" CHARACTERIZATION OF AMINOPEPTIDASE IN MIDGUT OF BODY LOUSE

PEDICUL US HUMANUS HUM ANUS

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A thesis submitted in partial fulfilment for the degree of Master of Science in the

JjJniversity of Nairobi i

1998

DECLARATION

I, Evah Adega Oduor, hereby declare that this thesis is my original work and the work described here has not been presented to any other University.

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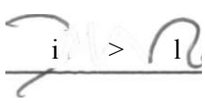
Signed  Date Z 3 > (t f S

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ABBREVIATIONS

LAP	-	Leucine Aminopeptidase
BSA	-	Bovine serum albumin
DMF	-	Dimethyl formamide
LpNA	-	Leucine para-nitroanilide
PAGE	-	Polyacrylamide gel electrophoresis
SDS	-	Sodium dodecyl sulphate
Tris	-	Tris-(hydroymethyl)-amino methane

*

UNIT ABBREVIATIONS

gm	**-**	**gram**
h	**-**	**hour**
M	**-**	**molar concentration**
min	**-**	**minute**
mg	**-**	**milligram**
ml	**-**	**millilitre**
mM	**-**	**millimolar**
nm	**-**	**nanometer**
pH	**-**	**-Log hydrogen ion concentration**
ug	**-**	**microgram**
ul	**-**	**microlitre**

SUMMARY

Body louse *Pediculus humanus humanus*, belong to the group of blood-sucking insects that act as vectors of several deadly diseases to either Mankind or his Livestock, resulting in massive economic losses, especially in third world countries where these diseases are prevalent. Body louse are host specific and feed exclusively on blood. Because of their feeding habits they are therefore prone to blood-borne control agents. Proteins are by far the most abundant nutrients of a bloodmeal. Hence the characterization of enzymes involved in bloodmeal digestion is crucial in understanding their physiological role within the body louse. In this study, Aminopeptidase was characterized from Midgut of human body louse *Pediculus humanus humanus* which had been dissected 24 hrs after bloodmeal. In crude midgut homogenate , the aminopeptidase showed optimum activity at pH 8.0 (Alkaline) using $^{-V}_{V}$ *Leucine para nitoanilide* as the substrate. Body louse midgut aminopeptidase is fairly thermally stable, incubation at 50°C for 150 min resulted in 80% remaining activity.

Supernatants from midgut homogenized in 1% Tween 20 showed 2.5 fold increase in enzyme activity, while midguts homogenized in 1% Triton X-100 showed 8.0 fold increase in enzyme activity. Aminopeptidase in the midgut of body louse is stimulated by bloodmeal and increases gradually with time after feeding. Maximal release is realized 48 hrs after bloodmeal. The activity persisted even 96 hrs after bloodmeal. The pattern of feeding in both female adults and newly hatched nymphs remained fairly similar.

Kinetic studies showed that the enzyme exists in two forms of soluble and membrane bound. However it exhibited different catalytic activity with regard to V-max but had the same Km value. The enzyme activity was inhibited by and 1'10 Phenanthroline and $Mn^{2'}$. The enzyme activity in homogenate supernatant was restricted to one peak after partial purification using Gel Filtration Chromatography on Superose 6. Only one peak of 67 KDa - 69 KDa was observed after separation of homogenates extracted with or without Triton X-100. Fractions purified from Superose 6 revealed one band of 69 KDa on Native PAGE with homogenates extracted in saline buffer and two bands of 67 KDa and 69 KDa with homogenates extracted in 1% Triton X-100. In-gel staining of the enzyme activity revealed one band of Mr*70,000. These results indicate that Aminopeptidase is one of the major digestive enzyme in the body louse

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Lice (phthiraptera) are wingless, dorsoventrically flattened permanent ectoparasites of birds and mammals (Harwood & James, 1979).

The thoracic and abdominal ganglia are fused into a single ganglionic mass in the thorax. The oesophagus opens into a huge midgut dominated by a capacious ventriculus. A short narrow posterior section connects the ventriculus to the hind gut. The posterior is so short therefore the ventriculus functions both as storage and digestive organ.

