
	JAS04684.1	Metalloproteinase type III 1b [*Crotalus adamanteus*]	67284		
3	sp|Q4VM07.1| VM3VB_MACLB	Zinc metalloproteinase-disintegrin-like VLAIP-B (Snake venom metalloproteinase)	68798	√	√
	JAS04447.1	Metalloproteinase type III 7 [*Agkistrodon piscivorus conanti*]	68638		
	sp|P0DM87.1|VM2_TRIST	Zinc metalloproteinase-disintegrin stejnitin (Snake venom metalloproteinase)	54401		
4	JAS04447.1	Metalloproteinase type III 7 [*Agkistrodon piscivorus conanti]*	68638	√	√
	sp|Q4VM07.1| VM3VB_MACLB	Zinc metalloproteinase-disintegrin-like VLAIP-B	68798		
5	sp|Q4VM07.1| VM3VB_MACLB	Zinc metalloproteinase-disintegrin-like VLAIP-B (Snake venom metalloproteinase)	68798	√	√
	sp|P0DM87.1|VM2_TRIST	Zinc metalloproteinase-disintegrin stejnitin (Snake venom metalloproteinase)	54401		
	JAS04675.1	Metalloproteinase type III 5 [*Crotalus adamanteus*]	69463		
	JAS04447.1	Metalloproteinase type III 7 [*Agkistrodon piscivorus conanti*]	68638		
	XP_023086434.2	disintegrin and metalloproteinase domain-containing protein 20-like [*Piliocolobus tephrosceles*]	84212		
6	sp|Q4VM07.1| VM3VB_MACLB	Zinc metalloproteinase-disintegrin-like VLAIP-B (Snake venom metalloproteinase)	68798	√	√
	JAS04447.1	Metalloproteinase type III 7 [*Agkistrodon piscivorus conanti*]	68638		
	sp|P0DM87.1|VM2_TRIST	Zinc metalloproteinase-disintegrin stejnitin (Snake venom metalloproteinase)	54401		
7	-	Not identified		√	√
8	pdb|1REO|A	Chain A, Ahplaao	55097	√	√
	AAQ16182.1	L-amino acid oxidase [*Trimeresurus stejnegeri*]	58607		
9	pdb|1REO|A	Chain A, Ahplaao	55097	√	√
	sp|A0A024BTN9.1| OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
10	sp|A0A024BTN9.1| OXLA_BOTSC	L-amino acid oxidase Bs29	56341	√	√
11	pdb|1REO|A	Chain A, Ahplaao	55097	-	√
	sp|A0A024BTN9.1| OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
	sp|P0C2D5.2|OXLA_PROFL	L-amino-acid oxidase (Okinawa Habu apoxin protein-1)	3601		
	sp|P0C2D6.1|OXLA_PROMU	L-amino-acid oxidase	2929		
12	pdb|1REO|A	Chain A, Ahplaao	55097	-	√
	sp|A0A024BTN9.1| OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
	BAP39915.1	L-amino acid oxidase [*Protobothrops elegans*]	57339		
	sp|P0DI84.1|OXLA_VIPAA	L-amino-acid oxidase	54714		
	sp|C0HJE7.2|OXLA_CRODU	L-amino acid oxidase bordonein-L	58882		
	sp|Q4F867.2|OXLA_DABSI	L-amino-acid oxidase	46343		
	sp|X2JCV5.1|OXLAA_CERCE	L-amino acid oxidase	58520		

(*Continued*)

Table 3. (Continued)

Spot no.	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Monovalent	Polyvalent
	sp|A8QL51.1|OXLA_BUNMU	L-amino-acid oxidase	58774		
	sp|P0C2D5.2|OXLA_PROFL	L-amino-acid oxidase (Okinawa Habu apoxin protein-1)	3601		
	sp|A0A2U8QPE6.1|OXLA_MICMP	L-amino acid oxidase	57079		
	XP_026523888.1	titin isoform X41 [*Notechis scutatus*]	3637718		
13	pdb|1REO|A	Chain A, Ahplaao	55097	-	√
	JAS04783.1	L-amino acid oxidase 1b [*Crotalus horridus*]	58587		
	sp|P0DI84.1|OXLA_VIPAA	L-amino-acid oxidase	54714		
	BAP39915.1	L-amino acid oxidase [*Protobothrops elegans*]	57339		
	sp|A0A024BTN9.1|OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
	sp|C0HJE7.2|OXLA_CRODU	L-amino acid oxidase bordonein-L	58882		
	JAV01888.1	BATXLAAO1 [*Bothrops atrox*]	56625		
	sp|Q4F867.2|OXLA_DABSI	L-amino-acid oxidase	46343		
	sp|P0C2D5.2|OXLA_PROFL	L-amino-acid oxidase (Okinawa Habu apoxin protein-1)	3601		
	sp|X2JCV5.1|OXLAA_CERCE	L-amino acid oxidase	58520		
	sp|A0A2U8QPE6.1|OXLA_MICMP	L-amino acid oxidase	57079		
14	pdb|1REO|A	Chain A, Ahplaao	55097	-	√
	sp|A0A024BTN9.1|OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
	sp|P0DI84.1|OXLA_VIPAA	L-amino-acid oxidase	54714		
	sp|C0HJE7.2|OXLA_CRODU	L-amino acid oxidase bordonein-L	58882		
	sp|P0C2D5.2|OXLA_PROFL	L-amino-acid oxidase (Okinawa Habu apoxin protein-1)	3601		
	JAV01888.1	BATXLAAO1 [*Bothrops atrox*]	56625		
	sp|A0A2U8QPE6.1|OXLA_MICMP	L-amino acid oxidase	57079		
15	pdb|1REO|A	Chain A, Ahplaao	55097	-	√
	sp|A0A024BTN9.1|OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
	sp|A0A2U8QPE6.1|OXLA_MICMP	L-amino acid oxidase	57079		
	XP_026523846.1	Titin isoform X1 [*Notechis scutatus*]	3675875		
16	pdb|1REO|A	Chain A, Ahplaao	55097	-	√
	sp|A0A024BTN9.1|OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
17	pdb|1REO|A	Chain A, Ahplaao	55097	√	√
	JAV01888.1	BATXLAAO1 [*Bothrops atrox*]	56625		
18	pdb|1REO|A	Chain A, Ahplaao	55097	√	√
	AAQ16182.1	L-amino acid oxidase [*Trimeresurus stejnegeri*]	58607		
	sp|A0A024BTN9.1|OXLA_BOTSC	L-amino acid oxidase Bs29	56341		
	sp|P0DI84.1|OXLA_VIPAA	L-amino-acid oxidase	54714		
	JAV01888.1	BATXLAAO1 [*Bothrops atrox*]	56625		
	sp|A0A2U8QPE6.1|OXLA_MICMP	L-amino acid oxidase	57079		
	sp|A8QL51.1|OXLA_BUNMU	L-amino-acid oxidase	58774		
	sp|P0C2D5.2|OXLA_PROFL	L-amino-acid oxidase (Okinawa Habu apoxin protein-1)	3601		
	XP_026523888.1	Titin isoform X41 [*Notechis scutatus*]	3637718		

