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Isolation and enzymatic characterization of the snake venom hyaluronidase from *Naja melanoleuca* (Forest cobra) venom

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Abstract
Hyaluronidase is ubiquitous enzyme in snake venoms components, known originally as "spreading factor" because they cleave hyaluronan found in the extracellular matrix (E.C.M) of connective tissue, facilitating toxin diffusion into the tissue and blood circulation of the prey/victims. The purification procedure of a hyaluronidase from *Naja melanoleuca* venom is described. It involves basically size exclusion chromatography on Sephadex G-75 at pH 6.0, followed by re-chromatography of active fractions on ion exchange chromatography using DEAE-cellulose. The specific activity of purified enzyme was 0.25 TRU/mg against 0.03 TRU/mg for the crude venom, representing 8.62 purification fold. The purified hyaluronidase has molecular weight of approximately 54 KDa on SDS-PAGE. The enzyme had optimal pH 6 and temperature 38 ^{0}C respectively. Its K_M was found to be 0.381×10^{-3} mg/min at 38 ^{0}C. This research showed that *N. melanoleuca* hyaluronidase exhibited high affinity for hyaluronic acid.

Keywords: Enzymatic characterization, snake venom hyaluronidase, *Naja melanoleuca*

Introduction
Snakebites cause considerable morbidity and mortality worldwide with the highest burden found in South Asia and Sub-Saharan Africa [1]. The annual incidence of snakebites worldwide is about 5 million with about 100, 000 to 200, 000 deaths [2, 3]. In 2009, WHO listed snakebite as a neglected tropical disease, recognizing its importance alongside many infectious diseases [4]. Incidences of snakebites are common among farmers and cattle readers (Adaudi and Adeyanju, 1989). Bites occur more often while victims are farming, herding or walking; although, the spitting cobra may bite victims who rolled upon the cobra in their sleep [5].
Snake envenomation involves subcutaneous or intramuscular injection of venom into the prey/human victims. The pathology of envenomation includes both local and systemic effects such as neurotoxicity (pre/postsynaptic), myotoxicity, cardiotoxicity, coagulant (pro/anti), hemostatic (activating/ inhibiting), hemorrhagic, hemolytic and edema forming activities [6]. Snake venoms comprise complex mixtures of enzymatic and non-enzymatic proteins, peptides and small organic compounds, such as citrate, nucleosides and acetylcholine [7]. Hyaluronidase is ubiquitous enzyme in snake venoms components, known originally as "spreading factor" [8] because they cleave hyaluronan, a proteoglycan found in the extracellular matrix (E.C.M) of connective tissue, facilitating toxin diffusion into the tissue and blood circulation of the prey/victims [9]. Although they are not toxic [8], they indirectly potentiate the toxicity of venoms, contributing to local and systemic envenoming [10].
Hyaluronidase enzyme has been purified and investigated for its physio-chemical and biological activities form diverse sources of venom such as Bee [11], Scorpion [12], Spider [13] and Snake [14]. In this study, we report for the first time the purification of enzyme hyaluronidase from *Naja melanoleuc*a venom and partially characterized its physic-chemical properties.

Materials and Methods
Materials
Lyophilized *N. melanoleuca* was bought from department of Pharmacology, Ahmadu Bello University, Zaria. General laboratory chemicals were obtained from Biochemistry department,

Bayero University, Kano and Sigma Aldrich Chemical Company. The chemicals are: Sodium acetate, Acetic acid, Hydrochloric acid, Sodium chloride, Sodium hydroxide, Glycine, Bovine serum albumin, Comassie brilliant blue G-250, Sephadex G-75, DEAE-cellulose, 40% Acrylamide (37.5:1), Ammonium Persulfate (APS), Tris-free base, Sodium Dodecyl Sulfate (SDS), 2-Mecaptoethanol, Bromophenol Blue, Coomasie Brilliant Blue R-250.

Methods

Enzyme Purification

Gel-filtration on Sephadex G-75

Lyophilized *N. melanoleuca* venom (20 mg in 2 ml of sodium acetate buffer, pH 6.0) was applied to a Sephadex G-75 column (1.6 × 98 cm) equilibrated with 0.1 M NaCl. The column was eluted using sodium acetate buffer, pH 6.0 with a flow rate of 1 ml/min and 3 ml fractions were collected. Protein content and hyaluronidase activity were assayed. Fractions having enzyme activity were pooled.

Ion-exchange chromatography on DEAE-cellulose

Hyaluronidase samples from the Sephadex G-75 column were loaded onto sephadex G-25 column (1.6 × 35 cm), equilibrated in sodium acetate buffer, pH 6.0. Sub-fractions were then eluted stepwise using 0 a convex concentration gradient of sodium chloride solution (0 to 1M). Fractionation was carried out at 20 °C at a flow rate of 1 ml/min and 3 ml fractions were collected. Finally fractions having enzyme activity were pooled.