Commonly there are two orders, the Anoplura or the sucking lice, and the Mallopflaga or the chewing lice.

Lice are permanent obligatory ectoparasites which live and feed on their host throughout their development. They feed frequently both day and night particularly when the host is quiet. Feeding normally occurs in the dark and the host body temperature influences the feeding. Females begin to lay eggs a day or two after maturity which is reached in 16 to 18 days after oviposition. Eggs are laid near the body and the incubation period lasts from 5 to 7 days. The eggs hatch into nymphs which are recognisable as small editions of the adult living and feeding in the same way. There are three nymphal stages. All nymphal stages feed on blood and maintain contact with the host by relatively simple responses. The egg to egg cycle averages about three weeks.

Adult Louse

Eggs

Human Body Louse *Pediculus humanus humanus* (magnification x 30)

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1.2 Literature Review

1.2.1 Sucking Lice (Anoplura)

Sucking lice (Anoplura) are host specific permanent, obligatory ectoparasites of man and animals. Kim and Ludwig (1978) listed 486 species and recognised 15 families of which 6 are of greater medical and veterinary importance.

1. Haematopidiae, consisting of one genus, the common *Haemtopinus.*

2. **Linognathidae, including two genera of veterinary importance,** *Linognathus* **and** *Solenopotes.*

3-4. Pediculidae and Pthiridae, consisting of *Pediculus* and *Pthirus,* respectively, and containing the lice that commonly infest man.

5-6 Polyplacidae and Hoplopluridae, including, among others the genera Polyplax and *Hoplopleura,* respectively rodent parasites that may be involved in the transfer from rodent to rodent of such human and animal pathogens as those of tulareia and murine typhus.

1.2.2 Anoplura Affecting Domestic Animals

Sucking lice of veterinary importance belong to one of the three genera, *Haematopinus, Linognathus* and *Solenoptes.*

Swine have one species *Haematopinus suis.* This is the largest species of the entire group measuring 5 or 6 mm in length. It is cosmopolitan in distribution.

Five species of Anoplura infest cattle.

1. Linognathus vituli, the long-nosed cattle louse, is characterised by its small size (about 2mm in length), its slender body, and its long snout.

*

7 ***Solenopotes capillatus,*** known as the little blue cattle louse in the USA and the tubercle-bearing louse in Australia, is smaller, 1.2-1.5 mm in length; it generally resembles *Linognathus vituli.*

3. ***Haematopinus eurysternus,*** the short nosed cattle louse, is generally considered economically the most important louse infesting domestic cattle, *Bos taurus*, is its normal host through which it has been introduced in all parts of the world particularly cold and temperate areas. It is found widely distributed over the body of the host.

4. *Haematopinus quardripertusus*, the cattle tail louse, is found in the long hair around the tail and sometimes around the eyes and in the ears. Its normal host is the zebu or the humped cattle, *Bos indicus,* but it also infests hybrids between this species and Bos *taurus.* It is predominantly tropical or sub-tropical in distribution.

5. *Haematopinus africanus* also infests cattle

N

6. Haematopinus tuberculatus, the buffalo louse is primarily a parasite of the water buffalo, *Bubalis bubalus.* It will infest domestic cattle, usually when in association with the water buffalo.

7. Horses mules and asses are frequently infested by the horse sucking louse, *Haematopinus asini.* It resembles the hog louse except that the head is relatively longer and more robust.

8. Sheep may be infested by the foot louse, *Linognathus pedalis.* This species occurs in USA, parts of South America, New Zealand, Australia, and South Africa. Two other species of *Linognathus* occur in sheep, *L. ovillus* and *L. africanus,* the three sometimes occur on the same animal though on different parts of its body (Meleney and Kim, 1974)

9. Goats may be infested by *Limgticithus stenoposis*

Amonsz the Anoplura that affect wild mammals is *Pediculis mjobergi* which parasitizes monkeys. Ronald and Wagner (1973) reported a fatal infestation of two spider monkeys *A teles geoffroyi* in a St Louis Missouri, zoo, death apparently resulted from anaemia. Men who cared for these monkeys also became infested. *P. mjobergi* may be merely a subspecies of *P. humanus.*

Other animals that may be infested by louse are the rodents. *Polyplax spinulosa* and *Polyplax serratta* infest rats.

