ISOLATION AND CHARACTERIZATION OF L-AMINO ACID OXIDASE FROM *NAJA NIGRICOLLIS*

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DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Binta Kurfi and has not been presented anywhere for the award of a degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this thesis and the subsequent write up by Rabi Abdu (SPS/13/MBC/00020) were carried out under my supervision.

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Dr Binta Kurfi Date

APPROVAL PAGE

This thesis has been examined and approved for the award of **Master Degree in Biochemistry**.

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In the name of Allah, the most Gracious, the most Merciful. All venerations are due to most revered one the guiding light to humanity Prophet Muhammad (S.A.W), his family and his companions and those who follow their foot step in piety until the day of resurrections.

With great pleasure, I extend my special gratitude to my entire family for their untiring support, encouragement, prayers and all necessary support during my studies. I am forever grateful.

I am very lucky, honored to have my supervisor, Dr Binta Kurfi for her tireless support and imminent advice toward the successful completion of this Thesis. I must acknowledge my academic Fathers; our Noble lecturers who took us through this programme for their timeless effort for their contributions'.

Lastly, I will like to acknowledge the contribution of part funding given to me by the Venom Antivenom Research Study (VARS, AKTH. Bayero University, Kano) in the course of this research

DEDICATION

I dedicate this research project to my entire family.

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LIST OF ABBREVIATIONS

WHO……... ……World Health Organisation

Nn-LAAO……*Naja nigrocollis* L-amino acid oxidase

KDa……..KiloDaltons

NAD……...Nicotinamide Adenine Dinucleotide

α……..Alpha

β……Beta

FAD ……..Flavin Adenine Dinucleotide

FMN…….....Flavin Mononucleotide

γ ……Gamma

nAChR……N-acetyl choline receptor

L-AAO……L-amino acid oxidase

ADP……Adenosine diphosphate

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

PLA_2…….Phospholipase A_2

RGD……Disintegrins

ADP……Adenosine diphosphate

vWF…….Von Willebrand Factor

MPs……..Metalloproteases

SVMPs……..Snake Venom Metalloproteases

ACE…….Angiotensin Converting Enzyme

BPP……Bradykinin Potentiating Protein

nAChR……N-acetyl choline receptor

PLA_2…….Phospholipase A_2

PCR……Polymerase Chain Reaction

bp……. Base pair

Kbp……Kilo Base pair

Cdt venom ……*C. adamanteus* venom

CdtV…… *Crotalus durissus terrificus* venom

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ABSTRACT

L-amino acid oxidase is a flavoenzyme present in *Naja nigrocollis* exhibiting neurotoxic and cytotoxic properties. The enzyme was purified from *Naja nigricollis* venom using Sephadex G-75 and Diethylaminoethyl (DEAE) Cellulose column chromatography to apparent homogeneity. The *Naja nigricollis* L-amino acid oxidase, displayed flavoenzyme activity, which was about 3.79-folds higher than that of the crude venom. The purified *Naja nigricollis* L-amino acid oxidase migrated as a single protein band on analytical polyacrylamide gel electrophoresis with an apparent molecular mass of 44 KDa, under reducing conditions in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH and temperature for the enzyme activity were found to be 6.25 and 45°C respectively. The enzyme was stable at pH range 5.0-8.0 and at a temperature range of 40-50°C. The kinetic parameters, K_m and V_{max} were found to be 0.045mM and 5.5mmol/min. The flavoenzyme activity was inhibited by EDTA and lead II sulphate and slightly inhibited by potassium hydrogen phosphate. The concentration and ratio (percentage purity) of DNA of the venom and gland was 43.9pg and 1.058; 300.0pg and 1.00 respectively. The most effective treatment of snakebite so far is the application of antivenoms. To enhance the effectiveness of such treatments, the study of LAAO and other enzymes present in snake venom will help to understand how the complexity of snake venom evolved and will aid the development of novel therapeutics for treating snake bites. The findings of this study could have potentials for future development and or improving existing therapeutics for snake bites.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

In the biological ecology, poisonous organisms are recognized worldwide by the kingdom Animalia. These venomous organisms have toxic substances and a minute dose can be used against the prey as defense mechanism (Yuri, 2015). Venomous snakes are found all through the vast majority; rural areas of tropical and subtropical countries situated in Africa, Asia, Oceania and Latin America. (Ogala and Obaro,1999; Warrell, 1999; Chippaux, 1998; Theakston *et al.*, 2003). This is a problem peculiar in Nigeria among the rural communities of Nigeria savannah (Godfrey *et al.*, 2013) hence; envenomation as a result of snake bite is a public hazard (Ogunfowokan *et al.*, 2011; World Health Organisation WHO, 2010). There are about 3000 species with about 600 venomous species classified into families; *Elapidae, Viperidae, Crotalidae, Hydrophiidae, Actractaspididae* and *Colubridae* but in Nigeria, the most common venomous families are elapids and viperids (Abdulrazaq, 2013). These include the *Naja melanoleuca* (black cobra) and *N. nigricollis* (spitting cobra), and the viperid *Echis carinatus* (carpet viper) and *Bitis arietans* (puff adder) (Habib *et al.*, 2001). *Naja nigricollis* is the commonest in Nigerian states within the savannah terrain (Warrell, 1999).

The gland is located at the upper jaw producing toxic substance called venom synthesized in the cytoplasm of the secretory cells in the gland (Warshawsky *et al.*, 1973). It is a moderately sized, with a distinct head with two large venom glands. Its colour can vary depending on region of origin (Abriol and Sabrina 2016; Westhoff *et al.*, 2005; Abriol, 2007).

This species can grow generally to a length of 1.2 to 2.2 metres (Warrell *et al.*1977; Naja, 2008) and releases its venom in a fine spray or propelled mist-like fashion through fast undulating head movements (Westhoff *et al.*, 2005; Abriol, 2007).

Snake envenomation occurs when venom is introduced into the body system on exposure to snakebite whereby, biological active toxins can be in single venom making it highly toxic (Gutiérrez, 2012). They function synergistically to cause incapacitation (Vonk *et al.*, 2013). The venom blocks the ion channel (Casewell *et al.*, 2013; Kalia *et al.*, 2015) resulting to severe illness, paralysis and eventually death (Oukkache *et al.*, 2014; Hodgson and Wickranmaratna, 2002). The complexity of the venom determines protein integrity and maintains hemostatsis for the expression of biological activities determining its survival (Tashima *et al.*, 2012). A quantitative increase in the production of these secretions as well as a qualitative improvement of toxic proteins is promoted, increasing its gain in absolute discretion in defense against predators (Calvete *et al.*, 2009).

Venoms are extensively complex and rich with active components having specific target and functions (Casewell *et al.*, 2013) consisting of high and low molecular weight substances. (Roland, 1994; Bieber, 1979; Russell, 1980; Tu, 1988; Heise *et al.*, 1995; Halliday and Adler, 2002; Fry and Wuster, 2004; Kang *et al.*, 2011).

Snake venom LAAO (SV-LAAO) are usually homodimeric with cofactors FAD (Flavin Adenine Dinucleotide) or FMN (Flavin Mononucleotide) covalently linked to their chemical structure. (Du and Clemeson, 2002). The flavin present gives the distinct yellow colour relating to the presence of the pigment riboflavin present as the cofactor, a fact that facilitates its purification (Guo *et al.*, 2012).

They have diverse physiological function which exhibit coagulopathic, fibrinolytic manifestation of *Naja* envenoming (Amel and Fatima, 2015) hence, dysfunction in the digestion, immune response, complement activation, cellular differenticiation and hemostatsis (Qui, 2012; Tan and Swaminathan, 1992).

The present study was designed to isolated, purify and characterize L-amino acid oxidase from *Naja nigrocollis.*

1.2 Statement of Research Problem

The efficacy of the snake venom might be reduced to a minimal, by isolation of enzyme that increases the toxicity of the venom. However, snake venom 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 increasing incidence of envenoming in some Nigerian communities. The use of the enzyme in antivenom production has not been reported despite its crucial role in induction of platelet aggregation and the induction of apoptosis, hemorrhage, and cytotoxicity following envenomation. Current available antivenoms are not effective against local necrotic and hemorrhagic effects of snake venom which could lead to long term disability and disfigurement of victims. Therefore, there is a need for employment of new approaches towards production of more effective and safer antivenoms.

1.2 Research Justification

In Nigeria, snake venom envenomation has an incidence of approximately 497 per 100,000 people per year with a mortality of 12.2% considered as a socio-medical problem. In Northern part of the country, cobra species of the family *elapidae* is of socio-economic importance. The *Naja nigricollis* is the predominant specie of medical importance that is more densely populated in the Northern savannah. The enzyme L-amino acid oxidase from *Naja nigricollis* venom found in this locality and its potential role in the pathophysiology of envenomation is scanty. Snake venom molecules could act 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-venom, anti-cancer, antibacterial, antihypertensive and many others are being exploited and several drugs and clinical diagnostic kits derived from snake venom have been commercialized.

1.3 Aim and Objectives

This study is isolating, purifying and characterizing of L-amino acid oxidase from the African black-necked spitting cobra (*Naja nigricollis*) venom

Objectives: The objectives of the research are as follows:

i. To isolate and purify L-amino acid oxidase from *Naja nigricollis* venom.

ii. To characterize L-amino acid oxidase from *Naja nigricollis* venom

iii. To access the effect of some compounds on purified enzyme

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of snakes

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 (Roland, 1994; Garl and Roger, 1989; Phelps, 1981). They have successfully evolved into efficient predators and colonized various habitats from mangrove swamps, estuaries, freshwater lakes, streams, dunes, grasslands to forests (Godfrey *et al.,* 2013; Warren, 1999).

Snakes are the second largest group of the reptiles recognized with more than 20 families. It comprises of about 500 genera and up to 3,400 species 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. (Uetz, 1999; Alejandro, 2007).

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). Their elongated body shape and lack of limbs/ legless reptiles probably evolved to enable their smooth movement or locomotion 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 belling scales to travel, gripping surfaces. The skin is renewed by periodic moulting. Snakes have developed different modes of locomotion to adapt to different 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). Toxin obtained from a given species may also differ depending on geographical regions. In kingdom Animalia poisonous organisms are represented in many taxa. (Utkin, 2015).

The ecology and evolution of venomous species and signals of positive selection in toxins have been consistently documented reflecting the role of venoms as an ecologically critical phenotype (Margres *et al.*, 2013). Variation in snake venom composition is a ubiquitous phenomenon at all taxonomic level. Many factors including phylogeny, geographic origin, season, age and prey preference may influence venom composition. The composition of snake venom is also strongly influenced by environmental factors including habitat, climate and preys (Mendez, *et al.*, 2011; Reptile Venom Research, 2010).

2.2 Venomous Snakes

Snakes are carnivores; either venomous used to kill their prey before eating (Behler and King, 1979; Freiberg and Walls, 1984) or non-venomous by killing their prey by constriction (Behler and King, 1979), or swallow their prey whole and alive (Mehrtens, 1987).

According to the World Health Organization (WHO) in 2010, there are about 3000 species of snakes in the world and approximately 600 are classified as "venomous" that are potentially fatal (WHO, 2010). In Africa and Asia lethal snakebites are predominant and are caused by approximately 410 venomous species of snakes (Sitprija, 2006). Venom is a 'biochemical weapon frameworks', along these lines ordinarily capacity to encourage, or shield the creating creature from predation (Casewell *et al.,* 2013).

Snake venom is highly modified saliva that facilitates the immobilization and digestion of prey, and defends against a threat. It is injected by unique fangs after a bite but some species are also able to spit salivary secretions 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; Bauchot, 1994; Fry *et al.*, 2006).

Venomous snake species are classified into six families: *Elapidae, Viperidae, Crotalidae, Hydrophidae, Atractaspididae* and *Colubridae* (Halliday and Adler, 2002; Mebs, 2003; Fry and Wuster, 2004); whereby *Viperidae, Elapidae, Colubridae and Actraspididae* are species found in Nigeria and the most common poisonous snakes are the Elapids and Viperids (Habib *et al.* 2001; Akubue, 1997; Chippaux, 1988). The species carpet viper (*Echis ocellatus*), black-necked spitting cobra (*Naja nigrocollis*) and puff adder (*Bites ariettas*) are the most important snakes associated with envenomation (Abdulrazaq, 2014; Menaldo *et al.*, 2012; Habib *et al.*, 2001).

2.3 Naja nigricollis

Naja nigricollis belongs to the cobra genus *Naja* and family *Elapidae*. *Naja nigricollis* is documented to belong to the most diverse and widespread genus of cobras. It previously included two subspecies that have been moved to the species *Naja nigricincta* - the zebra spitting cobra (*Naja nigricincta nigricincta*) and the black spitting cobra (*Naja nigricincta woodi*) (Abriol, 2011). Its geographical range include Western, Eastern, Central and parts of southern Africa; the Middle-East, India, South-Eastern Asia and Indonesia (Luiselli *et al.*, 2000)

Naja nigricollis is mostly found in the savannah terrain in Nigerian States and a widely distributed African cobra. In the South East, *Naja nigricollis* are found in the tropical rain forest due to altered habitat to man-made farmlands, plantations, suburban areas, and a few fragmented forests (Luiselli, 2001; Abriol, 2007).

Naja nigricollis are strong build with about 1.2 to 2.2 m (3.9 to 7.2 ft) in length. The origin determines the colour which can be either blackish or pale grey with a yellow or reddish ventral side with a broad black neck band, and often do they have an orange or pinkish bar on the neck. This specie lives in savanna regions and semi-dessert and also found in coastal scrubs and dry grassland. It's often found in human surrounding hunting for pest animals like rats and mice. This species are good climbers found in trees. They hide in Termite Mountain, abundant rodent holes, hollow trees and trunks of heights up to 1800m. This subspecies is nocturnal (Luiselli, 2001).

The fang is relatively fixed thus the rapid movement of the entire head directs the venom stream. The ability of the specie to track, perform rapid cephalic oscillations that coordinate with the target's movements suggest a level of neural processing that has not been attributed to snakes, or other reptiles (Westhoff *et al*., 2010).

Venom spitting in cobras is a projectile mechanism; once the bolus of venom is expelled from the fang tip the snake has no ability to direct or modulate the trajectory of the venom for increased accuracy (Westhoff *et al*., 2005; Triep *et al.*, 2013).). 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). Snake venoms are synthesized and stored in venom glands (Al-Quraishy *et al.*, 2014). Venomous snakes constitute well-integrated systems to immobilize prey which is to generally optimized to subdue preferred prey more effectively than non-prey, and many venom protein families manifest positive selection and rapid gene family diversification (Aird *et al.*, 2015).