Determination of total protein concentration

This was determined by the method of Bradford [15] using bovine serum albumin as (BSA) as a standard.

Determination of Hyaluronidase Activity

This was determined based on the method of Tolksdorf *et al*. [16] and that of Kass and [17]. The assay measures the turbidity resulting from hyaluronic acid and albumin reagent in an acidic condition; hyaluronidase activity is measured as lack of turbidity. Standard curve of hyaluronic acid turbidity was constructed by dilution of hyaluronic acid stock solution (0.4 mg/ml) using 0.1M of sodium phosphate, buffer pH 4.2 containing 0.15M sodium chloride in to various concentrations in a test tubes ranging from 0.2 to 4.0 mg. Tubes were placed in a boiling water bath for 5 minutes. Tubes were allowed to cool at room temperature. 9ml of albumin reagent was added and allowed to stand for 10 minutes. Absorbance was read at 420 nm. Absorbance versus mg of hyaluronic acid was plotted as standard curve.

For hyaluronidase activity, 0.5ml of a 0.8 mg/ml hyaluronic acid solution was pipetted into series of test tubes. The tubes were Incubated at 37 ^{0}C for 4-5 minutes to achieve temperature equilibrium. For a blank, 1ml of 0.1m sodium phosphate buffer pH 5.3 with 0.15M sodium chloride) was added. For test, 0.5ml of enzyme fractions was added. The tubes were then incubated exactly 10 minutes and cooled in an ice bath to room temperature. 9.0ml of albumin reagent was added to each tube and incubated at room temperature for 10 minutes. Absorbance was read at 420nm.

Sodium dodecyl sulphate polyacrylamide-electrophoresis (SDS-PAGE)

SDS PAGE was performed according to the method of Lammelli [18]. The samples and the protein markers were run on 12% gel using Tris-glycine buffer pH 8.3 and SDS. The gel was stained using Silver stain to visualize the bands.

Optimum pH and Temperature Determination

The optimum pH value for enzyme activity was determined using buffers ranging from 2.0 to 10.0. The optimum temperature (0 – 60 °C) was studied at various temperatures using standard assay conditions. The assays were performed in triplicates. The conditions which provide the maximum activity would be used in further experiments [19].

Kinetic Studies

The kinetic parameters (Km and Vmax) of the purified hyaluronidase were determined by linear regression from Lineweaver-Burk plot with hyaluronidase (0.2mg–1mg) as substrate [19].

Results

Hyaluronidase Purification

When *N. melanoleuca* venom (20mg) was fractionated on a sephadex G-75 column, seven peaks were resolved. Fractions with the hyaluronidase activity (Fig. 1) were pooled. About 67% of the activity and 9% of the protein loaded onto the column were recovered in the pooled fraction. Sub-fractionating was proceeded using DEAE cellulose ion exchange chromatography. Of the three peaks resolved, only peak 2 showed hyaluronidase activity (Fig. 2). About 43% of the activity and 5% of the protein loaded were recovered in fraction. It has a specific activity of 12 times higher than the crude venom and account 40.5% of the total activity. A summary of the purification is given in table 1.

N. melanoleuca venom and the hyaluronidase active fractions derived from the two chromatographic steps were subjected to 12% SDS-PAGE, and gel was stained with coomassie Blue. Plate 1, Shows that there were numerous bands in crude venom and two bands in the gel filtration fraction, but only a single band at approximately 54 KDa in the DEAE cellulose fraction.

Table 1: Two stage purification profile of Hyaluronidase from *N. melanoleuca* venom

Purification Stage	Protein (mg/ml)	Total activity (TRU/ml)	Specific activity (TRU/mg)	Activity yield (%)	Purification Fold
Crude venom	13.380	0.395	0.03	100	1
Sephadex G-75	1.227	0.264	0.22	66.84	7.59
Cellulose DEAE-52	0.659	0.168	0.25	42.53	8.62

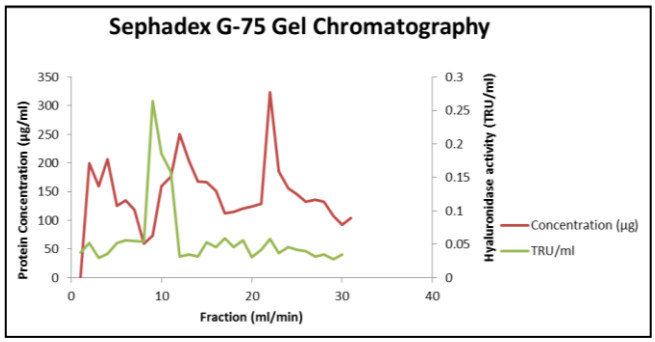

Fig 1: Elution profile of Hyaluronidase from *Naja melanoleuca* venom. Fractionation on sephadex G75: crude venom (20mg) dissolved in 5ml of sodium acetate buffer pH 6.0., was applied to the column (2 by 100cm) and elution was carried out at a flow rate of 1ml/minute at 25 °C with sodium acetate buffer pH 6.0.

