Abundance of Proteases in Nigerian Echis ocellatus Venom Revealed through Qualitative Proteomic Analysis

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ABSTRACT

Snake venom proteins are generally categorized into enzymatic and non-enzymatic classes**,** both of which play ~~a~~ crucial roles in the pathophysiology of envenoming. Echis ocellatus, a viper widely distributed across Nigeria, is responsible for a high proportion of snakebite-related morbidity and mortality in the region. Understanding the venom composition and its molecular complexity is essential for advancing venomics and improving antivenom design.In this study, comprehensive proteomic profiling was performed to characterize the enzymatic and non-enzymatic constituents of E. ocellatus venom. Liquid chromatography–mass spectrometry (LC-MS) analysis was conducted using the Advion TriVersa NanoMate nano-electrospray ionization (ESI) platform with chip-based LC-ESI-MS/MS technology. Proteins and peptides were identified and curated using coverage thresholds of ≥5%, ≥10%, and ≥20%. The enzymatic repertoire included numerous proteases, phosphodiesterases, phospholipase A_2 (PLA_2), arginine ester hydrolase, alkaline phosphomonoesterase, and 5′-nucleotidase. Notably, both phospholipases A_2 and B were detected, along with diverse snake venom metalloproteinases (SVMPs), which are key drivers of hemorrhage and tissue damage. Additional protein families identified included L-amino acid oxidases, cysteine-rich secretory proteins, venom nerve growth factors, as well as serine protease inhibitors, aminopeptidases, and acetylcholinesterases. In total, 163 proteins were identified, reflecting the high toxic potency and complexity of the venom. These findings provide valuable insight into *E. ocellatus* venom composition and support future development of improved antivenoms and venom-derived therapeutic candidates through quantitative proteomics.

Keywords: Snake venom, Echis ocellatus, Proteome, enzyme

INTRODUCTION

Echis ocellatus belongs to the Viperidae family of snake and predominantly distributed across Asia and Africa. Although relatively ~~a~~ small, it is a highly aggressive species with potent venom and responsible, for high~~er~~ mortality in rates in Nigeria's Savannah regions (Padial, 2006; Hughes, 1976). Snake venom is rich in a variety of toxins, mainly proteins and peptides, which are classified into enzymatic and non-enzymatic molecules. These toxins are the primary contributors to pathophysiological effects observed in the snakebite victims. For instance, proteases such as snake venom metalloproteinase (SVMPs) and snake venom serine proteases (SVSPs) can degrade the capillary basement membrane, leading intravascular bleeding - a hallmark haemorrhagic envenomation, especially, from *Viperidae* species (Kress, 1986; Bottrall *et al*., 2010; Preciado

and Pereañez, 2018). Snake venom also contains phospholipases, including phospholipase A_2 and phospholipase B, as well as phosphodiesterase. Phospholipases disrupt cellular membranes by where significantly reducing Na^+/K^+ ATPase activity, which ultimately damages ~~to~~ the lipid bilayer and leads to cell death is observed (Mukherjee 2014). Overall, snake venom effects can be categorized into neurotoxic, cytotoxic, haemotoxic and in some cases myotoxic (W.H.O. 2010).

Cytotoxic effects are typical of viper envenoming, leading to significant tissue~~s~~ damage. This occurs through the induction of neutrophil extracellular traps formation, which block blood vessels (Katkar *et al*., 2016). Clinically, this manifest into swelling that progresses to blistering, bruising, and in severe cases, systemic hypovolaemic shock (W.H.O. 2010). Haemotoxicity, another characteristic of Viperidae envenomation, can trigger hemostatic and/or cardiovascular pathologies. The degradation of capillary basement membrane often leads to a drop in blood pressure (Gutierrez *et al*., 2016). Haemostatic effects include bleeding at the bite site and hemorrhages in the gastro-intestinal tract, urinary tract and gums (Warrel, 1995). Typical of *Elapidae* envenomation, result primarily from presynaptic venom phospholipase A2 (PLA_2) activity (Dixon and Harris, 1999). This enzyme disrupts neuronal function by affecting axons and dendrites, causing Ca^{2+}, which leads to mitochondrial dysfunction and further neuronal pathology (Rigoni *et al*., 2007)**.**

Scientific research on venom protein identification, isolation and characterization has advanced significantly in recent years. Techniques ranging from traditional protein assays to modern proteomic approaches have been crucial in expanding the field of venomics (Tasoulis *et al*., 2022). These advancements have contributed to the discovery of therapeutic peptides and new protein molecules with potential biomedical applications. Proteomic studies characterized the venom of snakes from various regions. However, due to regional variations in venom composition and limited information on the venom of other *Echis ocellatus* from Nigeria, particularly from Kaltungo in Gombe State, proteomic analysis of this medically significant snake could significantly enhance regional venomics and toxin research.

MATERIALS AND METHODS

Animals and Ethic statement

The snakes were identified by a zoologist, Department of Zoology, Faculty of Science, Gombe State University, Nigeria. A voucher number (VEO:004) was assigned. The ethical approval was acquired from the National Veterinary Research Institute, Federal Ministry of Agriculture and Rural Development, VOM, Plateau State, Nigeria with a reference number: AEC/02/74/19.

Echis ocellatus venom

Extraction of the venom was performed on 30 adult snakes captured around Gombe, Gombe State. They were individually restrained and handled in a biosafety environment. The venom glands were stimulated by gently applying pressure while the fangs were positioned over a sterile container. The venom was collected, pooled and lyophilized and stored at -20°C.

Venom Protein Estimation

Exactly 500 µg venom was dissolved in 1 ml PBS**,** and the protein contents was estimated using quartz cuvette in an APEL PD-3000UV Spectrophotometer. The measurement involved recording the absorbance at near UV (280nm). Additionally, far UV absorbance (260 nm) was measured and the following formula was then applied to estimate the total protein concentration: Protein (mg/ml) = $1.55\ A_{280} - 0.76\ A_{260}$

Venom Arginine Ester Hydrolase Assay

The method described by Collins and Jones, 1972 was employed, utilizing α-benzoylarginine ethyl ester as substrate. The reaction volume was prepared to a total of 1.0 ml, consisting of 50 µl of a 50 µg venom solution and 0.95 ml of 0.8 mM α-benzoylarginine ethyl ester in 0.05 M Tris-HCl buffer at pH 7.8. The blank contained 1 ml 0.8 mM of the substrate prepared in 0.05 M Tris-HCl buffer, pH 7.8. The reaction was

monitored by measuring the increase in absorbance at 255 nm using APEL PD-3000UV spectrophotometer at every 60-seconds intervals, with an extinction coefficient of 815 $cm^{-1}M^{-1}$.

