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Isolation, Purification and Characterization of Hyaluronidase and Phospholipase A_2 Enzymes of *Echis ocellatus* and *Naja nigricollis* Snake Venoms

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ABSTRACT: Issues related to snakebite has for long been of high economic and medical importance. Management and treatment of snake envenomation has always been an issue, especially in remote areas, where anti-snake venom and facilities for its storage are not available, coupled with the high specificity and instability of anti-venom. Venom enzymes are usually responsible for so many tissue necrotic activity after bite. This research was aimed at isolation, purification and characterization of Hyaluronidase and Phospholipase A_2 (PLA2) Enzymes of *Echis ocellatus* and *Naja nigricollis* snake venoms. Isolation and purification of these enzymes were done using a two-step process which included gel filtration on Sephadex G-75, active fractions were applied to ion–exchange chromatography on DEAE (Diethylaminoethyl) cellulose. Individual active fractions of each venom, was then subjected to SDS–PAGE for molecular weight extrapolation. Enzyme characterization was done on the two isolated enzymes for the two snake venoms used. *N. nigricollis* enzymes were revealed to have an optimum temperature of 37 ºC and 55ºC, while that of *E. ocellatus* had 37 ºC and 40ºC, with a pH of 3.5 and 8.0 for both the venom enzymes. Velocity kinetics carried out shows that *N. nigricollis* PLA_2 has the highest V_{max} value of 30.567mg/min, while *E. ocellatus* PLA_2 however has the highest K_m value of 4.5378mg/ml. Purification and characterization done in this research has revealed/confirmed that these venoms contain Hyaluronidase and PLA_2 enzymes, giving a better understanding of the enzymes which will aid in the management and treatment of snake envenomation from these snakes.

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Keywords: *Echis ocellatus*; *Naja nigricollis*, Snake venom; Hyaluronidase; Phospholipase A_2; envenomation.

Envenomation by snake has for long been a serious medical, economic and social problem, causing a mortality rate of about a 100,000 annually. The risk of envenomation by snake is usually higher in rural areas than in urban areas, due to the fact that most of these snakes reside in these rural communities, where many farms, mountains and some other natural biotas are found. Snake venom is a crude mixture of different kinds of compounds, including enzymes such as; protease, phospholipase A, hyaluronidase, L-amino oxidase and phosphodiesterase. This venom is normally produced and stored in glands of venomous snakes, which is a modification of the salivary gland. These glands are however situated on each side of the head below and behind the eye of venomous animals (Hassan *et al*., 2020). Snakes are elongated, legless, carnivorous reptiles of the suborder Serpentes. Like all squamates, snakes are ectothermic, amniote vertebrates covered in overlapping scales (Mallow *et al.*, 2003). Many species of snakes have skulls with several more joints than their lizard ancestors, enabling them to swallow prey much larger than their heads with their highly mobile jaws (Kihiko, 2013) . Snakebite detrimental hemostatic and necrotic effects were shown to be directly involved in the proteolytic events caused by different venom enzymes and toxins present in snake venom, which subsequently results in blood coagulation, fibrinolysis and platelet

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aggregation among others. Snake venom enzymes also interfere with many normal body biological processes, distorting the immune systems and causing organs inflammation (Nasio, 2016). *Echis ocellatus* is one of the venomous viper snake species predominantly found in West Africa, commonly known as the West African carpet viper (Alirol *et al*., 2010). Its envenomation proves to be one of the most fatal snake bites in West Africa. Its venom has been reported to contain a prothrombin activating procoagulant, haemorrhagin and cytolytic fractions which causes haemorrhage, incoagulable blood, shock and local reactions/necrosis (Hasson *et al.*, 2003). Another snake of high economic and medical importance is the *N. nigricollis* which is also known as the black-necked spitting cobra. These species of spitting cobra are mostly found in sub-Saharan Africa. They are moderately sized snakes that can grow to a length of 1.2 to 2.2 m. They prey primarily on small rodents. They possess medically significant venom, like other spitting cobras, they can eject venom from their fangs when threatened (one drop over 7metres and more in perfect accuracy). Their neurotoxic venom irritates the skin, causing blisters and inflammation and can cause permanent blindness if the venom makes contact with the eyes and is not washed off immediately (Wüster *et al*., 2007). This research work was therefore carried out to isolate, purify and characterize Hyaluronidase and Phospholipase A_2 (PLA2) enzymes of *Echis ocellatus* and *Naja nigricollis* snake venoms.