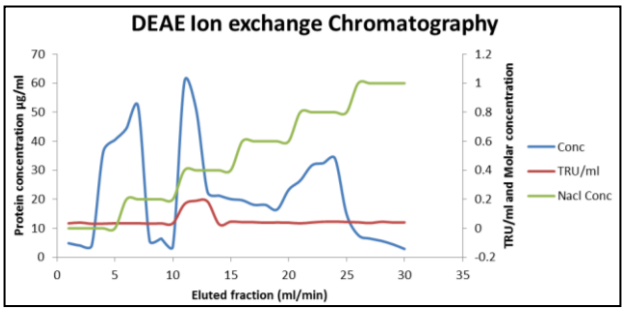

Fig 2: Elution profile of Hyaluronidase from *Naja melanoleuca* venom. Fractionation on DEAE-cellulose: pooled fraction from gel filtration (5ml) was applied to the column (30cm) and elution was carried out at a flow rate of 1ml/minute at 25ºC with convex sodium chloride gradient from 0.2M to 1.0M

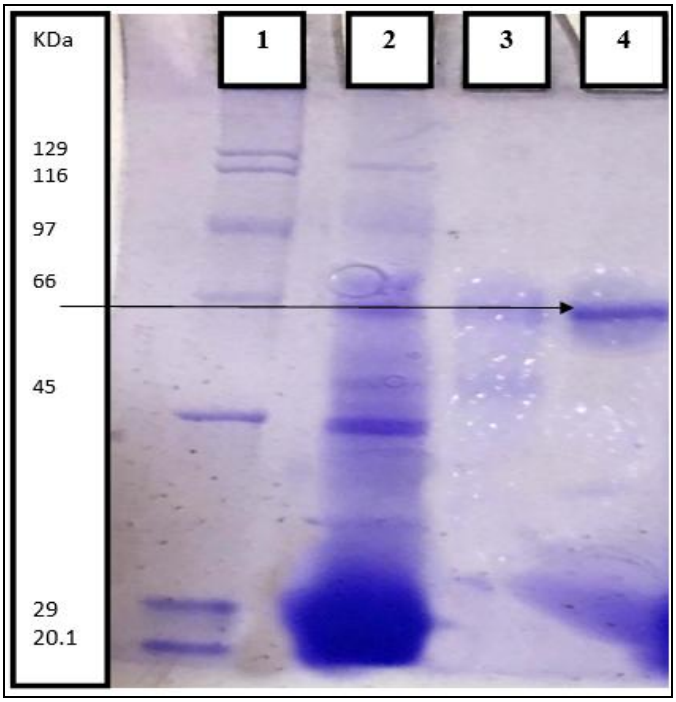

Plate 1: SDS-PAGE analysis of the Crude *N. melanoleuca* venom (Lane 2), active hyaluronidase fraction obtained from gel filtration (Lane 3) and active hyaluronidase fraction obtained from Ion exchange chromatography (Lane 4). Molecular marker is shown in Lane 1.

Hyaluronidase Characterization

The investigation of the physicochemical characteristics of the purified enzyme showed that the activity of the hyaluronidase increased as the pH was raised from 2 (Figure 3), and maximum activity was seen at pH 6 after which there was gradual decrease. Also hyaluronidase activity increased as the temperature was raised from 20, and the highest activity was observed at 38 ^{0}C (Figure 4).

When a fixed amount of purified hyaluronidase was incubated with increasing amount of hyaluronic acid for 10 min at pH 6 and 38^{O}C, the V_{max} and K_m were 0.025 $\times 10^{-3}$ mg/ml and 0.381×10^{-3} mg/min respectively as shown in figure 5.

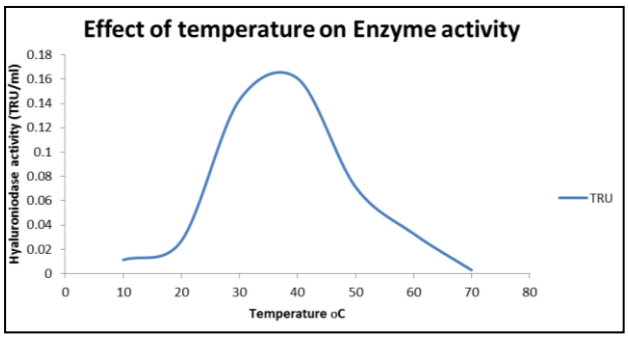

Fig 3: Effect of temperature on activity of hyaluronidase purified from *N. melanoleuca* venom.

