Original Article

Proteomic Analysis and Immunoprofiling of Persian Horned Viper Venom, *Pseudocerastes Persicus,* from Central Part of Iran

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ABSTRACT

Numerous species of venomous snakes of medical importance exist in Iran. *Pseudocerastes persicus* (*P. persicus*), one of the medically important snakes, also called the Persian horned viper, has a geographical spread that extends to the east, southwest, and central areas of Iran and is endemic across the wider region. As a result, this species is responsible for many snakebite occurrences. Venom from *P. persicus* found in the central province of Semnan contains phospholipase A2 and L-amino acid oxidase activities, and high toxic potency. The venom was fractionated by reverse-phase high-performance liquid chromatography (HPLC) and analyzed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and two-dimensional electrophoresis. Using liquid chromatography with tandem mass spectrometry (LC-MS/MS), a range of components were identified, consistent with the biochemical and toxicological properties of the venom. Proteins identified from 2D electrophoresis and shotgun methods included metallo- and serine proteases, phospholipases, oxidases, and Kunitz trypsin inhibitors, along with many other components at lower qualitative abundance. This study provides a more detailed understanding of the protein profile of Iranian *P. persicus* venom, which can be effective in the production of an effective antidote against it. The analysis of the resulting data shows that there is a wide range of proteins in the venom of the Persian horned viper. This information can provide a better understanding of how venom is neutralized by polyclonal antivenom. Considering the wide presence of this snake and its related species in Iran and surrounding countries, knowing the venom protein profile of this family can be of great support to antivenom producers such as Razi Vaccine & Serum Research Institute in the preparation of regional antivenoms.

Keywords: Proteomics, Pseudocerastes persicus, Venom

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1. Introduction

Over 400 species of venoumous snakes are known to science, categorized into four families Viperidae, Elapidae, Atractaspididae, and Colubridae (1). Their venoms are complex mixtures of peptides and proteins with a range of pharmacological and toxic properties (2) that can be grouped into 63 protein families (3). Accidental envenoming kills around 100,000 people *per annum*, and as a result of habitat and exposure risk, morbidity and mortality from snake bite is estimated to be four times higher in the countryside than in urban areas (4-5) with the consequence that incidence may be under-reported and that impact fall disproportionately on poorer groups in society. There are 155 known snake species in Iran, of which 27 are venomous (6). These come from four genera, Elapids, Vipers, Crotalidaes, and Hydrophids families that can be found in different provinces (7). The species of greatest medical importance belong to the Elapidae (*N. naja oxiana*) and the Viperidae (*Pseudocerastes persicus*, *Echis carinatus*, *Vipera lebetina*, *Vipera albicornuta* (8-9) that latter family comprises two subfamilies, Viperinae (the old vipers) and Crotalinae (the pit vipers) (10). Snakes of the *Pseudocerastes* genus are widespread, and the three species, *P. persicus*, *P. fieldi*, and *P. urarachnoides* (11), are distinguished from the true horned viper (genus Cerastes) (12) by horn-like structures above the eyes that contain several smaller scales. The genus Pseudocerastes is distributed across the Middle East, Arabia, and into Pakistan and Afghanistan (13-14,6) with isolated instances in Oman and the UAE to the south of the Persian Gulf (15). Morphologically, the taxonomic border between *P. fieldi* and *P. persicus* is not as clear as that between *P. urarachnoides* and other species of the genus (16). Based on its wide distribution across the provinces of Iran, *P. persicus* is of particular medical importance (17-18) and consistent with findings elsewhere that recent modelling has revealed villagers in rural areas are at particular risk from envenoming (19). The venom of *P. persicus* is typically yellow in color and possesses high hemorrhagic activity (20). Analysis shows major differences with the more limited enzymic properties of the venom of *P. fieldi* (*21)*, perhaps as a result of genetic segregation of the two species arisings from geographic separation (19-20). Given the medical importance of *P. persicus* and the need for effective therapies, this study undertook a detailed analysis of venom taken from *Pseudocerastes persicus* (*P. persicus)* sampled from Semnan province in Iran.

2. Materials and Methods

2.1. Chemicals and Materials

Chemicals, reagents for Sodium dodecyl sulfate polyacrylamide gel electrophoresis (DS-PAGE), materials for chromatography (Sephacryl S100, Protein A-Sepharose CL-4B) and immunologic analysis (Freund's adjuvant, anti-rabbit IgG-HRP, 3, 3′ diaminobenzidine tetrahydrochloride) were all purchased from Sigma. Materials for 2D electrophoresis were purchased from Bio-Rad.

2.2. Venom and Antivenom

Venom of *P. persicus* was prepared from the Department of Venomous Animals, Razi Vaccine and Serum Research Institute, Karaj, Iran. Samples were all lyophilized, taken-up in PBS (phosphate buffer saline) and clarified by centrifugation at 3500 rpm for 30 min at 4◦C using Sorvall RT600D. Supernatants of all venom samples were kept at -70°C prior to use. Three rabbits (3kg, 10 weeks of age) were immunized subcutaneously at one month intervals with *P. persicus* venom originating from Semnan. To do this, initially 60 µg venom was dissolved in 0.5 ml saline and then mixtrued 50:50 (v/v) with complete and incomplete Freund's adjuvant. Following this, each animal was injected with 1 ml of the above suspension. The first injection was delivered with Freund's complete adjuvant and the boosters were used incomplete adjuvant. At the end of immunization period, 5 ml blood was collected from each animal. To purify the IgG from immunized rabbit sera, serum was dialyzed against 50 mM Tris pH 7 (termed binding buffer) and loaded to a column of Protein A-Sepharose CL-4B that was previously equilibrated with the buffer. After washing the column to remove unbound components, bounded IgG was eluted with 100 mM citric acid buffer pH 3.0. Fractions of 1 ml were collected from the column and neutralized with 130-160 µl of 1 M Tris-HCl pH 9.0 to preserve the antibody. All fractions were analysed on 10% SDS-PAGE gels, and those containing purified IgG were pooled.