MATERIALS AND METHOD

Snake Venom Sample Collection: Lyophilized venom of *E. ocellatus* and *N. nigricollis* (400mg each) was purchased from the snake lab of Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Kaduna Nigeria and was aseptically transported and stored at -4^{0}C until used.

Enzyme Isolation: Hyaluronidase Enzyme Purification: Hyaluronidase from the two venoms were isolated and purified using a combination of two purification steps; gel filtration chromatography on sephadex G-75 and ion-exchange chromatography on DEAE cellulose.

Gel-Filtration on Sephadex-G75: Gel Preparation: The gel was prepared by dissolving 20g of sephadex G-75 in 100ml sodium acetate buffer, pH 6 for 24 hours at room temperature and mixed with a glass rod to make the swollen particles form slurry.

Filtration: The prepared gel slurry was then poured into a (10mm by 30cm) column plugged with cotton wool at the bottom. The column was first equilibrated with 0.1M sodium acetate buffer, pH 6.0, before the sample was applied. Fractions of 3ml each was collected at a flow rate of 1ml/122sec, after which total protein and enzyme activity were determined as previously described (Sutti *et al.*, 2014).

Ion Exchange Chromatography on DEAE Cellulose: Gel Preparation: DEAE-cellulose was prepared by dissolving 10g of the anion-exchanger in 100 ml of 0.1M sodium acetate buffer, pH 6.0.

Filtration: The slurry was poured into a 300mm column. Fraction with highest activity from the gel-filtration step was pooled and loaded onto the DEAE cellulose column equilibrated with the same buffer. Fractions were eluted under a linear concentration gradient of sodium chloride solution (1.0M). 3ml fractions were collected at a flow rate of 1ml per 1.33 minute and analyzed for total protein and enzyme activity. The fractions showing the highest activity were analyzed on SDS PAGE (Sutti *et al*., 2014).

Determination of Hyaluronidase Activity: Hyaluronidase activity was determined by a turbidimetric method. The working solution for this step consisted of a 200µL buffer (0.2 M sodium acetate, pH 6.0, containing 0.15 M NaCl), 200µL of substrate (hyaluronic acid from 1mg/mL in acetate buffer) and 100µL of enzyme (20 µg) to give a total reaction volume of 500µL. This mixture was incubated for 15 minutes at 37°C after which the reaction was stopped by adding 2 mL of hexadecyltrimethylammonium bromide in 2.5% NaOH. The resulting turbidity was read at 400 nm in a SpectraMax® 340 microplate reader after 30 minutes of incubation at room temperature. One unit of activity corresponded to the amount of enzyme that produced a 50% reduction in turbidity caused by 200µg of substrate under the conditions described above.

Purification of PLA_2 Enzyme: Gel Preparation: The two gels were prepared as previously described. 200mg of each of the snake venom sample was dissolved in 10ml equilibration buffer (0.05M Tris-HCl, pH 6.8); and was loaded on Sephadex G-75 column (2.6 X 50Cm). The sample dissolving buffer was used in equilibration of the Sephadex column and elution of the loaded samples. Fraction of 3ml was then collected at a flow rate of 30ml/hr using fraction collectors. Fraction with PLA_2 activity was recovered, pooled and directly applied to a DEAE cellulose column (1.6 X 25Cm) pre-equilibrated with the same buffer and eluted with a linear NaCl gradient from 0 to 1.2M in the same buffer (Hassan et al., 2020).

PLA_2 Activity Assay: A 0.5 ml of egg yolk suspension (4 mg ml^{-1}) was introduced into a clean test tube

containing 50µL of 1mM $CaCl_2$. To this, 100µL of venom fraction was added and incubated at 37°C for 1 h. Thereafter, the activity was stopped, by heating at 100°C for 2 min. A drop of phenolphthalein was added and then titrated against 2mM NaOH solution to an end point. The same procedure was carried out in the absence of the enzyme, to obtain the titre value for the blank, for an adequate comparison to deduce the effect of the enzyme on the yolk (deduction of any FFA released). The activity of phospholipase A_2 was defined as the amount of enzyme required to hydrolyze 1mg of FFA from the lecithin present in the egg yolk under the standard conditions (Chinyere *et al.,* 2016).