2.4 Snakebites and Envenomation

Envenomation leads to socio-economic hardship resulting to febrile illness and death especially in the poor rural populations and healthcare system of tropical and sub-tropical Africa, Asia, Oceania and Latin America (Pinho *et al*., 2005; WHO, 2005; Bucaretchi *et al.*, 2006; Gutierrez *et al*., 2006; Kalantri *et al.*,2006). Worldwide, there is an estimated 5.5 million snake bites per year which result in over 125,000 deaths (Cruz *et al.*, 2009). Snakebite is classified by the WHO as a neglected tropical disease thus envenoming is a significant major public health problem in tropical and subtropical regions (Ranawaka *et al.*, 2013) especially among rural communities of the Nigerian savannah (Abdulrazaq, 2013).

In subsistent farming populations of sub-Saharan Africa, snakebite is a significant cause of death and disability (Nicholas *et al.*, 2010). Therefore, the high impact of injuries and death worldwide in the sub-Saharan Africa in human and livestock is as a result of snakebites (Kipanyula and Kimaro, 2015). Snake bite depends on the geographical zone, occupation practice of the populace and the season. In Nigeria, snake-bite is at its peak during rainy season and intense farming activities (Ogala and Obaro, 1999; Warrell, 1999). In the rural populace, most of snake envenomation consults the traditional practitioners and rarely resort the modern medicine thus delay before adequate consultancy and diagnosis (Chippaux, 1988; Snow *et. al.*, 1994; Freiberg and walls *et. al.*, 1984). Therefore, incidence and prevalence of snake bite is not adequately captured (Chippaux *et al.*, 1996). In Nigeria, prevalence of envenomation is 48-603 per 100,000 household: morbidity is 100-120 and fatality is 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). Recent studies estimated over 314,000 envenomation, 6,000 amputations and 7,300 casualities yearly in Sub-Saharan Africa and one-fifth of the cases in Nigeria (Habib, 2013).

Snake Venom

Snake venom is a modified salivation that facilitates the immobilization and assimilation of prey, and shields against a danger. It is infused by exceptional fangs after a nibble however a few specie are likewise ready to spit containing zootoxins; the organs that emit the zootoxins is a modification of the parotid salivary organ found in other vertebrates on each side of the head, underneath and behind the eye and epitomized in a solid sheath (Bauchot, 1994). Snake venoms are synthesized and stored in venom glands (Al-Quraishy *et al.*, 2014). Venomous snakes constitute well-integrated systems to immobilize prey which is to generally optimized to subdue preferred prey more effectively than non-prey, and many venom protein families manifest positive selection and rapid gene family diversification (Aird *et al.*, 2015).

The spitting cobra in general can eject its venom towards its victim from a distance of approximately two meters (Triep *et al.*, 2013). This is a defense mechanism by streaming venom on the predator which must be highly accurate because the venom they spit is only an effective deterrent if it lands on the predator's cornea. Spitting cobras discharge orifice (the fang) is relatively fixed directing to the venom stream that requires rapid movements of the entire head. The cobra's ability to track and anticipate the target's movement and to perform rapid cephalic oscillations that coordinate with the target's movements suggests a level of neural processing (Westhoff *et al.*, 2010).

2.4.2 Effects of venom

Crude venom can be classified according to their structure, movement and segments. This prevalence is dependent on the species that can utilize to a great degree composition of various proteins and enzymes with a particular capacity which can combine three mechanism; Venoms can be generally separated into three groups: cytotoxins, neurotoxins and hemotoxins (Adukauskienė *et al*., 2011). Neurotoxins influence the sensory/nervous system either by hindering all nerve motivations resulting to incapacitation, extreme seizures and respiratory close down or by over-burdening the sensory system resulting in breakdown of the system. Nerve toxins are to a great degree fast acting. Hemotoxins attacks the red blood cells, acts as a specific cell toxins destroys blood vessels leading to internal and external bleeding. Cytotoxins initiate the absorption, annihilate cells and assault specific cardiovascular muscles causing heart failure. Animals that survive these bites seldom have any sequelae (after effects of the snake bite such as tissue damage). Their venoms, each unique, can affect multiple organ systems. The venoms have a predilection for the peripheral nervous system where the neuromuscular junction is a favorite target (Georgieva *et al.*, 2008; Aird, 2002).

2.4.3 Physiological Mechanism of Action

Snake envenomation has biologically dynamic toxins in single venom making it exceedingly lethal (Gutierrez, 2012). These toxins hinder the ion channels across the nerve layer sending a wave current to the nerve ends. The neurotransmitter which is the acetylcholine in the vesicles is discharged through the neurotransmitter to the postsynaptic receptor.

The acetylcholine binds to the receptor, the target cell reacts for a while and neutralized by the enzyme acetylcholinesterase. The acetylcholine can't be broken down , retained in the receptor prompting its biological impact on its prey (Hodgson and Wickranmanaratna, 2002). Because of the lavishness, heterogeneity and synergistic or opposing activity of various segments, the systems of every one of these impacts are not yet all completely known.

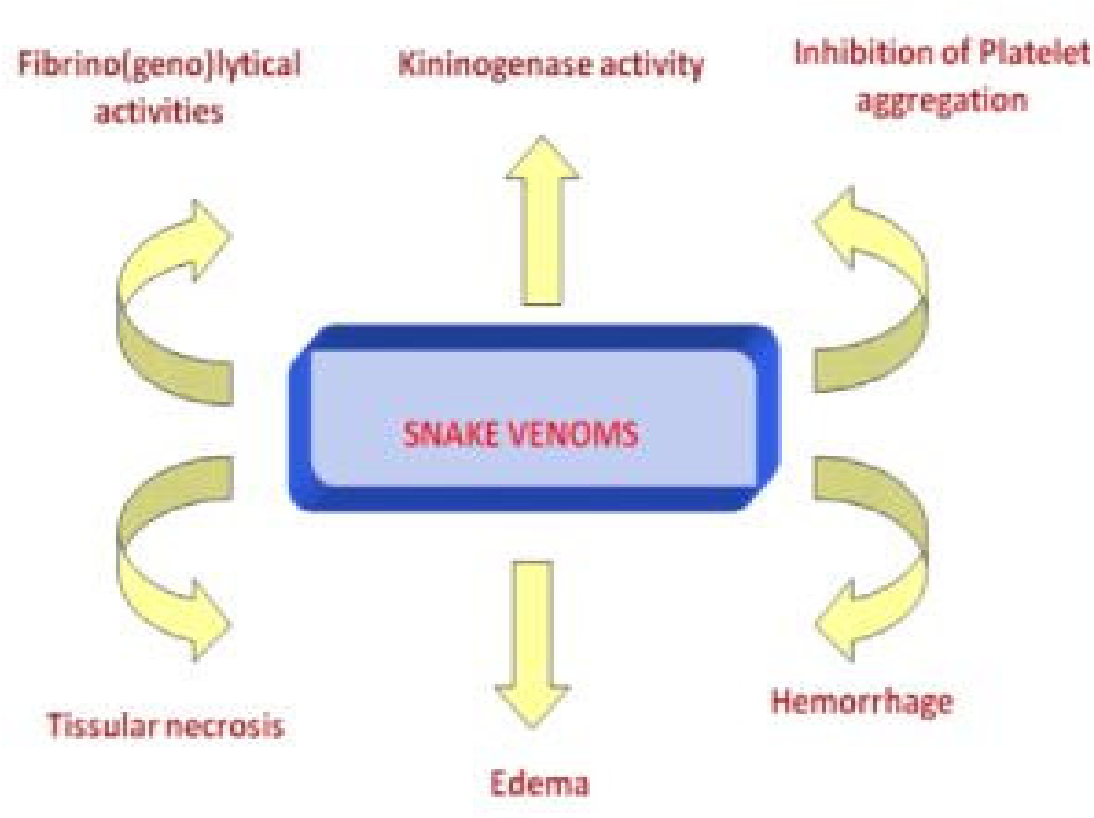


Figure 2.1: Pathophysiological impacts initiated by wind venoms

2.4.4 Biochemical Mechanism of Action

These venom influences the release of acetylcholine from the presynaptic layer is called β-neurotoxins and those influencing the postsynaptic film are called α-neurotoxins (Robert and Ludwig, 2004). They are opponents of postsynaptic nicotinic acetylcholine receptors (nAC hRs) in the neuromuscular neurotransmitter that predicament aggressively and irreversibly, forestalling synaptic acetylcholine (ACh) from opening the particle channel in this manner restraining particle stream and prompting loss of motion (Samson and Levitt, 2008). This venom are ordinarily quick following up on nerve tissue and neurotransmitters, frequently corrupting neurotransmitters or depolarizing the axonal layer for drawn out stretches of time, accordingly keeping apprehensive driving forces from being led. Cell digestion is hindered by inhibition of oxidative phosphorylation, which prompts a deficient supply of ATP for the cell. Mitochondrial electron transport is likewise hindered as Q-Cytochrome C, an electron acceptor protein in the Electron Transport Chain, is denatured (Rodríguez-Ithurralde *et al*., 1983). α-Neurotoxins, the snake poisons block post-synaptic acetylcholine receptors of snakes that lead to catecholamine discharge, loss of motion, respiratory failure and death (Koh *et al*., 2006).

2.4.5 Composition of Snake Venom

There are around 20 unique types of toxic enzyme found in snake venom yet not all snake species have these poisons. Most snakes have no less than six to twelve of these proteins in their venom (Peterson, 2006). Each one of the proteins has a particular capacity; either helps in the digestive related process or specialize in paralyzing the prey (Halliday and Tim, 2002). Snake venoms comprise of a blend of protein with or without synergist movement.

Snake venom proteins and polypeptides are characterized into super groups of enzymatic and non-enzymatic proteins. These super families demonstrate likenesses in their structures yet their organic capacities are plainly characterized. Different substances with toxc and deadly properties serve to immobilize the prey creature (Mattison, 2007).

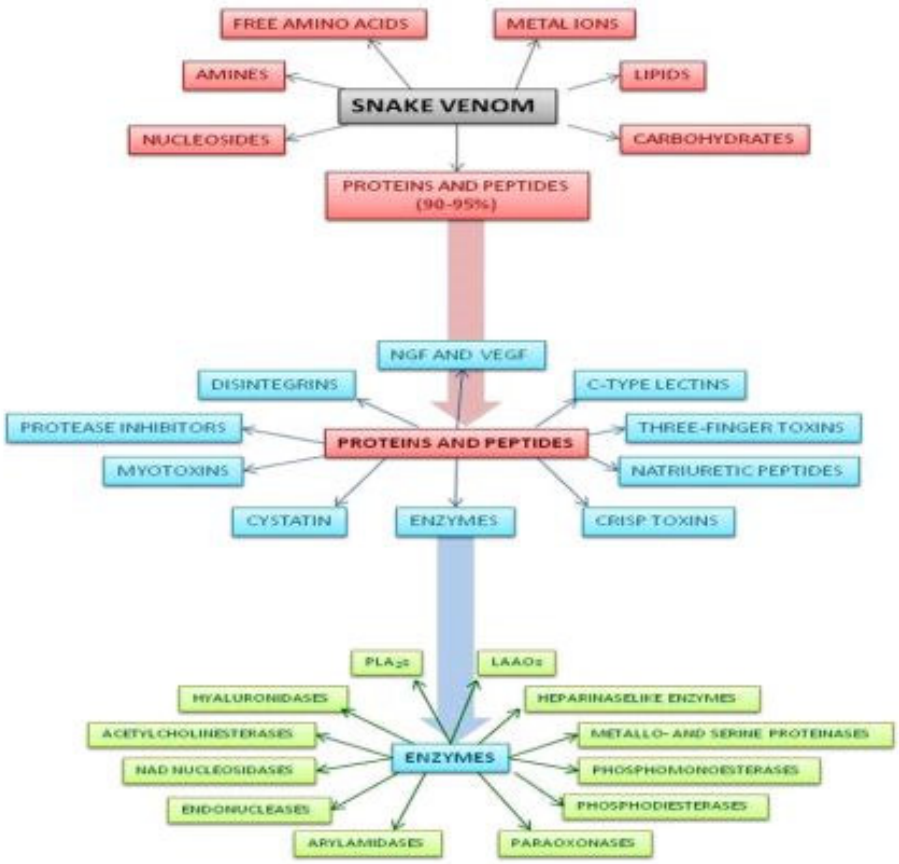


Figure 2.2: Composition of snake venom (Georgieva *et al.,* 2008)

2.4.6 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* (Suntravat *et al.*, 2010; Koh *et al*., 2006). Platelet function can be affected by venom components such as haemorrhagins and fibrinolytic activators, 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 L-amino acid oxidase, serine proteases, metalloproteinases, C-type lectins, disintegrins and phospholipases (Koh *et al*., 2006; Bon *et. al.*, 2000).

2.4.7 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 αß dimeric proteins or integrins (Hynes, 1992; Kamiguti *et al*., 1998).

2.5 L-amino Acid Oxidase (LAAO)

The enzyme LAAO is found in abundance in numerous organisms improving the level of toxicity during envenomation (Luiz *et al.*, 2014). These organisms include insects, snakes, fungi and bacteria (Du and Clemeson, 2002). The enzyme activity of this enzyme was discovered by Krebs from the liver and kidney tissue homogenates (Krebs, 1933) and was isolated from the tissues of rats. LAAO was first detected in snake venom of Vipera aspis in 1944 (Zeller *et al*., 1944).

LAAO are flavoenzymes that belongs to the class of oxidoreductases with enzymatic classification (E.C. 1.4.3.2) (Zuliani *et al*., 2009).

The LAAO present in snake venom has a multifunctional ability that possesses both clinical and biological manifestation which includes inhibition of platelet aggregation, induction of cell apoptosis hemorrhage, hemolysis, edema, cytotoxicity as well as antimicrobial, antiparasitic and anti-HIV activities (Tássia *et al*., 2014).

L-Amino acid oxidases (LAAO) catalyze the stereospecific oxidative deamination of L-amino acids. This is achieved by reaction of the enzyme with amino acid isomer used as a substrate (Campillo-Brocal *et al.,* 2015). The amino acid isomer is oxidized to an intermediate known as imino acid, with a concomitant reduction of the FAD cofactor. The

intermediate undergoes nonenzymatic hydrolysis, yielding α-keto acid and ammonia. The $FADH_2$ is reoxidized in the presence of molecular oxygen, thereby generating hydrogen peroxide (Izidoro *et al*., 2014). (Figure 2.3).