(*Continued*)

Table 3. (Continued)

Spot no.	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Monovalent	Polyvalent
19	sp|A0A024BTN9.1|OXLA_BOTSC	L-amino acid oxidase Bs29	56341	√	√
20	BAN82126.1	Serine protease, partial [*Ovophis okinavensis*]	9035	-	√
	JAV51428.1	Serine proteinase 12a [*Agkistrodon contortrix contortrix*]	28885		
	XP_026529526.1	Microtubule-actin cross-linking factor 1 isoform X1 [*Notechis scutatus*]	838459		
21	sp|P0C578.1|VSP2_OVOOK	Thrombin-like enzyme okinaxobin-2 (Fibrinogen-clotting enzyme)	2310	-	√
	JAV51428.1	Serine proteinase 12a [*Agkistrodon contortrix contortrix*]	28885		
	sp|I2C090.1|VCO3_OPHHA	Ophiophagus venom factor (Complement C3 homolog)	183812		
	XP_026526061.1	ALK and LTK ligand 1 [*Notechis scutatus*]	21543		
	sp|P85109.1|VSP1_GLOBR	Thrombin-like enzyme kangshuanmei (Fibrinogen-clotting enzyme)	26415		
	JAG68112.1	Dynamin-binding protein [*Boiga irregularis*]	90258		
22	JAV51428.1	Serine proteinase 12a [*Agkistrodon contortrix contortrix*]	28885	-	√
	BAN82126.1	serine protease, partial [*Ovophis okinavensis*]	9035		
	sp|E5L0E5.1|VSPPA_AGKPL	Venom plasminogen activator	28060		
	sp|Q5W958.1|VSP20_BOTJA	Venom serine proteinase-like HS120	27797		
23	BAN82126.1	Serine protease, partial [*Ovophis okinavensis*]	9035	√	√
	sp|Q9PSN3.1|VSP2_AGKBI	Thrombin-like enzyme bilineobin (Fibrinogen-clotting enzyme/Snake venom serine protease)	26461		
	BAN82122.1	Serine protease, partial [*Ovophis okinavensis*]	8080		
	pdb|2AIP|A	Chain A, Protein C activator	25090		
	sp|C0HLA2.1|VSP3_LACMR	Thrombin-like enzyme LmrSP-3	2942		
	ADI47563.1	Serine protease, partial [*Echis ocellatus*]	27233		
	sp|P0C5B4.2|VSPGL_GLOSH	Thrombin-like enzyme gloshedobin(Fibrinogen-clotting enzyme/Snake venom serine protease)	28597		
	sp|Q9DF66.1|VSP3_PROJR	Snake venom serine protease 3	28007		
	pdb|1OP0|A	Chain A, Venom serine proteinase	25318		
26	JAV51428.1	Serine proteinase 12a [*Agkistrodon contortrix contortrix*]	28885	√	√
27	XP_029142018.1	Zinc metalloproteinase-disintegrin jerdonitin [*Protobothrops mucrosquamatus*]	58843	√	√
	TSK34762.1	Disintegrin and metalloproteinase domain-containing protein 12 [*Bagarius yarrelli*]	146595		
	XP_032089254.1	ras GTPase-activating-like protein IQGAP1 [*Thamnophis elegans*]	189690		
28	XP_029142018.1	Zinc metalloproteinase-disintegrin jerdonitin [*Protobothrops mucrosquamatus*]	58843	√	√
	sp|P0DM87.1|VM2_TRIST	Zinc metalloproteinase-disintegrin stejnitin	54401		
	TSK34762.1	Disintegrin and metalloproteinase domain-containing protein 12 [*Bagarius yarrelli*]	146595		
29	XP_029142018.1	Zinc metalloproteinase-disintegrin jerdonitin [*Protobothrops mucrosquamatus*]	58843		
	sp|P0DM87.1|VM2_TRIST	Zinc metalloproteinase-disintegrin stejnitin (Snake venom metalloproteinase)	54401	√	√
	TSK34762.1	Disintegrin and metalloproteinase domain-containing protein 12 [*Bagarius yarrelli*]	215963		
	ETE65365.1	putative helicase senataxin, partial [*Ophiophagus hannah*]	146595		
30	XP_029142018.1	Zinc metalloproteinase-disintegrin jerdonitin [*Protobothrops mucrosquamatus*]	58843	√	√
	sp|P0DM87.1|VM2_TRIST	Zinc metalloproteinase-disintegrin stejnitin (Snake venom metalloproteinase)	54401		
31	XP_029142018.1	Zinc metalloproteinase-disintegrin jerdonitin [*Protobothrops mucrosquamatus*]	58843	-	√
34	JAS05371.1	Serine proteinase 9d [*Sistrurus miliarius barbouri*]	28266	√	√
	sp|P0DMH6.1|VSP_BOTFO	Snake venom serine protease	1729		
	sp|Q8AY78.1|VSP5M_TRIST	Snake venom serine protease 5	28117		
	sp|Q8AY79.1|VSPS2_TRIST	Beta-fibrinogenase stejnefibrase-2 (Snake venom serine protease)	28010		
	sp|Q5W958.1|VSP20_BOTJA	Venom serine proteinase-like HS120	27797		
	sp|Q71QH7.1|VSPP_TRIST	Snake venom serine protease PA	27933		

(*Continued*)

Table 3. (Continued)