Venom Phospholipase A_2 (PLA_2) Activity Assay

PLA_2 activity was determined using the acidimetric method described by Tan and Tan (1988), with egg yolk as the substrate.-A suspension was prepared in a conical flask, consisting of 5 ml of freshly prepared chicken egg yolk, 5 mL of 8.1 mM sodium deoxycholate and 5 ml of 18 mM calcium (II) chloride. The pH was adjusted to 9.87 using 1 M sodium hydroxide. The mixture was continuously stirred for 2 minutes to ensure homogeneity. Exactly 100 µg of venom, suspended in 100 µl PBS was added, and the pH decrease was monitored at 5 minutes intervals to estimate the reaction rate. PLA2 activity was expressed as the rate of pH decrease , with one corresponding to the liberation of 133 µmol of fatty acids.

Venom Phosphodiesterases (PDE) Activity Assay

The method described by Sulkowski and Laskowski (1970) was employed to estimate the phosphodiesterases activity spectrophotometrically using bis-p-nitrophenyl phosphate as the substrate. The reaction mixture was prepared in 24 well plate, with each well contains 400 µl of 100 µM Tris-HCl buffer (pH 9.0), 5 µM bis-p-nitrophenyl phosphate, and 10 µM $MgCl_2$. The mixture was pre- incubated at 37°C for 5 minutes prior to the addition of the venom (15 µg/µl), followed by an additional 5 minutes of incubation. The reaction was then arrested by adding 2 ml of 0.1 N NaOH, and the absorbance was measured at 400 nm using a molar extinction coefficient of 17,600. Enzyme activity was expressed in nmol of product per mg venom.

Venom Proteases Activity Assay

Protease activity was estimated as described by Neilier (2020), with minor modifications. The substrate was prepared by gently heating 2% azocasein at 50°C in a water bath for 10 minutes, after which the pH was adjusted using NaOH. The reaction mixture was composed of 450 µl of 100mM Tris-HCl buffer (pH 8.0) , 750 µl of 2% azocasein and 50 µg of venom suspended in 300 µl of PBS. This mixture was incubated at 37°C for 30 minutes. Following incubation, Following incubation, 750 µl was collected from the reaction mixture and mixed with 750 µl of 110 mM trichloroacetic acid to precipitate the proteins. After centrifugation at 20,000 g for 10 minutes, 750 µl of the supernatant was collected. To terminate the reaction, 750 µl of 500 mM NaOH was added. The absorbance was measured at 440 nm using an APEL PD-3000UV spectrophotometer.

Rate = Change in Absorbance at 440 nm = Absorbance of Test – Absorbance of Blank /Time

Venom Alkaline Phosphomonoesterase Activity Assay

A modified method by Lo *et al*. (1969) was used to determine alkaline phosphomonoesterase activity. The assay was performed in triplicate, with each reaction mixture containing 100 µl of 50 µg venom, 0.5 ml of 10 mM p-nitrophenylphosphate, 0.5 ml of 0.5 M glycine buffer (pH 8.5), and 0.3 ml of 0.01 M magnesium sulfate. After a 30-minute incubation at 37°C, 2 mL of 0.2 M sodium hydroxide was added to stop the reaction. This was allowed to stand for 25 minutes at 25°C, after which absorbance was measured at 400nm. An extinction coefficient of 18500 $cm^{-1}M^{-1}$ was used for calculations.

Venom 5'-Nucleotidase Activity Assay

To determine the 5'-Nucleotidase activity, we employed the method described by Heppel and Hilmore (1955) utilizing 5'-AMP as the substrate. The liberated inorganic phosphate was quantified using the ascorbic acid method outlined by Chen *et al*. (1956), with slight modifications. Each assay was conducted in duplicate, with an assay mixture containing 0.5 ml of 0.02 M of the substrate pH (8.5), 100 µl of venom (50 µg), 0.1 ml of 0.1 M magnesium sulfate and 0.5 ml of 0.2 M glycine buffer (pH 8.5). The mixture was incubated at 37°C for 10 minutes. The reaction was halted by adding 1.5 ml of 10% trichloroacetic acid to the reaction mixture.To estimate the liberated inorganic phosphate, a 1.0 ml mixture of ascorbic acid was prepared, containing 0.25 mL of 3 M sulfuric acid, 0.25 mL of 2.5% ammonium molybdate, 0.25 mL of 10% ascorbic acid, and 0.25 mL of deionized water. The source of the phosphate was KH_2PO_4. The mixture was allowed to

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stand at room temperature for 30 minutes before measuring the absorbance at 820 nm. A standard curve of inorganic phosphate was plotted using concentrations of 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml.

Necrosis Assay

The dorsal area of three mice was shaved, and they were intradermally injected with 50 µg of venom in 50 µl of PBS. The mice were then allowed to rest for 72 hours before being euthanized via CO_2 inhalation. After euthanasia, the dorsal skin was excised, and, the mean diameter (in mm) of the necrotic area was measured.

Coagulant Activity Assay

Coagulation time was assessed following the protocol described by Theakston and Reid (1983), with some modifications. Three venom doses (50, 100 and 200 µg) were prepared, each dissolved in 50 µl Milli-Q water and mixed with 500µl of citrated human plasma. The assay was conducted at 37°C. Clotting time was estimated in seconds by observing the appearance of fibrin networks or the immediate formation of clot.

Venom Qualitative Proteomics

Preparation of Gelfree Fractions and SDS-PAGE

Two GelFree snake venom samples were made with a GelFree 8100 Fractionation system containing 500 µg venom sample using a 12% and 8% Tris-Acetate Cartridge Kit with a separation range from 3.5 to 50 kDa each and a resolution between 10 kDa - 50 kDa and 35 kDa - 150 kDa respectively. For Gel-Free 1 using 12% Tris-Acetate Cartridge Kit, 14 fractions with approximately 150 µl each were recovered. In the Gel-Free 2 using 8% Tris-Acetate Cartridge Kit, an approximately 150 µl were recovered from 12 fractions.

In-Gel/ In-Solution Approaches

Two SDS-PAGE were prepared for each of the Gel-Free approach. These gels were made using 14 fractions from GelFree at 12% and 8% acrylamide concentrations, respectively (see Table 1). Both the SDS--PAGE gels were stained with the Pierce Silver Stain Kit (Thermo Scientific). SDS detergent was removed from GelFree 12% fractions 10–14 Gel-free 8% fractions 1–3, 6–12 using Pierce Detergent Removal Spin Column, 0.5 ml (Thermo Scientific). Fractions 3 and 6 from Gel-free 8% were mixed in one Eppendorf tube, and fractions 7 to 12 into another.

For LC-MS/MS injection (top-down analysis), GelFree 12% were reconstituted in 30 µl with 5% ACN with 0.1% FA. For Gel-free 8%, fractions 3–6 were reconstituted in 10 µl, and fractions 7–12 in 15 µl, with 3% ACN and 1% FA. For the in-solution approach, fractions generated with the Tris-Acetate Cartridge Kit were treated with 5% ACN and 0.1% FA without subjecting it to SDS-PAGE and then applied to the LC-MS/MS.