2.3. L-amino acid oxidase activity

The L-amino acid oxidase activity is commonly found in snake venom and can trigger a range of cellular effects that harms the envenomed individual. The assay for L-amino acid oxidase (LAAO) was based on a published protocol (http://www.worthington-biochem.com/LAO/assay.html) with a coupled reaction using peroxidase and L-leucine, monitoring the reaction at 436nm. To do this, 100 µl of 10 mg/ ml peroxidase was added to 2.9 ml of 200 mM triethanoleamine pH 7.6

containing 0.1% L-leucine and 0.0065% of o-dianisidine. One hundred µl of 2mg/ml diluted venom was added, the reaction was maintained at 25°C, and absorbance at 436 nm was monitored for 4-5 min.

2.4. Phospholipase A2 activity

Phospholipase A2 activity (PLA2) is another activity commonly found in snake venom. The PLA2 was assayed according to a published protocol (Abcam, ab133089). This assay was based upon the release of free thiols by hydrolysis of a substrate, a 1, 2-dithio analogue of diheptanoyl phosphatidylcholine. Thiol release was detected with 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). To carry out the assay, 10 µl of DTNB (10mM in 0.4M Tris-HCl pH 8), 5 µl of assay buffer (25mM Tris-HCl, pH 7.5, 10 mM CaCl2, 100mM CaCl2, 0.3 mM Triton X-100) and 10 µl of the venom sample were added to Microplate well. The reactions were initiated by addition of 200 µl substrate, and the absorbance of each reaction was recorded at 414 nm at 1 min intervals for 5 min, comparing against blank reaction.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All protein samples were analysed using SDS-PAGE based on the standard method (23). Venom samples were separated on 12% polyacrylamide gels using Mini Protean systems (BioRad); proteins were visualized with Coomassie Brilliant Blue R-250 or silver staining. This method was performed under non-reduced and reduced conditions for complete venoms.

2.6. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) and Western blotting

Lyophilized crude venom from *P. persicus* was dissolved in ultra-pure water to a concentration of 2 mg/mL and passed through a 0.2µm filter (Sartorius). Around 100 µl of prepared Sample was loaded to a C18 reverse phase HPLC column (100Å, 5 µm, 4.6 × 150 mm) previously equilibrated with solvent A (water, 0.1% TFA). Fractionation was carried out with a linear gradient of increasing acetonitrile concentration from solvents A (no acetonitrile) to B (60% acetonitrile, 0.1% TFA) at a flow rate of 1 ml/min for 80 min at room temperature and then monitored at 215 nm. Fractions were collected and lyophilized prior to further analysis. Western blotting was done using a wet transfer system (Bio-Rad) with a transfer buffer of 25 mM Tris, 192 mM glycine, and 20% methanol (v/v). Transfer was carried out at 100 volts for 1 h at 4°C on 0.45µm nitrocellulose membrane (Sartorius 11306). Membranes were blocked overnight with a 2% solution of skimmed milk in PBS and then washed three times with PBS. Membranes were probed with purified rabbit IgG raised against *P. persicus* venom, as described earlier. The IgG was diluted 1/100 in PBS and incubated over membranes for 1 h at room temperature. A conjugate anti-rabbit-HRP (1/5000) was added in PBS-0.1% Tween 20 and incubated across membranes for 1 h at room temperature. Finally, the membrane was incubated three times with PBS-0.1% Tween 20 before adding peroxidase substrate (10 ml DAB 0.05%, 10 µl 30% H_2O_2). Reactions were terminated by washing with water.

2.7. Two-dimensional gel electrophoresis

Venom samples of 750 µg from snakes found in Semnan, Iran, were dissolved in 185 µl of a sample buffer containing 8M urea, 2% CHAPS, 100 mM dithiothreitol (DTT), 0.5 % (w/v) Bio-Lyte ampholyte and 0.001% Bromophenol Blue, and separated by isoelectric focusing (IEF) on 11 cm strips, gradient pH 3–11 (Bio-Rad). The IEF was performed at 200 V for 1 h, 2000 V for a further hour, and 4000 V for 4 h using a Protean IEF cell (Bio-Rad) at 20°C. The current maximum was limited to 50 µA. Strips were rehydrated with sample buffer and then equilibrated for SDS-PAGE, firstly for 20 min with reduction buffer (6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol, 2% DTT), then for 20 min with alkylation buffer (3% iodoacetamide, 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol). Proteins were then separated in the second dimension on a Paya Pajohesh system using 15% SDS-PAGE gels. Samples were separated at 100 V for 4 h and then stained with Coomssie Brilliant Blue G-250. Sinaclon pre-stained protein markers were used to determine the molecular weights of separated venom components.