Spot no.	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Monovalent	Polyvalent
	XP_026540424.1	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1 isoform X1 [*Notechis scutatus*]	135476		
35	sp|P0DMH6.1|VSP_BOTFO	Snake venom serine protease	1729	√	√
	sp|E5L0E5.1|VSPPA_AGKPL	Venom plasminogen activator	28060		
	sp|Q8AY78.1|VSP5M_TRIST	Snake venom serine protease 5	28117		
	sp|Q5W958.1|VSP20_BOTJA	Venom serine proteinase-like HS120	27797		
	sp|Q71QH7.1|VSPP_TRIST	Snake venom serine protease PA	27933		
	sp|Q8AY79.1|VSPS2_TRIST	Beta-fibrinogenase stejnefibrase-2 (Snake venom serine protease)	28010		
36	JAS05372.1	Serine proteinase 9c [*Sistrurus miliarius barbouri*]	28221	√	√
	JAS05371.1	Serine proteinase 9d [*Sistrurus miliarius barbouri*]	28266		
	JAV51414.1	Serine proteinase 8 [*Agkistrodon contortrix contortrix*]	28242		
	sp|P0DMH6.1|VSP_BOTFO	Snake venom serine protease	1729		
	sp|P0C5B4.2|VSPGL_GLOSH	Thrombin-like enzyme gloshedobin (Fibrinogen-clotting enzyme/Snake venom serine protease)	28597		
	ADI47574.1	Serine protease, partial [*Echis coloratus*]	28437		
	sp|Q8AY78.1|VSP5M_TRIST	Snake venom serine protease 5	28117		
	sp|Q5W958.1|VSP20_BOTJA	Venom serine proteinase-like HS120	27797		
	sp|Q8AY79.1|VSPS2_TRIST	Beta-fibrinogenase stejnefibrase-2 (Snake venom serine protease)	28010		
	sp|Q8UUJ2.2|VSPUI_GLOUS	Snake venom serine protease ussurin;	26184		
	sp|Q71QH7.1|VSPP_TRIST	Snake venom serine protease PA	27933		
	XP_032092228.1	Vitelline membrane outer layer protein 1 homolog isoform X1 [*Thamnophis elegans*]	21236		
	JAI10638.1	Vacuolar protein sorting-associated protein 18 homolog [*Crotalus adamanteus*]	111967		
37	JAS05372.1	Serine proteinase 9c [*Sistrurus miliarius barbouri*]	28221	-	√
	JAS05371.1	Serine proteinase 9d [*Sistrurus miliarius barbouri*]	28266		
	sp|P0DMH6.1|VSP_BOTFO	Snake venom serine protease	1729		
	JAV51414.1	Serine proteinase 8 [*Agkistrodon contortrix contortrix*]	28242		
	sp|Q9PT41.1|VSPF5_MACLB	Factor V activator (Lebetina viper venom FV activator/Snake venom serine protease	28577		
	ADI47574.1	Serine protease, partial [*Echis coloratus*]	28437		
	sp|Q8AY78.1|VSP5M_TRIST	Snake venom serine protease 5;	28117		
	XP_023086434.2	Disintegrin and metalloproteinase domain-containing protein 20-like [*Piliocolobus tephrosceles*]	84212		
	sp|Q8AY79.1|VSPS2_TRIST	Beta-fibrinogenase stejnefibrase-2 (Snake venom serine protease)	28010		
41	-	Not identified		-	√
46	pdb|1BQY|A	Chain A, Plasminogen Activator	25590	√	√
	JAS04757.1	Serine proteinase 1f [*Crotalus horridus*]	28133		
	JAS04429.1	Serine proteinase 13e [*Agkistrodon piscivorus conanti*]	27985		
	JAS04417.1	Serine proteinase 18b [*Agkistrodon piscivorus conanti*]	27728		
	JAS04415.1	Serine proteinase 19b [*Agkistrodon piscivorus conanti*]	27782		
	sp|Q072L7.1|VSP_LACST	Snake venom serine protease	27796		
	sp|O13069.1|VSP2_BOTJA	Thrombin-like enzyme KN-BJ 2 (Kinin-releasing and fibrinogen-clotting serine protease 2)	26399		
	pdb|4E7N|A	Chain A, Snake-venom Thrombin-like Enzyme	28333		
	XP_032089049.1	Spectrin alpha chain, non-erythrocytic 1 [*Thamnophis elegans*]	263010		
	XP_032064352.1	Zinc finger protein 347-like [*Thamnophis elegans*]	169270		
	sp|Q9PT41.1|VSPF5_MACLB	Factor V activator/Lebetina viper venom FV activatorSnake venom serine protease	28577		
	ADI47574.1	Serine protease, partial [*Echis coloratus*]	28437		
47	JAS04757.1	Serine proteinase 1f [*Crotalus horridus*]	28133	√	√

(*Continued*)

Table 3. (Continued)

