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Isolation and Purification of a fibrinogenase from *Najanigricollis*venom

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Abstract
Snake venom metalloproteinases possessing fibrinogenolytic activity have been generating interest due to their clinical potential for the treatment of occlusive vascular diseases. In this study, a fibrinogenase from the venom of Najanigricollis was purified by chromatography on sephadex G-75 and DEAE-cellulose. Najanigricollisvenom crude preparation was separated into three peaks on sephadex G-75 and a single peak on DEAE-cellulose. The purified enzyme designatedNaja fibrinogenase, was homogenous by the criterion of SDS-PAGE, and consisted of a single chain with a molecular weight of 63 KDa. Purified metalloproteases had approximately 231 folds more proteinase activity than the crude venom. Preliminary information obtained on the purification profile of the enzyme would pave the way for characterization of the enzyme which would hopefully contribute to the wealth of information on these enzymes.
Keywords: Isolation, purification, fibrinogenase, Najanigricollis venom

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I. Introduction

Envenoming resulting from snakebite is a particularly important public health problem in rural areas of tropical and subtropical countries situated in Africa, Asia, Oceania and Latin America. Studies estimate that at least 421,000 envenomings and 20,000 deaths occur worldwide from snakebite each year, but warn that these figures may be as high as 1, 841,000 and 94,000 respectively. The highest burden of snakebite is in South Asia, South East Asia, and Sub-Saharan Africa. This is a problem to Nigeria too particularly the North-Eastern part of the country (Warrell, 1992;Chippaux, 1998; Swaroop and Grab, 1998; Theakston *et al*, 2003; World Health Organisation, 2010).

Snake venoms contain a mixture of biologically active proteins and peptides (90-95%) and also includes amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (5%) (Bieber, 1979; Russell, 1980; Tu, 1988;Heise*et al*, 1995; Fry and Wuster, 2004). These are produced by specialized glands and are toxic to the prey (Kochva, 1987).

Snake venom proteins may present different biological activities that affect physiological processes such as neurotransmission, the complement system and homeostasis (Stocker, 1990, Gold *et al*, 2002, Aird, 2002, Lewis and Gutmann, 2004).

The snake venom components are grouped into: enzymes that clot fibrinogen; enzymes that degrade fibrinogen; plasminogen activators; prothrombin activators; factor V activators; factor X activators;those with anti-coagulant activities including inhibitors of thrombin, phospholipases and protein C activators; enzymes with haemorrhagic activity; enzymes that degrade serine proteinase inhibitors; platelet aggregation inducers including direct acting enzymes, direct acting non-enzymatic components, and agents that require a cofactor;platelet aggregation inhibitors including - alpha-fibrinogenases, 5`-nucleotidases, phospholipases and disintegrins(Markland, 1998).

Snake venom molecules could act as (or be used as a prototype for) therapeutic agents (Volkers, 1998, Pal *et al*, 2002); research tools for use in the diagnosis of several diseases (Pal *et al*, 2002, Bailey and Wilce, 2001, Marsh, 2001); and/or in basic research for understanding physiological and pathological processes (Marsh, 2001, Andrews *et al*, 2001, Sher *et al*, 2000, Wisner *et al*, 2001).

Snake venom proteinases have been classified into various families, mainly serine proteases and metalloproteases (Matsui *et al*, 2000).

Snake venom metalloproteases are zinc-dependent endopeptidases classified into PI to PIV (Bjarson*et al*, 1995, Serrano, 1995 and Stocker *et al*, 1995) that induce haemorrhage by directly affecting capillary blood vessels and their interaction with endothelial cells (Serrano *et al*, 1995).They cleave basement membranes

leading to blood extravasion that occurs through gaps formed in endothelial cells. This ability also induces myonecrosis and plays a vital role in the significant local inflammatory response of the envenomation. (Serrano *et al*, 1995, Rucavado *et al*, 1995, Tans and Rosing, 2001).