2.8. In-gel Tryptic Digestion and Protein Identification by LC-MS/MS

All spots samples from 2DE gels and crude venom from Semnan province were excised from analytical gels, then dehydrated in 50% acetonitrile and rehydrated in 50 mM Tris pH 8.0, 10 mM DTT. Samples were heated for 15 min at 65°C, then reduced by addition of 15 mM iodoacetamide and incubated for 30 min in the dark at room temperature. Reduction was quenched by the addition of 10 mM DTT. Samples were again dehydrated with 50% acetonitrile and rehydrated in a solution containing trypsin and Lys-C solution. Proteolytic digestion was carried out overnight at ٣٧°C. The resulting peptides were purified by reversed phase extraction and analyzed by LC-MS. The LC-MS/MS analysis was

performed with an ABSciex TripleTOF 5600 machine (ABSciex) equipped with an electrospray interface with a 25 µm iD capillary and coupled to an Eksigent µUHPLC unit (Eksigent). Analyst TF 1.7 software was used to control the instrument and for data processing and acquisition. Acquisition was performed in Information Dependant Acquisition (IDA) mode for the 12 fractions from the pool and then analyzed in SWATH acquisition mode. For the IDA mode, the source voltage was set to 5.5 kV and maintained at 325°C, curtain gas was set at 27 psi, gas one at 27 psi and gas two at 10 psi. Identical conditions were used for the SWATH mode. Separation was performed on a reverse phase HALO C18-ES column 0.3µm i.d., 2.7 µm particles, 150mm long (Advance Materials Technology), which was maintained at 60°C. Samples were injected by loop overfilling into a 5µL loop. For the 60 min (IDA) and 120 min (SWATH) LC gradient, the mobile phase consisted of the following solvent A (0.2% v/v formic acid and 3% DMSO v/v in water) and solvent B (0.2% v/v formic acid and 3% DMSO in EtOH) at a flow rate of 3 µL/min. For data analysis, all runs were analyzed simultaneously with the Protein Pilot software. First Protein Pilot was run using a FASTA file with known proteins for *P. persicus*. At the time of analysis, very few proteins were available in Uniprot for this species. Therefore, Protein Pilot was repeated with combined proteomes of all the species that belong to the Viperidae family.

3. Results

3.1. Biochemical characterization

Analysis revealed high levels of LAAO activity in the venom samples, and proteolytic activity was previously estimated by our group to be to 11.56 U/ml (24), and the LD50 was 21.9 µg/mouse (25). The PLA2 activity was equal to 0.8 U/µg in venom sample from Semnan province.

3.2. Comparison of venom components in 1D electrophoresis

The SDS PAGE analysis of *P. persicus* venom from snakes from Semnan province in Iran is shown in Figure 1. Protein profile of collected venom from Semnan province was analyzed under non-reduced and reduced condition. The main groups of proteins under non-reduced treatment were detected in the area of 48-70kDa, 20-25kDa and in the range of 11-17kDa. On the other hand, under reducing conditions the position of proteins of venom changed and showed differences with the first condition.

3.3. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) analysis and immunoprofiling

The reverse-phase HPLC analysis of *P. persicus* venom from Semnan province generated 27 peaks, some well-resolved from other venom components, others present as minor or subsidiary peaks on the elution profile (Figure 2). When fractions were analysed by SDS-PAGE under non-reducing conditions, a wide range of proteins were detected (Figure 3A). Fraction 9, recovered from the column at an elution time of 25 min, contained the dominant protein species noted at around 25 kDa in initial SDS PAGE analysis (Figure 1). The most abundant group of proteins noted in early analysis (48 to 70 kDa, Figure 1) were recovered in RP-HPLC fractions 21 to 23 (Figure 3A). Proteins in an intermediate range of molecular weights eluted in fractions 12–14. They were well-resolved by SDS-PAGE (Figure 3A) despite the variability of their absorbance at 215 nm in the RP-HPLC elution profile (Figure 2). Heterogeneous protein mixtures were present in fractions 16–19 (Figure 3A), although the peaks eluting from RP-HPLC were well-separated (Figure 2). It was notable that some prominent peaks identified by RP-HPLC did not appear to contain proteins detectable by silver staining (*e.g.*, fraction 3, fraction 7), and others resolved imperfectly on SDS PAGE (fraction 15). Potentially, these could be peptides of a molecular weight too low for identification by SDS PAGE. Proteins were blotted from SDS PAGE gels for Western analysis with rabbit serum raised against the native venom components. The dominant groups of proteins identified in the initial SDS PAGE analysis were all recognized by rabbit antiserum confirming their immunogenicity (lanes labeled V, Figure 3B). However, recognition of some proteins isolated by RP-HPLC did not appear to be in proportion to their abundance. For example, the protein present in fraction 9 (high abundance, Figure 3A) was recognized only weakly in Western blotting analysis (Figure 3B); the converse was noted for species present in fraction 15, and the proteins of 25 to 35 kDa present in fractions 12 to 14 were undetectable with rabbit anti-venom.

3.4. 2D electrophoresis and proteomic analysis

Samples of the crude venom of *P. persicus* isolated in Semnan, Iran, were separated by 2D electrophoresis. High resolution separation of protein components was achieved. Proteins of less than 75 kDa could be classified into three groups according to their molecular weights and isoelectric points (Figure 4).