Spot no.	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Monovalent	Polyvalent
	pdb|1BQY|A	Chain A, Plasminogen Activator	25590		
	JAS04417.1	Serine proteinase 18b [*Agkistrodon piscivorus conanti*]	27728		
	JAS04429.1	Serine proteinase 13e [*Agkistrodon piscivorus conanti*]	27985		
	JAS04415.1	Serine proteinase 19b [*Agkistrodon piscivorus conanti*]	27782		
	JAV01826.1	BATXSVSP10 [*Bothrops atrox*]	28606		
	pdb|4E7N|A	Chain A, Snake-venom Thrombin-like Enzyme	26370		
	sp|Q6T5L0.2|VSPSH_GLOSH	Alpha-fibrinogenase shedaoenase (Snake venom serine protease)	26399		
	sp|O13069.1|VSP2_BOTJA	Thrombin-like enzyme KN-BJ 2 (Kinin-releasing and fibrinogen-clotting serine protease 2)	27876		
	sp|Q71QI0.1|VSP07_TRIST	Snake venom serine protease homolog KN7	28703		
	XP_015671556.1	Snake venom serine protease [*Protobothrops mucrosquamatus*]	28023		
	JAS04671.1	Serine proteinase 3b [*Crotalus adamanteus*]	28890		
	QHR82809.1	Serine protease 2 [*Vipera anatolica senliki*]	28084		
	sp|A8QL53.1|VSP1_NAJAT	Snake venom serine protease NaSP	31117		
	XP_026523831.1	Integrin alpha-4 [*Notechis scutatus*]	114850		
52	JAS05359.1	Cysteine-rich secretory protein 1c [*Sistrurus tergeminus*]	26787	-	√
53	ETE67131.1	Keratin, type II cytoskeletal 1, partial [*Ophiophagus hannah*]	240496	-	√
74	sp|A8E2V8.1|PA2A_TRIGS	Acidic phospholipase A2 Tgc-E6	15678	√	√
	sp|P0DJJ7.1|PA2A_OVOMO	Acidic phospholipase A2 Omo-E6	3261		
	JAV51451.1	Phospholipase A2 1a [*Agkistrodon contortrix contortrix*]	15952		
	sp|Q6EAN6.1|PA2A_SISTE	Acidic phospholipase A2 homolog sistruxin APrecursor	15419		
	XP_032088152.1	Group IIE secretory phospholipase A2-like [*Thamnophis elegans*]	17310		
	sp|Q7ZTA6.1| PA2AB_CROVV	Acidic phospholipase A2 Cvv-E6b	15429		
	AAB28455.1	Phospholipase A2 isozyme III, PLA2-III [*Trimeresurus gramineus*]	13716		
	JAV01879.1	BATXPLA5 [*Bothrops atrox*]	15504		
	sp|P06860.1|PA2BX_PROFL	Basic phospholipase A2 PL-X	13971		
	AAB28454.1	Phospholipase A2 isozyme IV, PLA2-IV [1] [*Trimeresurus gramineus*]	13783		
	sp|C0HJC1.1|PA2_BOTLA	Acidic phospholipase A2 BlatPLA2	13881		
75	sp|A8E2V8.1|PA2A_TRIGS	Acidic phospholipase A2 Tgc-E6	15678	√	√
	sp|P0DJJ7.1|PA2A_OVOMO	Acidic phospholipase A2 Omo-E6	3261		
	pdb|1C1J|A	Chain A, Basic phospholipase A2	13888		
	JAS04499.1	Phospholipase A2 1s [*Agkistrodon piscivorus conanti*]	15776		
	sp|P82896.1|PA2A5_TRIST	Acidic phospholipase A2 5	13870		
	sp|D6MKR0.1| PA2A6_CROHD	Acidic phospholipase A2 CH-E6	15498		
	sp|Q7ZTA6.1| PA2AB_CROVV	Acidic phospholipase A2 Cvv-E6b	15429		
	JAV51451.1	Phospholipase A2 1a [*Agkistrodon contortrix contortrix*]	15952		
	sp|P86907.1|PA2A_BOTAM	Acidic phospholipase A2	13858		
	sp|C9DPL5.1|PA2A1_BOTPI	Acidic phospholipase A2 BpirPLA2-I	13627		
	sp|C0HLF0.1|PA2_POROP	Basic phospholipase A2	14042		
	sp|C0HJC1.1|PA2_BOTLA	Acidic phospholipase A2 BlatPLA2	13881		
	sp|P86456.1|PA2A4_BOTAL	Acidic phospholipase A2 SpII RP4	13733		
	QHR82796.1	Phospholipase A2 3 [*Vipera anatolica senliki*]	17437		

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Table 4. List of non-immunoreactive proteins/peptides in *Ovophis monticola* venom.

	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Spot no.
1	AAZ75628.1	Kallikrein-Phi4, partial [*Philodryas olfersii*]	26827	45, 49, 50
2	BAN82001.1	Galactose binding lectin, partial [*Protobothrops flavoviridis*]	17654	80, 82, 84, 85
3	BAN82034.1	Serine protease, partial [*Protobothrops flavoviridis*]	22377	55
4	BAN82147.1	Cysteine rich secretory protein [*Ovophis okinavensis*]	26920	59, 60, 61, 62
5	BAN82148.1	Galactose binding lectin [*Ovophis okinavensis*]	18480	80, 82
6	BAN82149.1	C-type lectin alpha subunit [*Ovophis okinavensis*]	17686	87, 88
7	ETE59238.1	Fascin-3, partial [*Ophiophagus hannah*]	14941	48
8	ETE60526.1	Trichohyalin, partial [*Ophiophagus hannah*]	80507	51
9	ETE61374.1	Dynein heavy chain 8, axonemal [*Ophiophagus hannah*]	284552	72
10	ETE64295.1	Glycerol-3-phosphate acyltransferase 4 [*Ophiophagus hannah*]	50772	86
11	ETE66458.1	Helicase SRCAP, partial [*Ophiophagus hannah*]	494261	87
12	ETE70787.1	N6-adenosine-methyltransferase 70 kDa subunit, partial [*Ophiophagus hannah*]	59797	89
13	JAI12774.1	Leucine-rich repeat-containing protein 7-like [*Crotalus adamanteus*]	163679	45
14	JAS04407.1	Serine proteinase 6 [*Agkistrodon piscivorus conanti*]	28115	43,44, 48, 76
15	JAS04411.1	Serine proteinase 2 [*Agkistrodon piscivorus conanti*]	28333	49, 50, 55, 57,
16	JAS04568.1	Phospholipase A2 1b [*Boiga irregularis*]	16906	67, 72, 73, 78
17	JAS04670.1	Serine proteinase 3c [*Crotalus adamanteus*]	28849	45, 49, 50, 54, 55, 56, 57, 58
18	JAS04734.1	Cysteine-rich secretory protein [*Crotalus adamanteus*]	26612	59, 60, 61, 62
19	JAS04742.1	Serine proteinase 9d [*Crotalus horridus*]	28299	49
20	JAS04748.1	Serine proteinase 6 [*Crotalus horridus*]	28594	54, 55, 56, 57
21	JAS05249.1	Serine proteinase 2 [*Sistrurus tergeminus*]	28326	49
22	JAS05472.1	C-type lectin 2 [*Sistrurus miliarius barbouri*]	18147	87
23	JAS05484.1	Cysteine-rich secretory protein 1b [*Sistrurus miliarius barbouri*]	26772	61
24	JAV51425.1	Serine proteinase 15a [*Agkistrodon contortrix contortrix*]	28940	54, 55, 56, 57
25	JAV51455.1	C-type lectin 9a [*Agkistrodon contortrix contortrix*]	18657	80
26	pdb|1BK9|A	Chain A, Phospholipase A2	13964	72
27	pdb|1GMZ|A	Chain A, Phospholipase A2	13850	67
28	pdb|1JZN|A	Chain A, Galactose-specific lectin	16281	80, 82
29	pdb|1WVR|A	Chain A, Triflin	24782	59, 60, 61, 62
30	pdb|3JR8|A	Chain A, Phospholipase A2 bothropstoxin-2	13985	67
31	sp|A0A1I9KNP0.1|VSPH1_VIPAA	Vaa serine proteinase homolog 1	28909	49
32	sp|A8QL56.1|VSP1_OPHHA	Alpha- and beta-fibrinogenase OhS1	28637	45, 49, 50
33	sp|B0VXW0.1|OXLA_SISCA	L-amino-acid oxidase	58532	77
34	sp|B0ZT25.1|VSPH_PROJR	Snake venom serine protease homolog	28776	58
35	sp|C0HLA1.1|VSP2_LACMR	Thrombin-like enzyme LmrSP-2 (Snake venom serine protease)	3271	25, 32
36	sp|E5AJX2.1|VSP_VIPBN	Snake venom serine protease nikobin	28197	45, 49
37	sp|J3S832.1|VSPB_CROAD	Snake venom serine proteinase 11	28033	58
38	sp|J3S833.1|VSP2_CROAD	Snake venom serine proteinase 2	28298	49
39	sp|K4LLQ2.1|VSP_BOTBA	Thrombin-like enzyme barnettobin (Snake venom serine protease)	27567	38, 39
40	sp|O13057.1|VSP2_PROFL	Snake venom serine protease 2	28623	56
41	sp|O93421.2|VSPPE_GLOHA	Snake venom serine protease pallase	26031	49
42	sp|P0DJG8.1|CRVP_HELAG	Helicopsin	2618	61
43	sp|P0DL18.1|CRVP_OVOOK	Cysteine-rich venom protein okinavin	3496	59, 60, 61
44	sp|P0DM36.1|LECG_AGKPI	C-type lectin APL	16195	80, 82, 84, 85
45	sp|P81114.1|SLA4_TRIAB	Snaclec alboaggregin-A subunit beta	14357	81, 83, 86
46	sp|P81176.1|VSP1_GLOBL	Thrombin-like enzyme halystase (Snake venom serine protease)	26466	49

