

**BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON ALPHA-
FIBRINOGENASE FROM *NAJA NIGRICOLLIS* VENOM**

By

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Dedication

This work is dedicated to all victims of snakebite envenomation in Nigeria

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LIST OF ABBREVIATIONS

WHO.....	World Health Organisation
<i>N. nigricollis</i>	<i>Naja nigricollis</i>
KDa.....	KiloDaltons
ACE.....	Angiotensin Converting Enzyme
BPP.....	Bradykinin Potentiating Protein
NAD.....	Nicotinamide Adenine Dinucleotide
α	Alpha
β	Beta
γ	Gamma
nAChR.....	N-acetyl choline receptor
PLA ₂	Phospholipase A ₂
RGD.....	Disintegrins
ADP.....	Adenosine diphosphate
vWF.....	Von Willebrand Factor
MPs.....	Metalloproteases
SVMPs.....	Snake Venom Metalloproteases
ECM.....	Extracellular matrix
MMP.....	Matrix metalloprotease
LIBS.....	Ligand Induced Binding Site
SVTLE.....	Snake Venom Thrombin-like Enzymes
FBA.....	Fibrinopeptide A
FPB.....	Fibrinopeptide B
CRD.....	Carbohydrate Recognition Domain

CTLDS.....	C-Type Lectin Like Domains
GPIb.....	Glycoprotein Ib
LD ₅₀	Lethal Dose ₅₀
CNS.....	Central Nervous System
IgG.....	Immunoglobulin G
DNA.....	Deoxyribonucleic acid
TEMED.....	Tetra methylene diamine
TRIS.....	Tris(hydroxymethyl)aminomethane
ELISA.....	Enzyme Linked Immunosorbent Assay
SDS-PAGE.....	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
EDTA.....	Ethylene diamine tetra acetic acid
BSA.....	Bovine serum albumin
PBS.....	Phosphate buffered saline
ADAMS.....	A Disintegrin And Metalloproteinase-like protein
MDC.....	Metalloproteinase-Disintegrin-like domain-Cysteine rich terminus enzyme
CRISP.....	Cysteine-rich secretory protein

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ABSTRACT

A fibrinogenolytic enzyme, *Naja nigricollis* metalloprotease, was purified from *Naja nigricollis* venom using Diethylaminoethyl (DEAE) Cellulose, Sephadex G-75 and Heparin-Agarose column chromatography to apparent homogeneity. The purified *Naja nigricollis* metalloprotease migrated as a single protein band on analytical polyacrylamide gel electrophoresis with an apparent molecular mass of 65 KDa, under reducing conditions in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The *Naja nigricollis* metalloprotease, displayed fibrinogenase activity, which was about 9-folds higher than that of the crude venom. The enzyme was proteolytically active against human fibrinogen. The enzyme cleaved preferentially the α -chain, leaving the β -chain and γ -chain of fibrinogen intact. The fibrinogenolytic activity of the enzyme was inhibited by the chelating agent EDTA and 1, 10-phenanthroline and slightly inhibited by phenylmethylsulfonyl fluoride, aprotinin and beta-mercaptoethanol, indicating it is a metalloprotease. The inhibition by EDTA could be blocked by Zn^{2+} , but not by Ca^{2+} or Mg^{2+} and Co^{2+} . The optimum pH and temperature for the enzyme activity were found to be 7.5 and 45°C respectively. The enzyme was stable at pH 6.5-7.8 and at a temperature range of 20-60°C with activation energy (E_a) of 0.031 kJ/mol/K. The kinetic parameters, K_m and V_{max} were found to be 0.091 mg/ml and 0.00711 μ mol/min respectively. The purified *N. nigricollis* fibrinogenase has haemorrhagic activity and the polyclonal antibodies raised against the enzyme were capable of neutralizing the haemorrhagic effects of the enzyme in laboratory animals. The kinetic properties and alpha-fibrinogenolytic effect of the enzyme indicates its suitability as a possible prototype for development of defibrinogenating agents and development of diagnostic reagents for detection of fibrinogen. The anti-haemorrhagic effect of the antibodies suggests its suitability also as possible vaccine candidate.

CHAPTER ONE

1.0

INTRODUCTION

Snakes are animals found on every continent except Antarctica. Of the roughly 3,000 known species of snakes found worldwide, only 15% are considered dangerous to humans (Kasturiratne *et al.*, 2008). There are more than 600 known species of venomous snakes (about a quarter of all snake species) classified into several families: *Elapidae*, *Viperidae*, *Crotalidae*, *Hydrophiidae*, *Actroctaspididae* and *Colubridae* (Mebs, 2002; Fry and Wuster, 2004). The venomous snakes in Africa are known to belong to four main families- *Elapidae*, *Viperidae*, *Colubridae* and *Hydrophiidae* (Akubue, 1997).

Three species: black-necked spitting cobra (*Naja nigricollis*), carpet viper (*Echis ocellatus*), and puff adder (*Bitis arietans*), belonging to the first two families, are the most important snakes associated with envenomation in Nigeria (Habib *et al.*, 2001).

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 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, particularly the North-Eastern part of the country (Warrell, 1992; Chippaux, 1998; Theakston *et al.*, 2003; World Health Organisation (WHO), 2010).

Naja nigricollis (also known as black-necked spitting cobra) is a long medium bodied snake with a moderately distinct head; the shape of which is primarily due to two large venom glands found on each side of the head.

It is generally grey with a solid black hood and head. Its length ranges from 120 to over 280 cm. *Naja nigricollis* (family *Elapidae*) is found in Eastern Africa, Western and Eastern Kenya, widespread in Uganda, South-Western Africa, Tanzania, Rwanda, Burundi, Senegal, Namibia and Nigeria (Naja, 2008).

Snake venoms contain a mixture of biologically active proteins and peptides (90-95%), the remaining 5% include [polypeptide](#) toxins (molecular weight 5-10 KDa), compounds with low molecular weight (up to 1.5 KDa) including metals, peptides, lipids, nucleosides, carbohydrates, amines, and oligopeptides, which inhibit angiotensin converting enzyme (ACE) and potentiate bradykinin (BPP) (Bieber, 1979; Russell, 1980; Tu, 1988; Heise *et al.*, 1995; Halliday and Adler, 2002; Fry and Wuster, 2004). These are produced by specialized glands and most are toxic to the prey (Kochva, 1987). Inter- and intra-species variation in venom chemical composition is geographical and ontogenic (Halliday and Adler, 2002).

Enzymes ([molecular weight](#) 13-150 KDa), especially hydrolytic ones (Roland, 1994) constitute 80-90% of viperid and 25-70% of elapid venoms. Main snake venom enzymes (Roland, 1994) are (i) oxidoreductases: lactate dehydrogenase, L-amino acid oxidase and catalase (ii) transferases: alanine amino transferase (iii) hydrolases: phospholipase A₂, lysophospholipase, acetylcholinesterase, alkaline phosphatase, acid phosphatase, 5-nucleotidase, phosphodiesterase, deoxyribonuclease, ribonuclease 1, adenosine triphosphatase, amylase, hyaluronidase, NAD- nucleotidase, kininogenase, factor X activator, heparinase, α -fibrinogenase, β -fibrinogenase, α - β -fibrinogenase, fibrinolytic enzyme, prothrombin activator, collagenase, elastase (iv) lyases: glucosamine ammonium lyase.

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 that affect haemostasis 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 proteinases have been classified into various families, mainly serine proteases and metalloproteases (Matsui *et al.*, 2000). The snake venom metalloproteinases are zinc-dependent endopeptidases classified into PI to PIV (Bjnarson and Fox, 1995; Serrano *et al.*, 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 envenomation (Serrano *et al.*, 1995; Rucavado *et al.*, 1995; Tans and Rosing, 2001).

Haemorrhage production is a conspicuous toxic consequence of snake envenomation, 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). One of the components of the haemostatic system affected by snake venom metalloproteases is fibrinogen or factor II. 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 α (alpha), β (beta) and γ (gamma) (Cortalazzo *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; Ouyang and Huyang, 1979; Pandya and Budzynski, 1984; Kini and Evans, 1991; Swenson *et al.*, 2004).

Contrary to popular perception, snake venom molecules are not completely harmful, they also possess beneficial properties. 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).

The isolation of fibrinolytic enzymes from viper venoms led to the postulation that there may be a clinical application for these enzymes in the treatment of occlusive thrombi, such as those occurring in the great arteries and veins of cardiac and cerebral circulation as well as peripheral arteries and veins.

In the ensuing years a substantial body of literature has been generated on the identification and characterization of the fibrinolytic enzymes from a broad spectrum of snake species (Swenson *et al.*, 2005). Interestingly, these enzymes have been isolated from elapid venoms too (Kini and Evans, 1991).

Envenomation after snakebite is an underestimated and neglected public health issue responsible for substantial illness and death as well as socioeconomic hardship to impoverished populations living in rural and tropical Africa. Scarcity and delay of administration of anti-venom, poor health services and difficulties with transportation from rural areas to health centers are major factors that contribute to high fatality ratio of snakebite envenomation (Ribeiro *et al.*, 1995; Borges *et al.*, 1999; Pinho and Pereira, 2001; Mc Namee, 2001).

Polyclonal antibodies are a collection of immunoglobulin molecules that react against a specific antigen each identifying a different epitope. They are secreted by different B cell lineages within the body.

Snake antivenom is a biological product that typically consists of venom neutralising antibodies derived from a host animal. They are obtained from serum of animals hyperimmunised with venom of one or more species (Jones and Landon, 2002, 2003; Chotwiwatthanakun *et al.*, 2001). Antivenoms are produced by immunizing animals with whole venoms or isolated venom components (Gutiérrez *et al.*, 2011). They are the most effective pharmaceutical preparations in treatment of bites from venomous snakes (Theakston *et al.*, 2003; Fry *et al.*, 2003; Laing *et al.*, 2004). Antivenoms have been used successfully for more than a century and up to now constitute the only effective treatment for snakebites and envenomations by other poisonous animals (Morais and Massaldi, 2009).

Antivenoms are classified into two: monovalent and polyvalent. Monovalent antivenoms are antibodies raised against single snake specie while polyvalent are those containing neutralising antibodies raised against two or more snake species. Components of antivenom could consist of the entire antibody molecule (often IgG) or antibody fragments derived by digesting the whole IgG into Fab (monomeric binding) or F(ab)₂(dimeric binding).

The choice of animals for antivenom production depend on factors such as yield of antibodies, physicochemical characteristics of immunoglobulins, geographical distribution of animals, ease of handling, adaptability to the locality and risk of allergy. Animals currently used for antivenom production include horses and donkeys (Laloo and Theakston, 2003; Gutiérrez *et al.*, 2007; Gutiérrez and León, 2007). Other animals incude sheep, goat, rabbits and hens.

However, administration of equine (horse derived) antivenins aimed at the neutralization of toxins in humans is prone to potential risks due to activation of the immune system by the heterologous protein (Devi et al, 2002; Panfoli, 2010; Morais and Massaldi, 2009. This has led to the consideration of alternative animals for antivenom production. These include: sheep (León *et al.*, 2000; Căpitănescu *et al.*, 2008; Ferreira *et al.*, 2009), camelids (Khomehchian *et al.*, 2014), rabbits (Chanhome *et al.*, 2002), and lately chicken (Michael *et al.*, 2010; Aguilar *et al.*, 2014).

Anti-venom is the only specific antidote to snake venom and its timely administration completely reverses all systemic manifestations of envenoming (Premawardhena *et al.*, 1999; Theakston and Warrell, 2000; Johnston, 2003; Cheng and Winkel, 2001; Gutierrez *et al.*, 2006).

However, currently available antivenoms are not effective against the local necrotic effects of snake venom which can lead to long term disability and disfigurement (WHO, 2010).

Surveys in the more densely populated Northern savannah areas of Nigeria show that the spitting cobra, *Naja nigricollis*, is the predominant specie of medical importance (Pugh and Theakston, 1980). It has been suggested that antivenoms raised against venoms of snakes from a particular area should be given as first priority to snakebite patients in that area (Lalloo and Theakston, 2003).

In view of the above, confronting the neglected problem of snakebite and anti-venom scarcity in Nigeria requires intensive research on snake venoms from local species and subsequent production of potent anti-venom that could hopefully be a possible improvement over conventional anti-venoms.

Information on alpha-fibrinogenases from the African spitting cobra are scanty, hence the need for further research. Previous works on this venom were limited to purification of metalloproteases. In this work, attempts were made at purification of the specific metalloproteases (alpha-fibrinogenases) whose anti-coagulant activity makes some snake venoms excellent potential candidates for the design of new drugs for treatment of cardiovascular diseases. The use of the enzyme for the production of anti-venom has not been reported despite its crucial role in the pathophysiology of envenomation. Successful production of polyclonal antibodies against the enzyme is a very important step towards inhibition of the enzyme which induces local necrosis and haemorrhage at the site of a bite.

1.1 Research Justification

The research justification is therefore summarized as follows:

- i. Snake venom envenomation is a sociomedical problem with considerable magnitude affecting countries in Africa, Asia, Oceania and Latin America. The highest burden is in Sub-Saharan Africa and South Asia. Recent studies estimate that at least 421,000 envenomings and 20,000 deaths occur worldwide from snakebite each year but warns that these figures may be as high as 1, 841, 000 to 2.5 million envenoming and 94,000 to more than 100,000 fatalities as many cases of snakebite go unreported. This is a problem to Nigeria too especially in the Northern part of the country with an incidence of 497 per 100,000 people per year with a mortality of 12.2%.
- ii. In Nigeria, more severe cases of snakebite envenomation are inflicted by cobra species of the family elapidae. Surveys conducted in the more densely populated northern savanna show that *Naja nigricollis* is the predominant specie of medical importance.
- iii. Information on fibrinogenase from *Naja nigricollis* venom found in this locality and its potential role in the pathophysiology of envenomation is scanty.
- iv. Reasons for high levels of snakebite mortality in tropical developing countries include anti-venom scarcity and the triple problems of inadequacy, inaccessibility and inequality in healthcare.
- v. The use of *Naja nigricollis* fibrinogenase for the production of anti-venom has not been reported despite its crucial role in the pathophysiology of envenomation.

vi. Snake venom molecules could act as or be used as prototype for therapeutic agents, research tools for use in diagnosis of several diseases or in basic research for better understanding of physiological and pathological processes. Today, several medicinal properties of snake venom such as anti-cancer, antibacterial, antihypertensive and many others are being exploited and several drugs and clinical diagnostic kits derived from snake venom have been commercialized. Proteinases with fibrinolytic and anti-thrombolytic activity which form the focus of this research find potential application in drug development for treatment of thrombotic disorders which result in fatal heart attack and stroke.

1.2 Statement of Research Problem

- The intravenous administration of animal-derived antivenom is the mainstay and only specific treatment of snakebite envenoming. However, snake venoms are diverse therefore the efficacy of antivenoms is geographically and biologically restricted hence the need for concerted efforts towards the production of effective antivenom against local species to deal with the high incidence of envenoming in some Nigerian communities and to tackle the antivenom scarcity.
- The use of the enzyme in antivenom production has not been reported despite its crucial role in inducing necrosis following envenomation. Current available antivenoms are not effective against local necrotic effects of snake venom which could lead to long term disability and disfigurement of victims.

Hence the need for employment of new approaches towards production of more effective and safer antivenoms which is the use of purified relevant toxins like enzymes as antigens instead of the whole venom.

- Plasminogen activators were the only agents approved for the treatment of cardiovascular diseases. Venom enzymes especially metalloproteases act by a completely different mechanism from these and may have certain advantages over them; this further suggests their therapeutic potential. Several have been tested in vivo with promising results.

1.3 Aims of the Study

This study is aimed at isolation, purification and characterization of alpha-fibrinogenase from the African black-necked spitting cobra (*Naja nigricollis*) venom; also it is aimed at production of polyclonal monospecific antibodies as potential vaccine candidates with the hope of contributing to the wealth of information on snake venom components. This is needed for rational design and production of potent anti-venom.

1.4 Objectives of the Study

The specific objectives of the study were as follows:

- To isolate and purify alpha-fibrinogenase from *Naja nigricollis* venom
- To characterize alpha-fibrinogenase from *Naja nigricollis* venom
- To develop polyclonal antibodies against alpha-fibrinogenase from *Naja nigricollis* venom
- To evaluate the anti-haemorrhagic activity of polyclonal antibodies against the purified alpha-fibrinogenase

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Snakes

Snakes belong to the Phylum *Chordata*, Order *Squamata*, Sub-order *Serpentes* and Class *Reptilia*. Linnean taxonomy places all modern snakes within the Sub-order *Serpentes*, part of the Order *Squamata*, though their precise placement within Squamates remains controversial. Snakes are considered to be the second largest group of reptiles. More than 20 families are currently recognized, comprising about 500 genera and about 3,400 species (Uetz, 1999, Alejandro, 2007).

Living snakes are found on every continent except Antarctica (Underwood, 1979; Conant and Collins, 1991; Mattison, 1995; Halliday and Adler, 2002; Alejandro, 2007), in the Pacific and Indian oceans, and on smaller land masses—exceptions include some large islands, such as Ireland and New Zealand, and many small islands of the Atlantic and Central Pacific (Phelps, 1981; Roland, 1994). They have successfully evolved into efficient predators and colonized various habitats from mangrove swamps, estuaries, freshwater lakes, streams, dunes, grasslands to forests (Garl and Roger, 1989).

The origin of snakes has been traced to lizard-like ancestors that probably evolved some 100-150 million years ago during the lower to mid-Cretaceous period. This is supported by oldest 'snake-like' fossils found in some sandstone beds of Algeria (Rage, 1984; Kochva, 1987; Harris, 1991; Heise *et al.*, 1995; Fry and Wuster, 2004). The diversity of modern snakes appeared during the Paleocene period (66 to 56 Million years ago). Snakes are elongate, legless, reptiles. Their long body shape and lack of limbs probably evolved to enable their smooth movement in dense vegetation and forest.

They range in size from the tiny, 10cm-long thread snake to the reticulated python up to 8.7 metres in length (Mehrtens, 1987; Murphy and Henderson, 1997).

Generally snakes have highly flexible bodies with no eyelids, shoulder and sternum. Some traces of the pelvis and horn-like claws at the base of the tail which resemble the hind limbs, can still be seen in some primitive snakes. The skin of snakes is covered in scales with a smooth and dry texture. Most use specialized bell-shaped scales to travel, gripping surfaces. The skin is renewed by periodic moulting.

Snakes have developed different modes of locomotion to deal with particular environments. These include lateral undulation, sidewinding, concerting movement and reticular locomotion with each mode being discrete and distinct from the others. Transitions between these modes are abrupt (Gray, 1946; Cogger, 1991).

Snakes use smell to track their prey. They smell by using their forked tongues which gives them a sort of directional sense of smell and taste simultaneously. Snake vision varies greatly, from only being able to distinguish light from dark to keen eyesight, but the main trend is that the vision is adequate although not sharp, and allows them to track movements. Snakes can detect other animals approach by detecting faint vibrations in the air and on the ground (Cogger, 1991).

Although a wide range of reproductive modes are employed by snakes, all snakes employ internal fertilization (Capula *et al.*, 1989). Most species lay eggs, but most abandon their eggs after laying (Capula *et al.*, 1989). Some species are ovoviparous while others have been confirmed to be fully viviparous (Capula *et al.*, 1989; Cogger, 1991).