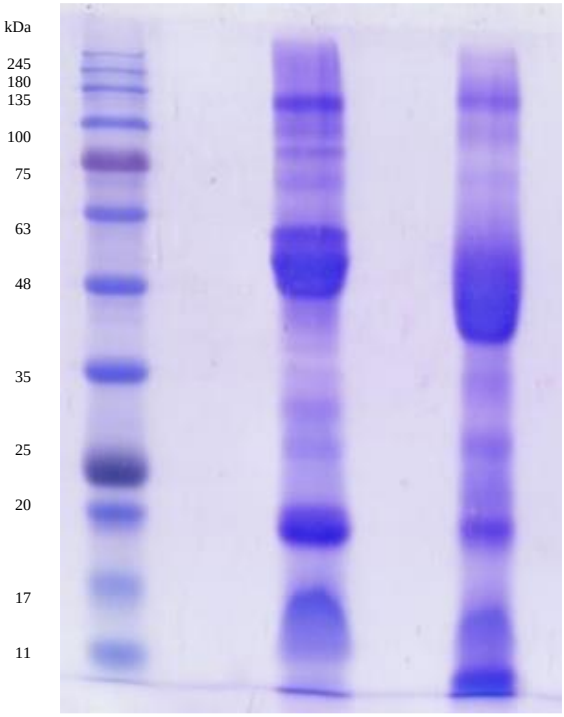


Fig. 1- SDS-PAGE analysis of *Pseudocerastes persicus* venom from Semnan province.
Sample of each well was equall to 10 µl of 2 mg/0.5 ml crude venom. From the left: Lane 1- marker with shown molecular weights 2- Crude venom, non-reduced conditions, Semnan province 3- Crude venom, reduced conditions, Semnan province.

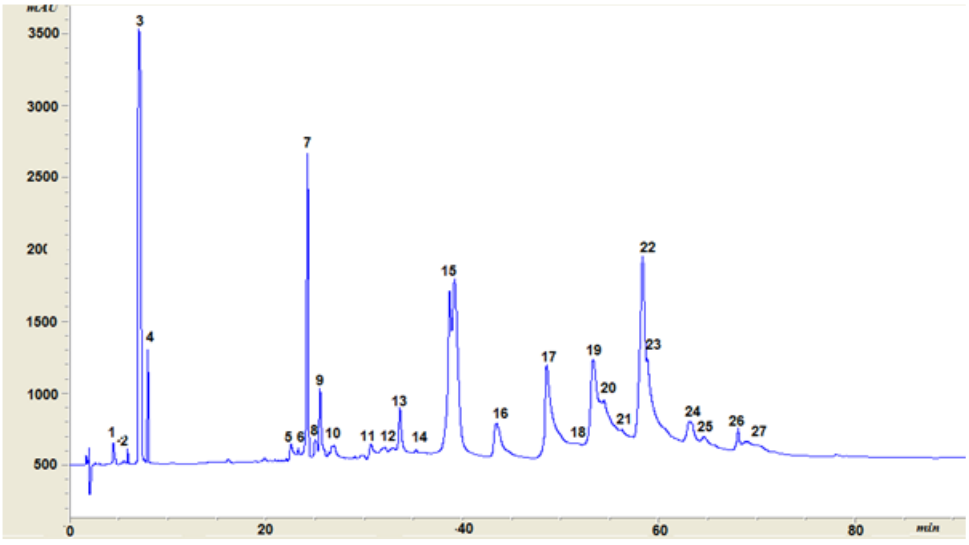
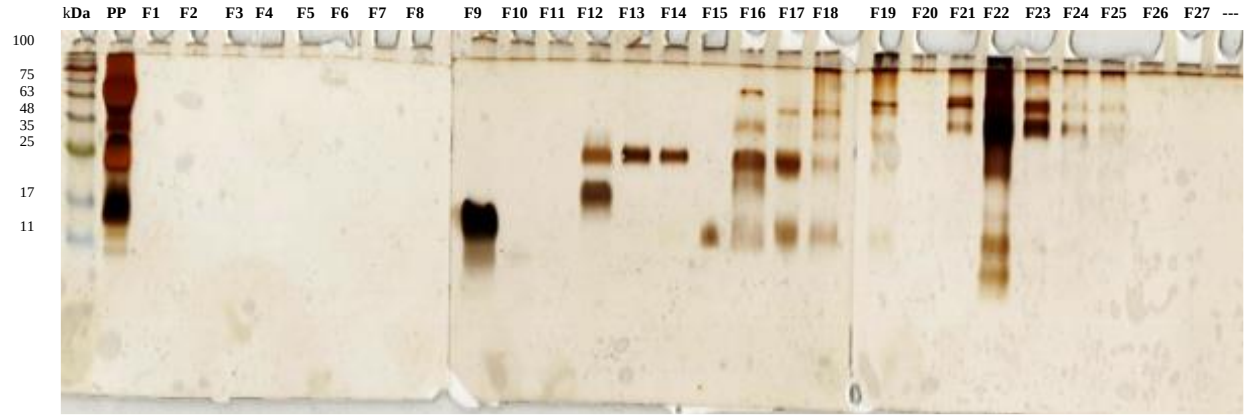


Fig.2- Reverse-phase HPLC separation of venom proteins from *Pseudocerastes persicus* from Semnan province. Solid trace shows elution profile as detected at 215 nm; fractions as numbered. (Lanes labelled V, Figure 3B). However, recognition of some of the proteins isolated by RP-HPLC did not appear to be in proportion to their abundance. For example, the protein present in fraction 9 (high abundance, Figure 3A) was recognized only weakly in Western blotting analysis (Figure 3B); the converse was noted for species present in fraction 15 and the proteins of 25 to 35 kDa present in fractions 12 to 14 were undetectable with rabbit anti-venom.