(*Continued*)

Table 4. (Continued)

	Protein/peptide accession	Description [*Organisms*]	MW (Da)	Spot no.
47	sp|P82981.1|VSP2_AGKCO	Thrombin-like enzyme contortrixobin/Fibrinogen-clotting enzyme (Snake venom serine protease)	25396	54, 55, 56, 57, 58
48	sp|Q27J47.1|VSPPA_LACMU	Venom plasminogen activator LV-PA	28044	44
49	sp|Q71QJ4.1|VSP04_TRIST	Snake venom serine protease homolog KN4	28685	49, 50, 55, 56, 57
50	sp|Q7SZE2.1|VSPD_GLOUS	Bradykinin-releasing enzyme KR-E-1 (Snake venom serine protease)	25335	49
51	sp|Q7T229.1|VSPH_BOTJR	Snake venom serine protease homolog	28636	54, 55, 56, 57
52	sp|Q7ZT99.1|CRVP_CROAT	Cysteine-rich venom protein catrin	26629	59
53	sp|Q7ZTA0.1|CRVP_AGKPI	Cysteine-rich venom protein piscivorin	26664	60, 61, 62
54	sp|Q8AY81.1|VSPST_TRIST	Thrombin-like enzyme stejnobin (Fibrinogen-clotting enzyme/Snake venom serine protease)	29309	25, 32
55	sp|Q91053.1|VSP1_GLOUS	Thrombin-like enzyme calobin-1 (Snake venom serine protease)	28889	49
56	sp|Q9YGJ2.1|VSP1_GLOHA	Snake venom serine protease pallabin	28662	49
57	XP_015671564.1	Snake venom serine protease serpentokallikrein-1 [*Protobothrops mucrosquamatus*]	88822	56
58	XP_023418723.1	Disintegrin and metalloproteinase domain-containing protein 17 [*Cavia porcellus*]	92703	64
59	XP_024069019.3	Disintegrin and metalloproteinase domain-containing protein 17 [*Terrapene carolina triunguis*]	99769	64
60	XP_025414344.1	Disintegrin and metalloproteinase domain-containing protein 9 [*Sipha flava*]	138126	54
61	XP_026527653.1	Laminin subunit alpha-1 [*Notechis scutatus*]	331363	80
62	XP_026535629.1	Dynein heavy chain 8, axonemal [*Notechis scutatus*]	511281	66
63	XP_026540213.1	Regulatory solute carrier protein family 1 member 1 [*Notechis scutatus*]	37705	80
64	XP_026541175.1	N-acetylated-alpha-linked acidic dipeptidase-like protein [*Notechis scutatus*]	81911	50
65	XP_028906446.1	Disintegrin and metalloproteinase domain-containing protein 17 [*Ornithorhynchus anatinus*]	94942	63
66	XP_032078796.1	Laminin subunit alpha-1 [*Thamnophis elegans*]	339446	85
67	XP_032080246.1	Centromere-associated protein E [*Thamnophis elegans*]	308791	59
68	XP_032085798.1	60S ribosomal protein L6 isoform X1 [*Thamnophis elegans*]	30312	42, 81
69	XP_032088226.1	Forkhead-associated domain-containing protein 1 [*Thamnophis elegans*]	137802	81
70	XP_032091805.1	Glial fibrillary acidic protein [*Thamnophis elegans*]	52228	88
71	XP_039181676.1	Snake venom serine protease-like isoform X1 [*Crotalus tigris*]	25811	49
72	XP_039181680.1	Snake venom serine proteinase 12-like [*Crotalus tigris*]	24539	56

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are degraded due to non-functional cross-linked structures, leading to coagulopathy and hypofibrinogenemia [30, 31]. However, in terms of enzyme proportion, our findings contrast with previous reports on the transcriptomic analysis of *O. okinavensis* venom glands, which mainly contained SVSP (93.1%) and relatively little SVMP (4.2%) [32]. It remains unclear whether the microenvironment within the venom gland might preferentially activate or interfere with the functioning of newly synthesized enzymes.

Compared with SVMP and SVSP, we detected smaller amounts of PLA_2 and LAAO in *O. monticola* venom. PLA_2 hydrolyzes phospholipids at the sn-2 position, generating fatty acids and lysophospholipids [33]. Group II PLA_2 is expressed exclusively in the venoms of the Viperidae [34]. We found both acidic and basic PLA_2 subtypes in *O. monticola* venom. It is noteworthy that the acidic PLA_2 (sp|P81478.1|PA2A2TRIGA and sp|P82896.1|PA2A5TRIST were present in high quantities partially contributing to more acidic properties of the venom. These were previously found to trigger oedema [35]. PLA_2 elicits inflammatory responses through the overproduction of pro-inflammatory cytokines (such as TNFα, IL-1β and IL-6) largely by immunocompetent cells (monocytes, neutrophils and mast cells) [36].