2.2 Venomous Snakes

All snakes are strictly carnivorous. Some snakes have a venomous bite, which they use to kill their prey before eating it (Behler and King, 1979; Freiberg and Walls, 1984). Other snakes are non-venomous and kill their prey by constriction (Behler and King, 1979), others swallow their prey whole and alive (Mehrtens, 1987). More than 3,000 species of snake exist worldwide, and approximately 1,300 species are considered venomous (Hider *et al.*, 1991; Stafford, 2000) - about a quarter of all snake species. Venomous snakes are usually defined as those that have venom glands and specialized venom conducting fangs which enable them to inflict fatal bites upon their victims (Klemmer, 1968). The evolution of the venomous form is believed to have occurred during the Miocene period (less than 30 million years ago) (Harris, 1991).

Venom like all salivary secretions is a predigestant delivered through the fangs that initiates the breakdown of food into soluble compounds facilitating proper digestion. Snake fangs are curved teeth situated on the maxillary bone and vary from family to family. They can be grooved or canalized (Underwood, 1979; Jackson, 2002). During the bite, the snake may leave fang marks at the bite site on the victim's body. Fang marks are different among snake families and they can be used as signs in diagnosis of snakebites (Lim, 1971; Nishioka *et al.*, 1995). Therefore, Venomous snakes use venom primarily to immobilize or kill their prey rather than for self-defense (Mehrtens, 1987). It has been suggested that all snakes may be venomous to a certain degree, with harmless snakes having weak venom and no fangs (Fry *et al.*, 2006).

All the known advanced snake species are venomous- classified into five families: *Elapidae*, *Viperidae*, *Crotalidae*, *Hydrophidae*, *Atractaspididae* and *Colubridae* (Halliday and Adler, 2002; Mebs, 2003; Fry and Wuster, 2004).

The venomous snakes in Africa are known to belong to four main families- the *Colubridae*, *Elapidae*, *Viperidae* and *Hydrophidae* (Warrell, 1984)-but in Nigeria, the most common poisonous snakes are the Elapids and Viperids (Akubue, 1997). These include the *Naja melanoleuca* (black cobra), *Naja nigricollis* (spitting cobra), the Viperid *Echis Ocellatus* (carpet viper) and *Bitis Arietans* (puff adder).

Family *Elapidae* comprising of cobras, kraits, mambas and coral snakes is widely distributed in America, Africa, Asia and Australia. They are characterized by a small head with short, fixed fangs mounted at the front of the jaw (proteroglyphous). Species of the Elapid family have very generalized diets (Shine *et al.*, 2007).

Haemorrhagic venomous snakes are grouped into four families: *Elapidae*, found in West Africa, South-East Asia, America, Australia and New Guinea; *Colubridae*, found worldwide except Antarctica, extremely high latitudes of Eurasia, North America, Central and West Australia; *Crotalidae*, found in Asia and America, and *Viperidae*, found in Europe, Africa, South-East Asia and USA (Panfoli *et al.*, 2010).

2.3 *Naja nigricollis*

Naja nigricollis belong to Genus *Naja* as classified by Laurenti in 1768. *Naja nigricollis* is documented to belong to the most diverse and widespread genus of cobras. It has two sub species. Its geographical range include Western, Eastern, Central and parts of southern Africa; the Middle-East, India, South-Eastern Asia and Indonesia.

Naja nigricollis is the commonest and most widely distributed African cobra and is a familiar snake in Nigerian states within the savannah terrain. Specifically, *Naja nigricollis* are found in the South Eastern Nigeria where its habitat has been altered from a tropical rain forest to man-made farmlands, plantations, suburban areas, and a few fragmented forests (Luiselli, 2001; Abriol, 2007).

Although there are several other genera that share a common name, *Naja* are the most recognized and most widespread group of snakes commonly known as cobras. The genus *Naja* consist of 20-22 species, but has undergone several taxonomic divisions in recent years, so sources vary greatly. The spitting cobra's family consists of several other species such as mambas, coral snakes, tipands and kraits (Abriol, 2007).

Naja species are long relatively slender snakes. Most species are capable of attaining lengths of 6 feet or more. *Naja nigricollis* is a long medium-bodied snake with a moderately distinct head; the shape of which primarily is due to two large venom glands found on each side of the head. The snake is generally grey with a solid black hood. It can also be black with red blotches on the hood. It generally grows to a length of 120-220cm but has been known to attain a length of over 280cm. The average length was found to be 117cm. These sizes are subject to trends based on geographical location and sub-species. All have a characteristic ability to raise front quarters of their bodies off the ground and flatten their necks to appear larger to a potential predator. They lack left lungs, external and internal girdle and limb features (Abriol, 2007).

All species in the genus *Naja* are capable of delivering a fatal bite in human. Several *Naja* species, referred to as spitting cobras, have developed a specialized venom delivery mechanism. They possess erect fangs on each maxillary bone.

Their front fangs have a rifled opening in the front surface, which allows the snake to propel venom out of the mouth. While typically referred to as spitting, the action is more like squirting. The range and accuracy with which they can shoot their venom varies from specie to specie, but it is used primarily as a defence mechanism.

Spitting cobras spit their venom to a distance of at least three metres (Bogert, 1943; Abriol, 2007) with a maximum capacity of 57 consecutive spits (Abriol, 2007). Cobra spit in the eye may lead to acute snake venom ophthalmia with permanent blindness.

Naja nigricollis defend themselves by liberally spitting their venom in the face of a potential threat with only the slightest provocation as a defensive mechanism, thus given the common name “spitting cobra” (Westhoff *et al.*, 2005; Abriol 2007). They have developed features to differentiate between their aggressors’ hands and faces (Westhoff *et al.*, 2005; Abriol, 2007). However this aggressiveness is counterbalanced by the fact that *Naja nigricollis* is less prone to bite than other related species.

Naja nigricollis releases its venom in a fine spray or propelled mist-like fashion through fast undulating head movements (Westhoff *et al.*, 2005; Abriol, 2007). This irritant venom cause harmful effects such as extreme burning, loss of coordination and could result in necrosis on mucous membranes which can cause partial loss of vision and permanent blindness from destruction of the cornea of the eye (Warrell and Ormerod, 1976).

Spitting cobras are categorized as generalist predators. Their adaptive capability enables them to prey on several different species when exposed to different microhabitats. Most cobra species are nocturnal (Luiselli and Angelici, 2000; Luiselli *et al.*, 2002; Abriol, 2007).



Plate 2.1: *Naja nigricollis* (African spitting cobra). Adapted from: http://www.biodiversityexplorer.org/reptiles/squamata/serpentes/elapidae/naja_nigricollis.htm

2.4 Snakebites and Envenomation

Substantial illness and death results from envenomation from venomous snakes and represents an economic hardship on poor rural populations and healthcare system of tropical and sub-tropical Africa, Asia, Oceania and Latin America (Pinho *et al.*, 2005; WHO, 2005; Bucaretschi *et al.*, 2006; Gutierrez *et al.*, 2006; Kalantri *et al.*, 2006).

Venomous snakes are believed to cause 2.5-3 million bites worldwide per year with 100,000- 150, 000 deaths (White, 2000; Koh *et al.*, 2006). The global mortality rates from snakebites appear to be about 5% of the victims (White, 2000). The World Health Organisation (WHO) estimates that approximately 2, 500,000 venomous snakebites per year result in 125, 000 deaths worldwide, 100,000 of which are in Asia and approximately 20,000 deaths in Africa (Pinho *et al.*, 2005; Sitprija, 2006).

Snakebite remains a major public health problem in many countries even though it is difficult to be precise about the actual number of cases. It is estimated that the true incidence of snake envenomation could exceed 5 million per year (Chippaux, 1998; Hasson *et al.*, 2010) with an associated mortality level of 125,000 persons per year (Chippaux, 1998). About 100,000 of these develop severe sequelae (Chippaux, 1998). The global disparity in the epidemiological data reflects variations in health reporting accuracy as well as the diversity of economic ecological conditions (Chippaux, 1998). Accurate records to determine the exact epidemiology or even mortality of snakebite cases are generally unavailable (Hasson *et al.*, 2010,).

Snakebites are an occupational hazard especially among snake catchers, snake charmers, forest workers, farmers, fishermen and their children (Snow *et al.*, 1994; Chippaux, 1998).

The incidence of bites is high in warm regions, where snakes are abundant and economic activities are mainly agricultural, especially in the tropical developing countries. In developed or industrialised nations, snakebite typically occurs during recreational activities resulting in an increase in the frequency of unprovoked snakebite (Reid, 1978; Russell, 1980; Chippaux, 1982; Chippaux, 1998).

Snakebite is a common occurrence in many parts of the tropics and seems to vary according to the geographical zone, the occupational practices of the population and season. In Nigeria, it has been observed that the incidence of snakebite is seasonal and there is a peak during the rainy season- a time when frogs and toads emerging from their hibernation are preyed on by snakes that come in frequent contact with humans; when farmers engage in intense farming activities that also bring them frequently in contact with snakes in the bush (Ogala and Obaro, 1999; Warrell, 1999).

Snakebites are however not confined to bush encounters, towns are not spared venomous snakes and snakebites do occur in the capital cities of African countries (Chippaux and Bressy,1981), as a significant number of victims are known to be bitten in their abodes; some while sleeping indoors (Omogbai *et al.*, 2002).

In most developing countries, up to 80 % of people bitten by snakes (Chippaux, 1988; Snow *et al.*, 1994) first consult traditional practitioners and only subsequently resort to modern medicine, thus accounting for the long delays before they receive proper treatment (Chippaux, 1998).

In Africa, with a population of about 760 million, probably one million snakebites occur every year involving 500,000 envenomations, of which 40% are hospitalized. It is likely that about 20,000 deaths per year occur as a result, although less than 10,000 are reported by health services (Chippaux, 1998).

In Africa, the prevalence of snakebites is underestimated by health authorities, mainly because the reporting system is inaccurate. Moreover, the poor organization of health facilities in many countries complicates the management of patients and accounts for the great variation in the case fatality rates (Chippaux *et al.*, 1996). *Echis* species (*Viperidae*) are probably responsible for the greatest number of accidents and deaths by envenomation in Africa (Warrell and Arnett, 1976).

The incidence of snakebite in Nigeria was estimated at 48-603 per 100,000 from household surveys. Morbidity per 100,000:100-120, case fatality rate: 2.1-16% (Onuaguluchi, 1960; Warrell and Arnett, 1976; Pugh *et al.*, 1979; Pugh and Theakston, 1980; Harries *et al.*, 1984; Idoko and Ikwueke, 1984). A more recent study estimates that over 314,000 bites, 7, 300 deaths and nearly 6,000 amputations occur from snakebite annually in Sub-Saharan Africa with Nigeria having one-fifth of all cases (Habib, 2013).

In Nigeria, a study in the Benue valley estimated that the annual incidence of snakebites was up to 600 per 100,000 inhabitants and that the case fatality rate was 12.3%, mainly from *Echis ocellatus* bites (Pugh and Theakston, 1980). Ogala and Obaro reported a 3.9% mortality rate among children bitten by snakes in Zaria (Idoko and Ibekwe, 1984; Ogala and Obaro, 1999) recorded a mortality rate of 5.7% in 175 cases recorded in a two-year period in Makurdi, Nigeria while other researchers have also reported low mortality rates among victims of snakebites in different parts of the tropics (Swaroops, 1954; Onuaguluchi, 1960; Reid, 1972).

Most severe cases of snakebite envenoming are inflicted by species of the families *Elapidae* and *Viperidae* (Gutierrez *et al.*, 2006). They are also responsible for the high incidence of snakebites in West Africa.

The puff Adder, a common name of snake species of genus *Bitis*, is considered Africa's most dangerous snake as it is responsible for more fatalities than any other snake in Africa (Panfoli, 2010).

Most snakebites are “startled”, “escape” or “defensive” bites and the snake often injects an insufficient amount of venom or no venom at all into the victims as against ‘business’ bites in which large volumes of venom are injected (Omogbai *et al.*, 2002), and therefore about one third to half of the victims are safe due to such ‘false’ or ‘dry’ bites (Silveira and Nishioka, 1992; Young and Zahn, 2001a). Snakebite in the tropics have been suggested to be mainly “escape” bites in which only small amounts of the venom are injected into the victims (Reid, 1961; Reid, 1972). Only 50-70% of bites by venomous specie will actually cause envenoming (WHO, 2010).

2.5 Snake Venom Constituents

Snake venoms are secretory products of venom glands (Oron and Bdolah, 1973; Dong, 2004). Typical venom glands consist of three major cell types, namely basal cells, conical mitochondrial rich cells and secretory cells. Venom is only produced by secretory cells in the glands (Oron and Bdolah, 1973; Dong, 2004). The accessory glands prevent wasteful flow of the secretions. Venom secretion is regulated by the glands themselves and is independent of neural control. Venoms of elapids, viperids, and some groups of colubrid snakes are produced in venom glands (in other colubridae groups, venom is secreted from Durvenoy's glands). Venom is accumulated in the lumen of the glands and is expelled through the tubular fangs during the bite or spitting (Gans and Elliot, 1968; Kochva, 1987; Young and Zahn, 2001a; Young *et al.*, 2001b; Dong, 2004).

Venomous snakes use venom primarily to immobilize or kill their prey rather than for self-defense. The venom is modified saliva and a mosaic of antigens (Bouquet, 1979; Mehrtens, 1987). Venom like all salivary secretions is a predigestant that initiates the breakdown of food into soluble compounds facilitating proper digestion (Aird, 2002). The quantum and amount of the venom entering the victim's body during the bite determines the lethality of the venom of any particular specie of snake (Allon and Kochva, 1974; Dong, 2004). The age of the snake, diet, the season, the climate, the altitude and time of secretion into the glands are factors that affect the qualitative and quantitative composition, distribution and level of venom in a snake (Kochva and Gans, 1965; De Lucca *et. al.*, 1974; Chippaux *et al.*, 1991; Daltry *et. al*, 1996b; Sasa, 1999; Dong, 2004).

Snake venoms form a natural rich source of biologically active molecules. These include peptides and proteins (90-95%), few free amino acids, nucleotides, biogenic amines, free lipids, carbohydrates and metallic elements bound to proteins (5%) (Russell, 1980; Tu, 1988, Hider *et al.*, 1991; Heise *et al.*, 1995; Fry and Wuster, 2004), with important pharmacological activities, which have evolved to favour the survival of the snake in its particular environment (Bieber, 1979; Koh *et al.*, 2006). These molecules are usually similar in structure but not identical to that of prey physiological systems, and confer a formidable array of toxic properties on the venom (Kochva, 1987; Koh *et al.*, 2006).

Snake venom protein constituents may present different biological activities that affect physiological processes such as neurotransmission, the complement system and haemostasis (Stocker, 1990; Gold *et al.*, 2002). The main toxins from Elapid snake venoms that affect the CNS are neurotoxins and dendrotoxins.

The general observation from neurotoxin envenomation is the development of cranial nerve palsies, which are characterized by ptosis, blurred vision, difficulty in swallowing, slurred speech and weakness in facial muscle. Similarly, dendrotoxins have been demonstrated to block particular subtypes of voltage-dependent potassium channels in neurons. The β -neurotoxins, on the other hand, act presynaptically by affecting the release of acetylcholine via mechanisms that differ for different β -neurotoxins. They are responsible for high toxicity and ultimately respiratory paralysis. They act by causing the disappearance of acetylcholine containing vesicles, preventing the controlled release of acetylcholine and blocking impulse transmission (Koh *et al.*, 2006).

There are three main classes of venom components that initiate cycles of degeneration and regeneration of skeletal muscle: (a) myotoxins, (b) cardiotoxins and (c) phospholipases A₂.

Myotoxins are small polypeptides which specifically act on skeletal muscles (Ownby *et al.*, 1976; Koh *et al.*, 2006). They are also called myonecrotic toxins and are found in venoms from rattlesnakes and other pit vipers. Mytotoxin-a, a 4.6 KDa representative member binds specifically to the sarcoplasmic reticulum of muscles, causing a change in ion permeability of the sarcoplasmic reticulum (an important calcium regulatory system) leading to swelling and disintegration of both the sarcoplasmic reticulum and muscle fibrils.

Cardiotoxins act on smooth muscles. Cardiotoxins, also known as cytotoxins are found exclusively in the venom of cobras and ringhals (Jeyaseelan *et al.*, 1998; Chang *et al.*, 2000; Koh *et al.*, 2006), and are direct lytic factors and membrane-active polypeptides.

They are single-chain, highly hydrophobic, basic, short 60-65KDa polypeptides closely related to the α -neurotoxin that binds to nAChR, but cardiotoxins do not show any significant affinity for the receptors (Dufton *et al.*, 1991; Koh *et al.*, 2006). The main targets of cardiotoxins are on excitable cells. They cause depolarization and contracture of cardiac, skeletal and smooth muscles, and depolarization and loss of excitability of nerves (Forouhar *et al.*, 2003; Koh *et al.*, 2006). The mechanism of action of degeneration is most probably calcium dependent, involving the direct activation of calcium-dependent proteases and the eventual failure of mitochondrial respiration due to a calcium overload (Harris, 2003).

Phospholipase A₂ (PLA₂) found in venoms of a number of snake families, including Viperidae, Elapidae and Hydrophiidae. The PLA₂ can have myotoxic, cardiotoxic or neurotoxic actions (Harris *et al.*, 1975; Gutierrez *et al.*, 1984; Koh *et al.*, 2006). PLA₂ enzymes may be single-chain polypeptides of around 120 amino acid residues or mixtures of two to five complementary polypeptides, and are Ca₂₊ enzymes. These enzymes catalyze the hydrolysis of phospholipids at the *sn*-2 position of the glycerol backbone to release fatty acid and corresponding *1-acyl* lysophospholipid (Verheij, 1980; Koh *et al.*, 2006). PLA₂ from snake venoms exhibit a wide variety of pharmacological effects by interfering with normal physiological processes (Valentin and Lambeau, 2000; Koh *et al.*, 2006). PLA₂ triggers a cascade of inflammatory events characterized by increased microvascular permeability and oedema formation, leukocyte recruitment into tissues, nociception and release of inflammatory mediators which mimic a number of systemic and local inflammatory disorders in humans (kamiguti, 1998).

2.5.1 Snake venom enzymes

Snake venoms have been identified as the richest source of enzymes among venomous animals (Tan and Ponnudurai, 1992). At least 26 enzymes, most of them hydrolases, have been detected in snake venoms (Iwanaga and Suzuki, 1979). The major enzyme groups found in snake venom include phospholipases A₂ (PLA₂), serine proteinases, metalloproteinases, phosphomonoesterases, phosphodiesterases, acetylcholinesterases, L-amino acid oxidases, glycosidases, arginine esterases, hyaluronidases, 5' and NAD nucleotidases (Russell, 1980; Matsui *et al.*, 2000; Torres *et al.*, 2003; Fry and Wuster, 2004; Yamazaki and Morita, 2004). Generally enzymes in the venom have molecular mass ranging from 13,000 to 150,000 daltons (Hider *et al.*, 1991). Most of these are hydrolases and possess a digestive role.

Other protein compounds with an enzymatic profile in snake venoms include cysteine-rich secretory proteins, which inhibit smooth muscle contraction and cyclic nucleotide gated ion channels (Morris *et al.*, 1964).

2.5.2 Snake venom components affecting haemostasis

Snake venoms are rich sources of components that can affect hemostasis by causing changes in blood coagulation and platelet function. Venom components (coagulant enzymes) affecting the clotting system include activators of prothrombin (factor II), factor V and factor X, while anti-coagulants include protein C activators, inhibitors of prothrombin complex formation and fibrinogenases. Intermediates between the true coagulants and true anti-coagulants are the thrombin-like enzymes which bring about clotting *in vitro* but defibrination (anti-coagulation) *in vivo* (Koh *et al.*, 2006). Platelet function can be affected by venom components such as haemorrhagins and fibrinolytic activators (Kamiguti *et al.*, 1998), phospholipases and RGD-containing disintegrins.