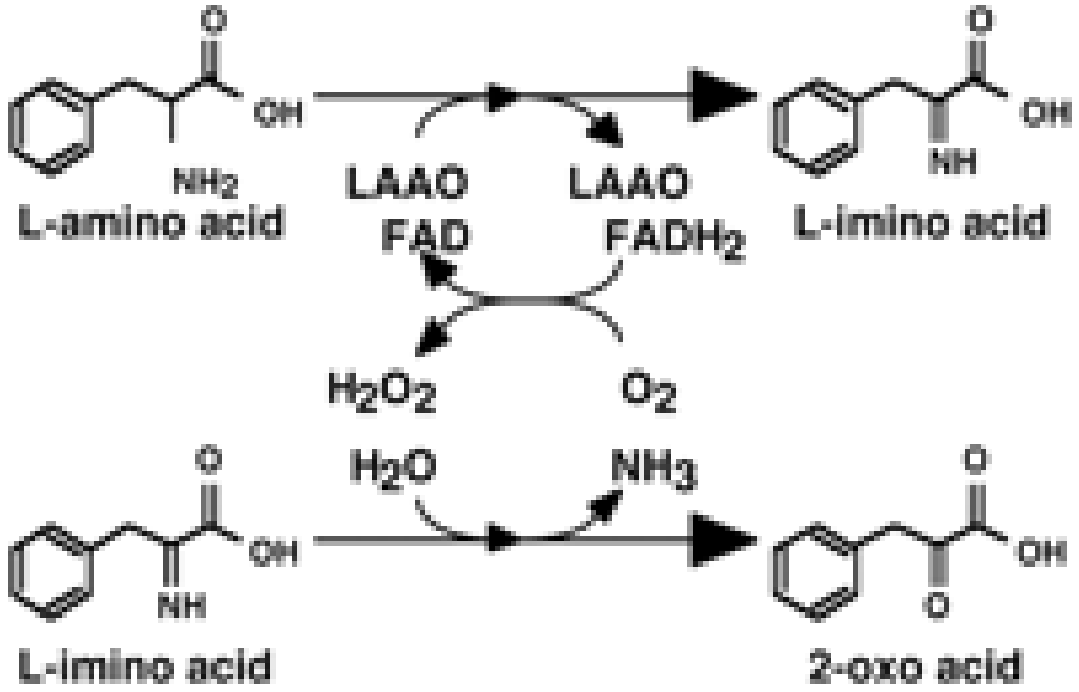


Figure 2.3: Mechanism of synthetic response catalyzed by L-amino corrosive oxidases (LAAO)

2.5.1 Flavoenzyme

Flavins are a yellow coloured compound with a basic structure of 7, 8-dimethyl-10-alkylisoalloxazine. The precursor of flavins is riboflavin also called vitamin B_2 (Edwards, 2014). Riboflavin-based coenzymes are tightly bound to enzymes catalyzing substrate oxidations and reductions, enable an enormous range of chemical transformations in biosynthetic pathways. Flavoenzymes catalyze substrate oxidations involving amine, alcohol oxidations and desaturations (Walsh and Wencewicz, 2013). Flavoenzymes catalyze a wide range of reactions essential for maintaining cellular processes.

Their striking chemical versatility is largely based on their ability to control the reaction of a flavin cofactor with O_2 (Massey, 1995).

More than 90% of flavin-dependent enzymes are oxidoreductases (Macheroux *et al.*, 2011) Flavoenzymes are proteins that undergo oxidation- reduction reaction (redox reaction), catalyze a wide range of biological reactions thereby can accept or donate one or two electrons. Their striking chemical versatility is largely based on their ability to control the reaction of a flavin cofactor with O_2. There are three major class of oxidoreductase: includes the dehydrogenases, that are characterized by poor or no reactivity with oxygen, the monooxygenases, that react very fast with O_2, forming a flavin adduct intermediate subsequently inserts an oxygen atom into the substrate molecule and the oxidases, that catalyze a rapid 2-electron transfer to O_2 to produce H_2O_2, typically—but not always—with no detectable catalytic intermediates (Mattevi, 2006). The biosynthesis of many classes of small molecule natural products, including polyketides, peptide scaffolds, and isoprenoids involve directed condensation of monomeric building blocks in a series of chain elongation reactions leading to release of nascent product frameworks (Fischbach and Walsh, 2006). The redox transformations are typically oxidations of scaffolds but less often can be reductive, While nicotinamide coenzymes (NAD(P)H/NAD(P)) are the most common coenzymes involved in redox transformations in primary metabolic pathways, flavin-dependent enzymes are heavily utilized in secondary pathways . Flavins, both in heme and nonheme contexts, overlap in their abilities to reductively activate and insert oxygen into natural product scaffolds. Flavin coenzymes are bound to substrate and does not dissociate from their protein partners (Valton *et al*., 2008). This tight binding is due to the ability of dihydroflavins (FMN and FAD) to undergo rapid, uncontrolled autoxidation outside the controlled microenvironments of enzyme active sites (Massey, 1994).

2.5.2 Oxidases

Oxidases are enzymes which catalyze a rapid transfer of two electrons to O_2 to yield H_2O_2 with no trace of catalytic intermediates. Flavins in its reduced state reacts with O_2 through electron-transfer process, generates a pair radical and also a rate limiting step (Sucharitakul *et al*., 2008). LAAO are flavoproteins of dimeric structure, with each subunit presenting a non-covalent bond with flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) forming a high energy phosphate bond. Hence, LAAO can be classification as FAD-dependent oxidoreductases. They are capable of catalyzing the stereospecific oxidative deamination of L-amino acid substrates to α-keto acids (Kommoju *et al*, 2007).

2.5.3. Mechanism

First half reaction: The oxidases (flavin-dependent aldehyde) generate carboxylic acid. This is the first half reaction, producing free radicals.

The reaction can be of different categories which are as follows: (a) H^+ and substrate carbanion, (b) H• and C• radicals, or (c) H^- and a carbonium ion like transition state, within this large substrate class, with hydride transfer as the most common mechanism.

Second half reaction: This is reoxidation of bound $FADH_2/FMNH_2$, generated by the reductive half reactions. The transformation in biosynthetic pathways is the reoxidation of one-electron that reacts with O_2. This is one-electron transfer from FlH_2 to give a product of superoxide anion and the flavin semiquinone, FlH•. The two products formed reacts to form radical recombination flavin hydroperoxide. This is the simplest outcome which is release of H_2O_2 by elimination and proton transfer (Palfey and McDonald, 2010). (Figure 2.4).

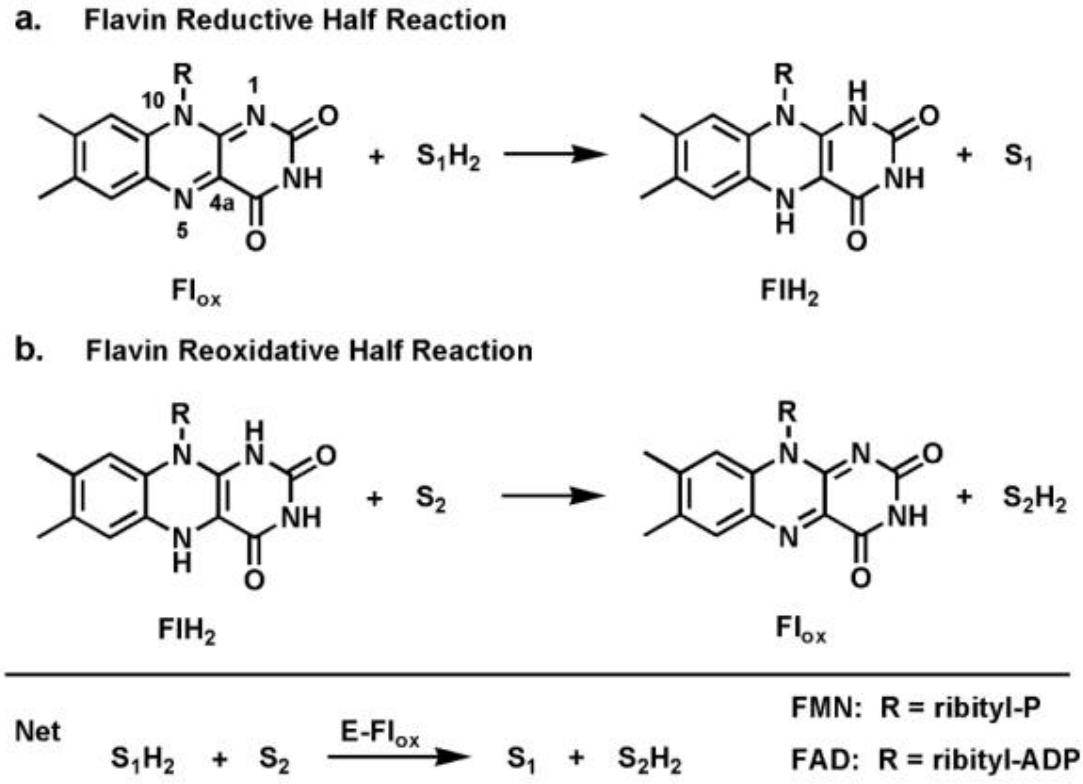


Figure 2.4: Two redox half reactions in flavoenzyme catalysis: (a) flavin reductive half reaction; (b) dihydroflavin reoxidative half reaction.

2.5.4. Effect of Hydrogen Peroxide

Louis Jacques in 1818 discovered hydrogen peroxide (H_2O_2) with both reducing and oxidizing properties. H_2O_2 is a free radial with reactive oxygen species (ROS). It has the potential of damaging cellular homeostasis during the enzymatic reaction. This potential damage to proteins, lipids and nucleic acids results in potentially toxic and a central signaling compound (Findrik *et al*., 2006).

The hydrogen peroxide generated in the chemical reaction is responsible for the multifuncti onal ability in the clinical and biological manifestation during envenomation (Jyotirmoy and ‘Dbasish, 2013).

H_2O_2 is transported by free diffusion through the lipid bilayer of membranes. Most membrane systems that are less permeable can be regulated by changes in membrane composition and channel proteins (Gerd *et al*., 2006), hence results in necrosis or apoptosis. Necrosis is the interaction between the plasma cell membrane and hydrogen peroxide produced resulting to cell death. The most common morphological changes were chromatin condensation, reduction and disintegration of nucleolus volume, and others. It also seems to be involved in the cytotoxic mechanisms of the enzyme which may ultimately represent another defense mechanism of the organism in response to the environment (Ande *et al*., 2008). The formation and diffusion into the cellular membrane in the human system will result to oxidative stress (Fox, 2013)

Oxidative stress is the imbalance between oxidants and antioxidants in favor of the oxidants which are formed as a normal product of aerobic metabolism but during pathophysiological conditions can be produced at an elevated rate. Both enzymatic and no enzymatic strategies are involved in antioxidant defense, and antioxidant efficacy of any molecule depends on the co-oxidant (Anu *et al.*, 2014). These led to hypoxia known to stimulate mitochondria to release ROS (mROS), under hypoxic conditions, mitochondria participate in a ROS burst generated at complex III of the electron transport chain (Liu *et al.*, 2002). Stress can be defined as a process of altered biochemical homeostasis produced by psychological, physiological, or environmental stressor (Dimitrios *et al.*, 2003). Exposure to stress may result in higher energy negative balance and may ultimately result in reduction in adaptation mechanisms, increase in the susceptibility to infection by pathogens, decline in productivity, and finally a huge economical loss (Kock *et al.*, 1987).

A typical human cell contains about 150,000 bases that can suffer from oxidative damage (Beckman and Ames, 1997).

Mutagens such as oxidants (free radicals or hydrogen peroxide) can damage DNA. Mutagen produces multiple forms of damage thus results to base modifications, particularly of guanosine, and double-strand breaks (Cadet *et al.*, 1999). During oxidative lesions, there will be double-strand breaks, as these are difficult to repair and can produce point mutations, insertions, deletions from the DNA sequence, and chromosomal translocations (Valeria and Povirk, 2003).

2.6 TOXICOLOGICAL RELEVANCE OF SNAKE VENOMS

2.6.1 Myotoxicity

Snake envenomation induces prominent local tissue damage that often results in permanent disability and systemic alterations associated with haemorrhage, coagulopathies, cardiovascular shock and renal failure. Clinical reports indicate that, in humans, the main invalidating effect is the irreversible disruption of muscle tissue (Gopalakrishnakone *et al.,* 1984). Tissue necrosis is a relevant local effect caused after snakebites, it is considered as a serious consequence in severe cases of envenomation. When myonecrosis appears, tissues are altered leading to the gangrene and infections （Montecucco *et al.,* 2008). This type of complication can be the cause of amputation. Indeed, myotoxins of snake venoms affect mainly the plasma membrane of muscle cells to which they bind through their cationic sequence. Molecular mechanism by which they caused the muscle tissue damage is not yet fully elucidated. Myonecrosis is due to the myotoxins that induce irreversible damage of skeletal muscle fibers. These molecules bind to the plasma membrane of muscle cells and alter its permeability and integrity (Falconi *et .al.,* 2000).

The induced muscle tissue damage could be due to the penetration of myotoxins into muscle cells by endocytosis, probably through membrane receptors onto the surface of muscle cells or following hydrolysis of phospholipids causing membrane disruption.

These molecules enter into the cytosol, reach and alter the membrane of mitochondria and sarcoplasmic reticulum of muscle cells. The intracellular effect of these toxins occurs only after their initial action on the plasma membrane, which marks the onset of degenerative events (Hamza *et al*., 2010).

2.6.2 Treatment of snake venom envenomation

The complexity of the snake venoms and their induced effects after envenomation makes difficult their treatment. However, more attention is given to loco-regional disorders that sometimes lead to amputations and permanent disabilities. Human suffering attributable to snake bites remains a public health problem in many countries of the world, several people over the world are known to be envenomed and some of them are killed or maimed by snakes every year (Kasturiratne *et al.,* 2008). Preventive efforts should be aimed towards education of regions at-risk to reduce contact with snakes and to understand snakes' behavior (Warrell *et al.,* 2013; Snow *et al.*, 1994).