1.2.3 Anoplura Affecting Man

Sucking lice that affect man are of the genus *Pediculus* and *Pthirus.*

l.The crab louse, *Pthirus pubis* also called the pubic louse is easily recognised by crab like"-appearance. It is 1.5-2.Omm long, nearly as broad as long, and greyish white. Its middle and hind legs are much stouter than those of the head and body louse. It infests the pubic regions particularly but also the armpits and more rarely the hairy parts of the body, such as the moustache, the beard and the eyelashes. The lice are .temdrkably stationery in their habits, often remaining attached for days at one point with mouth parts inserted into the skin. Continued defecation during this time results in accumulation of excrementous materials around the insect. The pruritus caused by their bites is very intense and discoloration of the skin often results if the infestation continues for sometime.

The female louse deposits her eggs on the coarser hair of the body where the parasites occur. The number of eggs per female is apparently quite small, usually not

more than 30. The life cycle requires not more than a month under normal conditions. Crab louse infestation known as pthiriasis is characteristic of human adults, children under the age of puberty not usually being affected. It is in a sense a venereal disease, often showing a marked increase in incidences in subcultures where sexual laxity prevails.

2 Head louse. The head louse, *Pediculus humanus capitis,* is grey in colour but tends to resemble the colour of the hair of the host. The male averages 2mm in length and the female 3mm. This form occurs on the head, about the ears and the occiput, but in heavy infestations it may establish itself on other hairy parts of the body. In severe infestations the hair may become matted with eggs, parasites, and exudate from the pustules that originate from the louse bite; a fungus may grow in the whole fetid mass, forming ^carapace like covering under which large numbers of lice may be found.

The number of eggs deposited by the female ranges between 50-150. These are glued to the hair and hatch in five to ten days. Development is very rapid. There are three moults and three weeks usually covers the entfre cycle from egg to egg. Lice are easily disseminated by slight physical contact stray hairs and similar means; therefore, slight infestations may occur even under the best sanitary conditions particularly among school children. As in the case with crab louse crowding under unsanitary conditions aids in development of massive infestations. Both the crab louse and the head louse are capable of serving as hosts in which the rickettsiae of typhus and spirochetes of relapsing fever can proliferate.

3. Body louse. The body louse, *Pediculus humanus humanus* is most common where clothing comes in contact with body rather continuously, for example on underwear, the fork of the trousers the armpits, the waistline, neck and shoulders. Usually it stays on the clothing and makes contact with the body while feeding; thus, it has a very suitable private environment, which its host has created for it. In heavy infestations some lice may remain on the body after all clothing has been removed.

The normal spread of lice is by the transfer from an infested to non-infested host. This is followed by a rapid build up in the population on the new host. Lice firmly attach their eggs to the hairs of their host or to the clothing of their host. A female body louse may lay 50 to 150 eggs in its life time. When eggs are laid near the body, the incubation period lasts from 5 to 7 days and may be longer at lower temperatures. Eggs do not hatch above 38° C or below 23° C. The eggs hatch into nymphs which are recognisable as small editions of the adult living and feeding in the same way. Maturity is reached in 16 to 18 days after oviposition. There are three nymphal stages, all nymphal stages feed on blood and maintain contact with the host by relatively simple responses. Females begin to lay eggs a day or two after maturity. The egg to egg cycle averages about three weeks.

1.3 Pediculosis.

The presence of lice in any part of the body is called Pediculosis. Louse bites may produce certain systematic disturbances such as general tiredness , irritability, depression and pessimism, and body rash. The intense discomfort that some persons **feel** when lice are biting may persist for several days. Typically a red papule will

develop at the site of each feeding puncture. The skin may "weep" and swellings may occur. In time, sensitisation may develop. The skin of persons who continually harbour lice becomes hardened and deeply pigmented.