As a result, it is common to find consumption of clotting factors and blood incoagulability accompanied by hemorrhage in victims of snakebite (Kamiguti *et al.*, 1998). It appears that for every factor involved in the blood coagulation cascade, there is a counterpart among the snake venom compounds that could either activate or inactivate the factors. These activators or inhibitors usually belong to various families such as serine proteases, metalloproteinases, C-type lectins, disintegrins and phospholipases (Koh *et al.*, 2006).

2.5.3 Mechanism of haemostasis

Blood fluidity in the circulation is maintained by the non-thrombogenic properties of the intact blood vessel walls. Damage to blood vessels triggers a prompt response of hemostatic reactions to prevent hemorrhage (Bithell, 1993; Kamiguti *et al.*, 1998). These reactions include contraction of the vessel wall itself due to the action of released vasoactive agents, adhesion and aggregation of circulating platelets to form a hemostatic plug and activation of clotting factors leading to the formation of fibrin clots. In order to allow full tissue healing, the clots are subsequently removed by the fibrinolytic enzyme, plasmin. In situations where any component of these mechanisms is altered, hemostasis is compromised and the result could be either thrombosis or hemorrhage (bleeding due to platelet and/or clotting factor deficiencies).

In small blood vessels, platelets alone can arrest bleeding. In their inactive form, platelets are discoid but once activated they become round, extend numerous pseudopods and then aggregate. This occurs when platelets are exposed to ADP, thrombin, adrenaline, collagen, and other agonists. Each agonist stimulates platelets via a specific receptor, whereby the receptor for collagen belongs to the superfamily of $\alpha\beta$ dimeric proteins or integrins (Hynes, 1992; Kamiguti *et al.*, 1998).

Indeed, the first reaction of platelets to vessel damage is their adhesion to the adhesive proteins, von Willebrand factor (vWF) and collagen on the exposed subendothelium. The respective platelet receptors for these proteins are the glycoprotein (gp) Ib/IX complex (Baumgartner *et al.*, 1978; Weiss *et al.*, 1986; Kamiguti *et al.*, 1998) and $\alpha_2\beta_1$ integrin also known as the gpIa/IIa complex (Santoro *et al.*, 1988; Kamiguti *et al.*, 1998). Engagement of these receptors stimulates platelets to secrete their granular contents and in particular ADP, which promotes activation of platelet $\alpha_{IIb}\beta_3$ integrin or gpIIb/IIIa. This receptor then binds the RGD-containing ligands (fibrinogen and von Willebrand factor) and thereby promotes platelet aggregation, resulting in the formation of a platelet plug which stops bleeding. Thus, the mechanism by which platelets act clearly depends on their surface receptors for vessel wall and plasma proteins and on normal content and release of their granular ADP stores.

For haemotoxic venoms, conspicuous toxic consequence of snake envenoming is haemorrhage production, 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 effect on endothelial cells (Kamiguti *et al.*, 1996; de Roodt *et al.*, 2003).

2.5.4 Snake venom proteases

Based on sequence, snake venom proteinases have been classified into various families, mainly serine proteases and metalloproteinases (Matsui *et al.*, 2000).

(i) Metalloproteases which are Ca^{2+} or Zn^{2+} dependent for their hydrolytic activity and are inhibited by metal chelating agents (EDTA and 1,10-phenanthroline)

These include factor X activator, prothrombin activator, $\alpha(\beta)$ -fibrinogenases, hemorrhagic proteases), (ii) serine proteases which include factor V activator, protein C activator, plasminogen activator, kinin-releasing and thrombin-like enzymes, β -fibrinogenases). Factor X activator and prothrombin activator being present in some snake venoms as serine proteases. Serine proteinases, including fibrinogenolytic enzymes are very abundant in Viperidae venoms in which they may account for 20% of their total protein content (Wisner *et al.*, 2001; Chernyshenko, 2010). The unique specificity of snake venom proteinases makes them potentially useful in research of fibrinogen-depletion (Koh *et al.*, 2006; Gardiner and Andrews, 2008; Chernyshenko, 2010) and limited proteolysis.

Snake venoms are rich sources of 20-100 kDa metalloproteinases (MPs) which, along with the transmembrane ADAMS (desintegrin-like and metalloproteinase-containing proteins), are members of the reprotolysins subfamily M12-family of metalloproteinases. In general, SVMPs (Snake venom metalloproteinases) have been classified into three basic structural classes, P-I to P-III (Bjarnason and Fox, 1995; Fox and Serrano, 2005; Fox and Serrano, 2008; Torres *et al.*, 2012). These metalloproteinases are synthesized in the venom gland as large multidomain proteins, including a proenzyme domain and a highly conserved zinc-proteinase domain (HEBXHXBGBXH) (Fox and Serrano, 2005; Ramos and Selistre-De-Araujo, 2006; Torres *et al.*, 2012). The P-I (PIa) metalloproteinase class includes proteins with molecular masses between 20–30 kDa that have low or no hemorrhagic effect, but strong direct-acting fibrino(geno)lytic activity, and contain only a metalloproteinase domain.

Class P-II (P-IIa, P-IIb, P-IIc, P-IId, and P-IIe) comprises proteins with molecular masses of 30–60 kDa that contain metalloproteinase and disintegrin-like domains.

Class P-III (P-IIIa, P-IIIb, P-IIIc, and P-IIId) includes a cysteine-rich domain, a metalloproteinase domain, and a disintegrin-like and a lectin-like domain (Bello *et al.*, 2006; Fox and Serrano, 2008; Torres *et al.*, 2012).

Comparison of amino acid sequences have indicated that, despite differences in their molecular sizes, all these enzymes may be related through a common ancestral gene encoding a prodomain, metalloproteinase, disintegrin and cysteine-rich domains (Kamiguti *et al.*, 1998). The prodomain contains a conserved sequence PRCGVDP, called a cysteine-switch, which is responsible for the enzyme activation and is lacking in the mature protein. A multi-domain structure characterizes the large hemorrhagic metalloproteinases; the N-terminal region possessing a zinc-containing metalloproteinase domain is followed by a disintegrin-like and a C-terminal cysteine-rich domain. Moreover, disintegrin-like regions of MDC enzymes possess a high sequence homology to the venom disintegrins. Disintegrins contain an RGD sequence, but a KGD sequence has also been reported (Kamiguti *et al.*, 1998). In contrast, the disintegrin-like regions of most venom MDC enzymes have a conserved ECD sequence overlapping the region where an RGD sequence occurs in the homologous disintegrins. However, this is not a constant motif found in all disintegrin-like domains because some venom MDC enzymes have a DCD sequence instead of ECD (Kamiguti *et al.*, 1998).

Metalloproteinases are capable of inducing rapid local bleeding. These enzymes have a potent proteolytic effect on the extracellular matrix proteins. Their effects on the blood vessel wall components have been extensively studied (Bjarnason and Fox, 1994; Kamiguti *et al.*, 1998).

The arrest of bleeding from the damaged blood vessels, however, depends on normal function of blood platelets and clotting factors, which are the principal blood components involved in hemostasis. The extent to which these latter components are directly affected by venom hemorrhagic metalloproteinases has not been explored in detail.

Zinc metalloproteases are widely occurring and participate in a number of important biological, physiological and pathophysiological processes including haemorrhage. Snake venom proteases cleave plasma proteins in a specific manner and induce alterations in the blood coagulation cascade (Kamiguti and Sano-Martins, 1995; Kamiguti *et al.*, 1998).

Various studies have demonstrated that purified venom metalloproteinases alone cause local hemorrhage. Hemorrhagic metalloproteinases from crotalid and viperid venoms produce local bleeding by causing lesions in the walls of small blood vessels (Kamiguti *et al.*, 1998). It is believed that this is caused by proteolysis of components of the basal lamina of the microvasculature. These enzymes degrade all major proteins of the extracellular matrix (ECM) and in this respect they resemble the cell-secreted soluble matrix metalloproteinases (MMP). A positive correlation exists between the proteolytic activity of venom metalloproteinases and their hemorrhagic potencies. Larger enzymes are more potent than the smaller ones in degrading the extracellular matrix. However, the proteolytic attack of these enzymes on ECM proteins is slow, while the *in vivo* hemorrhagic effect of the venom occurs within minutes of the bite or experimental injection. This indicates that the mechanism of action of these enzymes may be considerably more complex.

It has been well documented that the venom hemorrhagic metalloproteinases, because of their broad substrate specificity, cause digestion of the extracellular matrix proteins and damage the integrity of blood vessels (Kamiguti *et al.*, 1998).

2.5.5 Disintegrins

Disintegrins are cysteine-rich low molecular weight (5-10 KDa) polypeptides, which contain an RGD sequence recognized by integrins. Since the large hemorrhagic metalloproteinases possess a disintegrin-like domain (MDC enzymes) it is thought that some disintegrins may originate from autoproteolysis of these enzymes (Takeya *et al.*, 1990; Kamiguti *et al.*, 1998).

Venom disintegrins, unlike other RGD-containing ligands (i.e. fibrinogen) bind to $\alpha_{11b}\beta_3$ integrin without requiring prior activation of this integrin. This is because disintegrins have a unique RGD-containing loop which can express the ligand-induced binding site (LIBS) on the β_3 subunit (Juliano *et al.*, 1996; Kamiguti *et al.*, 1998).

Functionally, disintegrins can be divided into three groups according to their integrin selectivity and the presence of specific and active motifs. They are (a) those interacting with RGD motif-dependent integrins, (b) leukocyte integrin-binding disintegrins and (c) the $\alpha_1\beta_1$ integrin-binding disintegrins. The first group includes most of the monomeric disintegrins that contain the RGD motif, as well as disintegrins without the RGD motif but with inhibitory activity against RGD-dependent integrins such as KGD, MVD, MGD and WGD disintegrins. The second group is represented by MLD motif – containing disintegrins which interact with $\alpha_4\beta_1$, $\alpha_4\beta_7$ and $\alpha_9\beta_1$ integrins. The last group consists of the recently discovered, KTS motif containing disintegrins, obtustatin and viperistatin.

These disintegrins are potent and selective inhibitors of $\alpha 1\beta 1$, characterized as specific receptors for collagen IV (Marcinkiewicz, 2005; Koh *et al.*, 2006).

Disintegrins have been also shown to interfere with other integrin-mediated cell functions; for example, inhibition of tumor cell-extracellular matrix adhesion (Sheu *et al.*, 1997; Beviglia *et al.*, 1995) and metastasis (Morris *et al.*, 1995), of adhesion of human umbilical vein endothelial cells to matrix proteins (Juliano *et al.*, 1996), and also of egg fertilization through inhibition of sperm-oolemmal adhesion (Bronson *et al.*, 1995).

2.5.6 Fibrinogenases

Fibrinogen is a 340 KDa protein which contains two disulfide-linked symmetric half molecules; consisting of two outer D- domains, each connected by a coiled-coil segment to a central E-domain (Cortelazzo *et al.*, 2010). Each half contains three chains designated as A, B or G with a molecular mass of 63KDa, 56KDa, and 47KDa, respectively (Hettasch and Greenberg, 1998).

Fibrinogenases are direct-acting zinc dependent metalloproteinases with disulphide bonds. They do not cleave off fibrinogen fibrinopeptides and therefore do not induce polymerization (Markland, 1988; Swenson, 2005; Chernyshenko *et al.*, 2010). Fibrinogenases especially the alpha type slow down blood coagulation, because truncated fibrinogen does not form as strong a fibrin clot as the native one (Kini, 2006). Most of them are inhibited by blood serum proteins and have affinity both for fibrinogen and for stabilized fibrin (Markland, 1988; Swenson and Markland, 2005; Chernyshenko *et al.*, 2010).

β -fibrinogenases are mostly serine proteinases that cleave the α -chain of fibrinogen moderately though some alpha specific ones exist (Jiao *et al.*, 2005; Samel *et al.*, 2002; Chernyshenko *et al.*, 2010). They are mostly 23-32KDa glycoproteins possessing fibrinolytic and arginine-esterase activity apart from fibrinogenase activity. They are more heat stable and less dependent on pH changes than metalloproteases (Matsui *et al.*, 1998; Jiao *et al.*, 2005; Swenson and Markland, 2005). Extensive sequence homology exists between the fibrin (ogen) olytic serine proteinases and the plasminogen activators and thrombin-like venom serine proteinases. However, they do not have thrombin-like activity, and β -chain hydrolysis takes part in different non-thrombin sites (Matsui *et al.*, 1998; Swenson and Markland, 2005).

Direct fibrin (ogen) olytic metalloproteases degrade preferentially the α -chain of fibrinogen followed by the β -chain. The enzymes that degrade the β -chain without fibrinolysis belong to the serine proteases group (Markland, 1998). Some metalloproteases have fibrinogenolytic or fibrinolytic activity and are named fibrinogenases (Utaiincharoen *et al.*, 1993; Hung *et al.*, 1994; Zhang *et al.*, 1995; Lee *et al.*, 1999; Lee and Park, 2000; Hung and Chiou, 2001; Felicori *et al.*, 2003; Jia *et al.*, 2003). These enzymes have been classified as α , β and γ -fibrinogenases based on their specificity for cleaving fibrinogen polypeptide chains (Pandya and Budzynski, 1984; Kini and Evans, 1991; Swenson *et.al*, 2004). The majority of fibrin (ogen) olytic enzymes are metalloproteases (Ouyang *et al.*, 1979; Mao *et al.*, 1995) with specificity directed preferentially towards the α -chain and with somewhat lower activity towards the β -chain (Markland, 1998).

Many of the venom fibrinolytic enzymes that have been characterized are zinc metalloproteinases and members of the metzincin family (Markland, 1998). Adamalysin II (Gomis-Ruth *et al.*, 1993; Gomis-Ruth *et al.*, 1994) was the first venom metalloprotease whose three dimensional structure was determined.

A lot of interest has been generated in snake venom direct-acting fibrinolytic metalloproteases because of their clinical potential for the treatment of occlusive vascular disease.

Some of these enzymes have been tested *in vivo* with promising results (Markland, 1998, Willis *et al.*, 1989; Ahmed *et al.*, 1990; Markland *et al.*, 1994; Mao *et al.*, 1995; Markland, 1996; Gasmi *et al.*, 1997). These studies have revealed that the highly purified fibrinolytic snake venom enzymes produced consistent thrombolysis. The venom enzymes act by a completely different mechanism from that of the plasminogen activators; the only agents presently approved for clinical use (Markland, 1998, Sherry, 1990; Collen and Lijnen, 1995), and may have certain advantages over the plasminogen activators.

2.5.7 Thrombin-like enzymes

Some snake venom proteinases, called thrombin-like enzymes, can hydrolyse the N-terminal end of α or and β -chain, and release either fibrinopeptide A (Shieh *et al.*, 1988; Au *et al.*, 1993; Magalhaes *et al.*, 1993; Nishida *et al.*, 1994; Hahn *et al.*, 1996), fibrinopeptide B (Herzig *et al.*, 1970; Guan *et al.*, 1984; Shainoff and Welches, 1988) or both (Pirkle *et al.*, 1986; Nikai, 1995), inducing fibrin clots in a similar way to thrombin.

Approximately 100 snake venom toxins have been identified as ‘thrombin-like’ enzymes [Pirkle, 1998; Koh *et al.*, 2006].

Thrombin is able to cleave both fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from fibrinogen and activating factor XIII (fibrin-stabilizing factor). While some actions of these snake venom thrombin-like enzymes (SVTLEs) mimic the effects of thrombin, they usually cleave FPA alone; only a few cleave FPB. Thus, without cleavage of both FPA and FPB, they are unable to activate factor XIII and the clots produced can easily be broken down. The failure of the clots to be cross-linked leads to a breakdown in the fibrinolytic system and effective removal of fibrinogen from the plasma.

2.5.8 Platelet aggregation inhibitors

Many snake venom toxins affect platelet function (Andrews *et al.*, 2001; Koh *et al.*, 2006). They can be grouped into a few major families, such as enzymes like serine proteinases, zinc-dependent PI-PIV metalloproteinases of the reprotolysin family and group II PLA2 isoenzymes as well as proteins with no enzymatic activity, such as C-type lectins, CRISP and disintegrins (Juarez *et al.*, 2004; Koh *et al.*, 2006). Of these, disintegrins and C-type lectins (Andrews *et al.*, 2000, Wisner *et al.*, 2002; Koh *et al.*, 2006) have been considered as useful modulators of platelet function.

2.5.9 C-type lectin-like proteins

C-type lectins such as the mannose-binding proteins bind a sugar moiety in the presence of Ca^{2+} and contain the carbohydrate recognition domain (CRD). C-type lectins are a class of proteins widely distributed in nature that display various functions in important physiological processes. The C-type lectin-like proteins are an important group of proteins among the haemorrhagic components in snake venom.

Most C-type lectin-like proteins in snake venom do not contain the classic calcium/sugar-binding loop and they have evolved to bind a wide range of physiologically important proteins and receptors (Liu *et al.*, 2005; Koh *et al.*, 2006). Based on their structural and functional entities, these proteins in snake venom have been classified into the true C-type lectins (contains the CRD domain) that bind a sugar molecule and the C-type lectin-like proteins with CRD-related non-carbohydrate-binding C-type lectin-like domains (CTLDs) that do not bind a sugar moiety (Drickamer, 1999; Koh *et al.*, 2006). They are further divided into CRD-containing proteins, factor IX/X-binding proteins and those that bind to the platelet receptors (Clemetson *et al.*, 2005; Koh *et al.*, 2006).

Snake C-type lectin-like proteins bind to a wide range of coagulation factors that are important in haemostasis and to platelet receptors and display both anti-coagulant- and platelet-modulating activities. They activate platelets by binding to von Willebrand factor or specific receptors such as GPIb, $\alpha 2\beta 1$ and GPVI. Heterodimeric GPIb-binding molecules mainly inhibit platelet functions, while the multimeric binding molecules activate platelets. Some tetrameric snake venom C-type lectin-like proteins activate platelets by binding to GPVI, while others affect platelet function via integrin $\alpha 2\beta 1$. Some act by inducing von Willebrand factor to bind to GPIb as well, or activate platelets via $\alpha 2\beta 1$ and GPIb (Clemetson *et al.*, 2005; Koh *et al.*, 2006).

While the earliest described C-type lectin, botrocetin, clearly activates platelets by inducing interactions between GPIb and von Willebrand factor, many GPIb-binding C-type lectins are described as inhibitory (Fukuda *et al.*, 2002; Koh *et al.*, 2006).

2.5.10 *Naja nigricollis* venom

The venom of *Naja nigricollis* retains the typical elapid neurotoxic properties while combining these with cytotoxic and significant anticoagulant effect.

Haemotoxic features of the venom are responsible for bite symptoms which include severe external haemorrhaging and tissue necrosis around the bite area. Death generally occurs due to asphyxiation due to paralysis of the diaphragm. The murine LD50 of *Naja nigricollis* is 2mg/Kg subcutaneous and 0.03mg/Kg intravenous. The average venom yield of this snake is 150-350mg (*Naja*, 2008).

2.6 Uses of Snake Venom

The broad spectrum of snake venom activities, including their biochemical, toxicological, physiological and pharmacological profiles results from the action of their constituents. Therefore, snake venoms are of biological interest as a potential source of active compounds.