Whatever the therapy used, it should include not only the neutralization of toxicity but also the other effects induced by venoms (haemorrhage, necrosis etc.). To treat snake envenoming, the production and clinical use of antivenom must be improved. Although antivenom was effective in the neutralization of systemic complications, it has limited effectiveness against the development of local damage, metabolic dysfunctions and tissue damage (Montecucco *et .al.,* 2008). Collaboration between physicians, epidemiologists and toxinologists should enhance the understanding and treatment of envenoming (Warrell *et al.,* 2013).

2.6.3 BENEFICIAL EFFECTS OF ISOLATED SNAKE VENOM COMPONENTS

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 therapeutic agents (Volkers, 1998; Pal *et al*., 2002), basic research tools for use in the diagnosis of several diseases (Bailey and Wilce, 2001; Marsh, 2001; Pal *et al*., 2002), and in basic research for understanding physiological and pathological processes (Sher *et al*., 2000; Andrews *et .al*., 2001; Marsh, 2001; Wisner *et al*., 2001).

2.7. Therapeutic Uses

Therapeutic uses of snake venoms include applications as antiviral agents, anti-parasitic medication, anti-mircobial agents and antitumor agents. LAAO presents an interest in treatment of diseases; they act as procoagulant, anticoagulant, and on platelet aggregation as pro- or antiplatelet (Chérifi *et. al.,* 2014). Some of these molecules are used in the treatment of peripheral vascular disorders, thrombotic diseases and as hemostatic agent in some cases of bleeding and thrombocytopenia such as observed in post-operative situations (White, 2005).

Hemotoxins in snake venom target the circulatory system, and typically attack the body's clotting ability and muscles. Other drugs have been developed from neurotoxins in snake venom, which are used to treat Alzheimer's and Parkinson's, as well as stroke and brain injuries (Lecia, 2015).

Disintegrins, proteins found in the venom of many vipers, for example, act as binding partners to integrins, which play a large role in several different cellular processes.

Eristostatin, one of those disintegrins, which normally bonds to platelets in a snake's victim and carries toxins throughout the circulatory system, also bonded to melanoma cells in mice and kept tumors from metastasizing into other parts of the body (Noah, 2013).

2.7.1 Diagnosis of diseases

A wider range of haemostatic factors found in snake venoms have been used in the development of laboratory diagnostics (Marsh and Williams, 2005). Components of snake venoms contain two categories of components that act antagonistically through activation or inhibition of coagulation factors and platelet aggregation (Sanchez *et al*., 1997).

2.7.2 Basic Research

Most of venom compounds possess interesting properties and are increasingly used in biomedical research. 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). Indeed, the specific nature of coagulant or anticoagulant properties of venoms makes them useful to better understand the haemostatic mechanisms (Chérifi *et al.,* 2014).

2.8 SNAKE VENOMICS

Snake venom consists of a wide range of proteins, with a complex proteome. Therefore it is not possible to visualize every component of a proteome using a single proteomic technique. Recent publications in the field of venomics have emphasized the need for multidimensional approaches to maximize the protein coverage (Fox and Serrano, 2008). Recently, the combinatorial peptide library ap proach (commercialized as Proteominer TM) has emerged as a powerful tool for mining below the tip of the iceberg, and complements the data gained using the snake venomics protocol towards a complete visualization of the venom proteome (Calvete, 2011).

A general scheme of the steps to be followed in a snake venomics analysis towards a complete visualization of the venom proteomes is shown below (Figure 2.5).

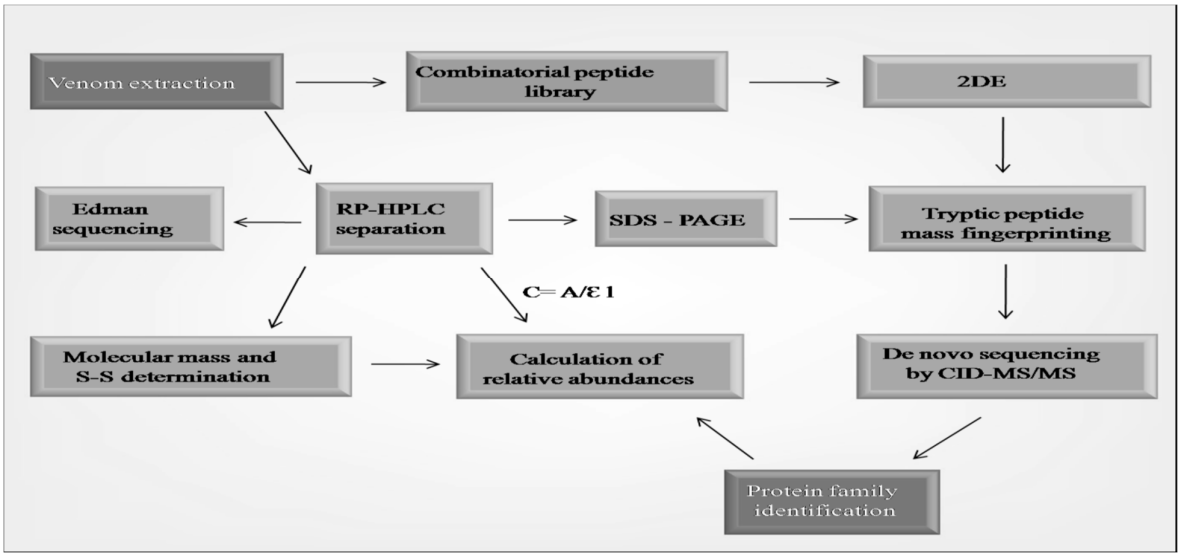


Figure 2.5: Scheme of the steps typically performed in a snake venomics analysis. CID: Collision induced dissociation; RP-HPLC: Reverse phase HPLC.

The proteomic approach has given rise to a comprehensive understanding of the venom complexity, composition, and relative abundance of different protein families, and provides insights to investigators to focus on different issues and identification of novel proteins (Nawarak *et al.,* 2003).

Studies have shown that the chemical composition of the venoms exhibit geographical variations and may be due to evolutionary environmental pressure acting on isolated populations (Alape-Giron *et .al.,* 2008). The snake venom composition is under genetic control and therefore proteome studies could serve as a tool to provide molecular markers for taxonomical purposes (Lomonte *et al.,* 2012; Calvete *et al.,* 2009).

However, besides varying between species, venom composition also differs within a species depending on age, season and temperature (Alape-Giron *et al.,* 2008).

CHAPTER THREE

MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Reagents and Chemicals

All reagents and chemicals used in this research were of analytical grade listed in Appendix I

3.1.2. Equipment and Apparatus

All the apparatus and equipment used in the research were of high quality and are listed in Appendix II with their capacity, specification and/or model where applicable:

3.1.3 EXPERIMENTAL ANIMAL

Important specie of snake *Naja nigricollis* (Nn, Black-necked spitting cobra) associated with envenoming in Nigeria (Habib *et al.*, 2001) was used in the study. The snake used was 1.70 meters long, black in colour and about three kilograms in weight. The two extracted venom glands A and B weight 1.21 kilograms and 1.80 kilograms respectively.

3.3. Methods

3.3.1. Reagents Preparations

All the reagent preparations are presented in Appendix III

3.3.2 Collection of Snake Venom

Naja nigricollis was caught from the wild at Rijiyar-zaki, Kano in the northern part of Nigeria. It was identified in the Zoology unit of the Biological Sciences department of Bayero University, Kano, Nigeria. The venom was collected fresh by saliva induction. The snake head was held and force onto a beaker covered with polythene. The tail was gently

touched and thus causes the snake to release its venom into the beaker by piercing the polythene with its fangs.

The venom was filtered to remove potential mucosal contaminants and extracted by manual stimulation, centrifuged, lyophilized and kept at –20°C at the AKTH laboratory, Bayero University, Kano.

3.3.3 Excision of Venom Gland

The snake (*Naja nigricollis*) was sacrificed three days after venom extraction. The gland was immediately placed into a pre-labelled 1.5 ml microcentrifuge tube having a screw cap, and dropped into liquid nitrogen. Samples were then stored at -80°C until use.

3.4 ENZYME PURIFICATION

LAAO from the venom was isolated by gel filtration and ion-exchange chromatography.

3.4.1 Gel filtration on Sephadex G-75

A chromatographic column was prepared by plunging some cotton wool into a 2 X 50 cm column. The slurry was then poured into the column packed with glass wool at the bottom. The column was first equilibrated with phosphate buffer pH 7.0, before the sample was applied. It was allowed to stand for 24hrs for compression. Crude venom (200 mg) was dissolved in 50ml phosphate buffer, pH 7.0 in a beaker. This was transferred to a centrifuge tube and the insoluble component was removed by centrifugation and 30ml of the recovered supernatant was loaded onto the packed column (2 X50cm). Thirty-four fractions at a flow rate of 1ml per 6 minutes were collected and analyzed for total protein and LAAO activity. The fraction with highest specific activity was pooled and further purified on a DEAE-cellulose column as described by Bordon (2012).

3.4.2 Ion-exchange Chromatography on DEAE-cellulose

DEAE-cellulose was prepared by dissolving 2.5g of the anion-exchanger in 100 ml of phosphate buffer, pH 7.0. The slurry was then poured into a 2.5 X 30 cm column.

The pooled fraction of sub-section 3.4.1 (14 ml) was loaded onto the column and eluted with a convex concentration gradient of sodium chloride solution (0.05 to 0.4M).

Eighteen fractions were collected at a flow rate of 0.5ml per 3 minutes and analyzed for total protein and enzyme activity. The fractions showing high specific activity were pooled.

3.5 Determination of Enzyme Activity (Kishimoto and Takahashi, 2001):

This was achieved by continuous spectrophotometric rate determination using L-Leucine as substrate (Lee *et al.*, 2011).

Principle

The reaction velocity is determined in a peroxidase coupled system by measuring the increase in absorption at 490nm resulting from the oxidation of L-leucine to 4-methyl-2-oxopentanoate.

$$L-Leucine \xrightarrow{L-amino\ acid\ oxidase} 4-methyl-2-oxopentanoate\ (ketoleucine) + ammonia + hydroperoxide$$

Conditions: T = 25ºC, pH = 7.6, A_{436nm}, Light path = 1cm

A leucine standard curve was prepared by making dilutions of the leucine standard (52mM) with distilled water to a total volume of 200 µL 250µl of 5IU/ml horseradish peroxidase, 250µl of 2mM orthopheneylenediamine reagent.

The contents were mixed and incubated at 37ºC for 1 hour and 100µl of 2.0M H_2SO_4 was added. The absorbance of the solution was measured at 490nm against reagent blank.

3.5.1 Procedure

For each sample, three test tubes were set up (Test in duplicate and Blank). For each tube, 200µl of each aliquot and distilled water was added to 200µl of 52mM L-leucine, 250µl of 5IU/ml horseradish peroxidase, 250µl of 2mM orthopheneylenediamine respectively. The mixture was incubated at 37ºC for 1hr and 100µl of 2.0M H_2SO_4 was added. The absorbance of the solution was measured at 490nm.

3.5.2 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 diluent to a total volume of 800 µL. The fractions were also analysed in duplicates by arranging test tubes labeled as test, standard and blank. Bradford reagent (200 µL) was added to all tubes Distilled water (700 µL) was dispensed into all tubes followed by 100 µL of fraction, BSA standard and distilled respectively.

The contents of each tube were mixed and incubated for 2 minutes at room temperature. The absorbance of the colored solution was measured against reagent blank at 490 nm.

3.5.3 Sodium Dodecyl Sulphate Polyacrylamide-Electrophoresis (SDS-PAGE)

The crude venom and pooled fractions from sub-sections 3.4.1 and 3.4.2 were subjected to SDS-PAGE using 14% gel by the method of Laemmli (1970) in the presence of protein standard.

Equal volumes (100 µL) of the samples and the dissolving buffer (glycerol, SDS, beta-mercaptoethanol and traces of bromophenol blue) were boiled at 100 ^{0}C 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. 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 electrophoresis tank was filled with the running buffer and the apparatus were 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, monitoring the bands followed by destaining with a mixture of acetic acid, methanol, and water for two hours. The gel was dried and stored.

3.5.4 Determination of Optimum Temperature

The optimum temperature was determined by measuring the LAAO activity at different temperature (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90) in duplicates.

200 µL fraction was subjected to the horseradish peroxidase and orthopheneylenediamine protein assay and the residual enzyme activity calculated.

3.5.5 Determination of Optimum pH

The optimum pH was determined by measuring their activities at different pH (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 respectively) in duplicates with 1.0M NaOH and 1.0M HCl to adjust the pH. 200 µL fraction was subjected to the horseradish peroxidase and orthopheneylenediamine protein assay and the residual enzyme activity determined.

3.5.6 Determination of Kinetic Parameters

The kinetic parameters determination was carried out by determining the enzyme activity at varying substrate (L-Leucine) concentration (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 mM) in duplicate in the presence of fixed enzyme concentration [E]. A double-reciprocal 1/V against 1/[S], a (Lineweaver-Burk) plot was used to determine K_m and V_{max} of L-amino acid oxidase.

3.5.7 Effect of some compounds on LAAO

The LAAO activity was determined by measuring the activities at its optimum pH and temperature in the presence of the following compounds; ($FeSO_4$, $MnCl_2$, urea, $CaCl_2$, $MgSO_4$, Pb_2SO_4, $(NH_4)_2NO_4$, Na_2CO_3, KH_2PO_4 and EDTA).

3.6 DNA extraction

3.6.1 Snake venom

Into a cryovial, 100mg of venom, CTAB (cetyl trimethylammonium bromide) (400µl) buffer with proteinase K (10µl) was added. The mixture was incubated at 65°C for 1 hour with vigorous vortexing at interval of 20 minutes. Exactly 400µl of phenol-chloroform-isoamyl alcohol was added, vortexed and spun for 10 minutes at 3000G.

The mixture was decanted; 400µl of phenol choloroform was added, spun for 10 minutes at 3000G and decanted. To the supernatant 1000ul of 100% ethanol and 4µl of 3.0M sodium acetate (pH 5.2) was added and incubated overnight at -20ºC.

The DNA mixture was spun for at 3000g for 10 minutes at 4ºC. The mixture was decanted, 400µl isopropanylethanol was added, spun at 4ºC for 5minutes. The procedure was repeated four times and decanted subsequently. The mixture was air dried.

3.6.2 Snake gland

The snake gland was cut into small pieces, mashed with liquid nitrogen. The mixture was sieved and the same procedure was followed as snake venom.