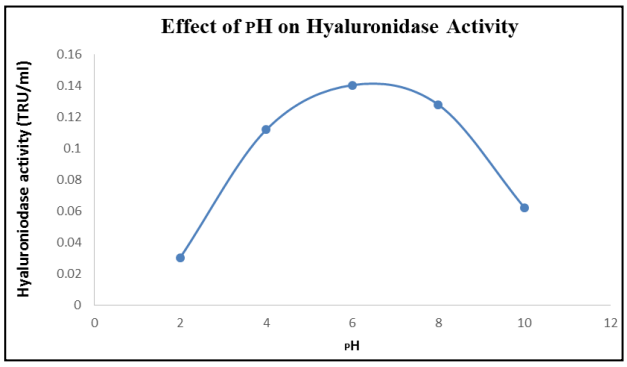

Fig 4: Effect of pH on activity of hyaluronidase purified from *N. melanoleuca* venom.

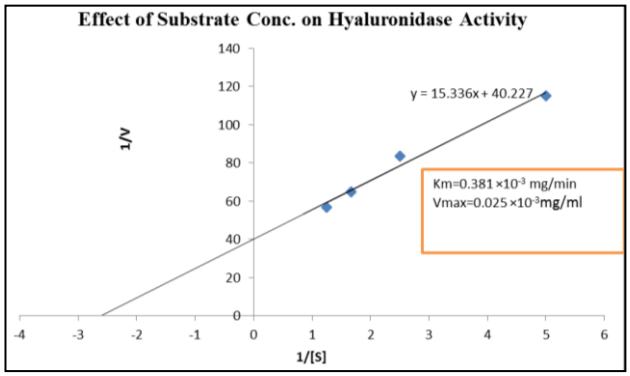

Fig 5: Lineweaber-Burk's plot for substrate specificity of hyaluronidase purified from *N. melanoleuca* venom.

Discussion

Pathophysiological mechanism of sanke venom in duced tissue damage and spreading facilitated diffusion of systemic toxins is currently gaining importance. Venom contains mixture of toxins and envenomation-induced effects are due to synergistic effects of these toxins. Metalloprotease and hyaluronidase are the main components involved in local tissue damage and gravitational spreading of the venom. The pathology involves the degradation of proteins and glycosaminoglycan's in the extracellular matrix (ECM)

surrounding the blood vessels and capillaries [20]

In this study, two successive steps of fractionation of *N. melanoleuca* venom through gel filtration on a sephadex G-75 column and Ion exchange chromatography on a DEAE cellulose resulted in purification of hyaluronidase enzyme as shown if figure 1 and 2. The specific activity of purified hyaluronidase enzyme was 0.168TRU/ml against 0.395 TRU/ml for crude venom, which presented almost a fold decrease in the activity, Grish and Kemparaju [19] reported similar case. Purified hyaluronidase represents 4.93% of the crude venom total protein, and has a specific activity of 0.25 TRU/mg with a purity of 8.72. In other similar research, Grish *et al*., [8], reported hyaluronidase represents 0.15% of the crude venom protein. The enzyme yield considered low after purification, mainly because the enzyme is present at low concentration in the total venom.

SDSPAGE under non-reducing conditions testified the purity of hyaluronidase enzyme. The enzyme was a monomeric protein as observed under the electrophoretic pattern under non-reducing conditions. Most of the hyluronidases isolated have been reported to be monomeric proteins [21]. The observed molecular mass of 54 KDa is well within the range of 33–110 kDa reported for hyaluronidases from venoms of snakes, bees, scorpions, stonefish and lizards [19].

The hyaluronidase enzyme isolated from N. melanoleuca showed maximum activity at pH 6. Hyaluronidases from snake, scorpions, bees are optimally active at 4-6 pH range *in vitro*, and they are either neutral (5-7 pH) or acidic (3-4 pH) [22]. From our characterization studies, it appears that the purified enzyme falls into the neutral range of hyaluronidases. It exhibited maximum activity at 38 °C and was completely denatured at and above 60 °C. The thermal conditions are highly important for the enzyme activity. Similar results were seen in purification of hyaluronidases from Venom of *Bungarus caeruleus* (Indian Common Krait). [23]

Result from Lineweaber plot (Figure 5) showed that purified hyaluronidase enzyme has K_m 0.381×10^{-3} mg/ml, exhibited low affinity of the hyaluronic acid for enzyme catalytic site compared to other hyaluronidases. This value was substantially lower than that reported of *Agkistrodon acurus* (6.2×10^{-3} mg/ml [23], which showed that the enzyme is highly specific to hyaluronic acid substrate.

Conclusion

In conclusion, this study presents the first purification of a hyaluronidase from the *Naja melanoleuca* venom. This enzyme exhibited high affinity for hyaluronic acid. Further inhibitory studies analyses might provide an insight for the better understanding of the role.

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