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

Therapeutic uses of snake venoms include applications as antihypertensive agents, anti-stroke medication, anticoagulants, anti-haemorrhagic agents, anti-angiogenic agents and neurotropic agents. A wider range of haemostatic factors found in snake venoms have been used in the development of laboratory diagnostics.

Venom proteins have been produced commercially to measure the levels of compounds associated with haemostatic disorders, such as fibrinogen, prothrombin, blood-clotting factors and protein (Lewis and Garcia, 2003).

2.7 Clinical Effects of Snake Envenomation

Snake venoms are considered to be most highly developed and complex of all known toxins produced by plants and other animals (Tu, 1996). They contain a large number of highly pharmacologically active substances and each of them has specific modes of actions. These various substances injected into the victim start acting independently, synergistically or antagonistically.

Snake venom proteins have evolved to target different tissues, organs and physiological systems. Hence a diversity of symptoms arises after a snakebite which will ultimately lead to failure of multiple tissues, organs, systems and often death (Torres *et al.*, 2003). Some of the major clinical symptoms are intense localized pain, loss of consciousness, drowsiness, headache, vomiting, inflammation, bleeding, shock, haemorrhage, necrosis and muscular paralysis (Campbell, 1979; Efrati, 1979; Reid, 1979; Russell, 1979).

The symptoms suggest that snake venoms affect various systems, particularly the central nervous system (CNS), cardiovascular system, muscular and vascular system (Koh *et al.*, 2006).

Neurotoxicity is the most pronounced effect of envenomation by snakes from the *Elapidae* family, while envenomation by snakes from the *Viperidae* family is usually characterized by local and, in severe cases, systemic effects (Torres *et al.*, 2012).

Envenoming by *Naja n. nigricollis* induces local necrosis, haemorrhage, complement depletion (Warrell and Ormerod, 1976), and respiratory arrest or paralysis (Chippaux *et al.*, 1991; Hasson *et al.*, 2012).

The venom of the *Naja n. nigricollis* consists of phospholipase A₂ (an anticoagulant enzyme which inhibits the prothrombinase complex by its binding to coagulation factor Xa (Stefansson *et al.*, 1990; Kerns *et al.*, 1999; Hasson *et al.*, 2010) and cardiotoxin (Bilwes *et al.*, 1994). Furthermore, in some cases envenoming by *Naja nigricollis* can induce corneal ulceration and anterior uveitis (Fung *et al.*, 2009; Hasson *et al.*, 2010).

The clinical manifestations of snakebites are dependent on two factors, the intrinsic toxicity and amount of venom injected. 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; Omogbai *et al.*, 2002). These local effects develop rapidly after the bite due to local effects of cytotoxic components such as metalloprotease, phospholipase A₂, and hyaluronidase. Consequently, a delay in the access to health facilities frequently results in drastic tissue damage and permanent disability.

Systemic effects of envenoming are also common and more immediately life threatening. In Elapid and hydrophid snakebites, neurotoxicity is a typical consequence, attributable to effects of pre and/or postsynaptically acting neurotoxins targeting neuromuscular junctions (Chiappinelli, 1991; Endo and Tamiya; 1991; Menez, 1991; Warrell, 1999; Koh *et al.*, 2006). These toxins cause progressive descending paralysis, which may become life-threatening when bulbar and respiratory muscles are involved. In envenoming by several Elapid snakes and some viperids, there is a generalized rhabdomyolysis, which may cause myoglobinaemia, hyperkalaemia, and acute renal failure (Gutierrez *et al.*, 2006).

Hemorrhagic manifestations following viper and spitting cobra bites are also common (Braud *et al.*, 2000; Matsui *et al.*, 2000; White, 2000). Haemotoxins include anticoagulants, procoagulants, fibrinolysins, haemorrhagins and haemolysins.

Four groups of myotoxins are found in snake venoms: small basic polypeptide myotoxins, local phospholipase A₂, myotoxins (myonecrotic toxins), general myotoxins (myoglobinuric toxins) and miscellaneous myotoxins. These are responsible for the local and systemic skeletal muscle damage characterized by skeletal muscle breakdown, muscle weakness, pain tenderness and myoglobinuria (Harris and Cullen, 1990; Gutierrez and Lomonte, 1997; Ownby, 1998; White, 2000). Snake venom nephrotoxins can cause primary or secondary kidney damage with clinical manifestations such as proteinuria, haematuria, haemoglobinuria, myoglobinuria, and renal failure (Sitprija and Boonpucknavia, 1979; Sitprija and Chaiyabutr, 1999).

2.8 Antivenoms

Antivenom is the only specific antidote to snake venom and its timely administration completely reverses all systemic manifestations of envenoming (Premawardhena *et al.*, 1999; Johnston, 2003; Pinho *et al.*, 2005; Gutierrez *et al.*, 2006; Sitprija, 2006). Adverse reactions are frequent (as high as 87%), may include anaphylactic shock, and cannot be predicted by sensitivity tests (Fan *et al.*, 1999; Premawardhena *et al.*, 1999). Snake venoms are immunochemically diverse; therefore, efficacy of antivenoms is geographically and biologically restricted.

Although an intravenous administration of antivenom prepared from Ig G of venom-immunised horses or sheep, is an effective treatment for systemic envenoming, the clinical consensus is that antivenom is of limited effectiveness against the effects of local envenoming that develop rapidly after a bite (Laloo and Theakston, 2003).

Such effects include severe pain, oedema, localized haemorrhage and necrosis (Warrell *et al.*, 1977; Hasson *et al.*, 2010) which often results in permanent scarring and deformity (Gutierrez *et al.*, 2006).

In Africa, as well as most other developing parts of the world where most snakebite occur, there is a crisis regarding production, distribution and accessibility of antivenoms (Theakston and Warrell, 2000; Cheng and Winkel, 2001a; Theakston *et al.*, 2003; WHO, 2010). This reflects a global lack of momentum for snakebite management.

Besides antivenom administration, the treatment of snakebite envenoming includes a number of additional interventions, such as maintenance of fluid, electrolyte balance, and good urine flow; administration of tetanus toxoid; assisted ventilation; dialysis; use of acetylcholinesterase inhibitors, preceded by atropine sulfate, in neurotoxic envenomations; early alkalinisation of urine by sodium bicarbonate in patients with myoglobinuria or haemoglobinuria; antibiotics in case of development of local infection; and surgical debridement of necrotic tissue. Atropine sulfate counteracts the muscarinic effects of acetylcholine, such as increased respiratory secretions, sweating, bradychardia, and colic (Cruz *et al.*, 2009).

New approaches have been developed to improve the quality of antivenoms, such as using DNA immunizations or the purified relevant toxins such as antigens instead of the whole venom; searching for other animal species from which it is possible to obtain antivenom, such as camels and hens; preparing antivenoms that combines antibodies with recombinant “nanobodies”, which, by having a low molecular mass, may reach tissue compartments more rapidly than conventional IgG fragments (Wilde *et al.*, 1996; Gutierrez *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals

General laboratory and inorganic chemicals were obtained from Aldrich Chemical Company, and were of analytical grade. The following chemicals used are products of Sigma (St. Louis, USA): human fibrinogen, human thrombin, tetramethylene diamine (TEMED), aprotinin, phenylmethylsulfonylfluoride, sodium phosphate monobasic and dibasic salts, bovine serum albumin, Bradford reagent, sodium carbonate, trichloroacetic acid, tyrosine, Folin and Ciocalteu reagent, acrylamide, metbisacrylamide, tromethamine (TRIS), hydrochloric acid, sodium dodecyl sulphate, ammonium persulphate, glycine, glycerol, beta-mercaptoethanol, bromophenol blue, sodium chloride, calcium chloride, acetic acid, sodium acetate, magnesium chloride, manganese chloride, zinc chloride, cobalt chloride, ethylene diamine tetra acetic acid, 1,10-phenanthroline, methanol, sodium hydrogen carbonate, anti-rabbit alkaline phosphatase conjugate, Alkaline phosphatase substrate and buffer, sephadex G-75, DEAE-cellulose, heparin agarose, comassie brilliant blue R250, Freund's complete and incomplete adjuvants.

3.2 Equipment

Centrifuge, UV-Visible spectrophotometer, electrophoresis tank and power pack, pH metre, water bath, ELISA washer, incubator, and plate reader, 22-G needle, dissection set, 19-G, 21-G needles, 1ml and 10ml syringes, chromatography columns (2.5 by 30cm, 1.7 by 15cm, 50cm), beakers, conical flasks, measuring cylinders, test tubes, test tube racks, glass wool, automatic pipettes, ELISA plates, glass rods.

3.3 Animals

Six young healthy rabbits (3.5- 4kg), 6 months old were purchased from the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. They were de-wormed and allowed to acclimatize for a week before the commencement of the experiment.

3.4 *Naja nigricollis* venom

Naja nigricollis was caught from the wild at Zaria in the northern part of Nigeria. It was 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 snakes were placed inside a beaker covered with polythene sheets. On fright, they attacked the polythene with their fangs releasing the venom, which was frozen and dried at 5°C. About 350mg of venom was obtained.

3.5 Enzyme Purification

3.5.1 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 2 by 100cm 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 (200 mg) 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. 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.

3.5.2 Ion-exchange chromatography on DEAE-cellulose

DEAE-cellulose was prepared by dissolving 5g of the anion-exchanger in 50 ml of phosphate buffer, pH 7.8. The slurry was then poured into a 2.5 X 30 cm column. The pooled sample (3ml) obtained from the gel-filtration step 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.

3.5.3 Affinity chromatography on heparin-agarose column

The pooled sample (3ml) obtained from the ion-exchange chromatography step was applied to a Heparin-Agarose column previously equilibrated with 10mM Tris-HCl + 5mM CaCl₂, pH 7, and eluted with 10mM Tris-HCl + 1M NaCl, pH 7. Ten 5ml fractions were collected at a flow rate of 1ml/minute. The isolated enzyme was considered *Naja nigricollis* fibrinogenase.

3.5.4 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 tyrosine standard (1.1mM) with distilled water to a total volume of 250 μL followed by addition of 625 μL of 500mM sodium carbonate solution and 125 μL Folin and Ciocalteu reagent. The contents were mixed and incubated at 37°C for 30 minutes. The absorbances of the green colored solutions were measured at 660 nm against reagent blank. A plot of absorbance versus μmole tyrosine was made. The fractions were assayed for fibrinogenase activity as follows:

Fibrinogen solution (0.5% in phosphate buffer), 0.2ml 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 °C and centrifuged for 10 minutes at 10,000g. Supernatant (250 µL) 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.

3.5.5 Determination of total protein concentration

This was determined by the method of Bradford (1976) using bovine serum albumin as standard (100µg/ml). A calibration curve covering the range 0 to 100 µg/ml standards were prepared by making dilutions in duplicate using water as a diluent to a total volume of 800 µL. The fractions were also analyzed in duplicates by arranging test tubes labeled as test, standard and blank. Distilled water (700 µL) was dispensed into all tubes followed by 100 µL of sample or fraction, 100µL BSA standard and 100 µL distilled water to the tubes labeled test, standard and blank respectively making a total volume of 800 µL. Bradford reagent (200 µL) 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.

3.5.6 Sodium dodecyl sulphate polyacrylamide-electrophoresis (SDS-PAGE)

The crude venom and pooled fractions from each chromatographic step were subjected to SDS-PAGE using 14% gels by the method of Laemmli (1970). Equal volumes (100 µL) of the samples and the dissolving buffer (glycerol, SDS, beta-mercaptoethanol and traces of bromophenol blue) were boiled at 100 degrees celsius for 3-5 minutes.

The resolving gel (1.5M Tris-HCl, pH 8.3+ 0.4% SDS, 30% acrylamide +0.8% metbisacrylamide, water, 10% ammonium persulphate and TEMED) was prepared, mixed, loaded onto the gel casting apparatus and overlaid with 0.1% SDS to isolate for polymerization for oxygen. This was poured off, rinsed with distilled water and blotted to remove excess water. A comb was inserted and the stacking gel (0.5M Tris-HCl, PH 6.8+0.4% SDS, 30% acrylamide +0.8% metbisacrylamide, water, 10% ammonium persulphate and TEMED) was prepared, loaded and allowed to polymerize for 45 minutes. The gel was then clipped to the electrophoresis apparatus with the running buffer (SDS-Tris-glycine buffer) followed by careful removal of the comb and loading of samples into the wells with a pipette tip. The bottom of the electrophoresis tank was filled with the running buffer and the apparatus was connected to the power supply and allowed to run at 40V for 40 minutes until the dye entered the resolving or separation gel, then increased to 80V until the dye reached the bottom of the gel. The power supply was turned off and the gel sandwich removed. The gel was stained with a staining solution (methanol, acetic acid, comassie brilliant blue R250) for two hours followed by destaining with a mixture of acetic acid, methanol, and water for two hours. The gel was dried and stored.

3.6 Characterisation of Purified *N. nigricollis* Fibrinogenase

3.6.1 Effect of temperature on activity of *N. nigricollis* fibrinogenase

The effect of temperature on the activity of the enzyme was determined by assaying at different temperatures (20, 30, 40, 50, 60, 70, and 80 °C) and comparing with control (37 °C). To test tubes arranged in duplicates for each temperature, 0.13ml of fibrinogen solution (0.5% in phosphate buffer) was dispensed into a centrifuge tube followed by 0.025ml of enzyme solution.

The mixture incubated for 10 minutes at the different temperatures and centrifuged for 10 minutes at 10,000g. Supernatant (125 μ L) was subjected to the Folin and Ciocalteu protein assay and the residual enzyme activity calculated.

3.6.2 Thermostability studies on activity of *N. nigricollis* fibrinogenase

Aliquots (25 μ l) of the enzyme were incubated for 30 minutes at different temperatures (20, 30, 40, 50, 60, 70 and 80 $^{\circ}$ C). These were allowed to cool at room temperature and assayed for residual fibrinogenase activity as previously described. To test tubes arranged in duplicates for each temperature, 0.13ml of fibrinogen solution (0.5% in phosphate buffer) was dispensed into centrifuge tubes containing the cooled pre-incubated enzyme solution, incubated at the assay temperature (37 $^{\circ}$ C) for 10 minutes and centrifuged for 10 minutes at 10,000g. Supernatant (125 μ L) was subjected to the Folin and Ciocalteu protein assay. The Arrhenius plot of logarithm of enzyme activity (Log V) versus the reciprocal of the absolute temperature (K^{-1}) was used to determine the energy of activation (Ea) of the partially purified enzyme.

3.6.3 Kinetic studies

The enzyme was assayed with varying substrate (fibrinogen) concentrations 0.1% to 1.0% in phosphate buffer pH 7.8. Dilutions of fibrinogen solution were made in a total volume of 0.13ml for each concentration. Enzyme solution (25 μ l) was added and incubated at 37 $^{\circ}$ C for 10 minutes. The mixture was centrifuged at 10,000g for 10 minutes. Supernatant (125 μ L) was subjected to the Folin and Ciocalteu protein assay. The Lineweaver-Burk plot was used to determine the kinetic parameters K_m and V_{max} .

3.6.4 Effect of metal ions and inhibitors on *N. nigricollis* fibrinogenase activity

The enzyme was assayed in the presence of 10 mM each of the following inhibitors: EDTA, 1, 10- phenanthroline, beta-mercaptoethanol, aprotinin, phenylmethylsulfonyl fluoride and 1mM each of the following metal ions: calcium, magnesium, cobalt, zinc and manganese. The activities were compared with that of control (assayed in the absence of inhibitor). To test tubes arranged in duplicates for each inhibitor, 0.025ml of enzyme solution and 0.025ml of inhibitor solution were mixed. Fibrinogen solution (0.5% in phosphate buffer) was dispensed into the tubes, incubated at the assay temperature (37 °C) for 10 minutes and centrifuged for 10 minutes at 10,000g. Supernatant (125 µL) was subjected to the Folin and Ciocalteu protein assay.

3.6.5 Effect of pH on *N. nigricollis* fibrinogenase activity

Optimum pH for activity was determined by incubating the reaction mixture in different buffers at varying pH values: acetate buffer; pH 4.5 to 6.5, phosphate buffer; pH 6.5 to 7.5 and Tris-HCl buffer, pH 7.5 to 8.5. To test tubes arranged in duplicates for each pH, 0.13ml of fibrinogen solution was dispensed into a centrifuge tube followed by 0.025ml of enzyme solution in a different buffer solution. The mixture was incubated for 10 minutes and centrifuged for 10 minutes at 10,000g. Supernatant (125 µL) was subjected to the Folin and Ciocalteu protein assay and the residual enzyme activity determined.

3.6.6 pH stability studies on *N. nigricollis* fibrinogenase activity

Aliquots (25µl) of the enzyme were pre-incubated for 30 minutes at different pH values ranging from 4.5 to 8.5.

To test tubes arranged in duplicates for each pH, 0.13ml of fibrinogen solution was dispensed into centrifuge tubes containing the pre-incubated enzyme solution, incubated at the assay temperature (37 °C) for 10 minutes and centrifuged for 10 minutes at 10,000g. Supernatant (125 µL) was subjected to the Folin and Ciocalteu protein assay and residual enzyme activity was determined.

3.6.7 Determination of fibrinogenolytic activity of *Naja nigricollis* fibrinogenase

The fibrinogenolytic activity of the enzyme was determined by the method of Oliveira *et al*, (1999). Fibrinogen and enzyme were mixed 1:100(w/w) and the mixture incubated at 37°C for different time intervals (0 to 90 mins). The reaction was stopped by the addition of an equal volume of denaturing buffer containing 2 % (w/v) SDS and 10% (v/v) beta-mercaptoethanol. The reaction products were subjected to SDS-PAGE.

3.7 Production of Monospecific Polyclonal Antibodies

3.7.1 Antigen preparation

Sterile and pyrogen free antigen was prepared by filtration of the purified enzyme through a low binding 0.22 micron filter. Antigen was prepared in such a way that it passes easily through a 20G needle. The antigen (500µg) was dissolved in 5ml of sterile PBS (phosphate buffered saline). The antigen solution (5ml) was mixed with 1ml of the adjuvant to give a concentration of 0.5mg/ml of complete Freund's adjuvant.

3.7.2 Immunisation

About 2 to 10 ml of pre-immune blood sample was collected from the ear artery and kept as naïve serum. Then 0.5ml of antigen solution was combined with 0.5ml of Freund's complete adjuvant and mixed thoroughly.

The sample was injected subcutaneously into 10 different sites. Booster injections using the Freund's incomplete adjuvant were given after 3 weeks (0.2ml per site). Blood (10ml) was collected from the animal with a 19G needle and allowed to clot and retract at 37°C overnight. The clotted blood was refrigerated for 24 hours before the serum was clarified by centrifugation at 2,500 r.p.m for 20 minutes and decanted. The titre was determined from the serum. This was repeated after 3 weeks until the titre reached acceptable levels.

3.7.3 Cross reaction (Titration)

Recognition of enzyme by polyclonal antibodies was evaluated by Enzyme Linked Immunosorbent Assay (ELISA) using the following protocol: Antigen was diluted to 0.1µg/25µL (4.0µg/ml) in a coating buffer (0.1M NaHCO₃, pH 8.6). Each well was coated with 25µL (0.1µg) of antigen in coating buffer by adding to wells of an ELISA plate, covered with plastic film and incubated at 4°C overnight or 37°C for one hour, the coating solution was removed and washed twice with PBS and the wells blocked by adding 50µL 3% BSA/PBS per well and incubated for one hour at 37°C, wrapped in plastic and blocking solution shaken off. Primary antibody preparation was serially diluted (1:10 to 1:10,000) in 1% BSA/PBS; 25µL was dispensed to each well. The plate was wrapped and incubated at 37°C for 1 hour, the antibody solution shaken off and washed 10 times with PBS. Anti-rabbit alkaline phosphatase conjugate at 1:1000 in 1% BSA, 25µL was added per well, the plate wrapped and incubated at 37°C for one hour, the antibody solution shaken off and washed 10 times with PBS.