The most characteristic symptoms of lice infestation is pruritus on the head. Continual scratching may lead to secondary eczema, impetigo-like lesions and enlarged posterior and auricular nodes. Impetigo Contagiosa, multiple abscesses of the scalp, eczema and secondary anaemia have been observed in some cases. In severe cases the scalp may be covered with epithelial debris and crusts so that the hair becomes matted. A child may become listless, pale and have a mild fever. The affected hair is often dull and without lustre (Rochese, 1946; Frazier, 1973; Epstein *et al.* 1977; Withowski, 1983; Alexander, 1984; Mumcouglu, 1991).

In livestock industry, infested animals are usually irritable, their hair matted and dull and their skin is damaged due to constant scratching . Lice infestation results in poor quality leather, weight loss, secondary infections and even death. In cattle, infestation my cause anaemia, loss of hair, extreme itching, intense self grooming and **hair**-ball accumulation which causes obstruction in the digestive tract (Steelman, 1976)

1.4 Louse-Borne Diseases.

The human body louse is a vector of louse borne diseases e.g typhus fever, trench fever and relapsing fever (Harwood & James, 1979)

1. Louse borne typhus: This disease is of ancient origin and wide distribution, chiefly in Europe, Africa, Asia, and higher altitudes of Mexico Central

and South America. The causative organism is the bacteria *Rickettsia prowazeki.* Whenever humans are concentrated in close quarters, and whenever excessive poverty or mass migration prevail, especially in time of war and famine, the disease may become rampant. Currently this occurs in refugee camps and among street dwellers within congested urban areas.

The disease is characterised by a high fever continuing about 2 weeks , backache, intense headache bronchial disturbances, mental confusion, stupor, a congested face and on the fifth or sixth day, by a red macular eruption on chest and abdomen , later spreading to other parts of the body, even to hands feet and face.

Pediculus humanus humanus is probably the sole agent in the transmission of the typhus organism from man to man although both the crab louse and the head louse can serve as host in which it can multiply. The louse acquires the parasite by way of blood meal . The rickettsia multiplies enormously in the epithelial cells of the midgut of the louse. These cells become distended after a few days and they rupture, releasing enormous numbers of rickettsiae into the lumen of the digestive tract which then appear in the louse faeces. The rickettsiae are pathogenic to both the louse and the human host. The infected lice die in 8 to 12 days because of damage to the gut epithelial cells. If a louse survives the infection, it remains infective for life.

The usual route by which man becomes infected is through faecal contamination, though it may be brought about by crushed contents of the louse. The bite of the louse is not directly involved because the rickettsia do not occur in the salivary glands. The parasite may remain alive and virulent in the louse's faeces at room temperature for more than 60 days; thus infection may be acquired through

respiratory passages by inhalation of minute particles of louse excrement.

2 Trench fever: This is a non-fatal disease characterised in overt cases by sudden onset of fever headache, dizziness, pain in the muscles and bones particularly in the legs with special tenderness of the shin lasting 24 to 48 hours followed at intervals of about 5 days by other attacks of fever of diminishing severity. The disease is wide spread even though in asymptomatic form, in louse infested areas of Europe, Africa, Asia and the highlands of Mexico and Central and South America.

The causative organism is the bacteria *Rochalimea quintana.* Unlike ***Rickettesia*** *prowze/ci,* this organism multiplies freely in the lumen of the digestive tract and not in the epithelial cells and it is not pathogenic to the louse. An infected louse remains infective for life. As in the case of louse borne typhus, the louse acquires the rickettsia in its blood meal and passes it on to man through the faeces or through the body contents of a crushed louse.

3. Relapsing fever: This is another of the three important diseases associated wiih lice. It has occurred in many parts of the world.