CHAPTER FOUR

4.0. RESULTS

4.1 Enzyme Purification

Naja nigricollis venom, initially fractionated on Sephadex G-75 column, displayed two peaks. The elution profile of this chromatography is shown in Figure 4.1. The pooled fractions showed 1.45 folds more L-amino acid oxidase activity than the crude venom with a yield of 59.80% (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. This step revealed 3.79 folds more L-amino acid oxidase activity than the crude venom with a yield of 6.95 % (Table 4.1).

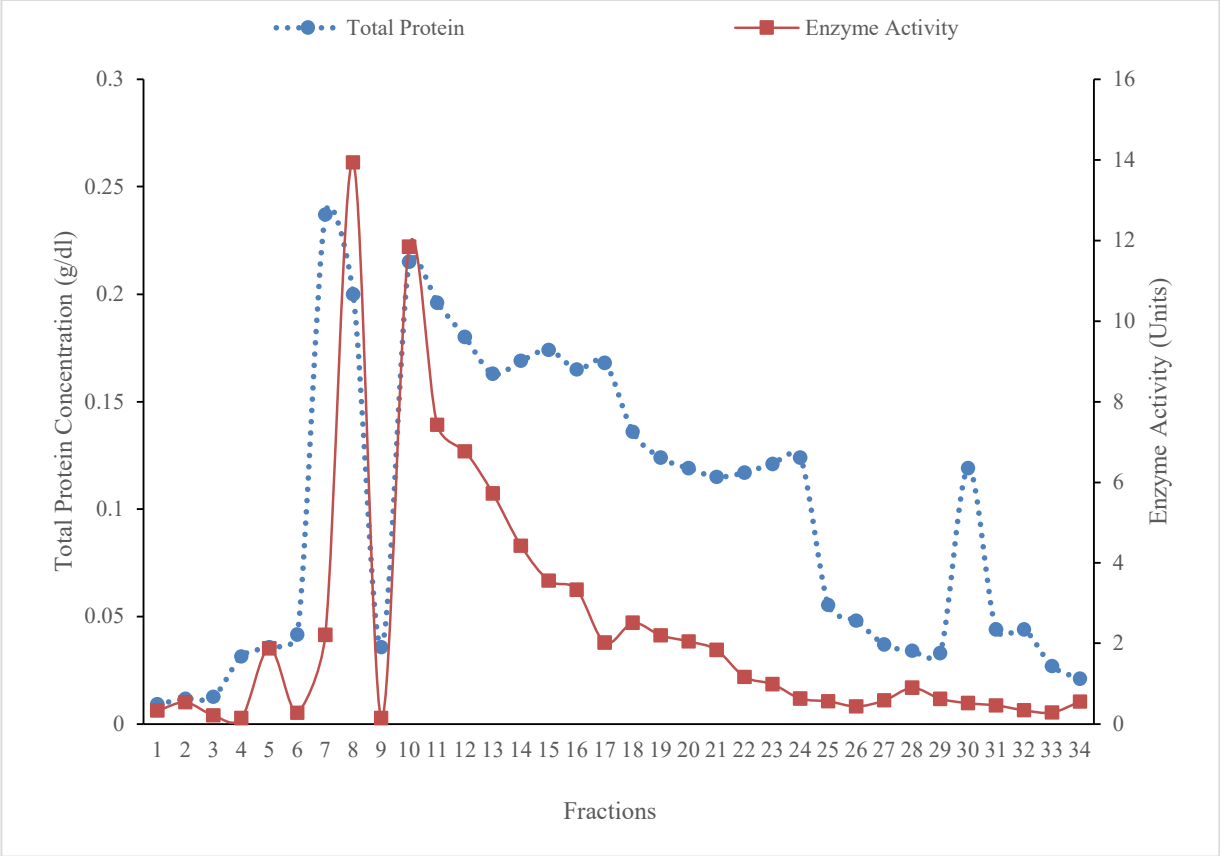


Figure 4.1: Elution profile of Nn-LAAO venom. Fractionation on sephadex G75: crude venom (200mg) dissolved in 40ml of phosphate buffer pH 7.0 and applied to the column (2 X 50cm) and elution was carried out at a flow rate of 1ml/6 minutes at 25^{o}C with phosphate buffer pH 7.0.

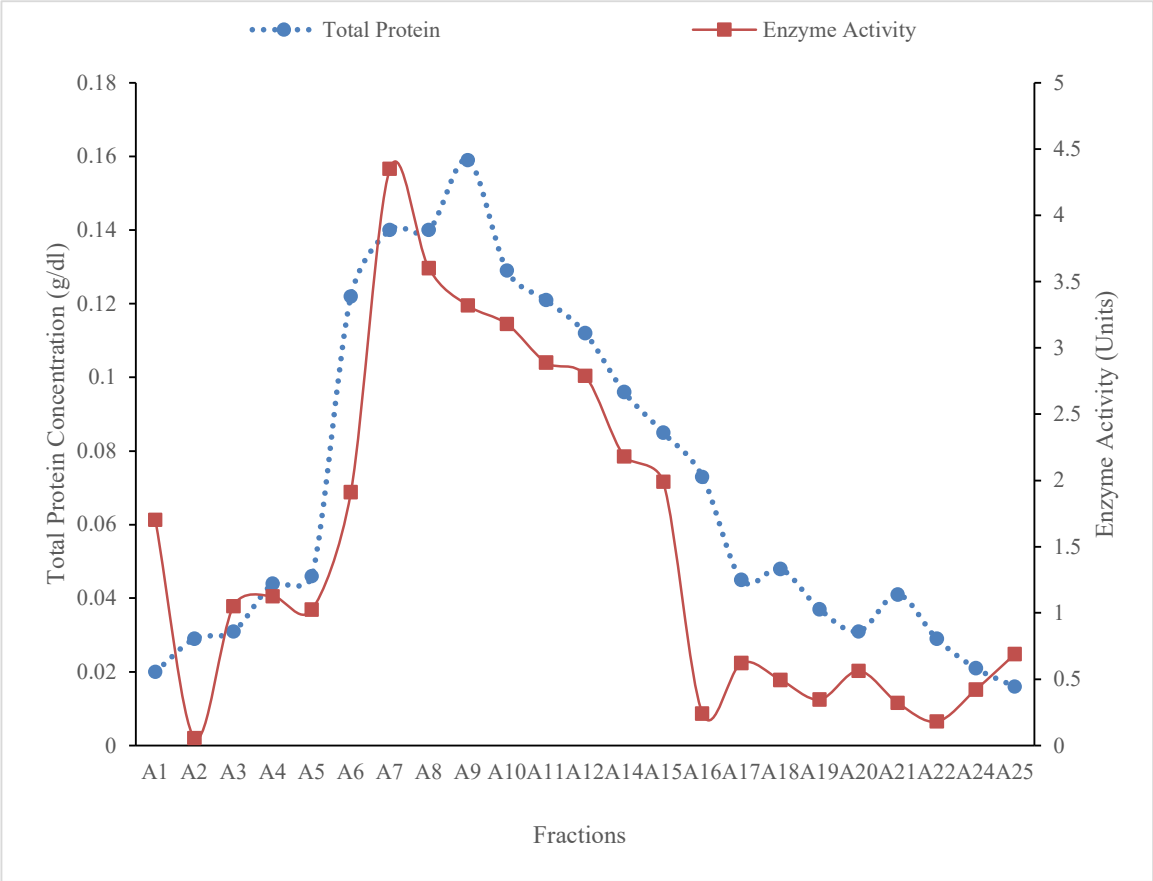


Figure 4.2: Elution profile of Nn-LAAO venom. Fractionation on DEAE-cellulose: pooled fraction from gel filtration (14ml) was applied to the column (2.5 by 30cm) and elution was carried out at a flow rate of 0.5ml/minute at 25ºC.

TABLE 4.1: Purification Profile of Nn-LAAO venom

PURIFICATION STEPS	TOTAL PROTEIN (mg/ml)	ENZYME ACTIVITY (mmoles/ml/hr)	SPECIFIC ACTIVITY (mmoles/ml/hr/ mg protein)	PURIFICATION FOLD	RECOVERY (%)
CRUDE VENOM	0.458	23.31	46.53	1	100
GEL FILTRATION ON SEPHADEX G-75	0.207	13.94	67.32	1.45	59.80
ION-EXCHANGE ON DEAE-52 CELLULOSE	0.009	1.62	176.24	3.79	6.95

4.1.2 Effect of Temperature on Nn-LAAO

The effect of temperature on Nn-LAAO activity has an optimum temperature of 45ºC (Figure 4.3) These activities of the enzyme started rising steadily from the ranges of 30 ºC to 45ºC until it attained its optimum temperature. The activity of the enzyme started declining at 50 ºC down to zero with little or no activity. There were no recorded activities till the last temperature of 80 -90ºC reading for the enzyme.

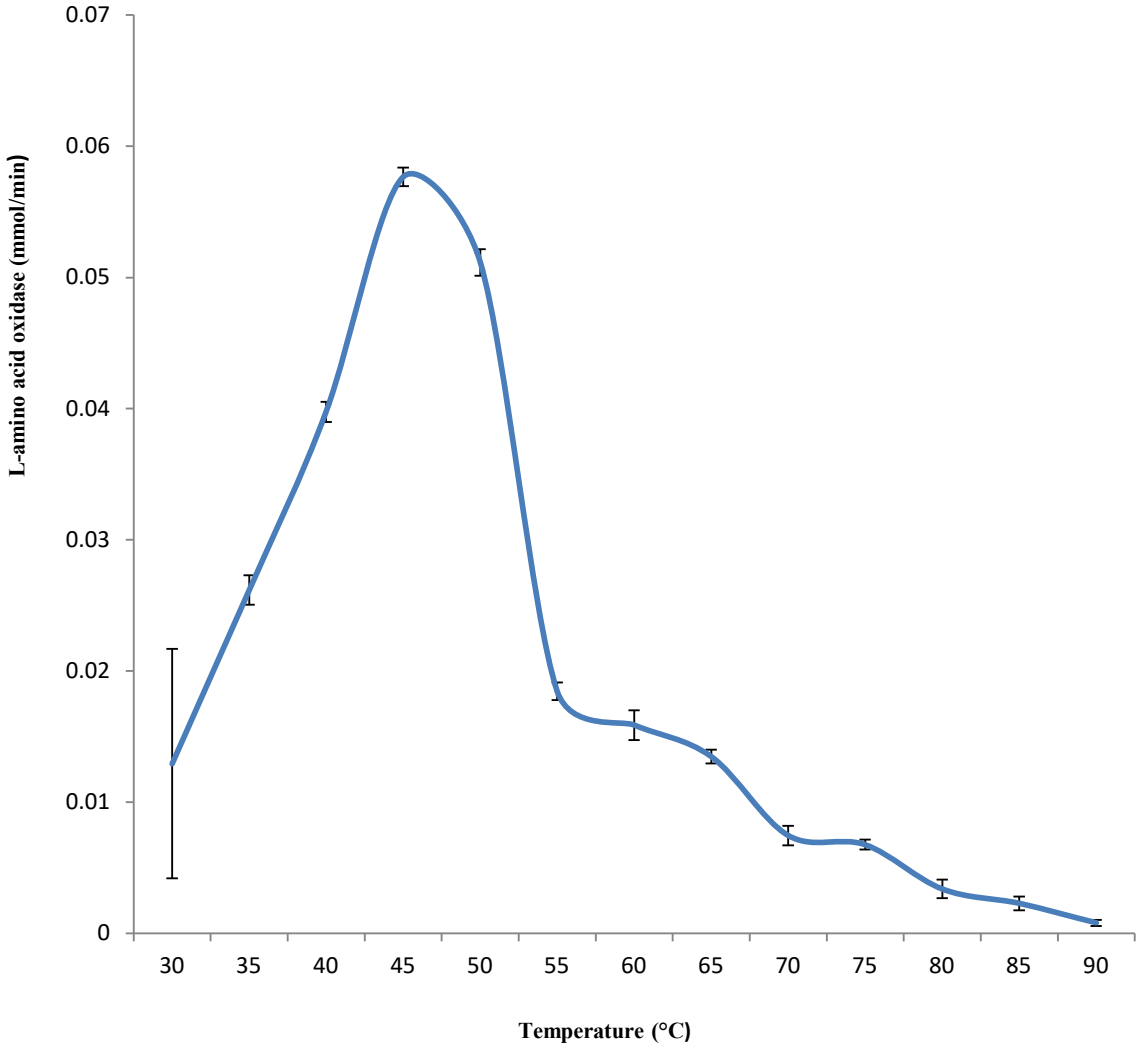


Figure 4.3: Effect of Temperature on Nn-LAAO Activity

4.1.3 Effect of pH on Nn-LAAO Activity

The pH of Nn-LAAO activity was at its optimium at 6.25 (Figure 4.4). The enzymatic activity of Nn-LAAO pH on was determined between the ranges of 2–10. The enzyme activity appears to rise steadily to its optimal and gradually declined downwards. Nn-LAAO experienced a sharp decrease in enzymatic activity between pH 2 and 5; and a decrease from pH 8.5 to 10 resulting in a strong decrease in activity.