LAAOs can act in concert with PLA_2 in local inflammatory reactions. LAAOs from *Calloselasma rhodosthoma* venom were shown to induce superoxide anion and hydrogen peroxide production by human neutrophils [37]. The most abundant LAAO in *O. monticola* venom is sp|P0C2D5.2|OXLA_PROFL, also known as Okinawa habu apoxin protein-1. This protein was first characterized from the venom of *Protobothrops flavoridis* to induce apoptosis in glioma cells [38]. The roles of both PLA_2 and LAAOs in snake venoms are multi-faceted. Their catalytic as well as cytotoxic properties have been extensively investigated for pharmaceutical potential against cancers and other diseases [39].

Due to the unavailability of homospecific antivenom to *Ovophis* spp. venoms, all pit viper envenoming victims usually receive either monovalent antivenom (raised against *T. albolabris* venom) or hematotoxic polyvalent antivenom (produced against venoms of *C. rhodostoma*, *D. siamensis* and *T. albolabris*) [16]. The latter gave considerably higher immunoreactive levels (30–50%) to *O. monticola* venom proteins than the former. Relatively greater levels of reactivity of the polyvalent antivenom was previously reported with the venoms of *C. rhodostoma*, *Hypnale hypnale* and *Trimeresurus hageni*, and even *Trimeresurus albolabris* when compared with those of monovalent antivenom [40]. With the combination of 2DE immunoblotting and LC-MS/MS analyses, we found that hematotoxic polyvalent antivenom reacted with a wider range of proteins and peptides accounting for 58% of the entire range of proteins and covering all major enzymatic groups. Nonetheless, we were able to observe that an array of LAAOs and SVSPs did not react with a monovalent antivenom specific only to *T. albolabris* venom. This finding suggests the shared antigenic epitopes particularly from *Ovophis*, *Calloselasma* and *Trimeresurus* venoms used to generate antivenom. In this context, proteomic analysis of Malayan pit viper *C. rhodostoma* venom revealed a similar SVMP dominance (41.17%), with other major constituents of snaclec (26.3%) and SVSP (14.9%) [41]. A study of phylogenetic relationships based on geographic distribution and mitochondrial and nuclear gene sequences also demonstrated that *O. monticola* is less distantly separated from *C. rhodostoma* than from *T. albolabris* [42]. Thus, the antivenom against immunogenic epitopes from *C. rhodostoma* venom should be further investigated for the possible adjunctive treatment of *O. monticola* bite victims.

Our current study revealed that 72 proteins (42% of venom proteins) were left unrecognized by both antivenoms. The majority of immunologically non-reactive proteins have low molecular mass. They include a number of SVSPs, PLA_2 and certain SVMP. The poor immunogenicity of these low molecular venomic proteins has been obviously reported, although some possess high toxicity [43, 44]. In addition, an array of non-enzymatic CTLs such as galactose-binding lectins, snaclec, alboaggregin A, and CRISPs such as triflin, okinavin, catrin, and piscivotin were found unrecognizable by antivenom. This reflects the difference in antigenic abundance between *O. monticola* venom and those venoms employed to generate horse immunoglobulins, as the proportions of CTLs and CRISPs in our *O. monticola* venom were only 1.6% and 1.2%, respectively. Nonetheless, their biological impacts on host cells and tissues should not be neglected. In the context of CTLs, alboaggregin A was shown to bind strongly with platelet glycoproteins IB and VI, and hence, activated platelet aggregation [45]. In addition, evidence of enhanced platelet activation and thrombotic microangiopathy-like symptoms has been documented with other related snaclecs [46]. In terms of CRISPs, the unique pdb|1WVR|A chain A triflin, as well as sp|P0DL18.1|CRVP_OVOOK okinavin from the related hime habu *O. okinavensis* were not reactive with the antivenoms. They have been previously described to have a calcium channel-impairing effect, leading to aberrations in muscle contraction [47, 48]. Considering the pathophysiological effects, our findings address the suite of protein targets which could be additional antigens for future antivenomic design. Furthermore, in order to alleviate the symptoms of mountain pit viper envenomation, these would

facilitate the development of specific drug schemes allowing patients to recover more quickly. The proteomic profile of *O. monticola* venom not only provides insight into the venomic phenotypes reflecting the evolutionary path among Viperid snakes, but also accelerates the discovery of novel candidates for medical and pharmaceutical use.

Supporting information

S1 Raw image.
(PDF)

S1 Table. List of all proteins found in *Ovophis monticola* venom.
(XLSX)

S2 Table. List of proteins in 89 spots in *Ovophis monticola* venom. Crude venom was subjected to 2DE gel. Proteins were separated in the first dimension in the pH range 3–10.
(DOCX)

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References

1. Organization WH. Fact sheet [Internet]: World Health Organization. 2021 17 May 2021.
2. Gutierrez JM, Calvete JJ, Habib AG, Harrison RA, Williams DJ, Warrell DA. Snakebite envenoming. Nat Rev Dis Primers. 2017; 3:17079. https://doi.org/10.1038/nrdp.2017.79 PMID: 28980622
3. Habib AG, Brown NI. The snakebite problem and antivenom crisis from a health-economic perspective. Toxicon. 2018; 150:115–23. https://doi.org/10.1016/j.toxicon.2018.05.009 PMID: 29782952

4. Malhotra A, Dawson K, Guo P, Thorpe RS. Phylogenetic structure and species boundaries in the mountain pitviper Ovophis monticola (Serpentes: Viperidae: Crotalinae) in Asia. Mol Phylogenet Evol. 2011; 59(2):444–57. https://doi.org/10.1016/j.ympev.2011.02.010 PMID: 21315823

5. Pandey DP, Chaudhary B, Ram Shrestha B. Documentation of a proven Mountain Pitviper (Ovophis monticola) envenomation in Kathmandu, Nepal, with its distribution ranges: implications for prevention and control of pitviper bites in Asia. J Venom Res. 2021; 11:1–6. PMID: 33747433

6. Chanhome L, Cox MJ, Wilde H, Jintakoon P, Chaiyabutr N, Sitprija V. Venomous snakebite in Thailand. I: Medically important snakes. Mil Med. 1998; 163(5):310–7. PMID: 9597848

7. Shibata H, Chijiwa T, Hattori S, Terada K, Ohno M, Fukumaki Y. The taxonomic position and the unexpected divergence of the Habu viper, Protobothrops among Japanese subtropical islands. Mol Phylogenet Evol. 2016; 101:91–100. https://doi.org/10.1016/j.ympev.2016.04.027 PMID: 27132943

8. Cox MJ, Hoover MF, Chanhome L, Thirakhupt K, Pongcharoen C. A Field Guide to the Venomous Snakes of Mainland ASEAN Nations: published and distributed by Merel J. Cox, Mark F. Hoover, Lawan Chanhome, Kumthorn Thirakhupt, and Chattraphas Pongcharoen; 2018.