Table 1: GelFree Fractions based on 12% and 8%

Lane	Gel 1	Gel 2
1	Marker (M)	M
2	Loading Buffer (LB)	LB
3	Snake Venom (SV)	F7
4	LB	F8
5	F1	F9
6	F2	F10
7	F3	F11
8	F4	F12
9	F5	F13
10	F6	F14

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Nano-LC-MS/MS and Data Analysis

The nano-LC-MS/MS set-up was as follows: Intact proteins were diluted in either 5% ACN/1% FA or 10% ACN/1% FA. Samples were loaded to an Acquity UPLC M-Class BEH C4 Trap Column (300Å, 5µm, 180µm x 20mm 2G V/M) at a flow rate of 5 µl/min using a nano-Acquity UPLC system (Waters). Protein separation was performed using an Acquity UPLC M-Class Bio-Resolve mAb Column (450Å, 2.7µm, 75µm x 250mm). The run consisted of three consecutive linear gradient steps: from 10 to 50% solvent B over 120 minutes, from 50 to 85% solvent B in 7 minutes, followed by an isocratic elution at 85 % B for 7 minutes, and finally re-equilibration to initial conditions (Solvent A was 0.1% FA in water, and solvent B was 0.1% FA in acetonitrile (CH3CN)). The column outlet was directly connected to an Advion TriVersa NanoMate (Advion) coupled with an Orbitrap Fusion Lumos™ Tribrid Mass Spectrometer (Thermo Scientific). The mass spectrometer was operated in data-dependent acquisition (DDA) mode. Survey MS scans were acquired in the orbitrap with the resolution of 120,000 (defined at m/z 200), with a user-defined lock mass of m/z 445.12 m/z for each Orbitrap scan. The top speed acquisition mode was set to fragment the most intense ions per scan using ETD-hCD, with fragments detected in the Orbitrap at a resolution of 120,000. The ion target count was set to 400,000 for survey scans and 50,000 for MS/MS scans. The Spray voltage for the NanoMate source was set to 1.60 kV, and the RF Lens was tuned to 30%. A minimal signal threshold of 50,000 was required to trigger the switch from MS to MS/MS. The spectrometer was operated in positive polarity mode, with singly charged state precursors ions excluded from fragmentation.

Mass spectra of the peptides were acquired and analyzed using Proteome Discoverer software v2.3.0.480 (Thermo Scientific) with ProSight node, utilizing the High/High Xtract/Respect deconvolution algorithms for a Top-Down approach. The Uniprot_Viperidae_canonical_2019_04 database, incorporated within the proteome discoverer, was used for the search. A three-tier search was conducted with precursor mass tolerances of 2 Da, 10ppm and 500 Da, and fragment mass tolerances of 10 ppm. A $-10\lg P$ score greater than 20% indicates relatively high confidence level in detected proteins, making them suitable for further analysis.

RESULTS

Protein Identification and Coverage Analysis

The total number of proteins identified from in-solution and in-gel digested samples, based on coverage percentage is presented in figure 1. In Figure 1A, 163 proteins were identified using $-10\lg P \geq 5\%$ coverage: 26 from in-solution digestion, 35 from in-gel digestion, with 102 proteins common to both. In Figure 1B, using $-10\lg P \geq 10\%$ coverage, 122 proteins were identified, 24 from in-solution and in-gel digestion, with 74 proteins common. In Figure 1C, the $-10\lg P \geq 20\%$ protein coverage criteria yielded a total of 61 proteins, 12 from in-solution and 11 from in-gel digestion. Overall, the highest number of proteins was identified using the $\geq 5\%$ coverage threshold. Visualization of the SDS PAGE indicated good quality separation of the proteins and the ranges of the molecular weights of the protein class was visible (Figure 2). Distinct banding patterns were observed between the fractions analyzed, indicating the presence of specific proteins in different fractions. A histogram showing proteome of 15 distinct protein families, along with other identified proteins in both In-solution and In-gel approaches, is presented in Figure 2. The predominant protein family in both approaches was SVMPs, with 8.3% more proteins identified in the In-gel approach. For LAAO, 11.1% more proteins were observed in the In-solution approach. A 14.3% higher abundance of SVSPs was observed in the In-gel approach. Snaclec, PLA_2 and CRVP were 2.9%, 36.4% and 17.6% more abundant in the in-gel approach, respectively. No differences were observed in the number of ASP, PK, 5'-nucleotidase, QPCT, PDE, AO and PLB. For other proteins, 18.2% more were identified using the In-solution approach.

Enzymatic and Necrotic Activities of Echis ocellatus Venom

A summary of enzymatic and necrotic activities of Echis ocellatus venom is presented in Table 2. The table shows activities of major enzymes activities present in the venom, including phospholipase A_2 (PLA_2, measured in µmol $min^{-1}ml^{-1}$), proteases (PRO, U/mg), arginine ester hydrolase (AEH, µmol min^{-1}), alkaline phosphomonoesterase (PME, µmol min^{-1} mg^{-1}), 5'-nucleotidase (5'Nuc, µmol/min/mg), phosphodiesterase (PDE, nmol/mg) and the necrotic effect necrosis (NEC, mm/50 µg) venom.

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The summary of identified and Characterized proteins/peptides in *Echis ocellatus* Venom is shown in Table 3. A total of 163 proteins/peptides were identified and characterized. The parameters included: protein coverage prediction (%), representing the proportion of amino acids in a specific protein sequence found in the peptide sequences, peptide-spectrum match (PSM) scores, which assess the likelihood that a peptide fragmentation was recorded in the LC-MS/MS experiment, with high scores indicating confident identification; and the number of distinct peptide sequences identified per protein group.