Haemorrhage is a conspicuous adverse consequence of snake envenoming, which can become systemic and potentially lethal. Haemorrhages are principally caused by metalloproteases(also called haemorrhagins), enzymes degrading proteins of extracellular matrix and components of the haemostatic system, that can also have cytotoxic effects on endothelial cells(Kamiguti*et al*, 1996, de Roodt *et al* ,2003).

Fibrinogen molecules are structures consisting of two outer domains each connected by a coiled segment to a central E domain. They are composed of two sets of three polypeptide chains termed A (alpha), B (beta) and G (gamma). (Cortelazzo*et al*, 2010).

Some metalloproteases have fibrinogenolytic or fibrinolytic activities and are named fibrinogenases. These enzymes have been classified as alpha, beta and gamma-fibrinogenases based on their specificity for cleaving fibrinogen polypeptide chains (Ouyang and Teng, 1976, Pandya and Budzynski, 1984, Kini and Evans, 1991, Swenson *et al*, 2004).

Successful production of polyclonal antibodies against these enzymes is a very important step towards inhibition of the enzyme as it plays a major role in envenomation. This may have relevance in clinical management.

While much information abounds on this enzyme from other snakes in other parts of the world types but there are scanty reports from Africa.

II. Materials And Method

***NAJA NIGRICOLLIS* VENOM**

Najanigricollis was caught from the wild at Zaria in the northern part of Nigeria. They were identified in the Zoology unit of the Biological Sciences department of Ahmadu Bello University, Zaria, Nigeria. The venom was collected fresh by inducing salivation. The snake pangs were placed inside a beaker covered with polythene sheets. On fright, they attacked the polythene with their fangs releasing the venom, which was collected, frozen and dried at 5 degrees Celsius.

ENZYME PURIFICATION

Gel-filtration on sephadex-g75

The gel was prepared by dissolving 5g of sephadex G-75 in 50ml phosphate buffer, PH 7.8 for 24 hours at room temperature and mixed with a glass rod to make the swollen particles form slurry. The slurry was then poured into a column packed with glass wool at the bottom. The column was first equilibrated with phosphate buffer, PH 7.8, before the sample was applied. Crude venom (200mg) was dissolved in 10ml phosphate buffer, PH 7.8 in a beaker. This was transferred to a centrifuge tube and the insoluble component was removed by centrifugation and 5ml of the recovered supernatant was loaded onto the glass column (50cm). Twenty seven fractions at a flow rate of 1ml per minute were collected and analyzed for total protein and enzyme activity. The fractions showing high specific activities were pooled.

Ion-exchange chromatography on DEAE-cellulose

DEAE-cellulose was prepared by dissolving 5g of the ion-exchanger in 50ml of phosphate buffer, PH 7.8. The slurry was then poured into a 2.5 X 30cm column. The pooled sample (5ml) was loaded onto the column and eluted with a convex concentration gradient of sodium chloride solution (0.05 to 0.3M). Thirty- five fractions were collected at a flow rate of 1ml per minute and analyzed for total protein and enzyme activity. The fractions showing high specific activity were pooled.

Determination of total protein concentration

This was determined by the method of Bradford (1979) using bovine serum albumin as standard (100ug/ml). A calibration curve covering the range 0 to 100ug/ml standard was prepared by making dilutions in duplicate using water as a diluent to a total volume of 800uL. The fractions were also analyzed in duplicates by arranging test tubes labeled as test, standard and blank. Distilled water (700uL) was dispensed into all tubes followed by 100uL of sample or fraction, 100uL BSA standard and100uL distilled water to the tubes labeled test, standard and blank respectively making a total volume of 800uL. Bradford reagent (200uL) was added to all tubes. The contents of each tube were mixed and incubated for 2 minutes at room temperature. The absorbances of the blue colored solutions were measured against reagent blank at 595nm.