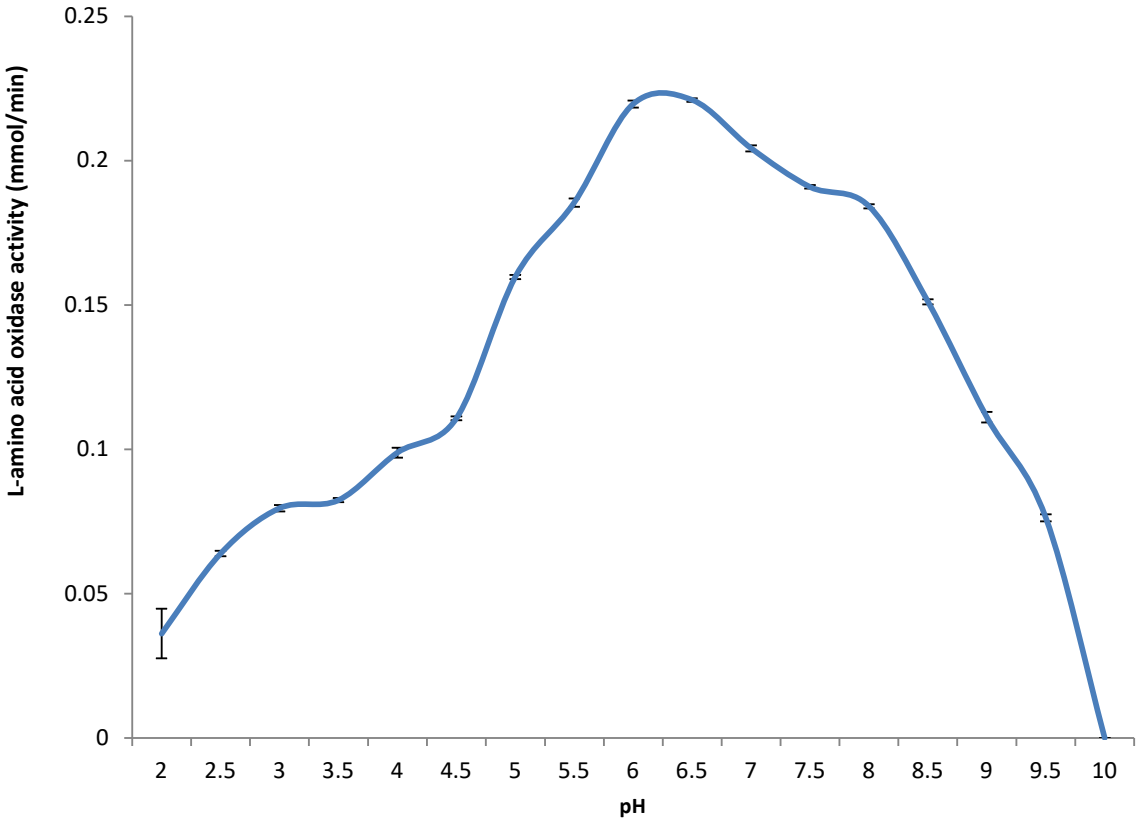


Figure 4.4: The effect of pH on Nn-LAAO activity

4.1.4 Effect of compounds on Nn-LAAO Activity

Nn-LAAO activity showed high activating effect with $(NH_4)_2NO_4$ (ammonium nitrate), Na_2CO_3 (sodium carbonate) and urea; while $FeSO_4$ (Iron II Sulfate), $CaCl_2$ (calcium chloride) and KH_2PO_4 (potassium hydrogen phosphate) has lesser stimulatory activity than the former while EDTA and lead II Sulphate (Pb_2SO_4) showed inhibiting effect.

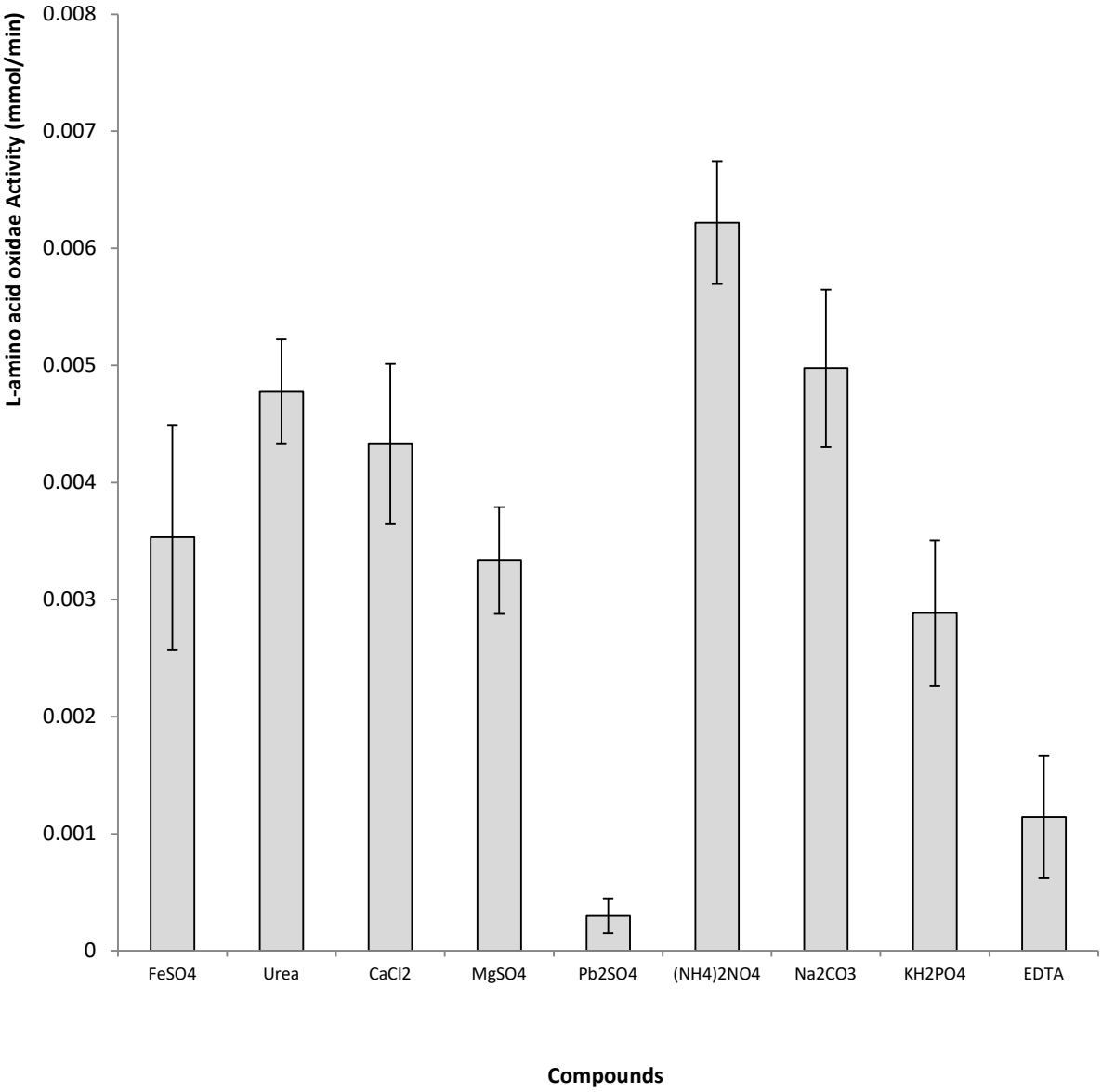


Figure 4.5 Effect of compounds on Nn-LAAO

4.1.5 Effect of Increase in Substrate Concentration in the Activity with Fixed Concentration of Nn-LAAO

Saturation of enzyme reaction was attained with increasing substrate (l-leucine) concentrations, a behavior typical of an apparent first-order enzyme reaction that obeys Michaelis-Menten (equation I). Using Lineweaver-Burke equation (equation II): the apparent maximum velocity (V_{max}) of the overall enzyme reaction and the apparent Michaelis-Menten constant K_m were experimentally calculated to be 5.5mmol/min and 0.045mM respectively shown in a graphical represented in Figure 4.6

$$V = \frac{V_{max}\,[S]}{K_m + [S]} \quad \text{..... Equation I: Michaelis–Menten equation}$$

$$1/V = \frac{K_m}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}} \text{.....Equation II: Lineweaver-Burk Equation}$$

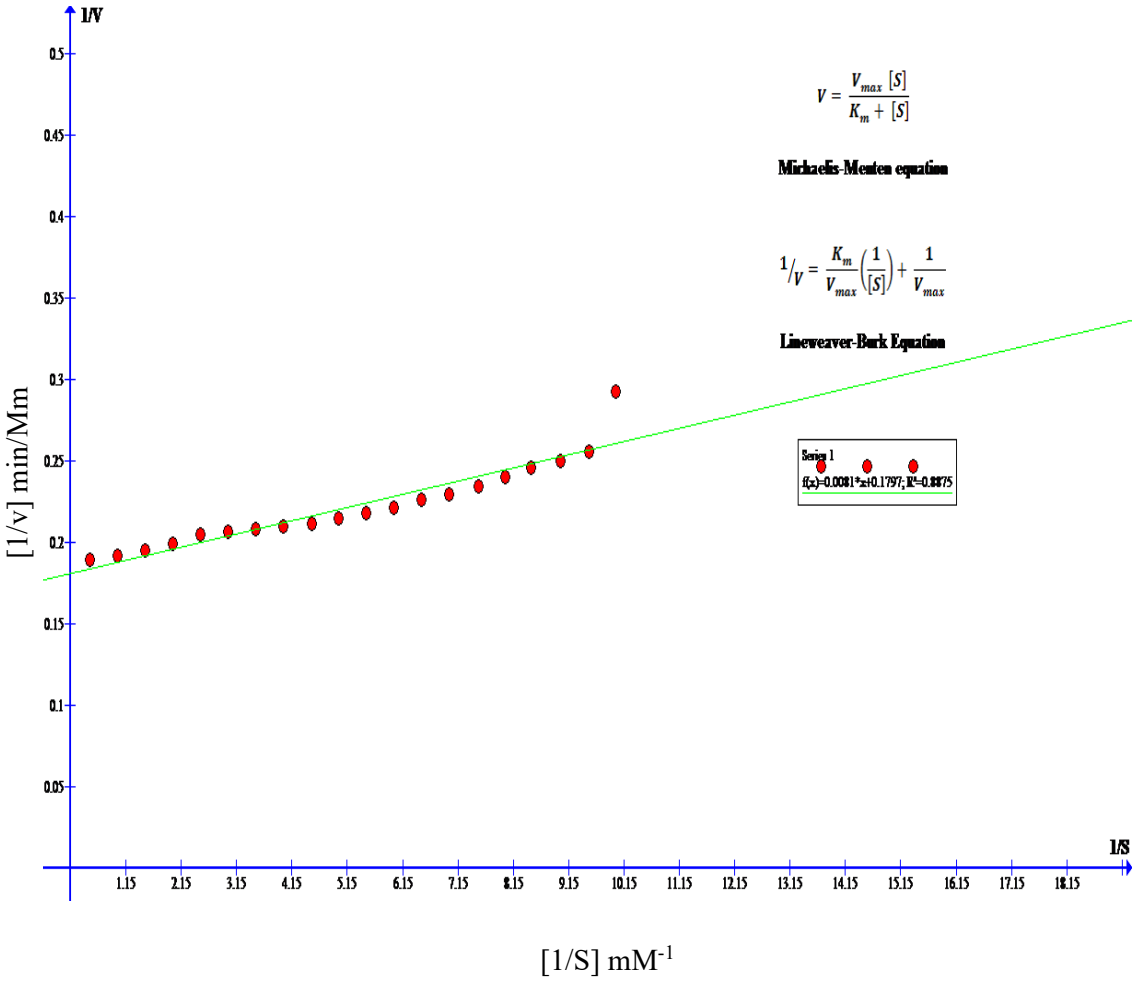
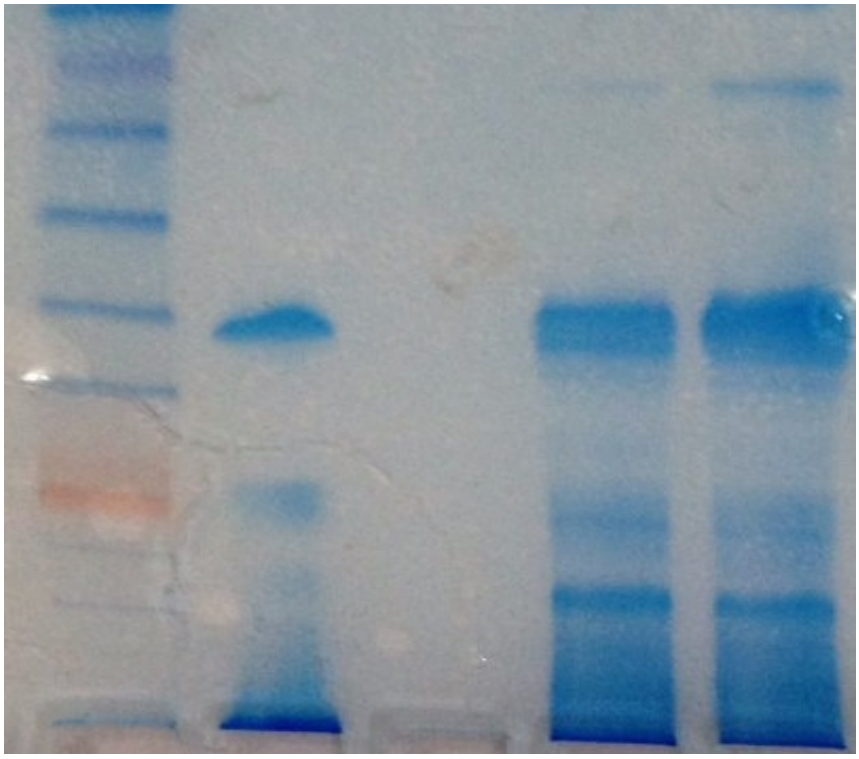


Figure 4.6 Linewaver-Burk graph of 1/V Against 1/S

4.1.6 Determination of Nn-LAAO Purity and its Molecular Weight

The purified fractions from DEAE and sephadex G-75 respectively were ran on SDS-PAGE. The apparent molecular weight of the purified enzyme was estimated from results of SDS-PAGE. The enzyme was found to be homogenous by the presence of distinct band pattern on SDS-PAGE, and consisted of a single chain (Plate 4.1) whose molecular weight was 44KDa.



L1 L2 L3 L4

PLATE 4.1: SDS PAGE of Purified Nn- LAAO
Key: L1= Protein marker, L2=purified enzyme from DEAE (44KDa), L3 and L4; other fraction from sephadex G-75 with relatively activity of the enzyme.

4.2 Quantification of DNA

DNA was extracted using the phenol-choroloform method and quantified. The concentration was 43.9ng and 300.0ng respectively; the purity was 1.059 and 1.00 respectively.

Table 4.2 Quantification of DNA in venom and gland

LAAOs	**Concentration**	**Purity**
Venom	43.9	1.059
Gland	300.0	1.00

CHAPTER FIVE

5.0 DISCUSSION

The purification of 200mg of crude Nn-venom yielded 1-fold approximately. The purified venom had 6.95% of the recovered protein which has 3.79-fold purification. The purification of 1 g of *C. adamanteus* and *C. adamanteus* venom was 4-fold greater (Bordon *et al.,* 2015; Raibekas and Massey, 1996). Significant differences in activity and protein concentration are observed even in snake venoms from the same species and region, and from *Crotalus durissus terrificus* venom (CdtV). This is associated with multiple forms of the LAAO and the venom composition that dependence on many factors such as the age and diet of the snake, climate, altitude and time of secretion into the glands (Alape-Giron *et al.,* 2008). These properties contribute significantly to the pathophysiology of envenomation.