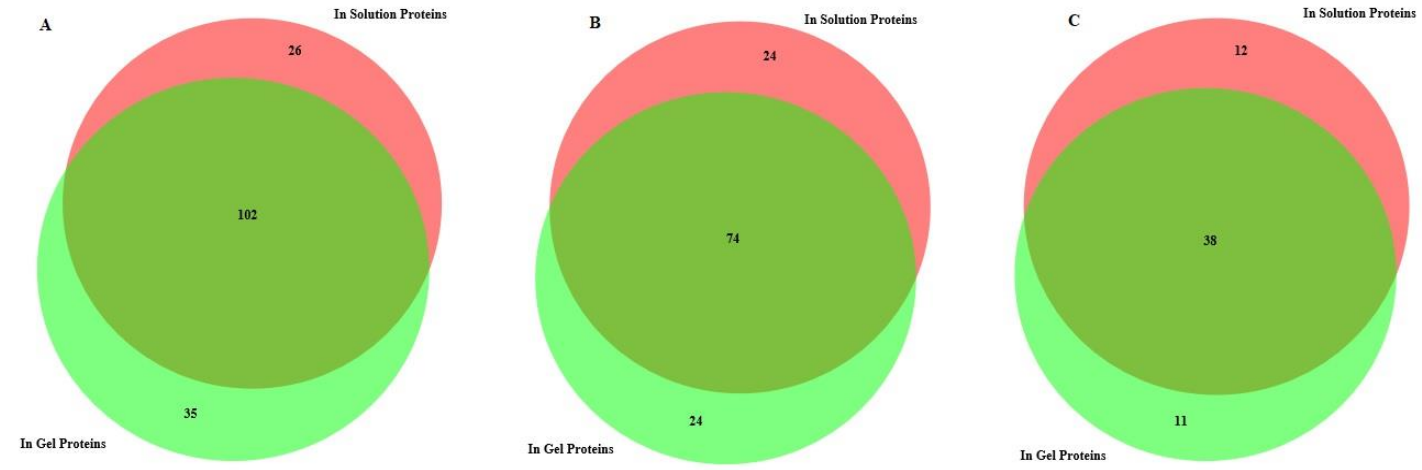

Figure 1: Number of identified proteins using in gel and in solution approaches. A. Proteins identified using −10lgP ≥ 5% coverage, B. proteins identified using −10lgP ≥ 10% coverage, C. proteins identified using −10lgP ≥ 20% coverage. The Venn were generated using BioVenn https://www.biovenn.nl/index.php. A web application for the comparison visualization using area proportional Venn diagrams.

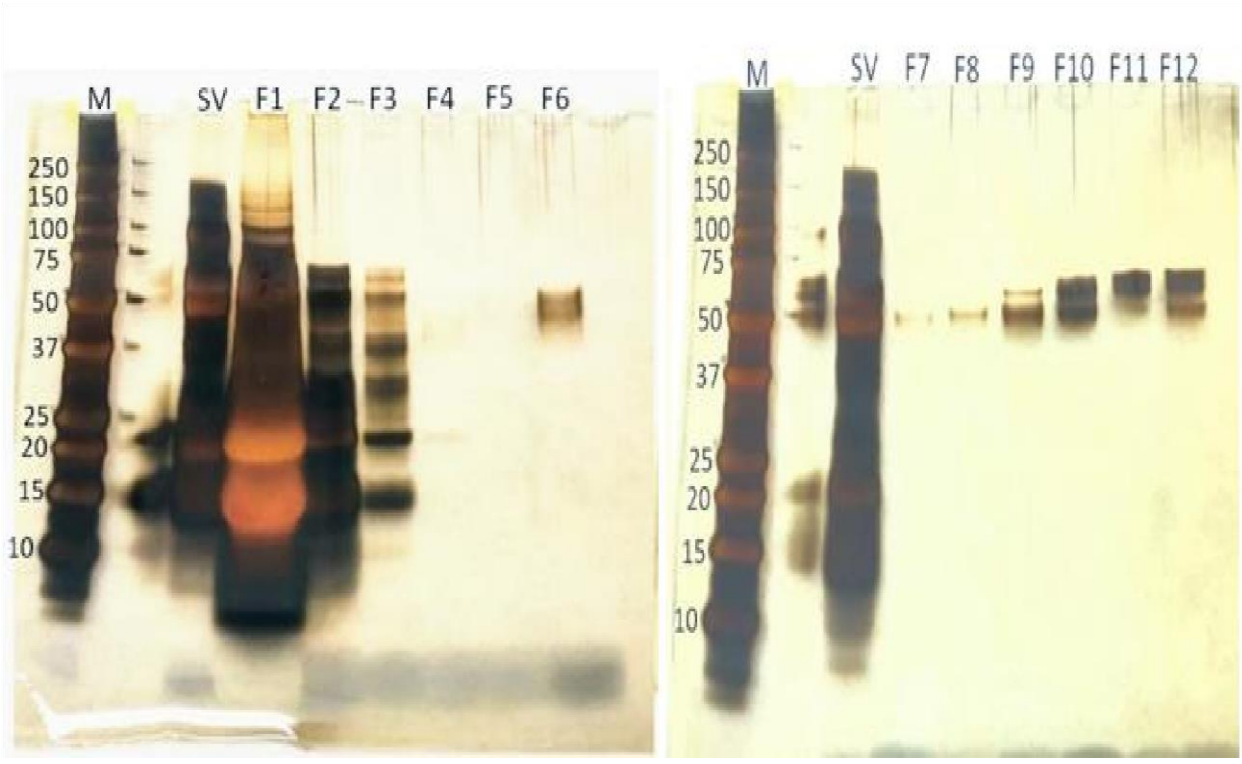

Figure 2: SDS-PAGE analysis of Echis ocellatus venom fractions.

Lane M: Molecular weight marker (protein ladder) showing protein sizes in kDa. Lane **F1**: Protein fraction 1 obtained from GelFree 8100 Fractionation system. Lane **F2**: Protein fraction 2 obtained GelFree 8100 Fractionation system. Additional lanes (F3, F4, F5, F6, F7, F8, F9, F10, F11 and F12) represent other venom fractions separated using the same procedure.The figure shows clear separation of proteins across different fractions, with distinct banding patterns indicating specific proteins present in each fraction.

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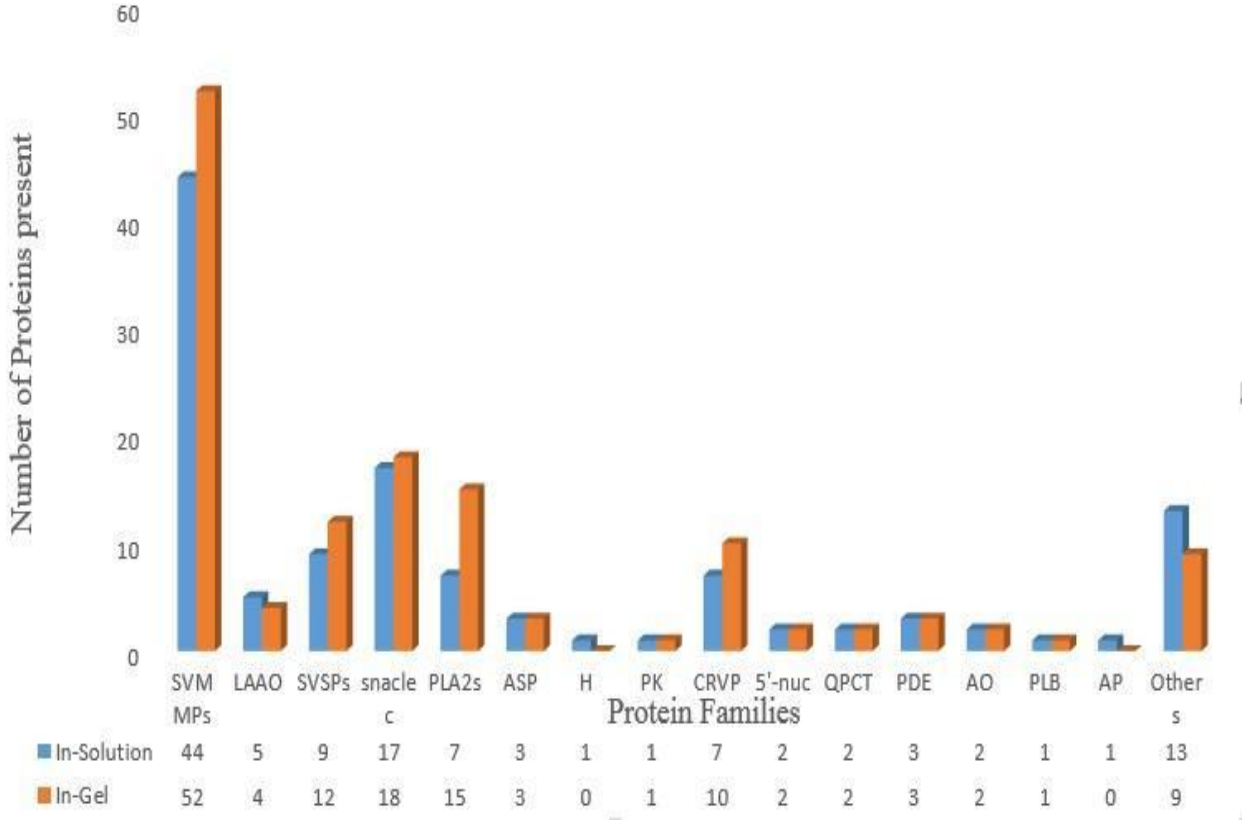