The causative organism is the bacteria *Borriela recurrentis.* The insect can acquire the pathogen by a single feeding on an infected person but cannot pass it on in the same way. Man acquires the parasite by crushing the louse, usually in the act of scratching to alleviate the irritation caused by the bite, and in this way releases the spirochetes, which then enter the excoriated skin or mucous membrane. After being ingested by the louse, the spirochetes pass through the stomach wall into the haemolymph. The digestive tract of the insect seems to be a hostile environment, but the ability of a sufficient number of spirochetes to survive there and pass on to the

haemolymph where they can multiply without being affected themselves and without damaging the host, indicates a highly successful adaptation of the parasite to its host. **Once** infected, louse remains so for life. Spirochetes are not found in the faeces. Consequently , transovarian transmission and transmission by faeces are not possible. Transmission from man to man is through transfer of lice.

The incubation period in the human is 3 to 10 days. The onset of the disease is sudden with headaches, chills, and fever and generalized pain. The fever remains high for several days and subsides abruptly followed by one or more relapses.

1.5 Bloodmeal Digestion

Blood-sucking insects are unusual in that their salivary secretions contain virtually no digestive enzymes. This is probably related to the need for the feeding insect to cause the least possible disturbance to the host. As a consequence digestive enzymes are produced in the midgut. "Batch digesters" such as the mosquitoes have few stored digestive enzymes in the unfed gut. The blood meal stimulates the gut epithelium into a synthetic round and digestive enzymes begin to appear in quantity a few hours after blood meal (Clements, 1992). The delay between the feeding and the beginning of digestion is likely to be prolonged by the range of protease inhibitors in the vertebrate serum which will bind and inactivate the first of the insect proteases to be produced. "Conveyor belt" digesters such as *Stomoxys calcitrans* can get digestion underway more rapidly. This insect has a store of digestive enzymes in the unfed gut. Allied to this most of the blood meal is stored in the non-digestive anterior region of the gut and is passed back in a limited stream through the digestive regions thus only

exposing the digestive enzymes to relatively few inhibitors at a time.

Proteins are far and away **the most** abundant **nutrients** in **the blood meal.** So, noi surprisingly ,the major digestive enzymes in the blood sucking insects are proteases. There have been a number of investigations on the digestive enzymes of haematophagus insects and they were reviewed excellently by Gooding (1972, 1975). Also there after a good many works on the presence and properties of enzymes from some haematophagus insects have been reported. The midgut (tissue and the lumen combined) of *Glossina morsitans morsitans* contains at least six proteolytic enzymes (Gooding & Rolseth, 1976). The combined action of all the proteolytic enzymes that occur within the midgut lumen could easily account for the complete hydrolysis of the proteins in the bloodmeal. Aminopeptidase is present within the posterior midgut cells and seems to have a digestive function when peptides resulting from protein digestion are absorbed from the midgut lumen and pass into the epithelia cells (Gooding, 1977b). In the gut of the horn fly *Haemotobia irritans* and in the stable fly *Stomoxys caicitrans* at least four proteolytic enzymes have been reported (Hori *et al.,* 1981). As in tsetse fly aminopeptidase is found in the epithelium of the posterior midgut but not in the lumen. In *Anopheles stephensi* the primary hydrolytic proteases i.e trypsin and chymotrypsin are insufficient for complete digestion of the blood meal however aminopeptidase plays an important role in secondary digestion of blood meal proteins (Billingsley, 1990). In *Rhodnius prolixus* there are at least four proteolytic enzymes that comprise part of an enzyme spectrum that is essential for digestion of blood meal (Houseman & Downe , 1983; Billingsley & Downe, 1985,1988). For haematophagus insects, two main hypothesis have been presented to account for the mechanism by