A

B

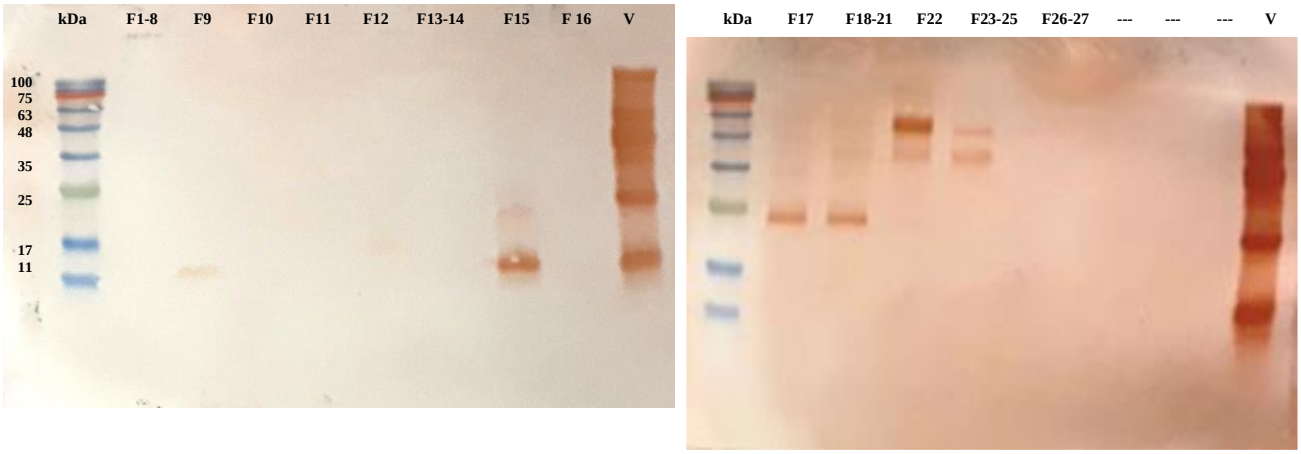


Fig. 3- Panel A Analysis of HPLC fractions by SDS-PAGE under non-reducing conditions. Samples from the left; marker with molecular weights indicated to the left of the image, crude venom from *Pseudocerastes persicus* (PP), fractions 1 to 27 from HPLC fractionation of the venom as labelled. Gel stained with silver nitrate. Panel B) Western blot of pooled or individual venom fractions from HPLC as labelled. Markers to the left of the panel, V crude venom. Samples were separated by SDS-PAGE under non-reducing conditions and probed with rabbit IgG raised against *Pseudocerastes persicus* (see Methods).

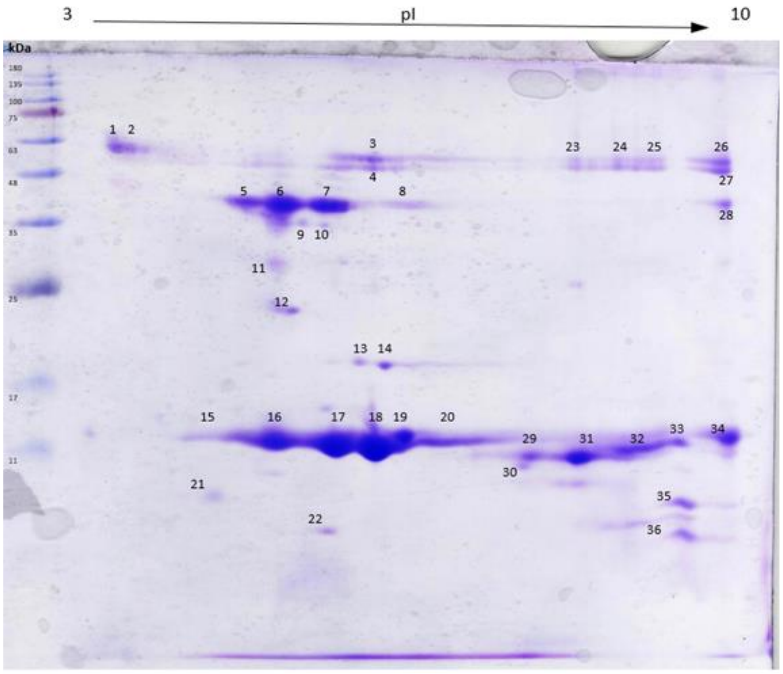


Fig.4. Analysis of *Pseudocerastes persicus* venom from Semnan province by two dimensional electrophoresis. Separation in the first dimesion was achieved by isoelectric focussing under reducing conditions; pH range is indicated at the top of the image. SDS-PAGE then followed, again under reducing conditions. Molecular weight markers are shown to the left of the image. Proteins were stained with Coomassie, numbered for reference purposes and excised for further analysis by LC-MS/MS.

The first group resolved at a molecular weight close to 63 kDa and possessed a range of isoelectric points from 3 to 10, clustering within four regions of the analysis. Proteins were numbered for later identification: species 1 and 2 lay at the acidic extreme; protein 3 possessed a pI closer to neutrality; while proteins 23–25 were more basic in character, the pI for 26 was 10. The second group of proteins (under 48 kDa) was dominated by three species with acidic pIs (labelled 5–7 in Figure 4) with other minor components of similar pI but slightly lower molecular weight (species 9 and 10), or similar molecular weight but with a pI closer to neutrality (protein 8). The second group of proteins (under 48 kDa) was dominated by three species with acidic pIs (labelled 5–7 in Figure 4) with other minor components of similar pI but slightly lower molecular weight (species 9 and 10), or similar molecular weight but with a pI closer to neutrality (protein 8). Minor venom components of similar pIs but molecular weights in the range 18–28 kDa comprised the third group (proteins 11 and 12; proteins 13 and 14). the fourth group in 2D electrophoretic analysis, proteins of 11 kDa or less, can be classify in two subgroups. First subgroup proteins with high abundance are at pIs in the acidic to neutral range (species 15–20), and the second ones are shown a further array of proteins of slightly lower molecular weight at the basic extreme (species 29–34).