SDS PAGE Electrophoresis: Chromatographic isolated fractions were evaluated by SDS-PAGE, performed on 12 % gels using a MiniVE 10 × 10 cm Vertical Gel Electrophoresis System (GE Healthcare, USA), according to Laemmli (Laemmli, 1970). Samples were prepared using reducing buffer containing SDS and β-mercaptoethanol, followed by heating at 100 °C for three minutes. After running (15 A, 120 V), the gels were stained with Coomassie brilliant blue R250. The molecular weight standard used was Unstained Protein Molecular Weight Marker (6.5-200kDa, Thermo Fisher Scientific, Inc., USA). The gel was washed with a buffer containing 0.015 M Tris–HCl, 5% formamide and 20% isopropyl alcohol, pH 7.9, and photo documented (Laemmli, 1970).

Physicochemical Characterization of the Enzyme: The optimum pH was determined by changing the buffers of the enzymatic turbidimetric assay as follows: 0.1 M sodium citrate, pH 2.0 to 8.0, 0.1 M sodium acetate, pH 4.0 to 6.0 and 0.1 M Tris–HCl, pH 5.5 to 8.0 (0.15 M NaCl was added to all buffers). The optimal working temperature of the enzyme was evaluated by adjusting assay temperature between 10 and 70°C. The thermal stability of the enzyme was tested by pre-incubating enzyme solution for 15 minutes under a temperature ranging from 25 to 80°C.

Initial velocity studies: The enzymes velocity studies were analyzed using standard procedures as described by Nwune, (2016). A Michaelis-Menten plot was done to check enzymes studies towards the model assumptions. The activities of hyaluronidase and phospholipase A_2 (V_0) were determined in the presence of various concentrations of substrates (0.4, 0.6, and 0.8mg/ml). The data obtained was then used to plot the Lineweaver-Burk plot to determine the kinetic parameters K_m and V_{max} (Hassan *et al.*, 2020)

Statistical Analysis: Some of the data obtained were presented as mean ± standard deviation of three determinations. The analysis of variance was used to compare the paired means; the $P < 0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Purification and molecular mass determination of Hyaluronidase and PLA_2: Result for Hyaluronidase and PLA_2 enzymes isolation from *E. ocellatus* and *N. nigricollis* snakes venoms are shown in Tables 1 to 4. Using a two-way purification step (gel filtration chromatography on sephadex G-75 and ion exchange chromatography on DEAE cellulose). Partially purified Hyaluronidase from *E. ocellatus* was shown to have a final total protein, enzyme and specific activities of 0.051mg/ml, 1.257TRU/mg and 22.115TRU respectively shown in Table 1. While that of *N. nigricollis* was 0.067mg/ml, 1.49TRU/mg and 22.129TRUas shown in Table 2.

Partially purified PLA_2 from *E. ocellatus* was shown to have a final total protein, enzyme and specific activities of 0.086mg/ml, 3.747(µmol/min), 46.154(mol/min/mg) as presented in Table 3. While that of *N. nigricollis* was revealed to have 0.078mg/ml, 2.496(µmol/min) and 32.100(mol/min/mg) respectively.

Molecular weight of the enzymes from the two venoms as determined by SDS-PAGE is shown in Figures 1 and 2. Hyaluronidase and Phospholipase A2 of *E. ocellatus* Venom were 30kDa and 14kDa respectively, while those of *N. nigricollis* Venom were 26kDa and 12.5kDa respectively.

Table 1: shows the purification steps for enzyme Hyaluronidase isolated from *E. ocellatus* venom using a two-step purification protocol (Gel filtration and ion exchange chromatography). Table 2: shows the purification steps for enzyme PLA_2 isolated from *E. ocellatus* venom using a two-step purification protocol (Gel filtration and ion exchange chromatography).

Physicochemical Characterization of Partially Purified Venom Enzymes: Results from the characterization of the partially-purified enzymes are presented in Figures 2a-2d, 3a and 3b and Table 5. The optimum temperature for *E. ocellatus* Hyaluronidase and *N. nigricollis* Hyaluronidase is 37^{o}C. While the optimum temperature for the *E. ocellatus* Phospholipase A2 and *N. nigricollis* Phospholipase A2 are 40^{o}C and 55^{o}C respectively. Also the optimum pH for the hyaluronisade and PLA2 from the two snake venoms are 3.5 and 8.0 respectively.