Alkaline phosphatase (substrate) in 5ml alkaline phosphatase developing buffer was added, 50µL per well, the plate was developed at room temperature and read at 15mins, 30mins and 1 hour.

3.7.4 Anti-haemorrhagic activity

A modified method described by Soto *et al*, (1988) was used to determine the anti-haemorrhagic activity. Eight animals were used and the protocol was as follows: group A animal was injected with 0.05ml-0.1ml of the enzyme on one thigh and 0.1-0.2ml of enzyme on the other; group B animal was injected with 0.1ml of enzyme and 0.05ml polyclonal serum on different sites; group C animal was injected with 0.1ml of enzyme and 0.05ml polyclonal serum on the same site; group D animal was injected with 0.1ml of enzyme followed by polyclonal serum (active immunization); group E was injected by polyclonal serum followed by 0.1ml of the enzyme crude venom only (passive immunization); group F animal was injected with polyclonal serum only; group G and H animals served as control. After 24 hours, the animal was sacrificed, the skin removed, diameter of haemorrhage measured and any appearance of oedema or necrosis noted.

CHAPTER FOUR

4.0

RESULTS

4.1 Enzyme Purification

Naja nigricollis venom, initially fractionated on Sephadex G-75 column, displayed two peaks, I and II. The elution profile of this chromatography is shown in Figure 4.1. The pooled fractions showed 1.56 folds more fibrinogenase activity than the crude venom with a yield of 53.7% (Table 4.1). Fractions with highest specific activity were pooled and further purified on a DEAE-cellulose column.

Pooled fractions obtained from gel filtration displayed a single peak after fractionation on DEAE-cellulose column. The elution profile of this step is shown in Figure 4.2. The pooled fractions from this step revealed 4.26 folds more fibrinogenase activity than the crude venom with a yield of 2.7 % (Table 4.1).

Fraction from the ion-exchange chromatographic step was applied to a Heparin-Agarose column. The non-adsorbed fraction displayed fibrinogenolytic activity and was considered as *Naja nigricollis* fibrinogenase. The elution profile of this step is shown in Figure 4.3. The purified *Naja nigricollis* fibrinogenase revealed 9 folds more fibrinogenase activity than the crude venom with a yield of 0.01 % (Table 4.1).

TABLE 4.1: Three-step Purification Profile of Fibrinogenase from *Naja nigricollis* Venom.

STEPS	TOTAL PROTEIN (mg/ml)	TOTAL FIBRINOGENASE ACTIVITY (μ moles/ml/hr)	SPECIFIC ACTIVITY (μ moles/ml/hr/mg protein)	PURIFICATION FOLD	RECOVERY (%)
CRUDE VENOM	0.360	16.84	46.78	1	100
GEL FILTRATION ON SEPHADEX G-75	0.1225	9.044	73.83	1.56	53.7
ION-EXCHANGE ON DEAE-52 CELLULOSE	0.0023	0.458	199.13	4.26	2.7
AFFINITY CHROMATOGRAPHY ON HEPARIN AGAROSE	0.000204	0.0910	446.48	9.54	0.01

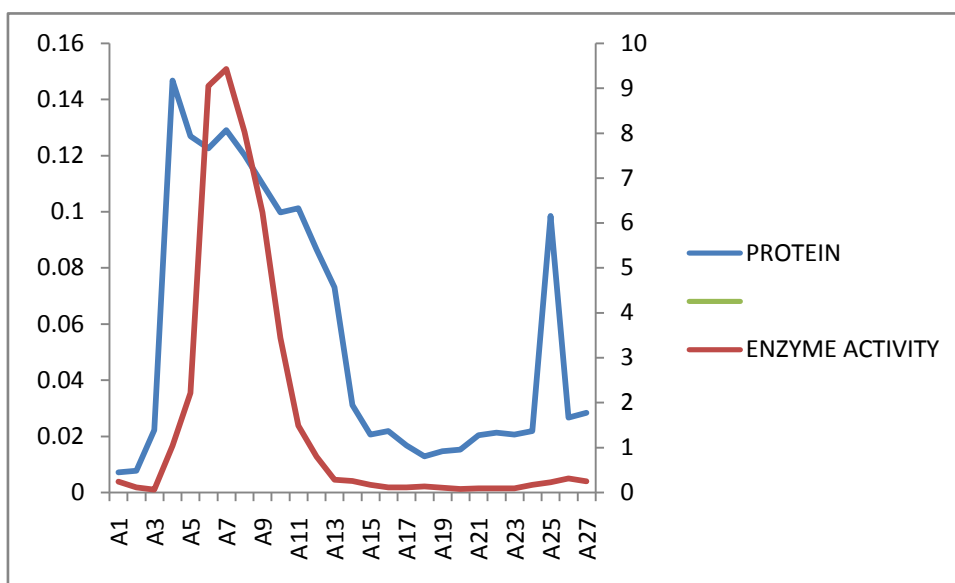


Figure 4.1: Elution profile of fibrinogenase from *Naja nigricollis* venom. Fractionation on sephadex G75: crude venom (200mg) dissolved in 10ml of phosphate buffer pH 7.8., was applied to the column (2 by 100cm) and elution was carried out at a flow rate of 1ml/minute at 25°C with phosphate buffer pH 7.8.

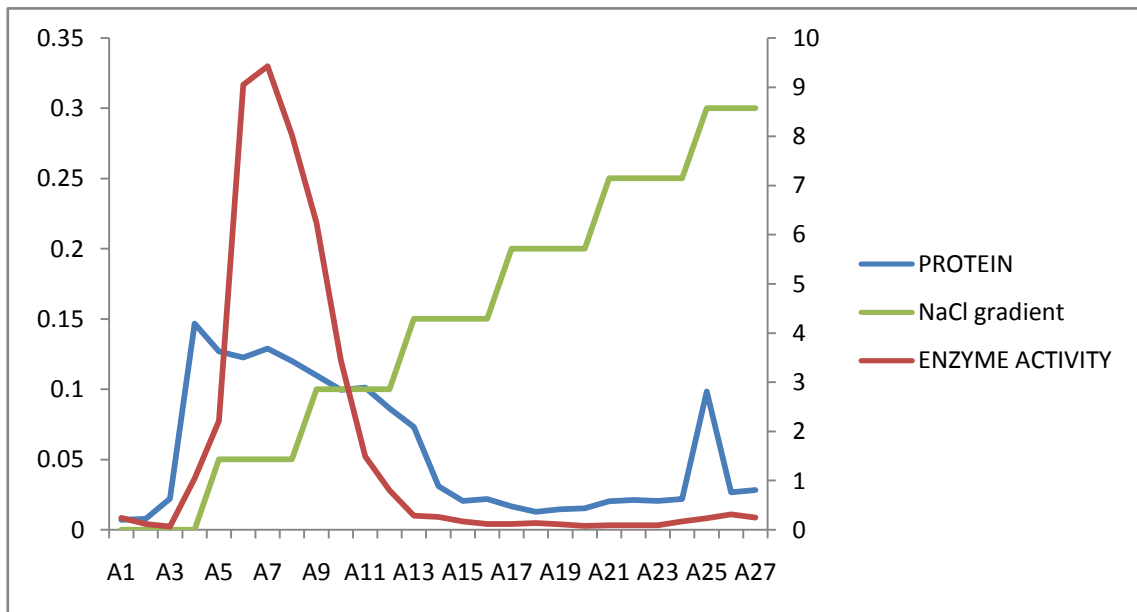


Figure 4.2: Elution profile of fibrinogenase from *Naja nigricollis* venom. Fractionation on DEAE-cellulose: pooled fraction from gel filtration (5ml) was applied to the column (30cm) and elution was carried out at a flow rate of 1ml/minute at 25 °C with convex sodium chloride gradient from 0.05M to 0.3M.

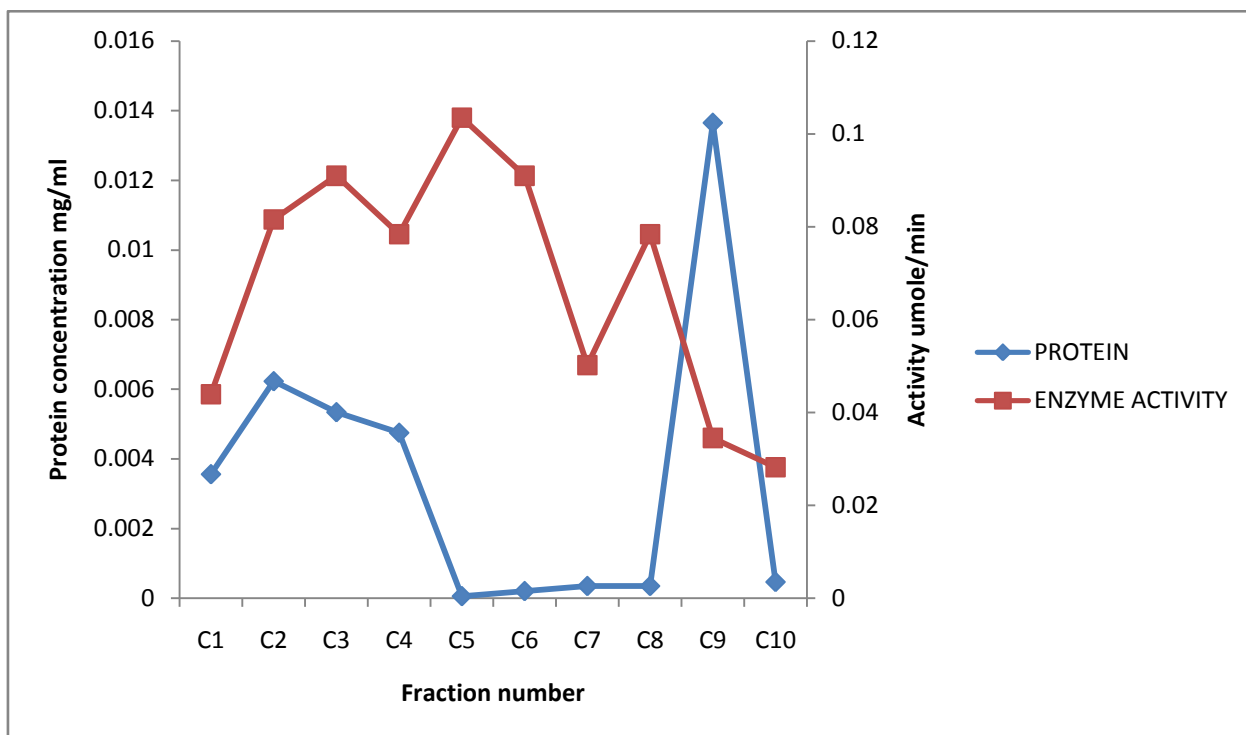


Figure 4.3: Elution profile of fibrinogenase from *Naja nigricollis* venom. Fractionation on Heparin-Agarose: the pooled fraction (2ml) from ion-exchange chromatography was applied to the column (1.7X15cm) and elution was carried out at a flow rate of 1ml/minute with 10mM Tris-HCl buffer containing 1M NaCl at pH 7.0.

4.2 Enzyme Characterisation

4.2.1 Effect of temperature on *N. nigricollis* fibrinogenase

The partially purified fibrinogenase showed a broad temperature range of activity with an optimum value of 40°C (Figure 4.4). The enzyme continued to show some activity even at temperatures above 40 °C with highest decline at temperatures above 60 °C representing a drop of more than 70%.

The thermostability studies (Figure 4.5) showed the enzyme to be stable at 20 to 60 °C. The activation energy (E_a) was found to be 0.00175 KJ/mol/K (Figure 4.6).

4.2.2 Effect of pH on *N. nigricollis* fibrinogenase

The fibrinogenase showed a broad range of activity over a wide pH range of 3.6 to 8.5 with an optimum pH of 7.5 (Figure 4.7). The enzyme was highly active over the narrow pH range of 6.5 to 7.8. Low enzyme activities were observed in alkaline pH above 7.8 and at acidic pH below 6.5.

The pH stability studies (Figure 4.8) showed the enzyme to be stable over a wide pH range with stability declining above pH 7.8 and below pH 6.5.

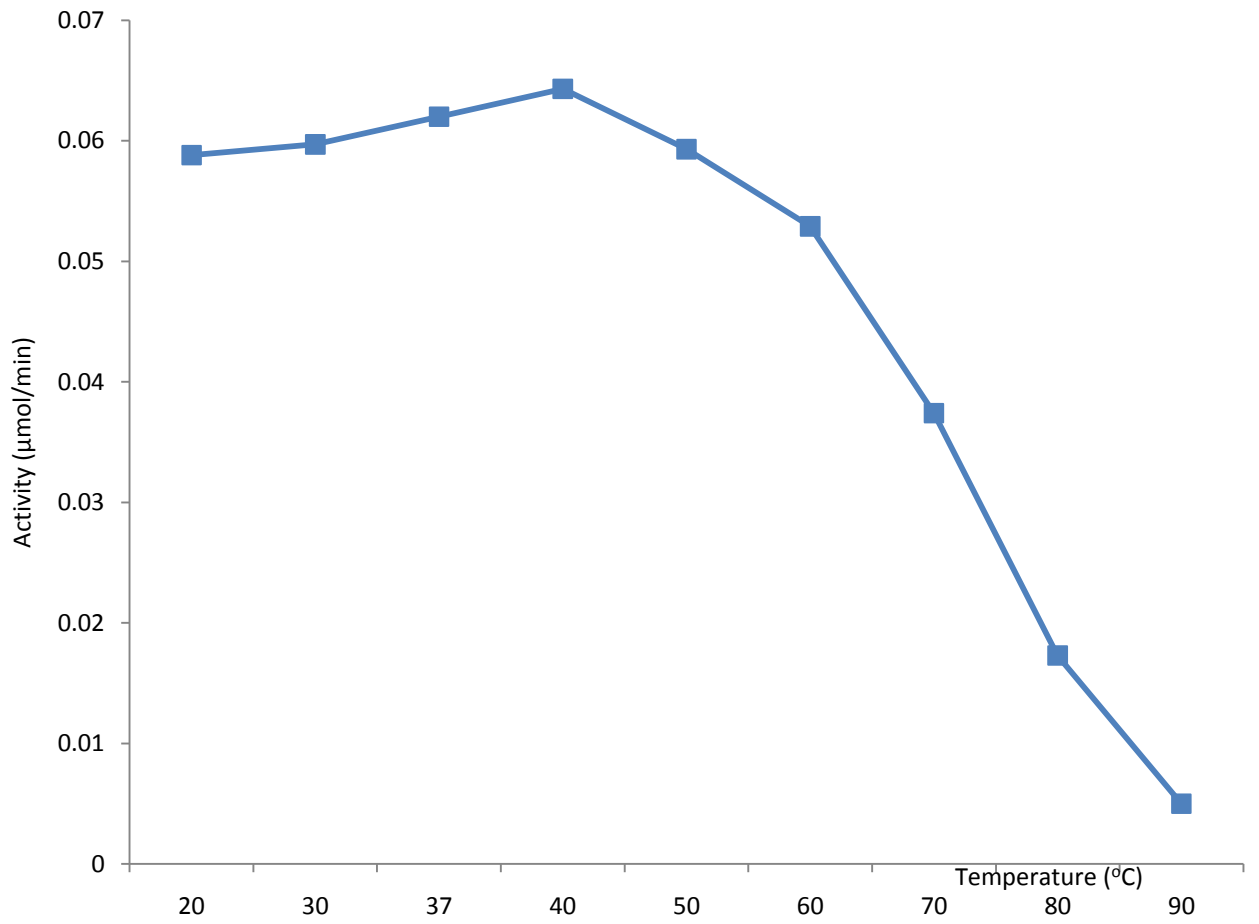


Figure 4.4: Effect of Temperature on the Activity of *N. nigricollis* Fibrinogenase

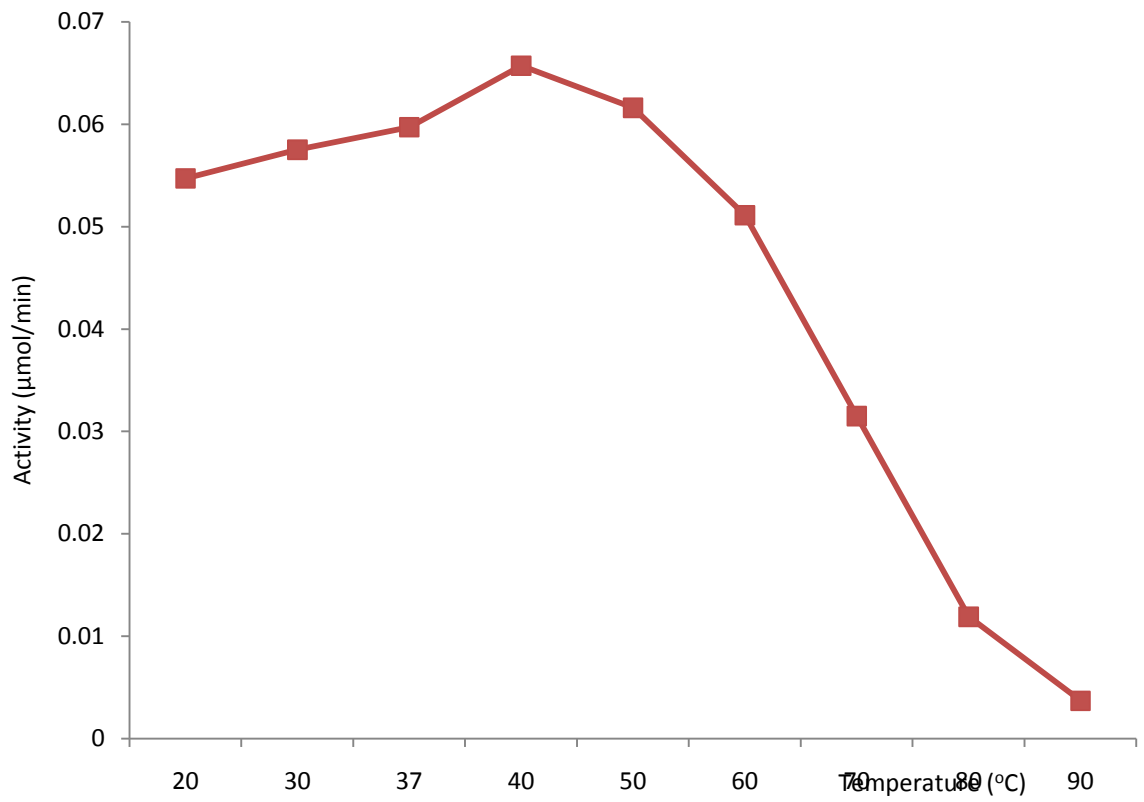


Figure 4.5: Thermostability Studies on *N. nigricollis* Fibrinogenase

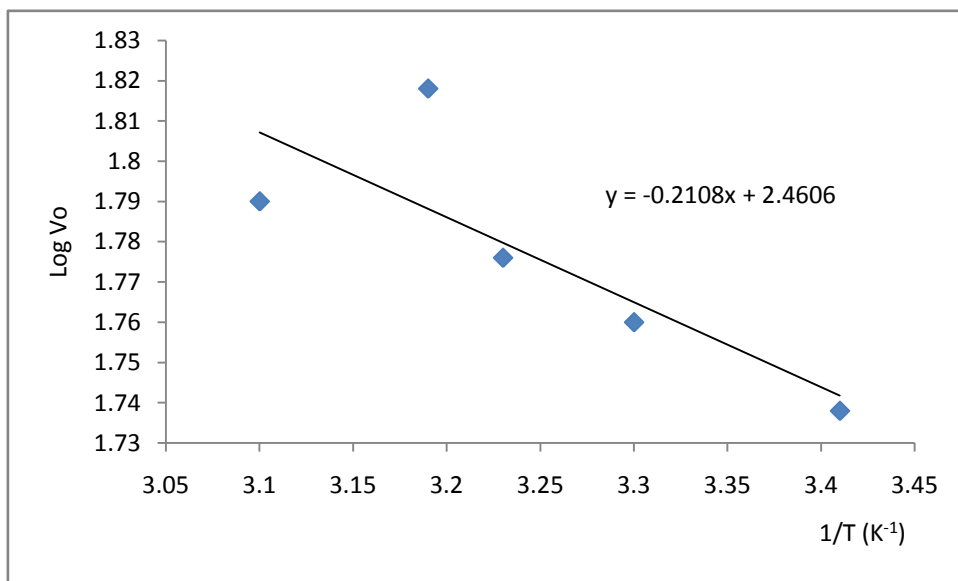


Figure 4.6: Arrhenius Plot for the Determination of Activation Energy for *N. nigricollis* Fibrinogenase