Figure 3. Protein families identified in *Echis ocellatus* venom. SVMPs: snake venom metalloproteinases; LAAO: L-amino acid oxidase; SVSPs: snake venom serine Proteases; snaclec: snake C-type lectin; PLA_2: Phospholipase A_2; ASP: aspartic proteases; H: hydrolase; PK: protein kinase; DI: disintegrin; cysteine-rich venom protein; 5'-nuc: 5'-nucleotidase; QPCT: glutaminyl-peptide cyclotransferase; PDE: phosphodiesterase; AO: amine oxidase; PLB: phospholipase B; AP: aminopeptidases; others: other proteins. Proteins were selected using $\geq -10\text{lgP}$ score with 5% score coverage.

Table 2. Summary of enzymatic and necrotic activities of *Echis ocellatus* venom. The table presents the activity levels of major enzymes present in the venom, including phospholipase A_2 (PLA_2, $\mu mol\ min^{-1}\ mL^{-1}$), proteases (PRO, U/mg), arginine ester hydrolase (AEH, $\mu mol\ min^{-1}$), alkaline phosphomonoesterase (PME, $\mu mol\ min^{-1}\ mg^{-1}$), 5'-nucleotidase (5'-Nuc, $\mu mol\ min^{-1}\ mg^{-1}$), phosphodiesterase (PDE, nmol/mg), and the necrotic effect (NEC, measured as mean diameter in mm per 50 µg of venom). Values represent the mean activity from [number of replicates] experiments.

Enzyme/Parameters	PLA_2 ($\mu mol\ min^{-1}\ ml^{-1}$)	PRO (U/mg)	AEH ($\mu mol\ min^{-1}$)	PME ($\mu mol\ min^{-1}\ mg^{-1}$)	5'Nuc (µmol/min/mg)	PDE (nmol/mg)	NEC (mm/50µg venom)
Activity	36.18± 0.40	8.43±0.15	84.70±0.80	15.83±0.35	3.44±0.05	3.85±0.04	10.33±0.25

Table 3. Identified and characterized proteins and peptides in *Echis ocellatus* venom. The table summarizes a total of 163 proteins/peptides identified, including protein coverage (%) indicating the proportion of amino acids in each protein sequence matched by identified peptides, peptide-spectrum match (PSM) scores reflecting the confidence of LC-MS/MS identifications, and the number of distinct peptide sequences detected per protein.

S/N	Accession No.	Description	Coverage % (In-Sol)	Coverage % (in-gel)	PSMs (In-Sol)	PSMs (in-gel)	Peptides (In-Sol)	Peptides (in-gel)
Enzymatic Proteins								
1	J7H670	L-amino acid oxidase Lm29	7	8	104	123	5	5
2	Q6WP39	L-amino-acid oxidase	11	6	114	161	7	5
3	Q4F867	L-amino-acid oxidase (Fragments)	25	24	505	625	14	13
4	P0DI89	L-amino-acid oxidase (Fragments)	40		6	-	2	0
5	B5U6Y8	L-amino-acid oxidase	54	52	1269	1002	28	25
6	A0A0B4U9L8	Metalloproteinase F1	--	5	0	8	0	2
7	J3S826	Metalloproteinase (Type II) 1c		8	0	4	0	1
8	E9JGG6	Metalloproteinase		12	0	15	0	1
9	Q3HTN1	Zinc metalloproteinase-disintegrin-like stejnihagin-A		7	0	1	0	1
10	E3UJL7	MP_IIx1 SVMP (Fragment)		12	0	2	0	1
11	A0A194ARK7	Metalloproteinase type III 8		6	0	2	0	1
12	Q8AWI5	Zinc metalloproteinase-disintegrin-like halysase		6	0	1	0	1
13	A0A194ASV3	Metalloproteinase type III 6b		6	0	1	0	1
14	J3S831	Snake venom metalloproteinase (Type III) 5		6	0	4	0	1
15	E9JGB7	Metalloproteinase		6	24	45	1	3
16	B8K1W0	Zinc metalloproteinase-disintegrin-like		9	3	136	2	6
17	A0A194ATS8	Metalloproteinase type II 5b		8	2	5	1	1
18	A0A194APN3	Metalloproteinase type III 4c		11	38	218	3	4
19	E9JG62	Metalloproteinase		5	32	91	3	5
20	R4NNL0	H3 metalloproteinase 1	5	5	50	219	4	4
21	E9JG36	Metalloproteinase (Fragment)	5	10	51	76	3	7
22	E9JGA5	Metalloproteinase (Fragment)	5	27	4	15	1	4
23	A0A1I9KNR6	Metalloproteinase of class P-III MPIII-1	5	5	12	18	3	2
24	E9JGC4	Metalloproteinase (Fragment)	5		6	55	2	1
25	T2HRS1	p-II metalloprotease (Fragment)	5		7	0	1	0
26	E9JG78	Metalloproteinase	6	13	58	361	3	6
27	C5H5D4	Zinc metalloproteinase-disintegrin-like	6		8	0	1	0