Table 1: Purification of Hyaluronidase from *E. ocellatus* Venom

Enzyme	Step	Total Protein (mg/ml)	Total Enzyme Activity (TRU/mg)	Specific Activity (TRU)	Purification Fold	Yield (%)
Hyaluronidase	Crude	0.208	2.131	10.227	1	100
	Gel filtration on sephadex G-75	0.124	1.969	15.840	1.5	92
	Ion exchange on DEAE -52 Celullose	0.051	1.257	22.115	1.4	52

Table 2: Purification Table for Hyaluronidase from *N. nigricollis* Venom

Enzyme	Step	Total Protein (mg/ml)	Total Enzyme Activity (TRU/mg)	Specific Activity (TRU)	Purification Fold	Yield (%)
Hyaluronidase	Crude	0.258383	3.879022	15.0126	1	100
	Gel filtration on sephadex G-75	0.185397	2.954326	15.93513	1.1	76
	Ion exchange on DEAE -52 Celullose	0.067343	1.490221	22.12882	1.5	50

Table 3: Purification of PLA_2 from *E. ocellatus* Venom

Enzyme	Step	Total Protein (mg/ml)	Total Enzyme Activity (µmol/min)	Specific Activity (mol/min/mg)	Purification Fold	Yield (%)
PLA_2	Crude	0.354742	4.267348	12.029441	1	100
	Gel filtration on sephadex G-75	0.184431	3.95723	21.456425	1.70	93
	Ion exchange on DEAE -52 Celullose	0.086305	3.746543	46.15385	2.15	86

Table 4: Purification Table for PLA_2 from *N. nigricollis* Venom

Enzyme	Step	Total Protein (mg/ml)	Total Enzyme Activity (µmol/min)	Specific Activity (mol/min/mg)	Purification Fold	Yield (%)
PLA_2	Crude	0.383654	4.027221	10.49701	1	100
	Gel filtration on sephadex G-75	0.164453	3.184615	19.36489	1.844801	79
	Ion exchange on DEAE -52 Celullose	0.077761	2.49615	32.10028	3.05804	62

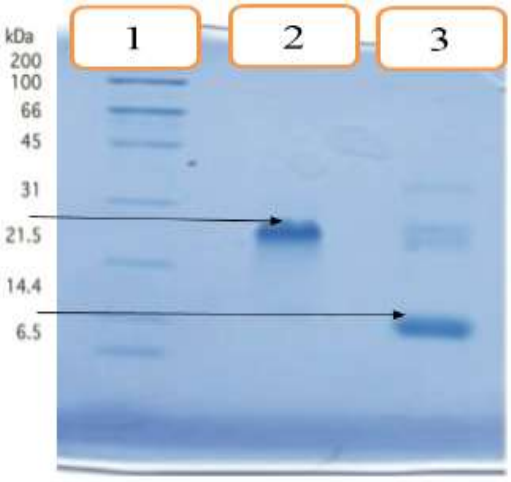

Fig 1: SDS-PAGE Analyses of Partially Purified Hyaluronidase and Phospholipase A_2 of *E. ocellatus* Venom
KEY: Lane 1 = Molecular weight marker, Lane 2 = Hyaluronidase, Lane 3 = Phospholipase A_2 Hyaluronidase – 30kDa PLA$_2$ – 14kDa

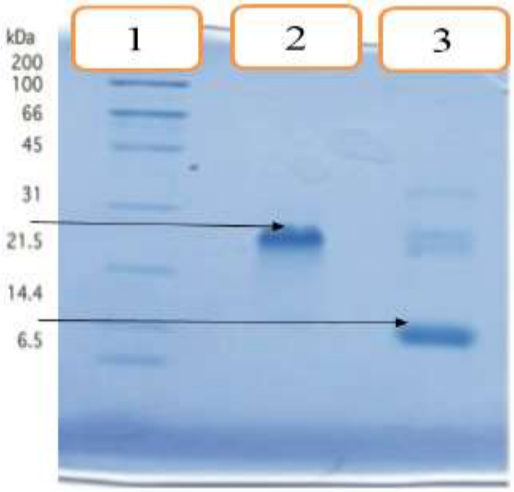

Fig 2: SDS-PAGE of Partially Purified Hyaluronidase and Phospholipase A_2 of *N. nigricollis* Venom
KEY: Lane 1 = Molecular weight marker, Lane 2 = Hyaluronidase, Lane 3 = Phospholipase A_2 Hyaluronidase – 26kDa; PLA$_2$ – 12.5kDa