Based on 2D electrophoretic analysis, other minor components were identified at lower molecular weights (proteins 21, 22, 35, and 36). The presence of low molecular weight peptides in the venom sample was suggested by staining at the dye front, components too low in molecular weight to be resolved on the 15% gels that were used for separation in the second dimension. Identification of protein components in the venom of *P. persicus* was carried out by top-down and bottom-up proteomic approaches using spots excised from 2D electrophoretic separation (top-down; Figure 3) and shotgun digestion of venom followed by liquid chromatographic separation of the resulting peptides and analysis by tandem mass spectrometry (bottom-up).Table 1 identifies proteins present in spots as designated in Figure 4. The LC-MS analysis revealed the presence of five dominant activities in the venom with evidence of multiple isoforms or closely related proteins within these criteria. Several proteins at the upper end of the molecular weight range (Figure 4) were identified as oxidases (Table 1; proteins 2, 3, 25). Other smaller species (spots 13 and 14) possessed this activity (Table 1) and with a similarity in peptide profile, might represent closely-related enzymes or proteolytic components of a larger oxidase present in the venom.

Table 1. Proteins identified [a] in *Pseudocerastes persicus* venom by electrophoretic separation.

Spot no.	Protein Family	Protein description	Species with the homologous protein	Accession number	No. of peptides
1	Peptidase S1	Serine Protease	*Vipera ammodytes ammodytes*	A0A1I9KNR8	8
2	Monoamine oxidase	L-amino-acid oxidase	*Echis coloratus*	A0A0A1WDN5	14
3	Monoamine oxidase	Amine oxidase	*Echis coloratus*	A0A0A1WCY6	5
4	Peptidase M12B	Metalloproteinase	*Echis carinatus leakeyi*	E9JGD0	4
5	Disintegrin	Metalloproteinase	*Echis carinatus sochureki*	E9JG32	5
6	Peptidase M12B	Group III snake venom metalloproteinase	*Echis ocellatus*	Q2UXQ9	6
7	Peptidase M12B	Metalloproteinase	*Echis carinatus sochureki*	E9JG34	12
8	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG93	1
9	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG58	2
10	Peptidase M12B	Metalloproteinase	*Echis pyramidum*	Q90500	2
11	Peptidase S1	Serine proteinase SP-5	*Vipera ammodytes ammodytes*	A0A1I9KNR5	3
12	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG68	9
13	Monoamine oxidase	Amine oxidase	*Echis coloratus*	A0A0A1WCY6	21
14	Monoamine oxidase	Amine oxidase	*Echis coloratus*	A0A0A1WCY6	24
15	Phospholipase A2	Ammodytin I2(C) variant	*Vipera ammodytes meridionalis*	Q6A3A7	4
16	Phospholipase A2	Ammodytin I1(C) variant	*Vipera aspis zinnikeri*	Q6A3J5	14
17	Phospholipase A2	Ammodytin I2(C) variant	*Vipera ammodytes montandoni*	Q6A3A0	30
18	Phospholipase A2	Ammodytin I2(C) variant	*Vipera ammodytes meridionalis*	Q6A3A4	28
19	Phospholipase A2	Ammodytin I2(C) variant	*Vipera ammodytes meridionalis*	Q6A3A7	10
20	Phospholipase A2	Ammodytin I2(C) isoform	*Vipera ammodytes meridionalis*	Q6A3A4	14
21	Phospholipase A2	Ammodytin I2	*Vipera aspis aspis*	Q6YCN9	1
٢٢	Phospholipase A2	Ammodytin I2	*Daboia russelii*	F2Q6F3	7

٢٣	Peptidase M12B	DSAIP	*Daboia siamensis*	A0A2H4Z2X4	5
٢٤	Peptidase M12B	Metalloproteinase	*Echis pyramidum leakeyi*	E9JGD0	8
٢٥	Monoamine oxidase	L-amino-acid oxidase	*Echis coloratus*	A0A0A1WDN5	6
٢٦	Peptidase M12B	Metalloproteinase	*Vipera ammodytes ammodytes*	A0A1I9KNR6	17
٢٧	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG93	10
٢٨	Peptidase M12B	Metalloproteinase	*Echis carinatus sochureki*	E9JG34	7
٢٩	PDGF/VEGF growth factor	Vascular endothelial growth factor F	*Echis coloratus*	A0A0A1WDQ8	9
٣٠	C-type lectin	C-type lectin-like protein 3B	*Macrovipera lebetina*	A0A0C5DM02	17
٣١	phospholipase A2	Ammodytoxin C variant	*Vipera aspis aspis*	Q6A363	5
٣٢	phospholipase A2	Ammodytoxin B isoform	*Vipera aspis aspis*	Q6A362	20
٣٣	phospholipase A2	Ammodytoxin B isoform	*Vipera aspis aspis*	Q6A362	19
٣٤	phospholipase A2	Ammodytin I1(C) variant	*Vipera berus berus*	Q6A3J2	13
٣٥	serine-type endopeptidase inhibitor activity	Kunitz/BPTI inhibitor-2	*Vipera ammodytes ammodytes*	A0A1S5QJK2	3
٣٦	serine-type endopeptidase inhibitor activity	Kunitz trypsin inhibitor protein 2	*Vipera renardi*	S4S375	1

[a] Proteins separated by 2D electrophoresis (Figure 4) were numbered (left column) and analysed by LC-MS/MS.