		batroxstatin-3						
28	Q90500	Metalloprotease (Fragment)	7	7	39	152	5	6
29	E9JG42	Metalloproteinase (Fragment)	7	8	1	33	1	1
30	E9JG29	Metalloproteinase	8	8	23	263	4	4
31	E9JG35	Metalloproteinase	8	15	50	196	5	6
32	E9JGC3	Metalloproteinase (Fragment)	8		32	55	4	1
33	E3UJL2	MP_III3 SVMP (Fragment)	8		3	0	2	0
34	E9JG53	Metalloproteinase (Fragment)	9	25	17	54	2	4
35	E9JGD0	Metalloproteinase (Fragment)	10	16	28	76	4	6
36	E9JGC9	Metalloproteinase (Fragment)	11	11	56	259	7	7
37	E9JGA3	Metalloproteinase (Fragment)	11	7	22	183	3	3
38	Q2UXQ1	Group III snake venom metalloproteinase	12	20	51	282	6	11
39	E9JG79	Metalloproteinase	12	10	95	111	7	5
40	E9JG31	Metalloproteinase (Fragment)	12	20	41	181	4	7
41	Q2UXQ3	Group I snake venom metalloproteinase	15	15	541	524	6	6
42	E9JG34	Metalloproteinase	16	19	119	586	10	12
43	E9JG45	Metalloproteinase (Fragment)	17	17	46	314	5	5
44	E9JG50	Metalloproteinase (Fragment)	17	13	55	110	4	4
45	E9JG63	Metalloproteinase (Fragment)	18	18	85	406	10	12
46	Q14FJ4	Zinc metalloproteinase/disintegrin	18	12	715	665	5	6
47	E9JG68	Metalloproteinase	19	17	83	407	11	10
48	Q2UXQ7	Group III snake venom metalloproteinase	20	28	136	535	14	16
49	A0A3G1E3T4	Venom metalloprotease PIII-SVMP EOC00089	25	22	250	566	13	13
50	E9JG93	Metalloproteinase (Fragment)	25	15	80	105	9	6
51	E9KJX9	Group III snake venom metalloproteinase (Fragment)	25	26	20	148	6	7
52	E9KJZ7	Group III snake venom metalloproteinase (Fragment)	26	36	167	600	14	16
53	Q6X1T6	Zinc metalloproteinase-disintegrin-like EoMP06 (Fragment)	27	33	264	805	18	25
54	E9KJZ0	Group III snake venom metalloproteinase (Fragment)	29	29	423	1052	17	17
55	Q2UXQ5	Zinc metalloproteinase-	30	30	352	812	16	17

		disintegrin-like EoVMP2						
56	Q2UXR0	Zinc metalloproteinase-disintegrin-like Eoc1	32	39	356	908	21	25
57	Q2UXQ9	Group III snake venom metalloproteinase	32	31	275	893	17	17
58	E9KJZ3	Group III snake venom metalloproteinase (Fragment)	36	58	166	878	13	18
59	E9KJY6	Group III snake venom metalloproteinase (Fragment)	37	40	453	1007	19	22
60	Q2UXQ0	Group III snake venom metalloproteinase	39	43	918	1002	20	24
61	E9KJZ6	Group III snake venom metalloproteinase (Fragment)	42	39	473	961	22	22
62	E9KJY1	Group III snake venom metalloproteinase (Fragment)	42	39	69	322	12	14
63	A0A0A1WD17	Snake venom metalloproteinase A (Fragment)	20	20	24	86	4	4
64	A0A1L8D649	BATXSVMPII7		8	0	1	0	1
65	A0A1L8D5W7	BATXSVMPIII22	6		1	0	1	0
66	A0A0A1WDU0	Venom metalloprotease PIII-SVMP	26	18	168	114	12	9
67	Q18DC8	Renin-like aspartic protease	69	43	625	195	14	11
68	Q18DC9	Renin-like aspartic protease	70	37	622	276	16	12
69	T1DKH1	2,4-dienoyl-CoA reductase, mitochondrial-like protein	5		3	0	1	0
70	A0A0F7ZAI9	Dual specificity mitogen-activated protein kinase kinase 3	5	5	3	8	1	1
71	P22640	Basic phospholipase A2 homolog		7	0	18	0	1
72	O42187	Basic phospholipase A2 B		13	0	65	0	1
73	Q7LZQ5	Phospholipase A2 II (Fragment)		19	0	6	0	1
74	A8E2V4	Acidic phospholipase A2		13	0	13	0	1
75	P04084	Acidic phospholipase A2 homolog vipoxin A chain		15	0	7	0	1
76	C0HLF0	Basic phospholipase A2		15	0	1	0	1
77	A0A0A1WCW3	Phospholipase A2 Group IIA e (Fragment)		22	0	8	0	1

78	A0A2I7YRZ0	Secretory phospholipase A2		12	0	7	0	1
79	A0A194AQ80	Phospholipase A2 1a	13	13	2	203	1	1
80	A0A0H3U1Y0	Phospholipase A2	13	13	1	2	1	1
81	Q6H3C5	Basic phospholipase A2 Ts-G6D49	15	15	4	34	1	1
82	P48650	Basic phospholipase A2 homolog ecarpholin	30	51	68	391	5	9
83	P0DJQ2	Basic phospholipase A2 homolog	35	65	38	87	1	2
84	Q4QT03	PLA2-32 (Fragment)	54	53	29	452	6	8
85	P0DM51	Basic phospholipase A2 BnpTX-1 (Fragment)	0	16	0	1	0	1
86	B5U6Y4	Phospholipase A2 homolog	64	78	145	1572	10	18
87	A0A2I7YS29	Serine endopeptidase		9	0	9	0	1
88	A0A1W7RJU0	Serine proteinase 9		6	0	2	0	1
89	E9JG21	Serine protease (Fragment)		12	13	48	1	2
90	E9JG04	Serine protease (Fragment)	12	15	22	199	3	4
91	E9JG20	Serine protease (Fragment)	12	12	19	156	2	2
92	B5U6Y3	Serine protease sp-Eoc49	14	9	20	167	3	3
93	E9JG09	Serine protease (Fragment)	18	14	15	59	4	3
94	E9JFZ8	Serine protease (Fragment)	19	28	69	148	5	6
95	E9JG12	Serine protease (Fragment)	32	32	104	496	8	8
96	D3YLG9	Serine proteinase (Fragment)	33	33	85	160	8	8
97	E9JG05	Serine protease (Fragment)	39	25	93	457	6	5
98	M9NCG3	Glutaminyl-peptide cyclotransferases	31	31	89	50	8	8
99	Q90YA8	Glutaminyl-peptide cyclotransferase	20	17	104	24	6	5
100	W8E7D1	Phosphodiesterase	15	11	106	87	12	9
101	A0A194AS02	Phosphodiesterase	12	12	131	70	9	8
102	U3TAI7	Phosphodiesterase	10	7	74	60	8	6
103	A0A068EPZ2	Amine oxidase	14	9	162	238	9	6
104	T2HQ57	Amine oxidase	11	12	161	239	8	7
105	F8S101	Phospholipase B	13	9	84	37	6	5
106	T1E6L9	Aminopeptidase	7		11	5	6	3
107	W8EFS0	5'-nucleotidase (Fragment)	38	25	92	39	14	8
108	A0A194APL9	Snake venom 5'-nucleotidase	13	8	28	37	7	4
Non-enzymatic Proteins								
109	P0DJC8	Snaclec aspercetin subunit alpha (Fragment)		24	0	1	0	1
110	I7ICN3	Snaclec rhinocetin subunit beta	5	7	37	65	1	2
111	C0HKZ6	Snaclec macrovipecetin subunit alpha	5		1	0	1	0