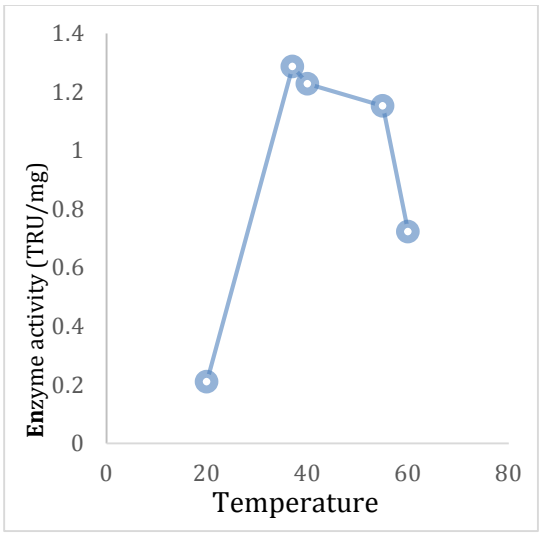

Fig 2a: Effect of Temperature on *E. ocellatus Hyaluronidase*

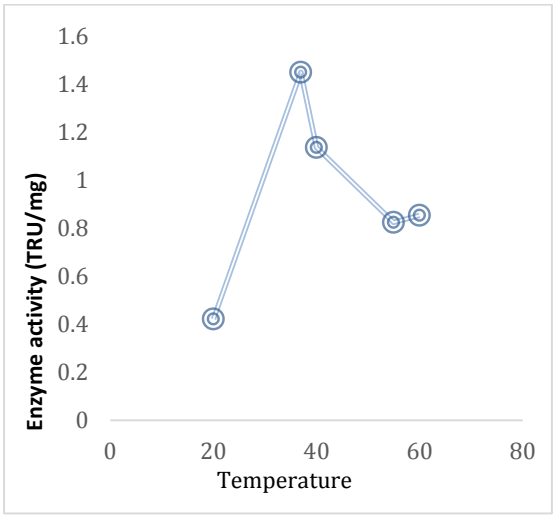

Fig 2b: Effect of temperature on *N. nigricollis Hyaluronidase*

Snake envenomation has been one of man's greatest health concern, for quite a long time, causing countless number of fatalities, infections and numerous health defects. Snake venom enzymes are responsible for so many detrimental bio-inflammatory effects encountered from snakebite (Nasio, 2016). PLA_2 enzyme for example has been shown to possess anticoagulant properties, hence inhibiting blood coagulation. PLA_2 also exhibits quite a high edematogenic and myotoxic activities, demonstrating its capacity to contribute to tissue damage after snake bite (Vineetha et al., 2017). Another important enzyme found in snake venoms of utmost importance is the Hyaluronidase, which aids in tissue degradation and necrosis, hence providing way/entry point for the other toxins enzymes into the body. In this study, Hyaluronidase and Phospholipase A_2 enzymes were isolated, purified and characterized from the venoms of *E. ocellatus* and *N. nigricollis.* Using a two-way step purification (gel filtration chromatography on sephadex G-75 and ion-exchange chromatography on DEAE cellulose).