Another large protein as a serine protease were identified (protein 1 and 11). Of the five groupings, proteins with metalloproteinase activity were represented most frequently in the analysis with 12 species, including the abundant species identified noted earlier with molecular weights close to 48 kDa (proteins 5, 6, 7; other metalloproteinases – proteins 4, 8, 9, 10, 12, 24, 26, 27, 28). The other dominant activity with abundant representation in the venom was PLA2, with numerous ammodytins identified in two clusters. Proteins 15, 16, 17, 18, 19, 20, and 21 possessed acidic or neutral pIs, while proteins 31, 32, 33, and 34 resolved at higher pIs in 2D electrophoresis (Figure 3). Two proteins with Kunitz trypsin inhibitor activity – the fifth set of activities – were identified (species 35 and 36). Other proteins recovered from 2D gels were unique representatives of other activities (Table 1). These and other activities were identified by shotgun digestion of crude venom (Table 2). Biological activities identified by this bottom-up approach but absent from the top-down analysis comprised 5' nucleosidase activity, a nerve growth factor, a peptide inhibitor of metalloproteinases, hyaluronidase, disintegrin, and a sodium channel protein (Table 2). Although the abundance of these species could not be determined from the analysis, the small size of several supports the idea advanced earlier that multiple venom components of significance evaded detection on the 15% gels used for 2D separation (Figure 3).

Table 2. Proteins identified in *Pseudocerastes persicus* venom by shotgun LC-MS/MS.

	Protein Family	Protein Description	Species with the homologous protein	Protein Accession No.	No. of Peptides
1	Amine oxidase	Amine oxidase	*Echis coloratus*	A0A0A1WCY6	28
2	5′ nucleotidase	5′ nucleotidase	*Macrovipera lebetina*	W8EFS0	11
3	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG96	8
4	C-type lectin	Snaclec-7	*Vipera ammodytes ammodytes*	A0A1J0CZM7	12
5	CRISP	Cysteine-rich secretory protein Dr-CRPB	*Daboia russelii*	F2Q6F3	7
6	phospholipase A2	Ammodytin I2(C) variant	*Vipera ammodytes montandoni*	Q6A3A0	14
۷	NGF-beta	Venom nerve growth factor	*Vipera ursinii*	V9I168	4
۸	metalloproteinase (M12B)	H3 metalloproteinase 1	*Vipera ammodytes ammodytes*	R4NNL0	13
۹	metalloproteinase (M12B)	Metalloproteinase of class P-II MPII-1	*Vipera ammodytes ammodytes*	A0A1I9KNT0	8
۱۰	metalloproteinase (M12B)	Metalloproteinase	*Echis coloratus*	E9JG68	12
11	C-type lectin	C-type lectin-like protein 3A	*Macrovipera lebetina*	A0A0C5DKL1	4
12	phospholipase B-like	Phospholipase B-like	*Echis coloratus*	A0A0A1WDU0	4
13	phospholipase A2	Ammodytoxin B isoform	*Vipera aspis aspis*	Q6A362	4
14	peptidase S1	Serine protease	*Echis coloratus*	E9JG21	4
15	peptidase S1	Serine protease	*Echis coloratus*	E9JG09	4
16	PDGF/VEGF growth factor	Vascular endothelial growth factor F	*Echis coloratus*	A0A0A1WDQ8	4
17	Peptidase M12B	Snake venom metalloproteinase F	*Echis coloratus*	A0A0A1WD12	۱۰
18	C-type lectin	C-type lectin-like protein 4B	*Macrovipera lebetina*	A0A0C5DGP5	٤
19	phospholipase A2	Vaspin basic subunit variant	*Vipera aspis zinnikeri*	Q6A351	٦
20	C-type lectin	C-type lectin I	*Echis coloratus*	A0A0A1WCD4	4
21	Peptidase M12B	Metalloprotease	*Echis pyramidum*	Q90499	٤
22	Peptidase M12B	Metalloprotease	*Echis coloratus*	E9JG69	12
23	C-type lectin	C-type lectin C	*Echis coloratus*	A0A0A1WDS6	3
24	phospholipase A2	Basic phospholipase A2 VRV-PL-VIIIa	*Daboia russelii*	D0VX11	2
25	C-type lectin	p31 alpha subunit	*Daboia russelii limitis*	K9JDK1	2
26	natriuretic peptide	Endogenous tripeptide metalloproteinase inhibitor	*Vipera ammodytes ammodytes*	A0A1I9KNP8	3
27	Glycosyl hydrolase 56	Hyaluronidase	*Macrovipera lebetina*	W8EEP5	2
28	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JGA1	5
29	C-type lectin	C-type lectin-like protein 4A	*Macrovipera lebetina*	A0A0C5E3E9	٢
30	Disintegrin	Disintegrin Dis-1	*Vipera ammodytes ammodytes*	A0A1I9KNR7	٢
31	C-type lectin	C-type lectin-like protein 3B	*Macrovipera lebetina*	A0A0C5DM02	٤
32	CRISP	Cysteine-rich secretory protein Dr-CRPK	*Daboia russelii*	F2Q6F2	٧
33	C-type lectin	Dabocetin alpha subunit	*Daboia russelii russelii*	K9JBU0	11
34	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG66	6
35	Peptidase M12B	DSAIP	*Daboia siamensis*	A0A2H4Z2X4	4
36	PDGF/VEGF growth factor	Vammin-1	*Vipera ammodytes ammodytes*	A0A1J0CZM5	3
37	phospholipase A2	Phospholipase A2-I	*Daboia siamensis*	Q7ZZQ1	1
38	BPTI/Kunitz inhibitor	Kunitz trypsin inhibitor protein 2	*Vipera renardi*	S4S375	1
39	C-type lectin	C-type lectin snaclec-1	*Vipera ammodytes ammodytes*	A0A1I9KNP6	1
40	Peptidase M12B	Metalloproteinase H4-A	*Vipera ammodytes ammodytes*	V5TBK6	4
41	peptidase S1	Serine protease	*Echis carinatus sochureki*	E9JG24	3
42	sodium channel	Sodium channel protein	*Causus maculatus*	A0A1B0Z7H4	1
43	PDGF/VEGF growth factor	Vascular endothelial growth factor A isoform 1	*Daboia russelii*	A0A223PK54	1
44	Peptidase M12B	Metalloproteinase	*Echis coloratus*	E9JG93	8