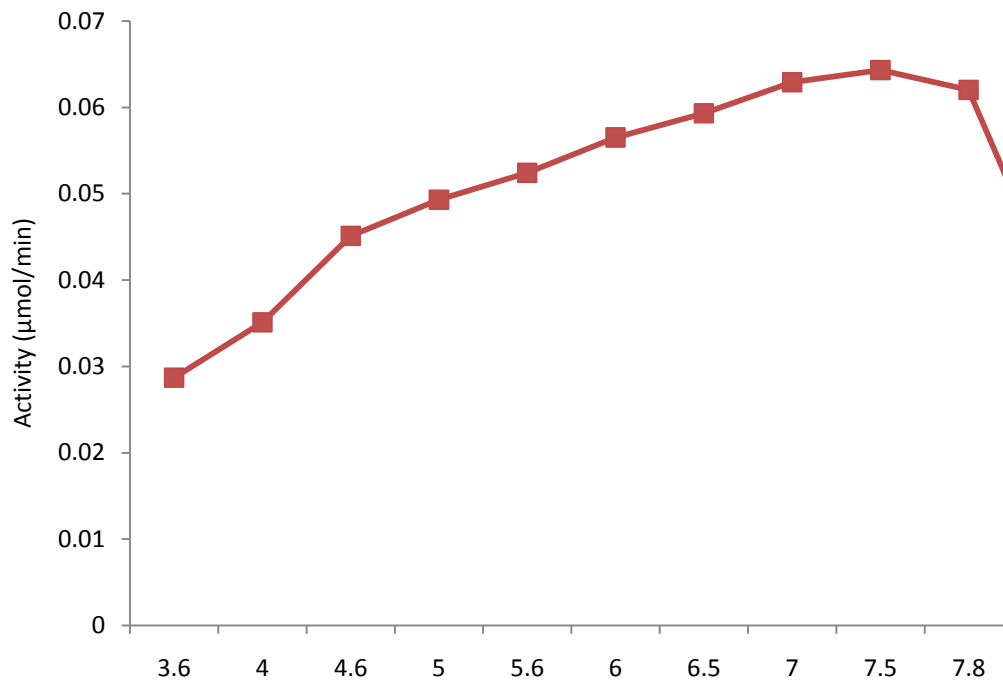


Figure 4.7: Effect of pH on Activity of *N. nigricollis* Fibrinogenase

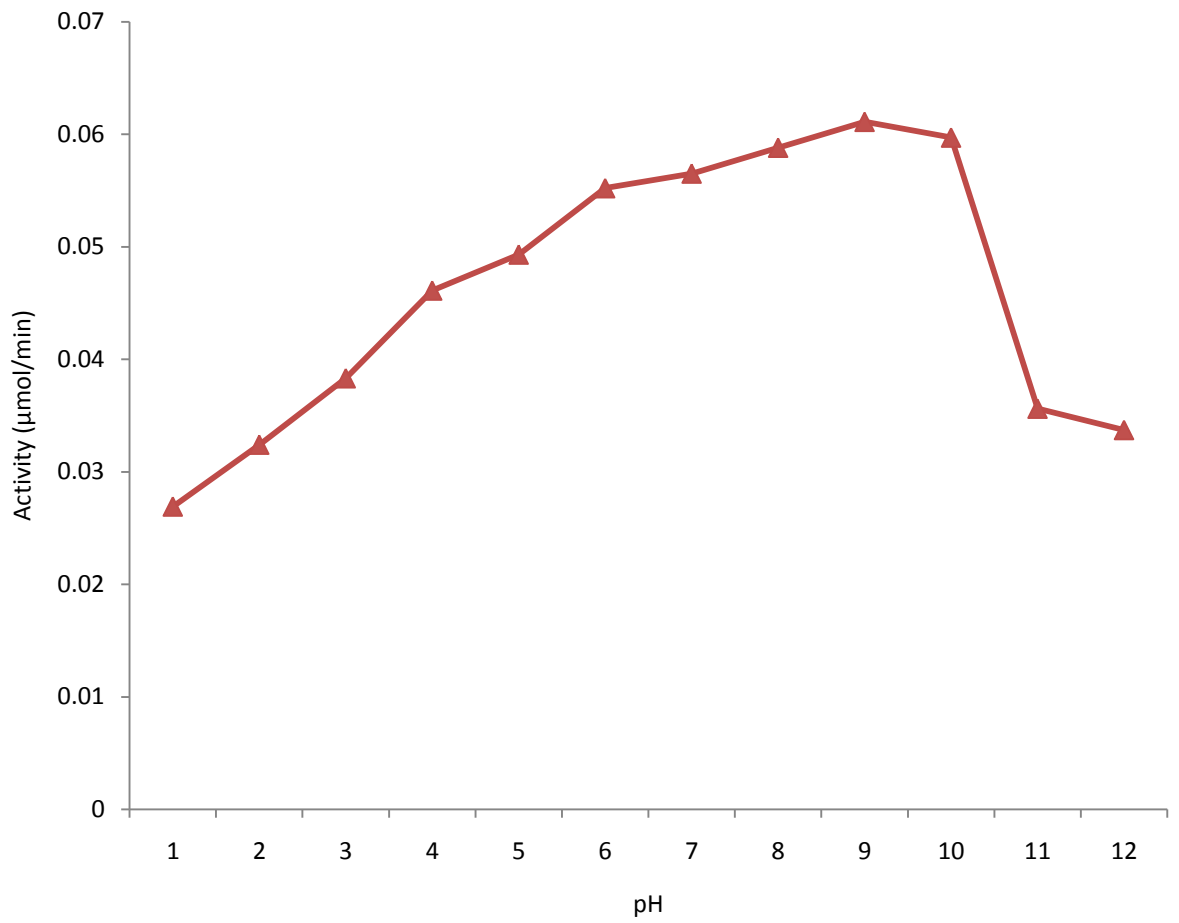


Figure 4.8: pH Stability Studies on *Naja nigricollis* fibrinogenase

4.2.3 Effect of substrate concentration

Steady state kinetic analysis from the initial velocity studies using fibrinogen as substrate revealed a K_m and V_{max} of 0.091 mg/ml and 0.00711 $\mu\text{mol}/\text{min}$ respectively (Figure 4.9).

4.2.4 Effect of metal ions and inhibitors

The enzyme activity was determined in the presence and absence of inhibitors and metal ions (Figure 4.10). The enzyme activity was inhibited by EDTA and 1, 10-phenanthroline. Inhibition by beta-mercaptoethanol, phenylmethylsulfonyl fluoride and aprotinin was very slight. The inhibition by EDTA could be prevented by zinc but not by cobalt, calcium or magnesium.

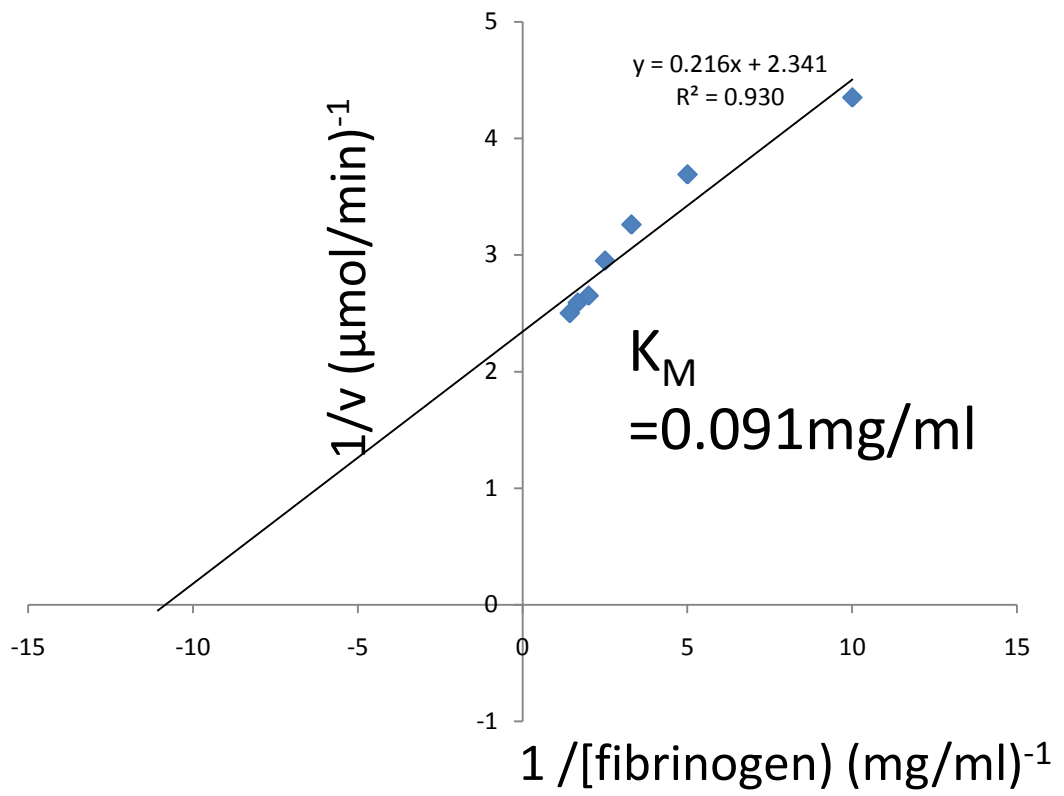


Figure 4.9: Lineweaver-Burke plot relating initial velocity data of the *N. nigricollis* fibrinogenase with fibrinogen concentration

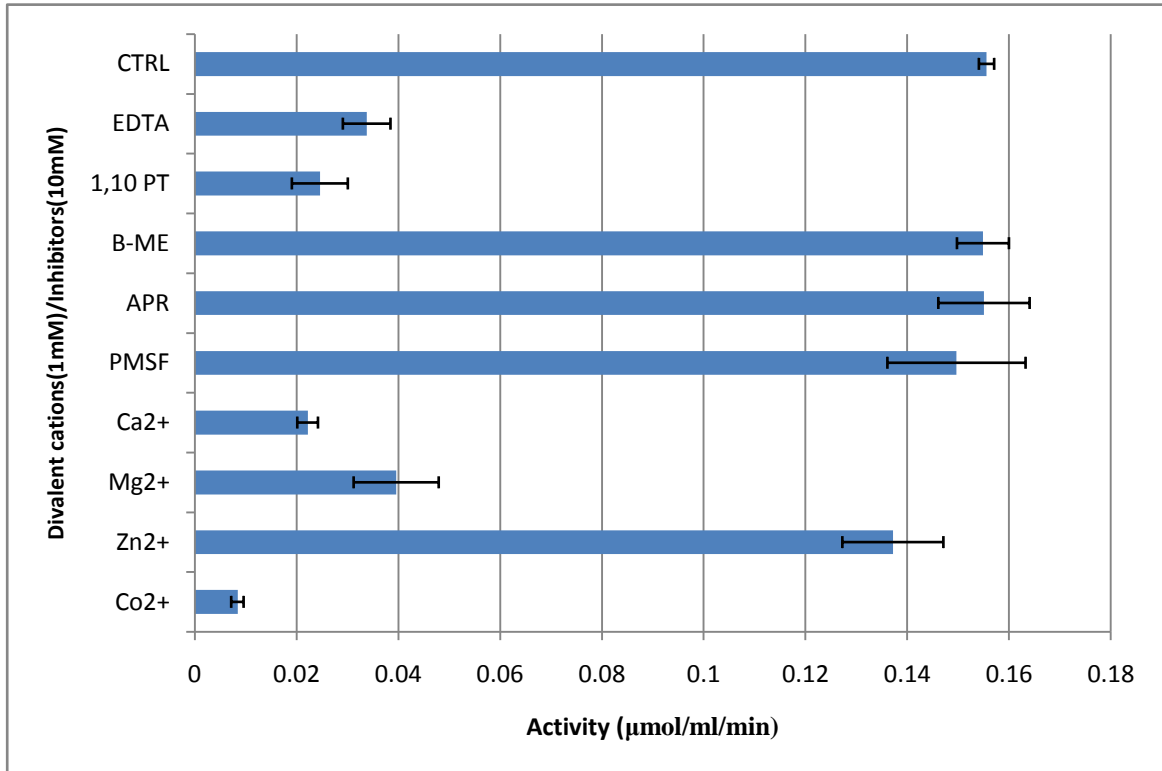


Figure 4.10: Effect of Metal Ions and Inhibitors on Activity of *N. nigricollis* Fibrinogenase

Key: 1, 10 PT=1, 10 Phenanthroline, B-ME=Beta mercaptoethanol, APR=Aprotinin, PMSF=Phenylmethylsulfonylfluoride

4.2.5 Molecular weight of *N. nigricollis* fibrinogenase

The apparent molecular weight of the partially purified enzyme was estimated from results of sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE). The enzyme was found to be homogenous by SDS-PAGE criterion, and consisted of a single chain (Plate 4.1) whose molecular weight was approximated to be 65KDa.

4.2.6 Fibrinogenolytic activity of *N. nigricollis* fibrinogenase

The ability of the enzyme to degrade fibrinogen was determined after incubation with fibrinogen for various time intervals using SDS-PAGE. The enzyme acted on the alpha-chain of fibrinogen only leaving the beta and gamma chains intact.

Plates 4.1 show the results of SDS-PAGE analyses of incubated mixtures of fibrinogen with the enzyme. As shown in the control lane (Lane 3), reduced fibrinogen was separated into α , β and γ -chains. When incubated with the enzyme (lanes 4, 5 and 6), the α -chain started to disappear after 30 minutes of incubation and completely disappeared within 60 minutes. The enzyme showed no effect on the γ -chain after 90 minutes of hydrolysis.

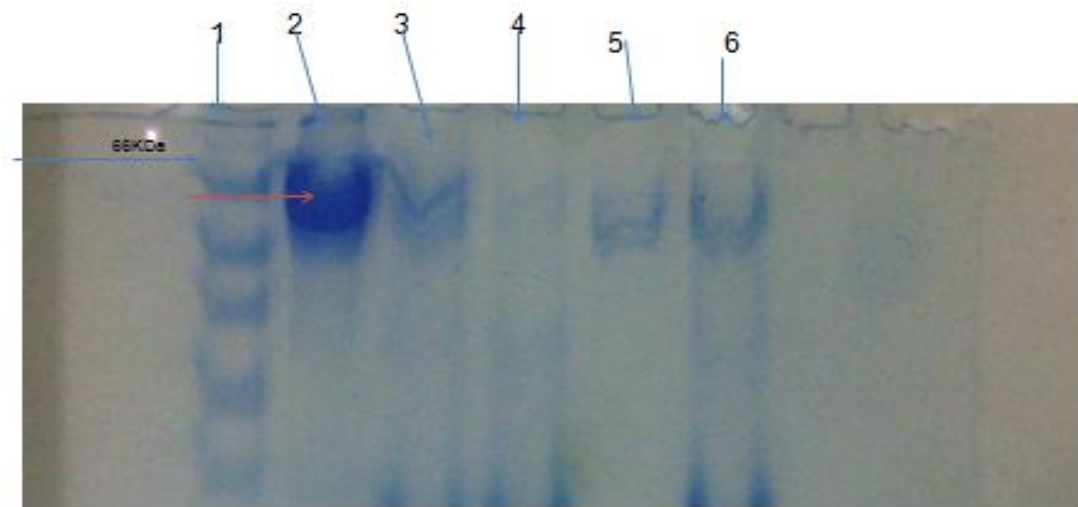


PLATE 4.1: Protein Electrophoregrams and Fibrinogenolytic Activity of Purified *N. nigricollis* Fibrinogenase

Key: Lane 1=molecular weight marker, Lane 2=purified enzyme (65KDa), Lane 3= α , β , and γ chains of fibrinogen, Lanes 4, 5, and 6=fibrinogen+enzyme at 90, 60 and 30 minutes incubation respectively.

4.3 Production of Polyclonal Antibodies

4.3.1 Agglutination reaction (ELISA)

Cross-reaction between the purified *Naja nigricollis* fibrinogenase and polyclonal antibodies raised against the purified *Naja nigricollis* fibrinogenase was confirmed and evaluated by ELISA as described in the methodology section. The titre was found to be acceptable by ELISA criterion (an absorbance of less than 1.000 at a dilution of 1 in 4000). The colour intensity yielded by the ELISA reaction decreased with increasing dilution in the following order: 1 in 10, 1 in 100, 1 in 1000, 1 in 4000, 1 in 10,000 and pre-immune (naïve) serum (Plate 4.3).

4.4 Anti-haemorrhagic Activity

4.4.1 Effect of polyclonal antibodies against *N. nigricollis* fibrinogenase

The effect of the polyclonal antibodies against the fibrinogenase was tested (Plates 4.4 to 4.11) as described in the methodology section. The observations made are summarized in Table 4.2.



Plate 4.2: Cross reaction by ELISA between purified *Naja nigricollis* fibrinogenase and polyclonal antibodies raised against the enzyme (after first booster dose): Rows 1,2,3,4,5 and 6 : 1 in 10, 1 in 100, 1 in 1,000, 1 in 4,000, 1 in 10,000 dilutions of polyclonal antibodies and neat(undiluted) polyclonal serum respectively.