Determination of protease (fibrinogenase) activity

This was determined by the method of Yun and Yuliang, 1991. A tyrosine standard curve was prepared by making dilutions of the working tyrosine standard (1.1mM) with distilled water to a total volume of 250uL

followed by addition of 625uL of 500mM sodium carbonate solution and 125uL Folin and Ciocalteu reagent. The contents were mixed and incubated at 37 degrees Celsius for 30 minutes. The absorbances of the green colored solutions were measured at 660nm against reagent blank. A plot of absorbance versuumole tyrosine was made. The fractions were assayed for fibrinogenase activity as follows: 0.2ml of fibrinogen solution (0.5% in phosphate buffer) was dispensed into a centrifuge tube followed by 0.1ml human thrombin solution (10u/ml) to form a clot. Fraction (0.9ml) was added to the clot formed, the mixture incubated for 10 minutes at 37 degrees Celsius and centrifuged for 10 minutes at 10,000g. Supernatant (250uL) was subjected to the Folin and Ciocalteu protein assay as described above for the tyrosine curve. The amount of tyrosine released in micromole was determined from the tyrosine standard curve.

III. Result

TABLE 1: PURIFICATION PROFILE OF FIBRINOGENASE FROM *NAJA NIGRICOLLIS* VENOM

STEPS	TOTAL PROTEIN mg/ml	TOTAL FIBRINOGENASE ACTIVITY Umoles/ml/hr	SPECIFIC ACTIVITY Umoles/ml/hr/mg protein	PURIFICATION FOLD	RECOVERY
CRUDE VENOM	0.480	36.84	76.75	1	100
GEL FILTRATION ON SEPHADEX G-75	0.098	27.71	282.75	3.68	75.2
ION-EXCHANGE ON DEAE-CELLULOSE	0.0011	19.549	17771.82	231.55	53