Nn-LAAO displayed optimum activity at 45^{o}C and was active over a temperature range of 30-45^{o}C. SV- LAAO enzymes mostly have optimum activity at 40-50^{o}C. Hence, subsequent exposure to temperatures greater than 55ºC results in a gradual decrease in activity caused by disrupt ions in hydrophobic interactions and hydrogen bonds between the different subunits of the enzyme (More *et al.*, 2010). As the temperature is elevating, the kinetic energy is increased to undergo the reaction, as it raised above its optimum, the kinetic energy of the enzyme and water molecules is increased; the structure of the enzyme molecule is disrupted gradually.

Nn-LAAO loose activity at high temperatures, by including an inactive form of the enzyme that is in reversible equilibrium with the active form; it is the inactive form that undergoes irreversible thermal inactivation to the thermally denatured state.

This is dependent on reversible active-inactve model transition (Daniel *et al.*, 2007; Michelle *et al.*, 2007; Peterson *et al.*, 2004). Therefore, as the temperature increases, enzyme activity deceases and vice verse (Ketnawa *et al.*, 2011; Michelle *et al.*, 2007). These changes involve alterations in the binding of the enzyme to substrates in the affinity of the flavin coenzyme for electrons (Samel *et al.*, 2006). There is decrease in enzyme activity as the temperature is above the optimum level which is caused by the equilibrium shift (Peterson *et al.*, 2007). The specific activity of some LAAO depends on the experimental temperature. These enzymes remain active for a variable period of time at a broad range of temperatures (30 to close to 45°C). Arrhenius equation describes the temperature sensitivity which is based on the energy required to initiate the reaction, termed the activation energy (Ea), (Von Lützow and Kögel-Knabner, 2009). The Arrhenius equation predicts exponentially increasing reaction rates with increasing temperature, assuming constant values of activation energy (Peterson *et al.*, 2004). *Naja naja oxiana* venom, Daboia russellii siamensis venom was reported to be stable at low temperatures (20ºC -70ºC) and loses its activity by heating at 70ºC (Abdelkafi-Koubaa *et al.*, 2014; Samel *et al.*, 2008). The wide range of temperature for the enzyme shows that the enzyme is capable of withstanding extreme conditions which is important for venom toxicity.

The Nn-LAAO optimal activity at pH 6.25 suggests that the enzyme would maximally hydrolyze proteins in alkaline conditions. At its optimum pH, Nn-LAAO has charged groups that may participate in binding them to each other or to other types of molecules.

The enzyme and the substrate attain an ionic equilibrium, permitting a better fit of the substrate in the active site of the enzyme and consequent maximum oxidation (Coles *et al.*, 1977). Ionic interactions are highly sensitive to change in pH; therefore, pH can either protonate or deprotonate a side group of amino acid residues at a catalytic site, thereby changing the level of enzymatic activity.

The presence of H^+ or OH^- ions will disrupt the bonds in the tertiary structure. However, it also affects the electrostatic attraction between the enzyme and substrate. The disruption to the bonds causes the enzyme to denature. For pH 2-5, H^+ bind to the carboxyl groups (COO^-) and H^+ bind to the unoccupied pair of electrons on the N atom of the amino (NH_2) while for greater pH 8-9, H^+ are removed from the COOH groups giving them a negative charge (COO^-), and H^+ are removed from the NH_3^+ groups removing their positive charge which results to the net charge on the molecule change (it becomes more positive) and the ionic (electrostatic) interactions with other molecules and ions are altered. This depends on reversible inactivation which is either spontaneous change in structure of the enzyme resulting to inactive configuration (Kapetaniou *et al.*, 2006). Also, there is a reversible inactivation in when the pH changes to values close to neutral thereby inducing inactivation, resulting in a spontaneous structural change of the enzyme to its inactive configuration and when the pH is lowered, the active conformation of the enzyme is restored. It is reported that there is a narrow range of pH in the oxidation of L-amino acids by LAAO (Pradiniwat and Rojunckarin, 2015; Butzke *et al.*, 2005). Optimum activity at alkaline pH between 6 -8.5 has been reported for LAAO venom (Sun *et al.,* 2010; Bordon *et al.*, 2015; Tan and Swaminathan, 1992). Therefore, snake venom has highly modified saliva which is alkaline in nature. Hence, 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 0.045mM is a clear indication of the high kinetic efficiency of the enzyme. Previous studies reported K_m values of 9.23 μM, 0.97 mmol/L, 0.06 mmol and 2.1 mM for LAAO *Crotalus durissus cumanensis* venom, *Lachesis muta* venom, *Cerastes cerastes* venom and *Agkistrodon blomhoffii ussurensis* venom respectively (Vargas *et al*., 2013; Bregge-Silva *et al*., 2012; Hanane-Fadila and Fatima, 2014; Sun *et al*., 2010).

It appears that the K_m value of the Nn-LAAO compares relatively less to other known snake venom LAAO implying higher affinity of this enzyme for its substrate (leucine), which might be related to different species. 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 catalysis of an enzyme can enhanced or slowed down or disrupted by the addition of compounds. They 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 Nn-LAAO exhibited inhibitory effect with lead II Sulphate and EDTA but not sodium, magnesium and calcium. This could be attributed to the dependence of LAAO on calcium and magnesium (Sun *et al*., 2010), which abolishes proteolytic and haemorrhagic activities due to structural alterations. These result in cofactors NAD or FAD reduction and inactivation of the enzyme. (Izidoro *et al*., 2014). This means that treatment for local effect of envenomation by *N. nigricollis* requires the incorporation of snake venom LAAO inhibitors for effectiveness. Lead II sulphate, a heavy compound can also block enzyme activity by binding to sulfhydryl groups that hold the structure of the protein portion of the enzyme together (Evans, 1984) and also, cofactors NAD or FAD are reduced, causing inactivation of the enzyme (Luiz *et al.*, 2014; Izidoro *et al.*, 2014). This means that treatment for local effect of envenomation by *N. nigricollis* requires the incorporation of snake venom LAAO inhibitors for effectiveness.

While some compounds can have stimulatory effect on Nn-LAAO, Urea appeared to have little or no effect on the activity of the enzyme. This could be attributed to the preference and affinity shown by both EDTA and ammonium sulfate are being high energy-dependent.

In accordance to several reports, the activity of the substrate l-Leu is increased through the addition of exogenous metal ions which includes Mg(2+), Mn(2+), Ca(2+), Ce(3+), Nd(3+), Co(2+) and Tb(3+) (Sun *et al*., 2010; Hofer, 1984).

SDS-PAGE analysis of the purified enzyme revealed a single protein band with an estimated molecular weight of 44KDa. Such observation indicates that the isolated enzyme is composed of a single polypeptide chain. When these toxins are treated under denaturing conditions, the molecular mass of each monomer determined by mass spectrometry is about 40–70 kDa This is close to LAAO from *N. Naja oxiana* venom which consisted of a single chain with a molecular weight of 57 Ka (Bordon *et* al. 2015; Abdelkafi-Koubaa *et al.*, 2014; Yab *et al.*, 2011; Zhong *et al.*, 2009; Samel *et al*., 2008). This variation in molecular mass among different LAAO might be related to the sites of glycosylation since these enzymes are considered to be glycoproteins. The carbohydrate moiety of the enzyme only plays a structural role or protects the enzyme against proteolysis since snake venoms are rich in proteolytic enzymes (Izidoro *et al*., 2014). This result indicates that Nn-LAAO is a non-covalently associated homodimer, as reported for most SV-LAAOs.

In the quantification of DNA, the concentration was 43.9ng of Nn-venom and 300.0ng in the Nn-gland. The purity showed 1.059 in the venom and 1.00 in the gland respectively. These showed that the DNA is more in the gland than lypolized venom and purity of the gland was a little more than the venom. Purification requires a reasonable source of the protein.

It is not difficult to appreciate that it is easier to purify a protein from a rich source than from a poor source (Selistre de Araujo *et al.*, 1996). Furthermore, a rich source is likely to produce a greater yield of the protein of interest. The quantification and purity measurements of DNA may aid laboratory scientists when troubleshooting PCR and help them to determine the best extraction method for each application (Boesenberg-Smith *et al.*, 2012; Schomburg *et al.*, 2002). Tissue samples and to a lesser extent whole cells have a protein content that greatly exceeds that of nucleic acid on a weight basis and purification of samples to a A260/A280 ratio represents an enrichment of nucleic acid that could be as much as 1 million fold (Held, 2001).

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

SUMMARY

The purified Nn-LAAO activity increased by 3.79 fold in comparison with the crude venom. It displayed optimum activity at pH 6.25 and was stable over a pH range of 5.5-8.5. Its optimum temperature was 45^{o}C and activity stable between 30-60^{o}C. The K_m and V_{max} were found to be 0.045mM and 5.57mmol/min respectively. The activity was inhibited by EDTA and lead II Sulphate; not affected by ammonium nitrate, sodium carbonate, and urea. Inhibition could be prevented by calcium chloride but not potassium hydrogen phosphate or magnesium sulphate.

These provide means of exploring the usefulness of the enzyme, assessing quality control criteria and standardization protocol for various applications.

Reliable measurement of DNA concentration and purity is important for many applications in molecular biology, especially array comparative genomic hybridisation (aCGH) where accurate determination of DNA concentration is critical. Impurities in DNA can lead to inaccurate measurement of DNA concentration and could potentially inhibit subsequent labelling reactions.

5.2 CONCLUSION

Nn-LAAO is a potentially toxic protein responsible for several biological activities. They catalyze redox reaction amino acids, generating hydrogen peroxide as a catabolic product. This reactive oxygen species so far seems to be the molecule responsible for the pharmacological effects of this class of enzymes. Nn-LAAO have been found to be valuable molecules with possible future applications to the treatment of many diseases and as models for the development of antivenom, antiviral, antitumor, antiparasitic, and antimicrobial drugs. However, the development of therapeutic agents based on the structure of widely characterized molecules previously isolated from snake venoms is gaining popularity in the search for future drugs.

5.3 RECOMMENDATIONS

- Comparative studies of the enzyme could take place in different snake species and enzyme can further be characterized
- More kinetic and inhibitory studies could be conducted

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APPENDICES

Appendix I: Reagents and Chemicals

REAGENTS	SOURCES
A. BUFFERS	
1. Tris-HCl Buffer	
i. Tris Reagent	
ii. Hydrochloric acid	May and Beaker LTD Dagenham England
. Phosphate Buffer	
i. Monosodium Phosphate (Sodium dihydrogen Orthophosphate)	Hopkin and Williams Chadwell Health Essex, England
ii. Disodium Phosphate (*di*-Sodium Hydrogen Phosphate)	Guangdong Guanghua Sci-Tech Co., Ltd.
B. REAGENTS FOR ENZYME ASSAY	
1. Tris-HCl Buffer	Guangdong Guanghua Chemical Factory Co, Ltd. China & May and Beaker LTD Dagenham England
2. Hydrochloric acid	
3. L-Leucine	Sigma-Aldrich, Japan
4. Horseradish peroxidase	Sigma-Aldrich, Germany
5. Orthophenylenediamine	Sigma-Aldrich, Life Science, Switzerland
C. SDS-PAGE ELECTROPHORESIS	
1. BIS-TRIS Buffer (Running Buffer)	Gene Script, USA
2. 4-20% Sodium Dodecyl Sulfate-Polyacrylamide Stacking Gel	Gene Script, USA
3. 2 X Protein Loading Dye	Ambion, USA
4. Commassie Blue Stain (G-30)	Bio-Rad
5. SeaBlue Plus 2 Protein Standard (Protein Marker)	Invitrogen, USA
D. OTHERS	
6. `sodium hydroxide	
7. Ammonium Sulphate	
8. Distilled Water	
9. Deionised Water	

10. Ferrous Sulphate
11. Manganese chloride
12. Urea
13. Calcium Chloride
14. Magnesium Sulphate
15. Lead Sulphate
16. Ammonium Nitrate
17. Sodium Carbonate
18. Potassium Hydrogen Phosphate
19. Ethylenediamine Tetraacetic acid

E: REAGENTS FOR DNA AND RNA EXTRACTION (DNA LABS KADUNA)

1. Liquid Nitrogen
2. CTAB (cetyl trimethylammonium bromide)
3. proteinase K
4. phenol-chloroform-isoamyl alcohol
5. 100% ethanol
6. 3M sodium acetate (pH 5.2)
7. Isopropanylethanol

F: REAGENT FOR PCR

1. Pure Water
2. Forward & Backward Primer

3. Deionized Ultrapure Water

4. Hot starch PCR Pre-mix

G: REAGENT FOR AGAROSE GEL

1. QDLE Agarose Powder

2. Tris Acetate EDTA (TAE) Buffer

Appendix II: Equipments and Apparatus

EQUIPMENTS/APPARATUS	CAPACITY/SPECS/MODELS
Cryovials	
Cryobox	
Pipette dropper	
Ice packs	
Insulated cooler	
Electronic Weigh Balance	B. Bran Sci & Instru. England LP202A (200g/0.01g)
Centrifuge	Soval legend thermofisher
Beaker (1000ml, 500ml, 200ml &100ml)	Permagold
Volumetric Flask (500ml, 100ml & 50ml)	E-Mil Boro A, England (1000ml); Permagold (500ml, 100ml & 50ml)
Stirrer (1m)	Permagold
Cuvette 1cm	Glass
Pipette (10ml)	Permagold
Mechanical Micropipette	
Chromatographic Column	
Water bath	
Spectrophotometer	Mirco lab- 3000
Funnel	E-Mil Boro A, England (75mm)
Spatula	
Plain Test Tubes (10 ml)	Micropoint Diagnostics
Plain Test Tubes (3ml)	Micropoint Diagnostics (LOT: 130113)
Vertical Electrophoresis Tank	Hoefer (27572 PC)

Power Supply Pack	BIO-RAD
Eppendrof tubes	
Incubator Shaker	
Staining Container (SDS PAGE)	
Heating Block Machine	
PCR Machine with Oil	PTC-100 Programmable Thermal Controller MJ Research,Inc.
Cold centrifuge 540	Eppendrof
DNA & RNA Ladders	NTB
Gel Doc1060	Bio-Rad
Refrigerator (-20^{o}C)	RL1
DNA & RNA Quantification spectrophotometer	QuantoPro
Centrifuge 5415 C	Eppendrof
Micro Centrifuge	LX-100 Model
Class 11 Biological Fume Cupboard	Class11 A2
UV Hood	
Face Shield	
Incubator Shaker	
Staining Container	
Heat Block (PCR)	

APPENDIX III:

Reagents Preparations

A. Buffers

1. Tris-HCl (20mM & 50mM) Buffer

i. The *Number of Moles* of the Tris-HCl Buffer was determined from the concentration (*mol/L*) and the volume needed:

$$Moles\ of\ Tris = Conc.\left(\frac{Mol}{L}\right) \times Volume\ (L)$$

ii. Grams of Tris to be dissolved was determine by multiplying *Number of Moles* with *Molecular Weight* of Tris (121.14 g/mol):

$$Grams\ of\ Tris = Moles\ of\ Tris\ (Mol) \times Molecular\ Weight\ (121.14 g/mol)$$

iii. The gram of Tris was dissolved in deionised water, one third (1/3) to one half (1/2) of the desired final volume.

iv. HCl (1M) and NaOH (1M) was used to adjust the pH as desired.

v. It is finally diluted to desired final volume with water.