9. Boda F, Banfai K, Garai K, Curticapean A, Berta L, Sipos E, et al. Effect of Vipera ammodytes ammodytes Snake Venom on the Human Cytokine Network. Toxins (Basel). 2018; 10(7). https://doi.org/10.3390/toxins10070259 PMID: 29941812

10. Tan CH, Palasuberniam P, Tan KY. Snake Venom Proteomics, Immunoreactivity and Toxicity Neutralization Studies for the Asiatic Mountain Pit Vipers, Ovophis convictus, Ovophis tonkinensis, and Hime Habu, Ovophis okinavensis. Toxins. 2021; 13(8):514. https://doi.org/10.3390/toxins13080514 PMID: 34437385

11. Davies EL, Arbuckle K. Coevolution of Snake Venom Toxic Activities and Diet: Evidence that Ecological Generalism Favours Toxicological Diversity. Toxins (Basel). 2019; 11(12). https://doi.org/10.3390/toxins11120711 PMID: 31817769

12. Chanhome L, Khow O, Omori-Satoh T, Sitprija V. Capacity of Thai green pit viper antivenom to neutralize the venoms of Thai Trimeresurus snakes and comparison of biological activities of these venoms. J Nat Toxins. 2002; 11(3):251–9. PMID: 12182545

13. Kumkate S, Chanhome L, Thiangtrongjit T, Noiphrom J, Laoungboa P, Khow O, et al. Venomics and Cellular Toxicity of Thai Pit Vipers (Trimeresurus macrops and T. hageni). Toxins (Basel). 2020; 12(1).

14. Danpaiboon W, Reamtong O, Sookrung N, Seesuay W, Sakolvaree Y, Thanongsaksrikul J, et al. Ophiophagus hannah Venom: Proteome, Components Bound by Naja kaouthia Antivenin and Neutralization by N. kaouthia Neurotoxin-Specific Human ScFv. Toxins. 2014; 6(5):1526–58. https://doi.org/10.3390/toxins6051526 PMID: 24828754

15. Gowtham YJ, Mahadeswaraswamy YH, Girish KS, K K. Cross-reactivity and neutralization of Indian King cobra (Ophiophagus hannah) venom by polyvalent and monovalent antivenoms. Int Immunopharmacol. 2014; 21(1):148–55. https://doi.org/10.1016/j.intimp.2014.04.012 PMID: 24815989

16. Asia WHORofS-E. Guidelines for the management of snakebites second edition 2016 22 October 2021.

17. Menezes MC, Furtado MF, Travaglia-Cardoso SR, Camargo AC, Serrano SM. Sex-based individual variation of snake venom proteome among eighteen Bothrops jararaca siblings. Toxicon. 2006; 47 (3):304–12. https://doi.org/10.1016/j.toxicon.2005.11.007 PMID: 16373076

18. Mackessy SP, Sixberry NM, Heyborne WH, Fritts T. Venom of the Brown Treesnake, Boiga irregularis: ontogenetic shifts and taxa-specific toxicity. Toxicon. 2006; 47(5):537–48. https://doi.org/10.1016/j.toxicon.2006.01.007 PMID: 16545413

19. Gibbs HL, Sanz L, Chiucchi JE, Farrell TM, Calvete JJ. Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult Dusky Pigmy rattlesnakes (Sistrurus miliarius barbouri). J Proteomics. 2011; 74(10):2169–79. https://doi.org/10.1016/j.jprot.2011.06.013 PMID: 21722760

20. Damm M, Hempel BF, Süssmuth RD. Old World Vipers-A Review about Snake Venom Proteomics of Viperinae and Their Variations. Toxins (Basel). 2021; 13(6). https://doi.org/10.3390/toxins13060427 PMID: 34204565

21. Vejayan J, Shin Yee LP, G., Ambu S, Ibrahim I. Protein profile analysis of Malaysian snake venoms by two-dimensional gel electrophoresis. The Journal of Venomous Animals and Toxins including Tropical Diseases. 2010;volume 16 (4):623–30.

22. Aung HT, Nikai T, Komori Y, Nonogaki T, Niwa M, Takaya Y. Biological and pathological studies of rosmarinic acid as an inhibitor of hemorrhagic Trimeresurus flavoviridis (habu) venom. Toxins (Basel). 2010; 2(10):2478–89. https://doi.org/10.3390/toxins2102478 PMID: 22069562

23. Tan NH, Fung SY, Tan KY, Yap MK, Gnanathasan CA, Tan CH. Functional venomics of the Sri Lankan Russell's viper (Daboia russelii) and its toxinological correlations. J Proteomics. 2015; 128:403–23. https://doi.org/10.1016/j.jprot.2015.08.017 PMID: 26342672

24. Shin J, Hong SY, Chung K, Kang I, Jang Y, Kim DS, et al. Solution structure of a novel disintegrin, salmosin, from Agkistrondon halys venom. Biochemistry. 2003; 42(49):14408–15. https://doi.org/10.1021/bi0300276 PMID: 14661951

25. Swenson S, Markland FS Jr. Snake venom fibrin(ogen)olytic enzymes. Toxicon. 2005; 45(8):1021–39. https://doi.org/10.1016/j.toxicon.2005.02.027 PMID: 15882884

26. Andrews RK, Gardiner EE, Shen Y, Berndt MC. Structure-activity relationships of snake toxins targeting platelet receptors, glycoprotein Ib-IX-V and glycoprotein VI. Curr Med Chem Cardiovasc Hematol Agents. 2003; 1(2):143–9. https://doi.org/10.2174/1568016033477559 PMID: 15320694

27. Markland FS Jr., Swenson S. Snake venom metalloproteinases. Toxicon. 2013; 62:3–18. https://doi.org/10.1016/j.toxicon.2012.09.004 PMID: 23000249