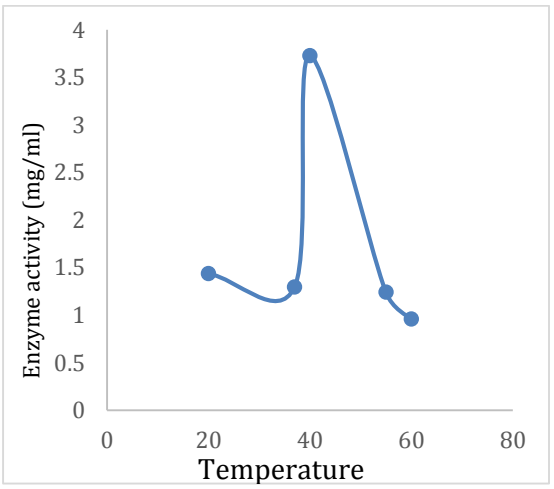

Fig 2c: Effect of Temperature on *E. ocellatus* PLA_2

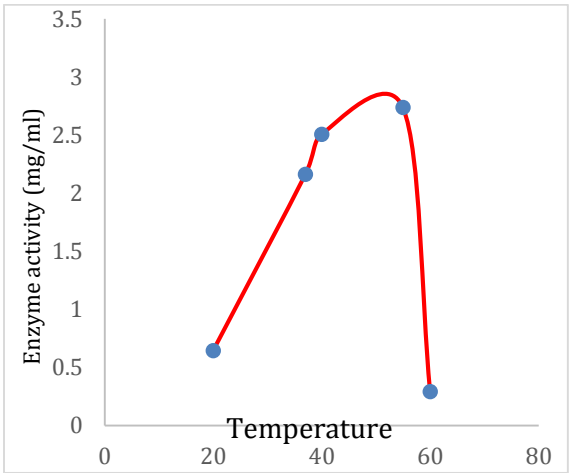

Fig 2d: Effect of Temperature on *N. nigricollis* PLA_2

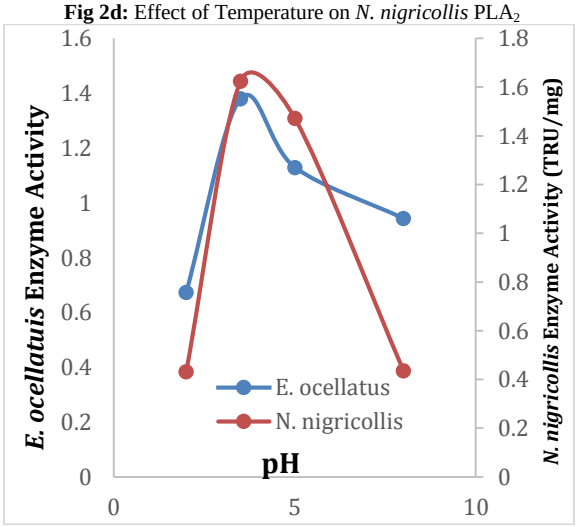

Fig 3a: Effect of pH on *E. ocellatus* and *N. nigricollis* Hyaluronidase

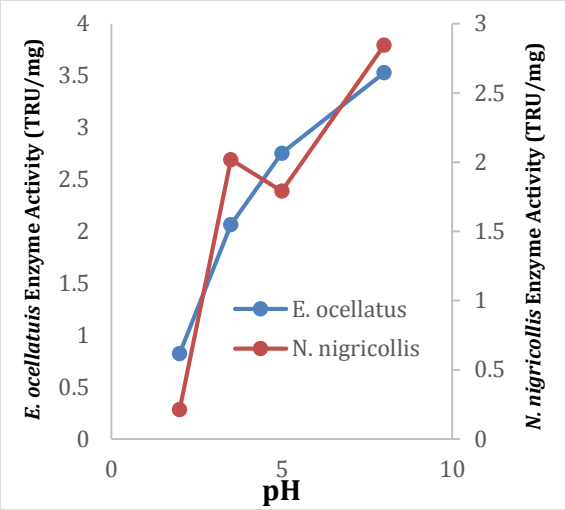

Fig 3b: Effect of pH on *E. ocellatus* and *N. nigricollis* PLA_2

Purified Hyaluronidase from *E. ocellatus* was shown to have a final total protein, enzyme, specific activities, purification fold and yield of 0.050956mg/ml, 1.257338TRU/mg, 22.115236TRU, 1.4 and 52 respectively. While that of *N. nigricollis* was revealed to have 0.067343mg/ml, 1.490221 TRU/mg, 22.12882 TRU, 1.5 and 50. Purified PLA_2 from *E. ocellatus* was shown to have a final total protein, enzyme, specific activities, purification fold and yield of 0.086305mg/ml, 3.746543(µmol/min), 46.15385(mol/min/mg), 2.15 and 86 respectively. While that of *N. nigricollis* was revealed to have 0.077761mg/ml, 2.49615(µmol/min), 32.10028(mol/min/mg), 3.05 and 86 respectively. Higher total protein and enzyme activity seen in *E. ocellatus* venom might suggest a higher physiological activity of its venom than in *N. nigricollis* venom.