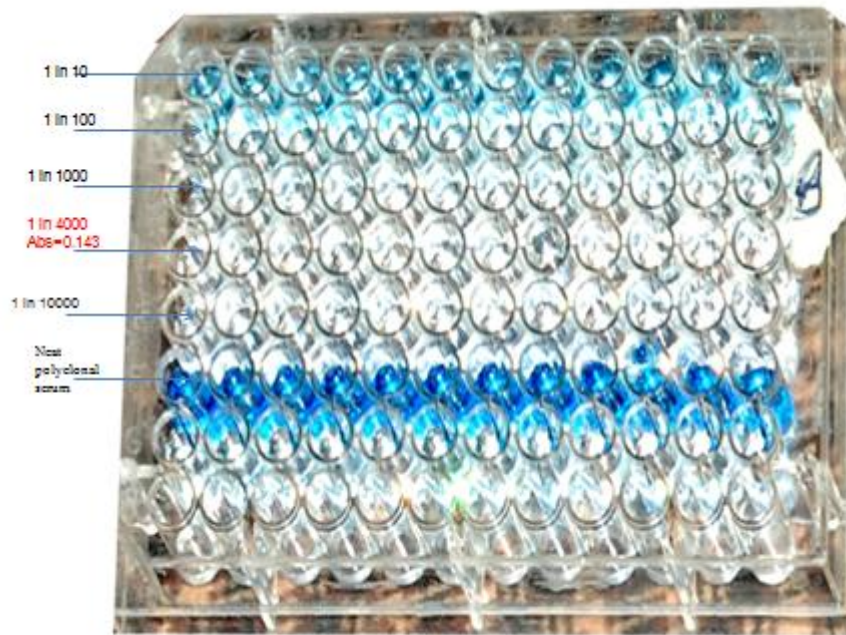


Plate 4.3: Cross reaction by ELISA between purified *Naja nigricollis* fibrinogenase and polyclonal antibodies raised against the enzyme (after second booster dose): Rows 1,2,3,4,5 and 6 : 1 in 10, 1 in 100, 1 in 1,000, 1 in 4,000, 1 in 10,000 dilutions of polyclonal antibodies and neat(undiluted) polyclonal serum respectively.

TABLE 4.2: Anti-haemorrhagic activity of polyclonal antibodies against *N. nigricollis* fibrinogenase

ANIMAL GROUP	TREATMENT	OBSERVATIONS
A	0.1ml of enzyme only	Moderate haemorrhage
	0.2ml of enzyme only	Severe haemorrhage
B	0.1ml enzyme and 0.05ml polyclonal serum on different sites	Mild haemorrhage indicating partial neutralization
C	0.1ml of enzyme and 0.05ml polyclonal serum on the same site	Mild haemorrhage indicating partial neutralization
D	0.1ml of enzyme followed by polyclonal serum (active immunization)	Mild haemorrhage indicating partial neutralization
E	polyclonal serum followed by 0.1ml of enzyme (passive immunization)	No changes observed indicating complete neutralisation
F	Polyclonal serum only	No changes observed
G	Control	No changes observed
H	Control	No changes observed



Plate 4.4: Control animal with no changes
Key: a=clear region

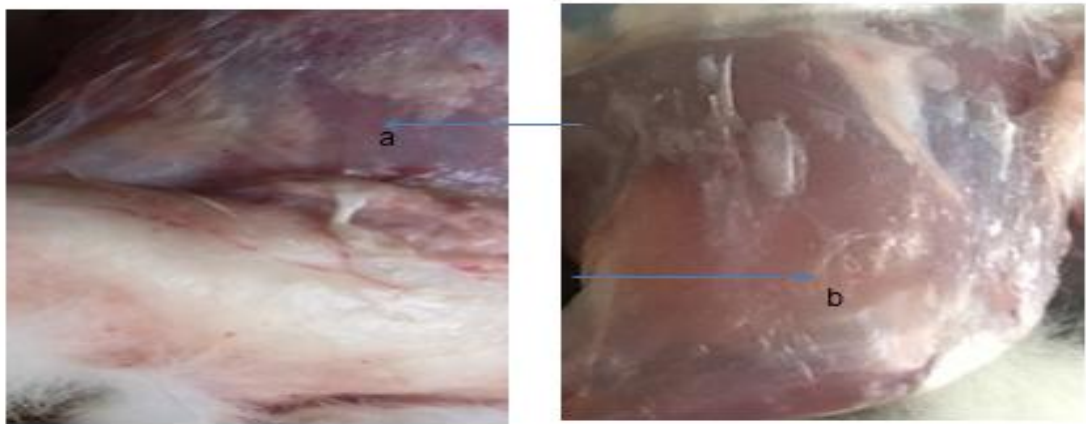


Plate 4.5: Shows complete neutralisation of haemorrhagic effect of purified *N. nigricollis* fibrinogenase when treated subcutaneously with 0.05ml of polyclonal serum followed by *N. nigricollis* fibrinogenase 0.1ml (passive immunisation).

Key: a= clear region indicating complete neutralization, b=clear region with no changes in control animal

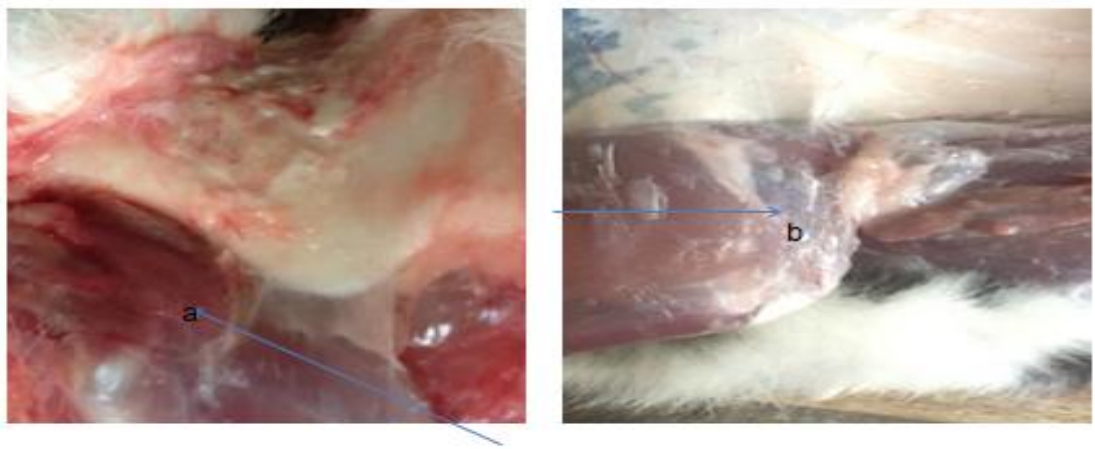


Plate 4.6: Shows partial neutralisation of haemorrhagic effect of purified *N. nigricollis* fibrinogenase when treated subcutaneously with 0.1ml of *N. nigricollis* fibrinogenase followed by polyclonal antibodies (active immunisation).

Key: a=region showing partial neutralisation, b=clear region with no changes in control animal

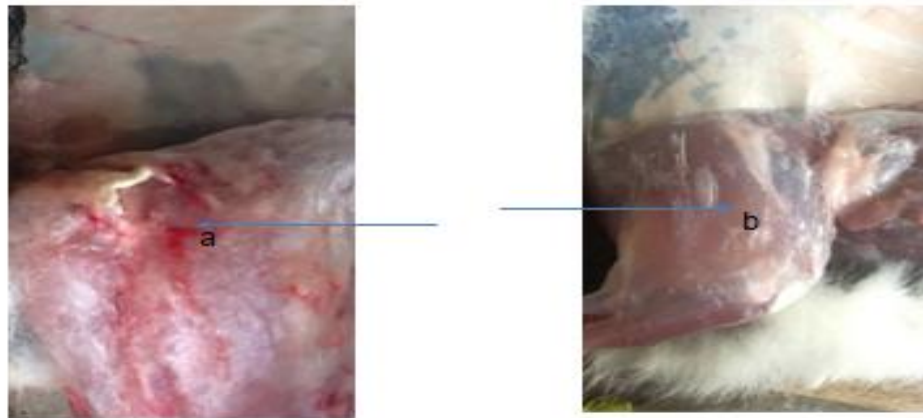


Plate 4.7: Shows partial neutralisation of haemorrhagic effect of purified *N. nigricollis* fibrinogenase when treated subcutaneously with *N. nigricollis* fibrinogenase and polyclonal antibodies at the same site.

Key: a=region showing partial neutralisation, b=clear region with no changes in control animal



Plate 4.8: Shows necrotic effect of purified *N. nigricollis* fibrinogenase when treated subcutaneously with *N. nigricollis* fibrinogenase.

Key: a=region shows necrosis, b=clear region with no changes in control animal

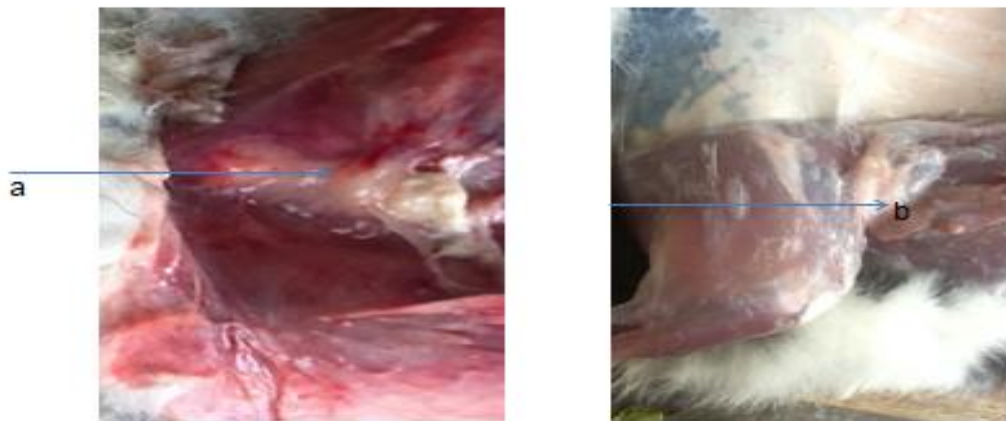


Plate 4.9: Shows partial neutralisation of haemorrhagic effect of purified *N. nigricollis* fibrinogenase when treated subcutaneously with *N. nigricollis* fibrinogenase and polyclonal antibodies on different sites.

Key: a=region showing partial neutralisation, b=clear region with no changes in control animal

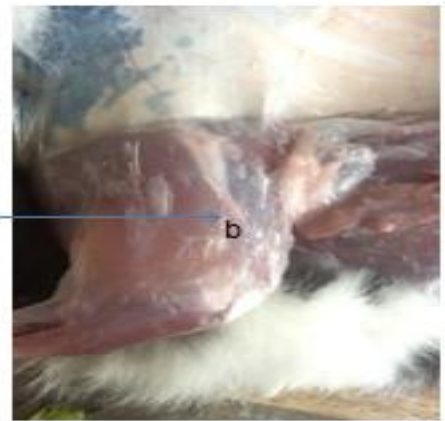
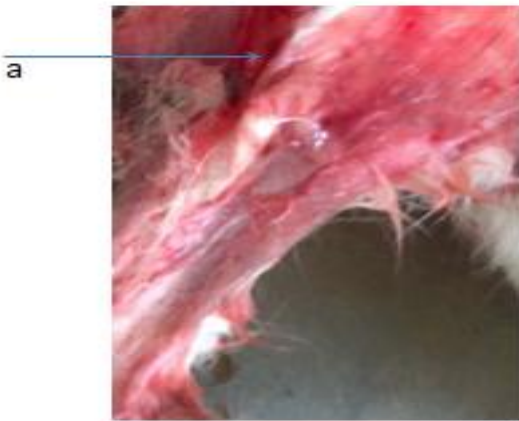


Plate 4.10: Shows moderate haemorrhage when treated subcutaneously with *N. nigricollis* fibrinogenase (0.1-0.2ml).

Key: a=region showing moderate haemorrhage, b=clear region with no changes in control animal

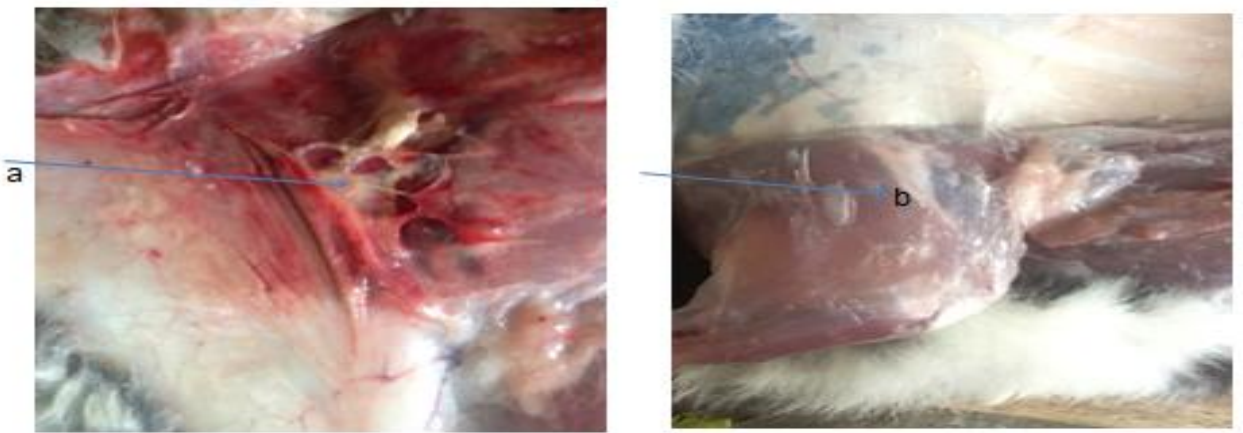


Plate 4.11: Shows severe haemorrhage and necrosis when treated subcutaneously with *N. nigricollis* fibrinogenase (0.05-0.1ml).

Key: a=region showing severe haemorrhage, b=clear region with no changes in control animal

CHAPTER FIVE

5.0

DISCUSSION

The purified *N. nigricollis* fibrinogenase had 9-fold more proteinase activity than the crude venom twice that reported for proteinase F1. This could be attributed to the presence of multiple forms of the enzyme each with its own unique properties due to differences in venom composition which are dependent on many factors such as the age and diet of the snake, climate, altitude and time of secretion into the glands.

The enzyme displayed optimum activity at 45°C and was active over a temperature range of 20-60 °C. Interestingly most snake venom metalloproteases enzymes display optimum activity at 40-50 °C. The wide range of temperature and pH of activity for the enzyme shows that the enzyme is capable of withstanding extreme conditions which is important for venom toxicity.

The very low activation energy (E_a) of 7.47×10^{-3} Kcal/mol/K or 0.031 KJ/mol/K suggests that proteolytic (fibrinogenolytic) activity by the enzyme would be thermodynamically favourable as less energy would be required to surmount the activated complex to form fibrinogen degradation products from fibrinogen which is important for inhibition of platelet aggregation by the enzyme.

The *N. nigricollis* fibrinogenase optimal activity at pH 7.5 suggests that the enzyme would maximally hydrolyze proteins in alkaline conditions. Optimum activity at alkaline pH has been reported for fibrinogenases (Evans, 1984,). A pH optimum of 8-10 and a pI of >10 was reported for proteinase F1 from *N. nigricollis* venom. Snake venom is highly modified saliva which is alkaline in nature. Fibrinogen or factor II, the substrate for fibrinogenase is a blood protein.

Therefore the site of catalysis in the envenomed victim provides the optimum pH for the activity of the enzyme; blood too being alkaline in nature.

The observed low K_m value of 0.091 mg/ml is a clear indication of the high kinetic efficiency of the enzyme. Previous studies by Torres *et al.*, (2012) and Chernyshenko *et al.*, (2010) reported K_m values of 14.59mg/ml and 8.5 μ M for fibrinogenases from *Bothrops moojeni* and *Echis multisquamatis* venoms respectively. It appears that the K_m value of the *N. nigricollis* fibrinogenase compares relatively less to other known snake venom fibrinogenases implying higher affinity of this enzyme for its substrate (fibrinogen). This property (low K_m value), though a disadvantage in terms of envenomation by *N. nigricollis*, could be considered a great advantage in terms of the clinical usefulness of these enzymes in the treatment of human diseases and as diagnostic reagents.

The effect of divalent metal ions on the activity of *N. nigricollis* fibrinogenase was studied. Divalent metal ions are involved in enzyme catalysis in a variety of ways which include activation of electrophiles or nucleophiles, bridging an enzyme with substrate together by means of coordinate bonds as well as holding reacting groups in the required three dimensional orientations (Advani *et al.*, 2010). The enzyme activity could be inhibited by calcium, magnesium and cobalt but not zinc. This could be attributed to the dependence of metalloproteases on zinc, one mole of zinc per mole of toxin (Panfoli *et al.*, 2010; Trape *et al.*, 2001), removal of which abolishes proteolytic and haemorrhagic activities due to structural alterations (Panfoli *et al.*, 2010; Bjarnason and Tu, 1978; Nikai *et al.*, 1984). Many of the venom fibrinogenolytic enzymes that have been characterized are zinc metalloproteases and members of the metzincin family (Markland, 1998, Stocker *et al.*, 1995).

Results from studies on the effect of inhibitors on the enzyme strongly suggest that the enzyme belongs to the metalloprotease family based on the observed inhibitory effect of the chelating compound EDTA and 1, 10-phenanthroline on the activity of the enzyme. Beta-mercaptoethanol, aprotinin and phenylmethylsulfonyl fluoride appeared to have little or no effect on the activity of the enzyme, indicating that it is not a serine protease but a metalloprotease. This could be attributed to the preference and affinity shown by both EDTA and 1, 10-phenanthroline for Zn^{2+} ; metalloproteases being zinc-dependent. Similarly, proteinase-F1 from *N. nigricollis* venom was reported to be inhibited by the metalloprotease inhibitor EDTA and not by inhibitors of serine, cysteine or acid proteinases (Evans, 1984). This means that treatment for local effect of envenomation by *N. nigricollis* requires the incorporation of snake venom metalloprotease inhibitors for effectiveness.

SDS-PAGE analysis of the purified enzyme revealed a single protein band with an estimated molecular weight of 65KDa. Such observation indicates that the isolated enzyme is composed of a single polypeptide chain. This is similar to proteinase F1 from *N. nigricollis* venom which consisted of a single chain with a molecular weight of 58 KDa (Evans, 1984). Molecular weights in the range of 36 to 52.5 KDa have been reported for colubrid and viperid fibrinogenases (Markland, 1998).

The purified *Naja nigricollis* metalloprotease was active on fibrinogen and displayed haemorrhagic and partial fibrinolytic activity. Snake venom metalloproteases could be haemorrhagic, fibrinolytic or both and are believed to share a common ancestral precursor (Markland, 1998).

SDS-PAGE analysis of the fibrinolytic activity of the enzyme demonstrated that the enzyme was able to degrade fibrinogen chains to some extent.

Only the alpha- chain was degraded leaving the beta- and gamma-chains intact even at longer incubation times.

The observed fibrinolytic activity of the purified enzyme implies that the enzyme could be useful clinically in the design of prototypes for therapeutic and diagnostic agents as well as other amazing properties possessed by snake venom fibrinogenases yet to be discovered.

The pattern of degradation observed suggests that the enzyme is an alpha-fibrinogenase. This could be supported by the fact that majority of the metalloprotease fibrin (ogen) olytic enzymes show specificity directed preferentially towards the α -chain with a few exceptions (whose preference is directed to the β -chain). Specificity for both chains is not absolute though, substantial degradation of the alternate chain does occur with increasing time though no reports of enzymes with unique gamma-chain specificity exist (Markland, 1998).

The same observation was reported for proteinase F1 from *N. nigricollis* venom (Evans, 1984) though the inhibition of platelet aggregation by proteinase F1 was independent of its action on fibrinogen (Kini and Evans, 1991). All fibrin (ogen) olytic enzymes are direct acting and do not rely on components in the blood for activity (Markland, 1998).

Initially, the majority of the fibrinolytic enzymes were purified from snakes of the Asian, North American, and central and South American Crotalid family (Markland, 1998). Interestingly, these enzymes could be purified from the venom of some elapid snakes too. This is supported by Evans, (1984), who reported the presence of a fibrinolytic proteinase from *Naja nigricollis* venom, designated proteinase F1.

The present work lends more support to the presence of fibrinogenolytic enzymes in Elapid snake venoms.