112	Q9PSM8	Snaclec coagulation factor IX/factor X-binding protein subunit B	9	20	4	20	1	2
113	Q6X5S0	Snaclec 7	11	5	4	7	2	1
114	Q7T2Q0	Snaclec EMS16 subunit beta	12	12	17	62	3	3
115	P81996	Snaclec echicetin subunit beta	12	12	119	378	1	1
116	B5U6Y7	Snaclec CTL-Eoc125	13	13	17	33	5	5
117	Q9PSM9	Snaclec coagulation factor IX/factor X-binding protein subunit A	22	33	75	405	3	5
118	Q6X5T0	Snaclec 1	56	49	124	129	5	5
119	B5U6Y6	Snaclec CTL-Eoc124	57	60	424	529	8	10
120	Q3BK16	Disintegrin CV-11-alpha		11	0	164	0	2
121	P0C6R6	Disintegrin ocellatin		55	0	35	0	5
122	M5BGY5	Disintegrin DS-AC		39	0	56	0	5
123	P0C6A4	Disintegrin EO5B	23	39	4	66	1	2
124	Q3BER3	Disintegrin EO4A	24	32	20	268	3	5
125	Q14FJ3	MLD-containing dimeric disintegrin subunit	45	83	6	230	2	6
126	F2Q6F2	Cysteine-rich seceretory protein Dr-CRPK	5	18	32	62	1	2
127	P0DMT4	Cysteine-rich venom protein	36	29	262	218	7	5
128	A0A194ARS4	Cysteine-rich secretory protein 1b	11	24	43	107	2	3
129	F2Q6F6	Cysteine-rich seceretory protein Pg-CRP	15	15	35	78	2	2
130	B7FDI0	Cysteine-rich venom protein (Fragment)	21	9	208	125	4	2
131	Q7LZ61	Coagulation factor X-activating enzyme heavy chain	11	10	49	170	7	7
132	A0A0K8RYY8	C-type lectin 3	5		2	0	1	0
133	I7JX23	C-type lectin like protein 3	11	11	24	40	2	2
134	A0A0A1WCD8	C-type lectin D	12	12	10	12	1	1
135	A0A0A1WDW9	C-type lectin J	19	19	90	100	4	4
136	A0A0A1WDS6	C-type lectin C	19		4	0	1	0
137	Q6X5T4	C-type lectin 2	20	20	11	24	4	4

138	A0A0A1WD30	C-type lectin G (Fragment)	35	15	127	135	3	2
139	A0A0F7Z3D0	Ubiquitin C		16	0	1	0	0
140	Q5KQS4	Antihemorrhagic factor Cmsf		6	0	5	0	1
141	P67863	Snake venom vascular endothelial growth factor toxin vammin		8	0	10	0	1
142	A0A1W7RIQ5	Calreticulin		9	0	1	0	1
143	T1DEE8	Kallikrein-CohPH-2		6	4	14	1	2
144	J3S4H6	Histone H3	5		2	0	1	0
145	J3S9R8	TraB domain-containing protein-like	5	5	1	1	1	1
146	J3SDL3	Tubulin alpha chain	6		2	0	2	0
147	J3RZQ4	Histone H2A	7		1	0	1	0
148	A0A1W7RGR8	Heat shock cognate protein 70	10		3	2	3	1
149	J3S3X5	Actin, cytoplasmic 1	12		4	0	4	0
150	P30894	Venom nerve growth factor	15	6	6	3	2	1
151	P01966	Hemoglobin subunit alpha	15		3	0	2	0
152	K9JDK1	p31 alpha subunit	18	18	24	99	2	2
153	P0CV91	Peroxiredoxin-4 (Fragments)	28		1	0	1	0
154	J3SF57	Putative ribosome biogenesis protein RLP24-like protein			0	7	0	1
155	A0A1W7RH08	78 kDa glucose-regulated protein	12		7	3	6	2
156	J3SE00	Angiotensin-converting enzyme	6		17	4	6	2
157	P13647	Keratin, type II cytoskeletal 5	10	0	3	0	2	0
158	E9JG07	Serine protease (Fragment)	11	15	74	451	3	4
159	P35908	Keratin, type II cytoskeletal 2 epidermal	30	3	30	1	15	1
160	P00761	Trypsin	36	25	41	48	6	4
161	P02533	Keratin, type I cytoskeletal 14	22	1	29	2	9	1
162	P35527	Keratin, type I cytoskeletal 9	39	6	40	3	14	2
163	P13645	Keratin, type I cytoskeletal 10	46	3	28	3	23	2

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DISCUSSION

To profile the venom proteomes of E. Ocellatus, the whole crude venom was subjected to in-solution and in-gel digestion before reverse-phase liquid chromatography ionization high-resolution mass spectrometry (LC-MS/MS) measurements to compare the venom proteome coverage and to observe which methods will reveal greater number of proteins. The study identified 159 and 161 venom proteins using in-solution and in-gel digestion protocols via the LC-MS/MS, respectively. Furthermore, three scenarios were examined based on the proteome −10lgP score percentage to which excludes less significant proteins. The selection criteria of −10lgP score ≥ 5%, ≥ 10% and ≥20% used revealed proteins that were able to be identified in both the approaches. Though the highest number of proteins were identified using the smallest percentage coverage −10lgP 5% score. The analysis revealed more proteins identified using −10lgP 5% in-gel approach. Furthermore, this study showed more proteome coverage can be attained using both in-gel and in-solution at FDR 0.05%. The results indicate that *E. ocellatus* contains enzymatic and non-enzymatic protein families. The enzymatic protein families detected include snake venom metalloproteinase (SVMP), phospholipase A_2 (PLA_2), phospholipase B (PLB), snake venom serine proteinase (SVSP), L-amino acid oxidase (LAAO), 5′-nucleotidase (5′NTD), glutaminyl-peptide cyclotransferase (GC), aminopeptidase (AP), phosphodiesterase (PDE), amine oxidases and aspartic proteases. These enzymes were the main culprit in fostering the venom effects and are the targets for antivenom prediction, production and venom neutralization (Bailon *et al*., 2020).