The above steps were used to prepare 20mM and 50mM Tris-HCl Buffer.

2. Phosphate Buffer (0.1 M); pH range 5.8 to 8.0.

4.56g of dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) was weighed and dissolved in 200ml of distilled water.

pH	**5.8**	**6.2**	**6.4**	**6.6**	**6.8**	**7.0**	**7.2**	**7.4**	**7.6**	**7.8**	**8.0**
ml of A	92.0	81.5	73.5	62.5	51.0	39.0	28.0	19.0	13.0	08.5	05.3
ml of B	08.5	18.5	26.5	37.5	49.5	61.0	72.0	81.0	87.0	91.5	94.7

The two solutions were mixed in the proportions indicated and the final volume was adjusted to 200 ml with deionised water. The pH of 8.0 was used in the research.

B. Reagents for Column Chromatography

Sephadex G-75: 5g of sephadex G-75 was dissolved to 50ml with 20mM Tris HCl buffer (pH: 7.0)

C. Reagents for Enzyme Assay

REAGENT

i. 5Mm L-Leucine (1331.7g/mol)

0.33g of L-Leucine was weighed and dissolved gradually in 500ml of 20Mm tris HCl.

ii. 2Mm Orthophenylenediamine/ peroxidase substrate (M.Wt 108.14g/mol)

0.11g of Orthophenylenediamine was weighed and dissolved gradually in 500ml of 20mMtris HCl

iii. 2M H_2SO_4 (M.Wt 98.1g/mol)

19.6ml of concentrated H_2SO_4 was measured into a measuring cylinder. 50ml of distilled water was measured in another measuring cylinder. 19.6ml of concentrated H_2SO_4 was gradually added and made up to 100ml mark with distilled water.

iv. Horseradish peroxidase

0.0025g of horseradish peroxidase was weighed and dissolved in 200ml of 0.1M phosphate buffer (pH8.0)

D. Reagent for Inorganic substances

i. 0.1M Ferrous Sulphate

0.278g of ferrous sulphate was weighed and dissolved into 10ml of distilled water

ii. 0.1M manganese chloride

0.20g of manganese chloride was weighed and dissolved in 10ml of distilled water

iii. 0.1M urea

0.060g of urea was weighed and dissolved in 10ml of distilled water

iv. 0.1M calcium chloride

0.147g of calcium chloride was weighed and dissolved in 10ml of distilled water

v. 0.1M magnesium sulphate

0.246g of magnesium sulphate was weighed and dissolved in 10ml of distilled water

vi. 0.1M lead sulphate

0.303g of lead II sulphate was weighed and dissolved in10ml of distilled water

vii. 0.1M ammonium sulphate

0.08g of ammonium sulphate was weighed and dissolved in10ml of distilled water

viii. 0.1M sodium carbonate

0.106g of sodium carbonate was weighed and dissolved in10ml of distilled water

ix. 0.1M potassium dihydrogen phosphate

0.174g of potassium dihyrogen phosphate was weighed and dissolved in10ml of distilled water

x. 0.1M EDTA (Ethylenediamine tetra acetic acid)

0.292g of EDTA was weighed and dissolved in10ml of distilled water

D. Preparation of agarose gel

1.5% of Agarose gel

1.5g of QDLE agarose powder was dissolved in 100ml of tris acetate (TAE) buffer and heated at 90^{o}C – 100^{o}C until dissolved completely. Poured into the rack until solidified.

G. Reconsistution of PCR Primer

ALL PREPARATIONS TOOK PLACE IN THE UV HOOD

Stock Solution

Forward primer: 113.6ul of ultrapure water was added to the forward primer

Backward primer: 117ul 0f ultrapure water was added to the backward primer.

Working solution

10 pica mol dilution of primers

5ul of primer was added to 45ul of ultra pure water: This was done for both the forward and backward primers.

Appendix IV:

Elution profile of *Naja nigricollis* L-amino acid oxidase from sephadex G75

FRACTIONS	OD_{595Nm}	PROTEINS	OD_{490nM}	Activity (Units)	Specific Activity
1	0.019	0.0092	0.05	0.335	36.41
2	0.024	0.0117	0.08	0.538	45.81
3	0.026	0.0127	0.032	0.214	16.88
4	0.063	0.0314	0.022	0.147	4.69
5	0.074	0.0358	0.28	1.88	52.4
6	0.085	0.0416	0.042	0.281	6.76
7	0.49	0.237	0.33	2.21	9.33
8	0.424	0.2	2.08	13.94	67.32
9	0.74	0.0358	0.022	0.147	4.11
10	0.431	0.215	1.768	11.84	55.1
11	0.401	0.196	1.108	7.42	37.88
12	0.369	0.18	1.011	6.77	37.63
13	0.328	0.163	0.853	5.72	35.06
14	0.339	0.169	0.659	4.42	26.13
15	0.356	0.174	0.531	3.56	20.45
16	0.331	0.165	0.497	3.33	20.18
17	0.338	0.168	0.302	2.02	12.04
18	0.279	0.136	0.375	2.51	15.8
19	0.253	0.124	0.329	2.2	17.78
20	0.244	0.119	0.306	2.05	17.22
21	0.231	0.115	0.275	1.84	16.02
22	0.239	0.117	0.174	1.17	9.96
23	0.248	0.121	0.148	0.99	8.2
24	0.253	0.124	0.094	0.63	5.08
25	0.109	0.0553	0.084	0.563	10.18
26	0.098	0.048	0.065	0.436	9.07
27	0.075	0.037	0.087	0.583	15.75
28	0.069	0.034	0.134	0.898	26.41
29	0.073	0.033	0.093	0.623	18.88
30	0.243	0.119	0.077	0.516	4.34
31	0.089	0.044	0.069	0.462	10.51
32	0.09	0.044	0.058	0.339	8.8
33	0.056	0.027	0.043	0.288	10.67
34	0.043	0.021	0.083	0.556	26.48

Appendix V:

Elution profile of *Naja nigricollis* L-amino acid oxidase from DEAE-cellulose

FRACTIONS	OD595nM	PROTEIN	OD490nM	ACTIVITY	SPECIFIC ACTIVITY
A1	0.04	0.02	0.254	1.702	85.09
A2	0.02	0.029	0.086	0.0576	19.87
A3	0.063	0.031	0.157	1.052	33.93
A4	0.089	0.044	0.168	1.126	25.58
A5	0.094	0.046	0.153	1.025	22.28
A6	0.249	0.122	0.285	1.91	15.65
A7	0.285	0.14	0.649	4.35	31.06
A8	0.288	0.14	0.532	3.6	25.74
A9	0.325	0.159	0.495	3.32	20.86
A10	0.264	0.129	0.472	3.18	24.67
A11	0.247	0.121	0.432	2.89	23.92
A12	0.229	0.112	0.416	2.79	24.89
A14	0.196	0.096	0.326	2.18	22.75
A15	0.173	0.085	0.297	1.99	23.41
A16	0.15	0.073	0.036	0.241	3.3
A17	0.092	0.045	0.093	0.623	13.84
A18	0.099	0.048	0.074	0.496	10.33
A19	0.075	0.037	0.052	0.348	9.42
A20	0.063	0.031	0.084	0.563	18.15
A21	0.083	0.041	0.048	0.322	7.84
A22	0.059	0.029	0.027	0.181	6.24
A24	0.043	0.021	0.063	0.422	21.11
A25	0.032	0.016	0.103	0.69	43.13

APPENDIX VI:

DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE Sample preparation (store at $0^{o}C$)

- Add the SDS sample buffer (Room Temperature) to the samples (equal volume of 4X buffer) still on ice, and boil at $100^{o}C$ 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 VII:

TERMS AND CALCULATIONS IN ENZYME PURIFICATION

Calculation of LAAO activity

1. Absorbance of sample=Absorbance of sample- Absorbance of blank
2. Determine mmoles of L-Leucine equivalent liberated using the equation derived from the L-Leucine standard curve.
3. Determine units of LAAO activity per ml of LAAO sample using the following equation:

Units/ml=mmole L-Leucine X reaction volume / Sample volume X reaction time X volume assayed

Where:

mmole L-Leucine= mmole of L-Leucine equivalent released

Reaction volume=total volume in ml of assay (1000µl=1.0ml)

Sample volume=volume in ml of LAAO sample (200µL) =0.2ml

Reaction time (minutes) of reaction incubation=1 hour

Volume assayed=volume (in ml) used in colorometric determination (0.2)

Specific enzyme act (µmol/enzyme/hour/mg protein) =units of enzyme (µmole/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

APPENDIX VIII:

Effect of temperature on L-amino acid oxidase activity

Temp	abs 1	Activity 1	abs 2	Activity 2	abs 3	Activity 3	MEAN	SD
30	0.122	0.018209	0.119	0.017761	0.019	0.002836	0.012935	0.008749
35	0.175	0.026119	0.168	0.025075	0.183	0.027313	0.026169	0.00112
40	0.262	0.039104	0.272	0.040597	0.265	0.039552	0.039751	0.000766
45	0.381	0.056866	0.388	0.05791	0.39	0.058209	0.057662	0.000705
50	0.348	0.05194	0.335	0.05	0.345	0.051493	0.051144	0.001016
55	0.124	0.018507	0.119	0.017761	0.128	0.019104	0.018458	0.000673
60	0.103	0.015373	0.101	0.015075	0.115	0.017164	0.015871	0.00113
65	0.094	0.01403	0.087	0.012985	0.09	0.013433	0.013483	0.000524
70	0.05	0.007463	0.055	0.008209	0.045	0.006716	0.007463	0.000746
75	0.048	0.007164	0.043	0.006418	0.045	0.006716	0.006766	0.000376
80	0.021	0.003134	0.019	0.002836	0.028	0.004179	0.003383	0.000705
85	0.012	0.001791	0.015	0.002239	0.019	0.002836	0.002289	0.000524
90	0.005	0.000746	0.007	0.001045	0.004	0.000597	0.000796	0.000228

APPENDIX IX:

Effect of pH on LAAO Activiy

pH	abs 1	Activity 1	abs 2	Activity 2	abs 3	Activity 3	MEAN	SD
2	0.309	0.046119	0.204	0.030448	0.215	0.03209	0.036219	0.008613
2.5	0.428	0.063881	0.422	0.062985	0.435	0.064925	0.06393	0.000971
3	0.542	0.080896	0.531	0.079254	0.528	0.078806	0.079652	0.0011
3.5	0.548	0.081791	0.551	0.082239	0.558	0.083284	0.082438	0.000766
4	0.672	0.100299	0.665	0.099254	0.65	0.097015	0.098856	0.001678
4.5	0.738	0.110149	0.747	0.111493	0.741	0.110597	0.110746	0.000684
5	1.07	0.159701	1.065	0.158955	1.075	0.160448	0.159701	0.000746
5.5	1.244	0.185672	1.232	0.183881	1.251	0.186716	0.185423	0.001434
6	1.479	0.220746	1.463	0.218358	1.472	0.219701	0.219602	0.001197
6.5	1.48	0.220896	1.477	0.220448	1.485	0.221642	0.220995	0.000603
7	1.376	0.205373	1.362	0.203284	1.368	0.204179	0.204279	0.001048
7.5	1.28	0.191045	1.275	0.190299	1.283	0.191493	0.190945	0.000603
8	1.236	0.184478	1.229	0.183433	1.238	0.184776	0.184229	0.000705
8.5	1.008	0.150448	1.01	0.150746	1.019	0.15209	0.151095	0.000875
9	0.748	0.111642	0.731	0.109104	0.755	0.112687	0.111144	0.001842
9.5	0.518	0.077313	0.502	0.074925	0.513	0.076567	0.076269	0.001222
10	0.209	0.031194	0.211	0.031493	0	0	0	0

APPENDIX X:

Effect of Compounds on LAAO Activity

Metals	abs 1	Activity 1	abs 2	Activity 2	abs 3	Activity 3	Mean	SD
$FeSO_4$	0.021	0.003134	0.031	0.004627	0.019	0.002836	0.015752	0.001055
Urea	0.029	0.004328	0.035	0.005224	0.032	0.004776	0.02111	0.000633
$CaCl_2$	0.028	0.004179	0.025	0.003731	0.034	0.005075	0.018982	0.000317
$MgSO_4$	0.023	0.003433	0.019	0.002836	0.025	0.003731	0.014654	0.000422
Pb_2SO_4	0.002	0.000299	0.001	0.000149	0.003	0.000448	0.00129	0.000106
$(NH_4)_2NO_4$	0.038	0.005672	0.042	0.006269	0.045	0.006716	0.027388	0.000422
Na_2CO_3	0.033	0.004925	0.038	0.005672	0.029	0.004328	0.022119	0.000528
KH_2PO_4	0.018	0.002687	0.016	0.002388	0.024	0.003582	0.012615	0.000211
EDTA	0.004	0.000597	0.008	0.001194	0.011	0.001642	0.004958	0.000422

APPENDIX XI:

Effect of Substrate Concentration on LAAO Activity

V	S	1/V	1/S
5.29	2	0.189036	0.5
5.21	1	0.191939	1
5.13	0.667	0.194932	1.49925
5.03	0.5	0.198807	2
4.88	0.4	0.204918	2.5
4.85	0.333	0.206186	3.003003
4.81	0.286	0.2079	3.496503
4.76	0.25	0.210084	4
4.72	0.222	0.211864	4.504505
4.65	0.2	0.215054	5
4.59	0.182	0.217865	5.494505
4.52	0.167	0.221239	5.988024
4.42	0.154	0.226244	6.493506
4.35	0.143	0.229885	6.993007
4.26	0.133	0.234742	7.518797
4.17	0.125	0.239808	8
4.07	0.118	0.2457	8.474576
4	0.111	0.25	9.009009
3.91	0.105	0.255754	9.52381
3.42	0.1	0.292398	10