28. Kini RM, Rao VS, Joseph JS. Procoagulant proteins from snake venoms. Haemostasis. 2001; 31(3–6):218–24. https://doi.org/10.1159/000048066 PMID: 11910188

29. Castro HC, Zingali RB, Albuquerque MG, Pujol-Luz M, Rodrigues CR. Snake venom thrombin-like enzymes: from reptilase to now. Cell Mol Life Sci. 2004; 61(7–8):843–56. https://doi.org/10.1007/s00018-003-3325-z PMID: 15095007

30. Hutton RA, Warrell DA. Action of snake venom components on the haemostatic system. Blood Rev. 1993; 7(3):176–89. https://doi.org/10.1016/0268-960x(93)90004-n PMID: 8241832

31. Yokoi H, Sakai A, Kodama T, Magome S, Nagayasu T, Tawara M, et al. Severe hypofibrinogenemia in patients bitten by Gloydius tsushimaensis in Tsushima Island, Nagasaki, Japan, and treatment strategy. Toxicon. 2020; 188:142–9. https://doi.org/10.1016/j.toxicon.2020.10.027 PMID: 33130186

32. Aird SD, Watanabe Y, Villar-Briones A, Roy MC, Terada K, Mikheyev AS. Quantitative high-throughput profiling of snake venom gland transcriptomes and proteomes (Ovophis okinavensis and Protobothrops flavoviridis). BMC Genomics. 2013; 14:790. https://doi.org/10.1186/1471-2164-14-790 PMID: 24224955

33. Kang TS, Georgieva D, Genov N, Murakami MT, Sinha M, Kumar RP, et al. Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. FEBS J. 2011; 278(23):4544–76. https://doi.org/10.1111/j.1742-4658.2011.08115.x PMID: 21470368

34. Lomonte B, Angulo Y, Moreno E. Synthetic peptides derived from the C-terminal region of Lys49 phospholipase A2 homologues from viperidae snake venoms: biomimetic activities and potential applications. Curr Pharm Des. 2010; 16(28):3224–30. https://doi.org/10.2174/138161210793292456 PMID: 20687875

35. Nakashima K, Nobuhisa I, Deshimaru M, Nakai M, Ogawa T, Shimohigashi Y, et al. Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A2 isozyme genes. Proc Natl Acad Sci U S A. 1995; 92(12):5605–9. https://doi.org/10.1073/pnas.92.12.5605 PMID: 7777556

36. Burin SM, Menaldo DL, Sampaio SV, Frantz FG, Castro FA. An overview of the immune modulating effects of enzymatic toxins from snake venoms. Int J Biol Macromol. 2018; 109:664–71. https://doi.org/10.1016/j.ijbiomac.2017.12.101 PMID: 29274419

37. Pontes AS, da SSS, Xavier CV, Lacouth-Silva F, Kayano AM, Pires WL, et al. Effect of L-amino acid oxidase from Calloselasma rhodosthoma snake venom on human neutrophils. Toxicon. 2014; 80:27–37. https://doi.org/10.1016/j.toxicon.2013.12.013 PMID: 24462716

38. Abe Y, Shimoyama Y, Munakata H, Ito J, Nagata N, Ohtsuki K. Characterization of an apoptosis-inducing factor in Habu snake venom as a glycyrrhizin (GL)-binding protein potently inhibited by GL in vitro. Biol Pharm Bull. 1998; 21(9):924–7. https://doi.org/10.1248/bpb.21.924 PMID: 9781840

39. Hiu JJ, Yap MKK. Cytotoxicity of snake venom enzymatic toxins: phospholipase A2 and l-amino acid oxidase. Biochem Soc Trans. 2020; 48(2):719–31. https://doi.org/10.1042/BST20200110 PMID: 32267491

40. Chaisakul J, Khow O, Wiwatwarayos K, Rusmili MRA, Prasert W, Othman I, et al. A Biochemical and Pharmacological Characterization of Phospholipase A2 and Metalloproteinase Fractions from Eastern Russell's Viper (Daboia siamensis) Venom: Two Major Components Associated with Acute Kidney Injury. Toxins. 2021; 13(8):521. https://doi.org/10.3390/toxins13080521 PMID: 34437392

41. Tang EL, Tan CH, Fung SY, Tan NH. Venomics of Calloselasma rhodostoma, the Malayan pit viper: A complex toxin arsenal unraveled. J Proteomics. 2016; 148:44–56. https://doi.org/10.1016/j.jprot.2016.07.006 PMID: 27418434

42. Alencar LRV, Quental TB, Grazziotin FG, Alfaro ML, Martins M, Venzon M, et al. Diversification in vipers: Phylogenetic relationships, time of divergence and shifts in speciation rates. Mol Phylogenet Evol. 2016; 105:50–62. https://doi.org/10.1016/j.ympev.2016.07.029 PMID: 27480810

43. Correa-Netto C, Teixeira-Araujo R, Aguiar AS, Melgarejo AR, De-Simone SG, Soares MR, et al. Immunome and venome of Bothrops jararacussu: a proteomic approach to study the molecular immunology of snake toxins. Toxicon. 2010; 55(7):1222–35. https://doi.org/10.1016/j.toxicon.2009.12.018 PMID: 20060013

44. Madrigal M, Pla D, Sanz L, Barboza E, Arroyo-Portilla C, Corrêa-Netto C, et al. Cross-reactivity, antivenomics, and neutralization of toxic activities of Lachesis venoms by polyspecific and monospecific antivenoms. PLoS Negl Trop Dis. 2017; 11(8):e0005793. https://doi.org/10.1371/journal.pntd.0005793 PMID: 28787445

45. Dörmann D, Clemetson JM, Navdaev A, Kehrel BE, Clemetson KJ. Alboaggregin A activates platelets by a mechanism involving glycoprotein VI as well as glycoprotein Ib. Blood. 2001; 97(4):929–36. https://doi.org/10.1182/blood.v97.4.929 PMID: 11159519

46. Long C, Liu M, Tian H, Li Y, Wu F, Mwangi J, et al. Potential Role of Platelet-Activating C-Type Lectin-Like Proteins in Viper Envenomation Induced Thrombotic Microangiopathy Symptom. Toxins (Basel). 2020; 12(12). https://doi.org/10.3390/toxins12120749 PMID: 33260875

47. 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. https://doi.org/10.1046/j.1432-1033.2002.02940.x PMID: 12047379

48. 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. https://doi.org/10.1093/jb/mvn174 PMID: 19106157