It was observed that snake venom metalloproteinases (SVMPs) represent the major components of *E. ocellatus* venom proteins and are the most abundant in the enzymatic protein families. The SVMPs are the primary enzymes contributing to the toxicity of crotalid venoms such as those found in North America (Markland and Swenson, 2013). This family of proteins were differentiated into three classes based on their molecular weight (size) and domain properties. Class I (P-I) contain a metalloproteinase (M) domain with 20 – 30 kDa molecular weight; they are the smallest. Class II (P-II) has a pro-domain, proteinase, and disintegrin-like domain connected by a short spacer region to the carboxyl terminus of the domain. P-II has a molecular weight range of 30 – 60 kDa. Class III (P-III) is the largest class with 60 – 100kDa molecular weight, contain pro-domain, proteinase, disintergrin-like domain, and cysteine-rich domain on the carboxy side of the disintegrin-like domain (Markland and Swenson, 2013; Oyama and Takahashi, 2017). Distinct protein belonging to P-III, P-II, and P-I SVMPs (Table 2) were identified.

Several metalloproteinase isoforms were also identified which includes E9JG34 (P-III), E9JG68 (P-III), E9JGB7 (P-III), E9JG79 (P-III), E9JG35 (P-III), E9JG78 (P-III), E9JG62 (P-III), E9JG29 (P-III), E9JG26 (P-III), E9JGH1 (P-II), E9JGH3 (P-II) and E9JGG6 (P-I). Metalloproteinase isoforms identified here were associated with metallopeptidase and toxin activity. SVMPs are famous for their hemorrhagic activity, have fibrinolytic activity, act as prothrombin activators, activate blood coagulation factor X, possess apoptotic activity, inhibit platelet aggregation, pro-inflammatory activity, and can inactivate blood serine proteinase inhibitors (Markland and Swenson, 2013).

The snake venom PLA_2 is classified as secretory PLA_2 ($sPLA_2$) from groups I (*Elapidae* and *Hydrophiidae* snakes) or group II (*Viperidae* and *Crotalidae* snakes) based on their structure, catalytic mechanisms, localization, and evolutionary interactions (Cedro *et al*., 2018). Symptoms observed the moment after snake bites such as oedema, necrosis, thrombogenesis, hypotension, inflammation and pain were mainly caused by PLA_2, phosphodiesterase, 5’ nucleotidase, L-amino acid oxidase, peptides, and C-type lectins which even the human system’s immune is rapidly overshadowed (Oyama and Takahashi, 2017). The likes of these heavy deleteriousness is compared to the pro-inflammatory effects due to imbalances in renin, Angiotensin Converting Enzymes (ACE) and aldosterone which also exacerbate lung injury caused by SARS-COV-2 infections, contributing to the reduction of the pulmonary function and the increase of fibrosis and inflammation, the immune systems likewise cannot ordinarily withstand all these (Mohammed *et al.,* 2021). This venom proteomic study reveals PLA_2 proteins with a range of 13-16 kDa and phospholipase B proteins (F8S101 Phospholipase B and A0A0A1WDU0 phospholipase B like). Preliminary enzymatic screening of the venom using various assay methods reveals the presence of major viper toxins. These include venom phosphodiesterases, phospholipase A_2, alkaline phosphomonoesterase, 5’-nucleotidase, proteases. The activities of these venom proteins were further confirmed by the necrotic activities of 10.3 mm/50 µg venom.

With its diverse functions, PLA_2 was reported to cause rapid necrosis of skeletal muscle fibres, thus being myotoxic as reported earlier (Teixeira *et al*., 2003).

The non-enzymatic proteins identified include C-type lectin/lectin-like (CTL), cysteine-rich secretory protein (CRISP), snake venom nerve growth factor (NGF), disintegrin (DIS), snake venom vascular endothelial growth factor, and snaclec proteins. Furthermore, the venom also exhibited the presence of other non-enzymatic proteins of biological significance. In total, 163 proteins and peptides were detected in the venom. It was well established that regional and intra specie variations play a significant role in determining venom composition (Reeks *et al.,* 2015). Previous proteomic studies on *Echis* venom found in regions other than Nigeria showed the abundance of some of these venom proteins (Alape-Girón *et al*., 2008; Mukherjee 2014; Trummal, 2014; Mukherjee *et al*., 2016; Kalita *et al*., 2017; Leonel *et al*., 2021). These findings are consistent with the main consequences of the local (edema, swelling, bleeding, and pain) and systemic (blood coagulation) manifestations of snakebite by *Echis ocellatus* in Nigeria.

Therefore, the integrative analysis of *Echis ocellatus* venom reveals a strong correlation between proteomic composition and enzymatic function. LC-MS/MS profiling identified 163 proteins and peptides, with SVMPs and PLA_2 as the predominant toxins. The structural abundance of these enzymes aligns with their measured biological activities, confirming their central role in the venom's pathophysiology, including hemorrhage, myonecrosis, and thrombotic effects. The comparative use of in-solution and in-gel digestion methods enhanced proteome coverage, demonstrating the value of combining approaches for comprehensive venom characterization. Functional assays validated the proteomic data, emphasizing that the most abundant proteins are also the most biologically active. These findings have direct implications for antivenom development. Targeting structurally and functionally dominant enzymes such as SVMPs and PLA_2 can improve antivenom efficacy and guide treatment strategies for *Echis ocellatus* envenomation. Future studies should explore correlations between venom proteomics and clinical outcomes to optimize therapeutic interventions.

CONCLUSION

The analysis of Echis ocellatus venom proteomics via in-solution and in-gel digestion methods revealed significant insights into its proteome and enzymatic activities. Especially, snake venom metalloproteinases (SVMPs) emerged as predominant, contributing to the venom's enzymatic activities such as hemorrhage, fibrinolysis, and prothrombin activation. The comparison between in-solution and in-gel digestion methods showed different degrees of protein identification efficiency, with in-gel digestion demonstrating higher proteome coverage in certain protein families like SVMPs and C-type lectins. These findings underscore the importance of selecting appropriate method choice in venom proteomics studies, where the selection of digestion method can influence the depth of proteome coverage and the identification of critical venom components. Thus, the use of both digestion methods to draw a achieve better proteome coverage. Moreover, this study contributes to the understanding of regional variations in venom composition, emphasizing the need for tailored antivenom strategies based on regional venom profiles. Future research directions could focus on correlating venom proteomic profiles with clinical outcomes to enhance antivenom efficacy and treatment protocols for snakebite envenomation caused by *Echis ocellatus*.

Ethical statement

This study was conducted in strict accordance with internationally accepted guidelines for the care and use of laboratory animals. All experimental procedures involving animals and the handling of snake venom were reviewed and approved by the Nigerian National Veterinary Research Institute under approval number AEC/02/74/19.

All animals were housed, fed, and monitored following the standards outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council) and relevant national regulations.

Collection, handling, and extraction of snake venom were performed by trained personnel using appropriate safety and welfare protocols to ensure minimal harm to the snakes and researchers. Venom extraction followed accepted herpetological guidelines and did not involve procedures that would cause lasting injury to the snakes.

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