Table 5: Result for Enzyme Velocity Studies

Enzymes	Kinetic Parameters	*
E. ocellatus	K_m (mg/ml)	0.749
Hyaluronidase	V_{max} (tru/min)	2.256
N. nigricollis	K_m (mg/ml)	1.386
Hyaluronidase	V_{max} (tru/min)	4.212
E. ocellatus PLA_2	K_m (mg/ml)	4.538
	V_{max} (mg/min)	20.534
N. nigricollis	K_m (mg/ml)	1.189
PLA_2	V_{max} (mg/min)	30.567

Results from the SDS-PAGE analysis of the two isolated venoms enzymes revealed proteins with molecular weight of 30KDa and 14kDa respectively for Hyaluronidase and PLA2 from *E ocellatus*, while that of *N. nigricollis* were 26kDa and 12.5kDa. These correspond with the studies done by (Ketelhut *et al.*, 2003), in which he revealed that PLA_2 isolated from *Bothrops jararacussu* snake venom has a molecular weight of 15kDa and that of (MARUÑAK *et al.*, 2007), which reported that the PLA_2 isolated from snake has molecular weight 15.6kDa. Similarly (Kolarich *et al.*, 2005) also reported isolation of snake venom hyaluronidase from *Vespula vulgaris* with molecular weight of 15kDa. Understanding the characteristics of Hyaluronidase and PLA_2 from snake venoms is important for venom researchers, as it would help in the production and management of effective therapeutic antivenom, considering the role of the enzymes in envenomation. That was one of the reasons why enzyme characterization was done in this research on the two isolated enzymes for the two snakes venom used. Temperature optimization assay was done, in which Hyaluronidase and PLA_2 from *E. ocellatus* was found to be more active and viable at optimum temperatures of 37ºC and 40ºC respectively. While those of *N. nigricollis* were shown to have optimum temperatures of 37ºC and 55ºC respectively. The optimal pH for hyaluronidase and PLA2 from *E. ocellatus* and *N. nigricollis* were 3.5 and 8.0 respectively.This is similar to what was observed by (Ushanandini *et al.*, 2006), which showed Hyaluronidase and PLA_2 to have an optimal temperature of 35ºC and 37ºC and a pH of 4 and 8. The increase in body temperature of snake bite victims above the normal physiological temperature could also make the condition favorable for PLA_2 to exert its hydrolytic function effectively. Research done by (Sutti *et al.*, 2014) on purification and characterization of a hyaluronidase from venom of the spider *Vitalius dubius* also reported a similar enzyme physicochemical conditions with this research. Kinetic of enzyme help in revealing the various enzyme kinetics velocity parameters in the presence and absence of substrate or inhibitor. Velocity kinetics carried out showed that *N. nigricollis* PLA_2 had the highest V_{max} value of 30.567mg/min, while *E. ocellatus* Hyaluronidase has the lowest V_{max} of 2.2563tru/min. *E. ocellatus* PLA_2 however had the highest K_m value of 4.5378mg/ml. These results are consistent with the results of the work done by (Chinyere *et al.*, 2016) on the effects of aqueous root extract of *Annona senegalensis* on *Bitisarietans* venom protease and phospholipase A_2 activities.

Conclusion: Both *E. ocellatus* and *N. nigricollis* snake venoms were shown to contain Hyaluronidase and PLA_2 enzymes, which contributes to the various detrimental snake bite effects. Furthermore, *N. nigricollis* enzymes had temperature optima of 37ºC and 55ºC, while that of *E. ocellatus* had 37ºC and 40ºC, with a pH of 3.5 and 8.0 for both the venom enzymes. *Naja nigricollis* PLA_2 had the highest V_{max} value of 30.567mg/min, while *E. ocellatus* PLA_2 had the highest K_m value of 4.5378mg/ml. Data obtained from this research will aid in the selection of

appropriate inhibitors for the snake enzymes which could serve as the key for the management and treatment of snake envenomation.

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