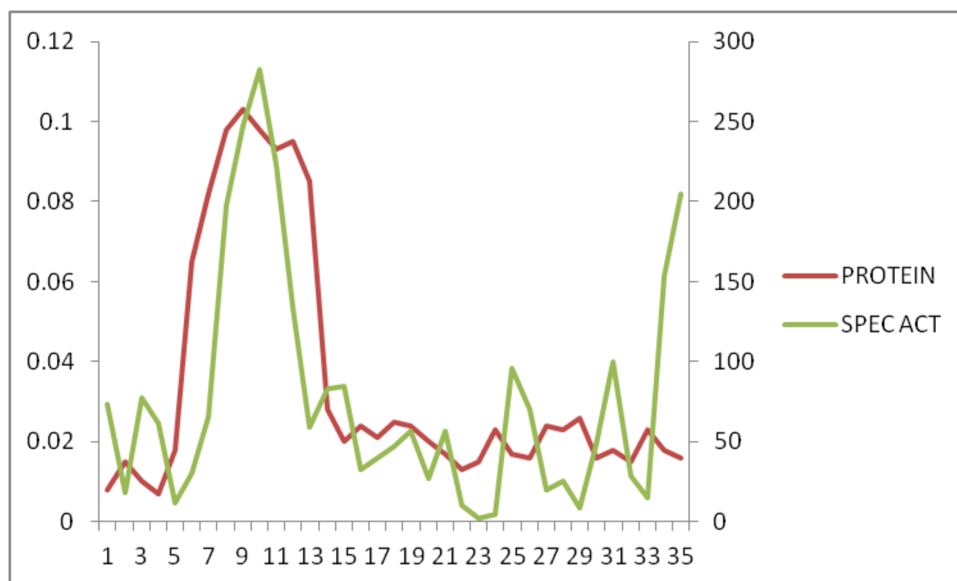

FIG 1: Elution profile of*Najanigricollis* fibrinogenase on sephadex G-75

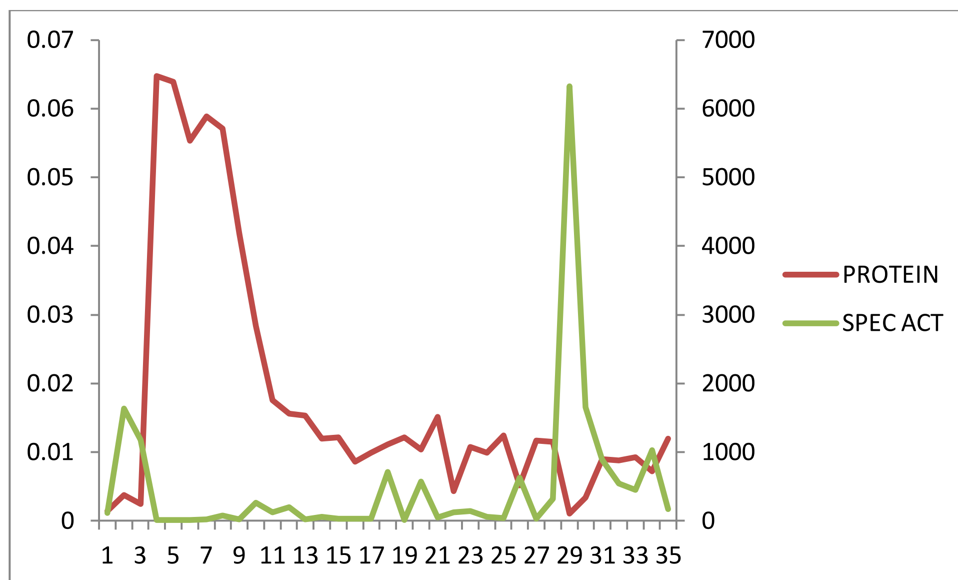

FIG 11 : Elution profile of*Najanigricollis* fibrinogenase on DEAE-cellulose

IV. Discussion

Snakevenom fibrinogenases are present in large quantities in venom of vipers and some elapids (Markland, 1998). The presence of fibrinogenases in such large quantities implies that the major targets of activity are structural proteins. Bites by viperids and elapids, such as African spitting cobras and some Asian cobras, induce local necrosis (Warrell, 1999; Gutierrez and Rucavado, 2000; White, 2000; Eric et.al, 2002; Gutierrez and Rucavado, 2003).These local effects develop rapidly after the bite due to local effects of cytotoxic components such as metalloprotease, phospholipase A2, and hyaluronidase.

Fibrin (ogen) olytic enzymes are direct acting and therefore do not rely on blood components for activity. This is supported by reports by Kini and Evans, 1999 of a fibrinogenase(proteinase-F1) from*Najanigricollis* venom that inhibits platelet aggregation in the absence of fibrinogen.

In the present work, a metalloprotease active on fibrinogen from*Najanigricollis* venom was isolated and purified. The*Najanigricollis* crude preparation separated into three distinct peaks I, II and III with proteolytic activities. The protease peaks suggest the presence of multiple forms of the enzyme. Peak I and II, were stable and were used for the purification. Ion exchange chromatographic separation of the enzyme by elution on DEAE-cellulose by convex concentration gradient produced a single active peak (figures I and II). The partially purified enzyme showed 231 times more proteinase activity than the crude *Najanigricollis* venom. However, a 15-fold increase in proteinase activity was reported by Evans, 1984.

The partially purified fibrinogenase was analysed using SDS-PAGE. The enzyme appeared as a single protein band with molecular weight of 63KDa suggesting the presence of a single polypeptide chain. These findings are similar to those of Evans, 1984 who reported the presence of a 58KDa fibrinogenolytic proteinase from the *Najanigricollis.*

Snake venoms proteases especially fibrinogenases have potential medical roles for the treatment of human diseases like occlusive vascular disease. These enzymes have been purified from several different snake species and have shown promising results (Markland, 1998). These studies have revealed that the highly purified fibrinolytic snake venom enzymes produced consistent thrombolysis. The venom enzymes act by a completely different mechanism than the plasminogen activators; the only agents presently approved for clinical use (Markland, 1998), and may have certain advantages over the plasminogen activators.

It is important to obtain them with high degree of purity and devoid of other pharmacological effects to help develop therapeutic agents and anti-venom antibodies.

More extensive research is therefore required to isolate each of them and identify their chemical and biological properties in order to fully understand theirproteomic/ venomics.

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