These enzymes are used as defibrinogenating agents for treatment of occlusive vascular diseases (Markland, 1998), diagnostic agents for detection of fibrinogen (Markland, 1998, Purves and Naidoo, 1992).

The observed haemorrhagic activity of the purified enzyme is attributable to the fact that snake venom metalloproteinases are capable of inducing rapid local bleeding because they have a potent proteolytic effect on all the major components of the extracellular matrix proteins (ECM). The broad substrate specificity of these enzymes is well documented and is believed to be a contributing factor to the haemorrhagic activity of the enzymes (Kamiguti *et al.*, 1998).

Anti-venoms, though effective for treatment of systemic envenoming, are however not effective for treatment of these local effects (Lalloo and Theakston, 2003) due to rapid activity of toxins and the inability of antivenom IgG to cross the blood/tissue barrier (Warrell, 1992; Gutierrez *et al.*, 1998).

Purified *N. nigricollis* fibrinogenase was haemorrhagic when tested on laboratory animals. Interestingly, the 65 KDa purified enzyme also showed 38 folds more proteinase activity than the crude venom. The molecular weight of the purified enzyme could be considered within the upper limit of the range for molecular weight of fibrinogenases (20 to 58 KDa) as reported by Markland, 1998. It has been postulated that a positive correlation exists between the proteolytic activity of venom metalloproteinases and their hemorrhagic potencies. Larger enzymes are more potent than the smaller ones in degrading the extracellular matrix.

The *in vivo* hemorrhagic effect of the venom occurs within minutes of the bite or experimental injection (Kamiguti *et al.*, 1998). The molecular weight of the purified enzyme suggests that the enzyme is capable of inducing haemorrhage rapidly following envenomation by *N.nigricollis*.

Interestingly, the polyclonal antibodies raised against the enzyme were capable of neutralizing the haemorrhagic and necrotic effects of the purified fibrinogenase in laboratory animals.

These findings could lend support to the development of new approaches aimed at improving anti-venom therapy which include the use of purified relevant toxins as antigens instead of the whole venom (Wilde *et al.*, 1996; Gutierrez *et al.*, 2006). This is very important in view of the adverse reaction or anaphylactic shock, economic and ethical problems (Panfoli *et al.*, 2010) associated with the different approaches currently used to inhibit haemorrhagic venoms.

In the present work, the properties displayed by the purified enzyme are clearly different from those shown by proteinase F1, the only representative fibrinogenase of the Elapid family that has been characterized. Moreover, polyclonal antibodies have not been raised against this enzyme. The novelty of this study results lies in the revelation of new properties by the purified *Naja nigricollis* fibrinogenase coupled with the anti-haemorrhagic effect shown by polyclonal antibodies raised against the enzyme. Variation in snake venom composition due to several factors could be responsible for the presence of multiple forms of the enzyme, each with its own unique properties that contribute significantly to the pathophysiology of envenomation.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

- The purified *N. nigricollis* fibrinogenase showed 38 folds more proteinase activity than the crude *N. nigricollis* venom.
- The purified *N. nigricollis* fibrinogenase displayed optimum activity at pH 7.5 and was stable over a pH range of 6.5-7.8.
- The optimum temperature of activity for the purified *N. nigricollis* fibrinogenase was found to be 40°C. The activity of the enzyme was found to be stable over a temperature range of 20-60 °C.
- The kinetic parametres K_m and V_{max} for the purified *N. nigricollis* fibrinogenase were found to be 0.091mg/ml and 0.00711 μ mol/min.
- The activity of the purified *N. nigricollis* fibrinogenase could be inhibited by EDTA and 1, 10-phenanthroline; it was not affected by PMSF, beta-mercaptoethanol, and aprotinin. Inhibition could be prevented by zinc but not calcium, manganese or cobalt.
- The purified *N. nigricollis* protease showed partial fibrinogenolytic activity with preference for the alpha chain of fibrinogen.
- The purified *N. nigricollis* fibrinogenase migrated as a single protein band on analytical SDS-PAGE with an apparent molecular mass of 65 KDa.

- Polyclonal antibodies raised against the purified fibrinogenase neutralised the haemorrhagic effect of the enzyme.

6.2 Conclusion

A 65 KDa fibrinogenase was isolated and purified to apparent homogeneity from the venom of *Naja nigricollis*.

Biochemical characterization of the purified *N. nigricollis* protease revealed a novel 65 KDa haemorrhagic fibrinolytic metalloprotease with properties clearly different from those reported other fibrinogenases.

Monospecific polyclonal antibodies which cross-reacted with the purified *N. nigricollis* fibrinogenase were raised in laboratory animals.

The monospecific polyclonal antibodies raised against the purified *N. nigricollis* fibrinogenase neutralised the haemorrhagic effect of the enzyme in vivo.

6.2 RECOMMENDATION.

The search for more fibrinogenases in the same snake specie should be considered to provide a basis for comparison which would lead to revelation of more useful properties of these enzymes.

There is need for further characterization of the purified enzyme using advanced analytical techniques. This would pave the way for possible isolation and cloning of the alpha-fibrinogenase gene.

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APPENDICES

APPENDIX I: Elution profile of *Naja nigricollis* fibrinogenase from sephadex G75

FRACTION	OD _{595nm}	PROTEIN	OD _{660nm}	ENZ ACT	SPEC ACT
A1	0.024	0.0072	0.09	0.242	33.61
A2	0.026	0.0078	0.043	0.116	14.87
A3	0.074	0.0222	0.025	0.066	2.97
A4	0.49	0.1467	0.35	1.042	7.1
A5	0.424	0.1269	0.744	2.215	17.45
A6	0.409	0.1225	3.04	9.044	73.83
A7	0.431	0.129	3.168	9.424	73.05
A8	0.401	0.1201	2.698	8.024	66.81
A9	0.367	0.1099	2.096	6.236	56.74
A10	0.333	0.0997	1.157	3.441	34.51
A11	0.338	0.1012	0.501	1.49	14.72
A12	0.289	0.0865	0.269	0.8	9.25
A13	0.244	0.0731	0.113	0.289	3.95
A14	0.104	0.0311	0.097	0.26	8.36
A15	0.069	0.0207	0.064	0.173	8.36
A16	0.073	0.0219	0.044	0.119	5.43
A17	0.056	0.0168	0.045	0.119	7.08
A18	0.043	0.0129	0.051	0.138	10.7
A19	0.049	0.0147	0.041	0.11	7.48
A20	0.051	0.0153	0.029	0.078	5.1
A21	0.068	0.0204	0.035	0.094	4.61
A22	0.071	0.0213	0.035	0.094	4.41
A23	0.069	0.0207	0.034	0.091	4.4
A24	0.073	0.0219	0.064	0.173	7.9
A25	0.329	0.0985	0.087	0.232	2.36
A26	0.089	0.0267	0.123	0.314	11.76
A27	0.095	0.0284	0.094	0.251	8.84

APPENDIX II: Elution profile of *Naja nigricollis* fibrinogenase from DEAE-cellulose

FRACTION	OD _{595nm}	PROTEIN	OD _{660nm}	ENZ ACT	SPEC ACT
B1	0.01	0.0023	0.179	0.458	199.13
B2	0.02	0.0047	0.082	0.22	46.809
B3	0.024	0.0056	0.166	0.423	75.536
B4	0.348	0.0818	0.363	1.079	13.191
B5	0.383	0.0901	0.848	2.522	27.991
B6	0.387	0.091	0.755	2.246	24.681
B7	0.313	0.0736	0.428	1.274	17.31
B8	0.26	0.0612	0.301	0.894	14.608
B9	0.198	0.0466	0.129	0.329	7.06
B10	0.165	0.0388	0.029	0.078	2.01
B11	0.059	0.0139	0.081	0.216	15.54
B12	0.09	0.0212	0.151	0.386	18.208
B13	0.08	0.0188	0.038	0.1	5.319
B14	0.091	0.0214	0.086	0.229	10.701
B15	0.084	0.0198	0.055	0.147	7.424
B16	0.09	0.0212	0.091	0.245	11.557
B17	0.014	0.0033	0.019	0.05	15.152
B18	0.067	0.0158	0.089	0.238	15.063
B19	0.054	0.0127	0.027	0.072	5.669
B20	0.063	0.0148	0.063	0.169	11.419
B21	0.059	0.0139	0.056	0.151	10.863
B22	0.063	0.0148	0.024	0.063	4.257
B23	0.075	0.0176	0.039	0.104	5.909
B24	0.052	0.0122	0.056	0.151	12.377
B25	0.07	0.0165	0.102	0.26	15.758
B26	0.047	0.0111	0.07	0.188	16.937
B27	0.032	0.0075	0.18	0.458	61.067
B28	0.026	0.0061	0.043	0.116	19.016
B29	0.024	0.0056	0.034	0.091	16.25
B30	0.015	0.0035	0.044	0.119	34
B31	0.031	0.0073	0.095	0.254	34.795

APPENDIX III: Elution profile of *Naja nigricollis* fibrinogenase from heparin agarose

FRACTION	OD _{595nm}	PROTEIN	OD _{660nm}	ENZ ACT	SPEC ACT
C1	0.012	0.00356	0.017	0.0439	123.315
C2	0.021	0.00623	0.03	0.0816	13.098
C3	0.018	0.00534	0.034	0.091	17.041
C4	0.016	0.00475	0.029	0.0784	16.505
C5	0.002	0.000058	0.039	0.1035	1784.48
C6	0.007	0.000204	0.034	0.091	446.078
C7	0.012	0.00035	0.019	0.0502	143.429
C8	0.012	0.00035	0.029	0.0784	224
C9	0.046	0.01365	0.013	0.0345	2.527
C10	0.016	0.000466	0.01	0.0282	60.515

APPENDIX IV: DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE

Sample preparation (store at 0°C)

- Add the SDS sample buffer (Room Temperature) to the samples (equal volume of 4X buffer) still on ice, and boil at 100°C immediately for 3-5 minutes.

Procedure

- Assemble the gel casting apparatus making sure that the sandwich of glass plates and spacers will make a good seal. Look out for chips or cracks.
- Prepare the resolving gel and mix
- Load the apparatus with the resolving gel solution
- Top with 0.1% SDS to isolate polymerization for oxygen.
- After polymerization, pour off the 0.1% SDS and rinse with distilled water
- Remove any water droplets from the inside of the casting apparatus with a paper towel.
- Insert the comb for the stacking gel
- Prepare the stacking gel solution
- Vortex and load the stacking gel taking care not to introduce air bubbles around the comb (some bubbles can be removed by pipetting up and down).
- Allow the stacking gel to polymerise completely(45 minutes)
- Remove the glass and gel sandwich from the casting gel apparatus.
- Clip the sandwich to the electrophoresis apparatus with X1 SDS electrophoresis buffer before carefully removing the comb from the gel.
- Carefully load the samples into the bottom of the wells using a pipette tip.
- Fill the bottom of the electrophoresis apparatus with 1X SDS electrophoresis buffer and connect the apparatus to the power supply.
- Run the gel at 40V until the dye enters the separation gel (40 minutes), then increase the current to 80V.
- When the dye reaches the bottom of the separation gel, turn off the power supply and remove the gel sandwich.
- Carefully open the sandwich by using one of the spacers to pry the plates apart
- Gently cut away the stacking gel and place the separation gel in a small plastic container for staining
- Cover the gel with fixing solution and shake gently for 15 minutes
- Pour off the fixer and cover the gel with staining soln. shake gently for at least 2 hours
- Pour off the staining solution and cover the gel with wash solution. Destain for at least 2 hours (it is usually necessary to change the wash solution at least once)
- The gel can be stored in water or dried down between sheets of cellulose on a drying frame.

APPENDIX V: TERMS AND CALCULATIONS IN ENZYME PURIFICATION

Calculation of protease activity

1. Absorbance of sample = Absorbance of sample - Absorbance of blank
2. Determine μ moles of tyrosine equivalent liberated using the equation derived from the tyrosine standard curve.
3. Determine units of protease activity per ml of protease sample using the following equation:

Units/ml = μ mole tyrosine X reaction volume / Sample volume X reaction time X volume assayed

Where:

μ mole tyrosine = μ mole of tyrosine equivalent released

Reaction volume = total volume in ml of assay (285 μ l = 0.285 ml)

Sample volume = volume in ml of protease sample (25 μ L) = 0.025 ml

Reaction time (minutes) of reaction incubation = 10 minutes

Volume assayed = volume (in ml) used in colorimetric determination (0.25 ml)

E.g. Units/ml = 0.0275μ mole X 0.285 ml / 0.025 ml X 10 minutes X 0.25 ml

= 0.125 μ mole/ml/min

i.e. * μ mole tyrosine X 4.56 μ mole/ml/min.

Specific enzyme act (μ mol/enzyme/hour/mg protein) = units of enzyme (μ mole/hour) / total mg protein

Total activity = specific activity X total mg protein

Recovery/yield (%) = total activity of a given fraction / total activity of original mixture

Fold purification = specific activity of a given fraction / original specific activity

Unit definition

One fibrinogenase unit will hydrolyse fibrinogen to produce color equivalent to one μ mole (181 μ g) of tyrosine per minute at PH 7.5 at 37°C (colour by Folin and Ciocalteu reagent).

APPENDIX VI: EFFECT OF PH ON ENZYME ACTIVITY

pH	0D	ACT
3.6	0.074	0.0287
4	0.09	0.0351
4.6	0.121	0.0451
5	0.133	0.0493
5.6	0.141	0.0524
6	0.152	0.0565
6.5	0.16	0.0593
7	0.169	0.0629
7.5	0.173	0.0643
7.8	0.167	0.062
8	0.098	0.0383
8.5	0.091	0.0356

APPENDIX VII: pH STABILITY STUDIES

pH	OD	ACT
3.6	0.069	0.0269
4	0.083	0.0324
4.6	0.103	0.0383
5	0.124	0.0461
5.6	0.133	0.0493
6	0.148	0.0552
6.5	0.152	0.0565
7	0.158	0.0588
7.5	0.165	0.0611
7.8	0.161	0.0597
8	0.092	0.0356
8.5	0.087	0.0337

APPENDIX VIII: EFFECT OF TEMPERATURE ON ENZYME

T °C	T K	OD	ACTIVITY
20	293	0.158	0.0588
30	303	0.161	0.0597
37	310	0.167	0.062
40	313	0.173	0.0643
50	323	0.16	0.0593
60	333	0.142	0.0529
70	343	0.096	0.0374
80	353	0.044	0.0173
90	363	0.013	0.005

APPENDIX IX: THERMOSTABILITY STUDIES

T ^{°C}	T(K)	1/T	OD	$v \times 10^{-3}$	LOG $v \times 10^{-3}$
20	293	3.41	0.147	0.0547	1.738
30	303	3.3	0.154	0.0575	1.76
37	310	3.23	0.161	0.0597	1.776
40	313	3.19	0.177	0.0657	1.818
50	323	3.1	0.166	0.0616	1.79
60	333	3.1	0.137	0.0511	1.708
70	343	2.92	0.081	0.0315	1.498
80	353	2.83	0.031	0.0119	1.076
90	363	2.76	0.009	0.0037	0.568

APPENDIX X: DETERMINATION OF KINETIC CONSTANTS

[S]	1/[S]	OD	v	1/v
1	1	0.149	0.0552	18.1
0.9	1.1	0.157	0.0584	17.1
0.8	1.25	0.164	0.0611	16.4
0.7	1.43	0.179	0.0666	15.02
0.6	1.67	0.173	0.0643	15.55
0.5	2	0.169	0.0629	15.9
0.4	2.5	0.152	0.0565	17.7
0.3	3.3	0.138	0.0511	19.6
0.2	5	0.122	0.0451	22.17
0.1	10	0.099	0.0383	26.1

APPENDIX XI: EFFECT OF METAL IONS AND INHIBITORS

	OD	ACT
Co ²⁺	0.0222	0.0084
Zn ²⁺	0.3707	0.1372
Mg ²⁺	0.1071	0.0396
Ca ²⁺	0.0603	0.0222
PMSF	0.4039	0.1497
APR	0.4187	0.1551
B-ME	0.4187	0.1549
1,10		
PT	0.0665	0.0246
EDTA	0.0911	0.0338
Control	0.4187	0.1556

APPENDIX XII: IMMUNISATION OF RABBITS

- Withdraw the contents of the adjuvant ampoule using 10ml syringe with a 1 inch 21g needle. Add to a 50ml vial preferably with a narrow neck.
- Dissolve 50mg (0.05g) antigen in 5ml sterile PBS, PH 7.0-7.3 or 0.5mg per ml i.e. 2.5mg in 5ml. Maintain or create sterility if at all feasible. Draw the solution into the syringe.
- Insert needle into the adjuvant vial and forcibly drive solution into the vial. Withdraw about half of the mixture into the syringe and force it into the adjuvant vial again.
- Repeat the mixing procedure until a smooth stable, milky, water-in-oil emulsion forms. Mixture may seem to thicken as the emulsion forms. Check if the emulsion is complete by expelling a drop from the syringe onto the water surface in a beaker. If the drop remains on the surface and does not disperse when the beaker is agitated slightly the emulsion is adequate.(stable for days at 2-8°C)

PROCEDURE

- Allow 4 young healthy female rabbits (3.5-4kg) to acclimatize for a week.
- Day 0: prepare sterile and pyrogen free antigen and keep at Room temperature.
- Day 1: collect naïve serum (2-10mls) into a plain container from the ear artery. Inject SC FCA+Antigen (0.1ml per site) at 10 sites using insulin syringes.
- Day 21: booster injection with ICA. Increase dose to 0.2ml per site. Collect 10ml of blood from central ear artery with a 19g needle; allow clotting and retracting at 37 °C overnight. Refrigerate clotted blood for 24hrs. Decant serum and clarify by centrifugation at 2,500 rpm for 20 minutes. Determine the titre from the serum (ELISA).
- Day 42: Collect blood for Antibody titration and boost with ICA.

APPENDIX XIII: ELISA (AGGLUTINATION REACTION)

- Dilute the Ag to 0.1µg/25µl or 4µg/ml in coating buffer (0.1M NaHCO₃, PH 8.6).
- Coat each well with 25µl Ag in coating buffer by adding to wells of a pre-treated ELISA plate, covering with plastic film and incubating at 4 °C overnight or 37 °C for 1 hour.
- Shake out coating solution with PBS twice.
- Block wells by adding 50µl PBS per well and incubate for 1 hour at 37 °C wrapped in plastic.
- Shake blocking solution (without washing or drying) and add primary Antibody preparation diluted in PBS 25µl/well (1:10, 1:100, 1:1000, *1:4000,1:10,000 and undiluted or neat)
- Wrap plate and incubate at 37 °C for 1hour.
- Shake off Antibody solution and wash ten times with PBS
- Add 25µl of anti-rabbit alkaline phosphatase(1:1000 in PBS)
- Wrap and incubate for 1 hour at 37 °C. Shake off and wash 10 times with PBS
- Add substrate solution(PNPP) IN 5ml ALP developing buffer, 50µl/well
- Develop plate at RT and read at 15 minutes, 30 minutes and 1 hr.
- If absorbance or OD>1 at 1:4000, continue booster dose.

APPENDIX XIV: ANTI-HAEMORRHAGIC ACTIVITY

Protocol

ANIMAL GROUP	TREATMENT
A	Enzyme only
B	Enzyme and antibody on different sites
C	Enzyme and antibody on same site
D	Enzyme followed by Antibody (active)
E	Antibody followed by enzyme (passive)
F	Antibody only
G	Control
H	Control

- Sacrifice after 24h and remove skin.
- Measure diameter of haemorrhage; note the appearance of oedema and necrosis.