4. Discussion

Snake venoms are ancient cocktails of pharmacologically-active proteins and peptides (26-27). Of the 2.7 million snakebites estimated to occur annually, the toxicity of these proteins is thought to result in between 81000 and 137000 deaths (4). Proteomic approaches (28) have allowed elucidation of the composition of snake venoms, the biological properties of their constituent components and their antigenicity, supporting the development of therapeutics to reduce morbidity and mortality (29-30). In this study, the venom proteome of *P. persicus* was explored using approaches that have been used by others to characterize many other snake venoms, including those from kraits and cobras (31-32). The significance of *P. persicus* arises from its wide geographic distribution across the region (33-34) and locally with particularly common occurrence in western and central provinces of Iran (35). It is evidenced that the biological activities present in the venom may not be shared within the genus (20-36) and with other vipers found in the region (*e.g.*, *Eristicophis macmahonii*) that are related at the phylogenetic level (37). The composition of venom from these vipers has been understood incompletely; however, this is important for designing an efficient immunological therapeutic (21). Our study was focused on analyzing samples from *P. persicus* found in Semnan province. The obtained results were in good agreement with the work of others and in common with the results found by Ali *et al.*, concering that proteins within the venom can be grouped by activity. Proteins with PLA2 activity were found commonly with a range of molecular weights and isoelectric points, suggesting the presence of multiple species and isoforms. The PLA2 is thought to have an important role in early tissue injury and mortality arising from the disturbance of homeostasis and the induction of paralysis (38). It means we have 4 groups of PLA2 in venom of vertebrates and invertebrates, where two groups are seen in snake venoms. Aside from PLA2 catalytic activity, a property that is highly dependent on amino acids present in the active site at position 49 and calcium, can be seen in snake PLA2s with entirely independent inflammatory and bactericidal effects (40-42). For these reasons, the development of neutralizing anti-PLA2 antibodies are thought to be crucial in the development of effective antivenom preparations (43). Metalloproteases were also well-represented and consistent with other studies (21), and found across a wide range of isoelectric points. Zinc-dependent proteases from snakes have been classified into three groups according to domain structure (44), and the metalloproteinases from *P. persicus* venom enzyme falling within a PIII sub-group that contains metalloproteinase, disintegrin-like and cysteine-rich domains (45-46). The metalloproteases of viperid snakes plays diverse roles in venom toxicity, including pronounced hemorrhagic activity (46) and interference at numerous stages of the blood clotting cascade (47); however, the range of metalloproteases in the *P. persicus* venom is substantially greater than the closely-related species (e.g., *P. fieldi* and *Eristicophis macmahonii)* (21); in these species, coagulopathic effects may be mediated by PLA2s or bioactive mediators of low molecular weight (20-48). By comparing with the PLA2s and metalloproteases, serine proteases were represented infrequently in the venom of *P. persicus* in this analysis, a finding of some surprise, given that these enzymes are abundant in the venom of some other viper species (47). Viperid serine proteases are typically glycoproteins with variable molecular weights (26–67 kDa) according to the carbohydrate moieties attached (50); however, they have evolved commonly among venomous animals through gene duplication and enhanced evolution (51). In snakes, their thrombin-like activities place them within the S1 family of peptidases (52). Matching a modest abundance of serine proteases in the venom of *P. persicus*, a small set of Kunitz inhibitors were detected (53). Comprising 50 to 60 amino acids, these proteins have three highly conserved disulfide bonds (54-55) to stabilize their conformation. As with many other venom components (56-57), it has been proposed that the Kunitz inhibitor from *P. persicus* may be a multi-functional protein with therapeutic potential (58). Proteomic analysis reveals a wide range of proteins present in the venom of *P. persicus* with diverse biological activities. This should support better understanding of envenomation and the therapeutic opportunities afforded by polyclonal antivenom. This is important given the distribution of *P. persicus* across Iran and the wider region. With these considerations in mind, the Razi Vaccine and Serum Research Institute has progressed the development of a polyvalent antivenom. Further research is required to establish whether the venom components of *P. fieldi* and other related species are similar to those identified in our study but irrespective, there are likely benefits in developing antivenom preparations against *P. fieldi* and other snake species of importance in Iran.

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Authors' Contribution

A.N. and M.S. conceptualized the study, contributed the resources and were involved in the supervision, project administration, data interpretation and analysis. M.S and A.N. accomplished all experiments and analyzed and interpreted the data. A.N. contributed to recruiting and writing the manuscript. F.T. was involved in HPLC. TE measured enzyme activity. A.Z. was involved in resource preparation. All authors read and approved the final manuscript.

Ethics

All animal inoculations and bleeding activities were fully compliant with general animal ethics. Moreover, animal housing was according to standard operating procedures of the Razi Institute, which were mainly based on normal farming procedures.

Conflict of Interest

The authors declare no conflict of interest.

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