which the levels of proteases within the midgut are regulated after blood meal: a hormonal mechanism and a secretogogue mechanism (Gooding, 1975) At present, the secretogogue mechanisms appear to account for best regulation in *Aedes aegypti* (Briegel & Lea 1975), mosquitoes (Gooding, 1973) *Rhodnius prolixus* (Garcia & Garcia, 1977), *Glossina morsitans morsitans* (Gooding 1974a, 1977a), *Stomoxys calcitrans* (Lehane ,!977; Spates, 1979) and *Haematobia irritans* (Hori & Kuramachi, 1982) The profile of proteases in the body louse midgut have not been studied in detail and only trace amounts to trypsin have been reported by Borovsky and Schlein (1988). There are advantages in targeting digestive enzymes for vaccine production. Vaccine production is an expensive and time consuming business and, of course, there is always the chance that at the end of the day the effect on the insect may not be that required. Targeting of digestive enzymes can take away some of this uncertainty. There are a wide range of enzyme inhibitors available which could be used to mimic the effects of a successful vaccine. Effects on insects such as fecundity and longevity can be measured and used to help develop models predicting the likely impact of successful vaccine. Such preliminary work would build confidence prior to the long haul required to produce a successful vaccine.

A significant amount of work of this sort has been carried out in herbivorous pest insects where there is much interest in the possibility of genetically engineering their host plants to express potent trypsin inhibitors (e.g. Christeller *et al.,* 1992). Similar work on blood sucking insects would lay a useful foundation. (Spates, 1979) administered soybean trypsin inhibitor at the high rate of 3mg per ml with whole blood and found that this inhibited the egg production in *Stomoxys calcitrans* by 71%.

Another advantage of targeting proteases is that they are one of the most **thoroughly** studied and understood groups of molecules. Unfortunately little of this knowledge has been gained in insects, but nevertheless the general knowledge of these molecules would be a head start on the trail to produce a vaccine (Lehane,1994)

1.6 Economic Significance.

Sucking lice (Anoplura) are a major problem in both human and veterinary health world wide, both as debilitating agent and as vectors of disease. The number of cases of head and body louse infestations throughout the world was estimated to run into hundreds of millions (Taplin and Meinkin, 1987). Infestation has increased world wide since the middle sixties (Gratz, 1977a). Six million people were infested in the USA in 1975(Anon, 197£). A substantial rise was also noted in Israel (Ramussen, 1984).

Sucking lice cause irritation to man and domestic animals resulting in considerable economic losses (Steelman, 1976; Alexander, 1984). Infested animals are not only irritable but their hair is matted and dull and their skin colour is damaged due to constant scratching. Lice infestation results in poor quality leather, weight loss, secondary infections and even death. In cattle, infestation may cause anaemia, loss of hair, extreme itching, intense self grooming and hair ball accumulation which causes obstruction in the digestive tract. Significant losses in heifers may be caused by moderate to heavy infestations by cattle lice. The hog louse *Haematopinus suis* next to hog cholera is considered the worst enemy of swine (Steelman, 1976).

The human body louse *Pediculus humanus* is a vector of louse-borne typhus

(Rickettsia prowazeki), trench **fever** *(Rochalimea quintana)* and louse-borne relapsing fever (*Borriela reccurentis).* Typhus is especially considered endemic in all continents except **Australia.** In USA losses to **livestock** in 1965 due to lice infestation was estimated to **be** 100 million **dollars** (Steelman, 1976).

Skin irritation caused by lice infestation stimulates grooming and this grooming enhances lice removal but also causes excessive rubbing leading to hair losses and secondary infections. Host parasitic interaction between the mouse and its louse Polyplax serrata has been extensively studied. Grooming was very effective in removal of lice infestation (Bell *et al.,* 1962) and lice exhibited resistance to rats even when grooming was limited (Bell *et al.,* 1966)

Animal pathogens may be transmitted by lice for example *Rickettsia typhi* causing murine-typhus and is transmitted among rodents by the rat louse *Polyplax spinulosa* (Steelman, 1976).

1.7 Method of Control

1.7.1 Chemical Control

Currently lice are controlled by treating infested humans and animals with topically applied insecticides (Pediculocides). These are

in the form of powder, lotion shampoo, gel and spray formulations. Pediculocides containing active ingredients DDT, lindane, pyrethrin, pyrethroids, nialathion and carbaryls are being widely used. Most of the formulations have limited ovicidal activity and need to be applied more than once in order to destroy the entire

population. Side effects such as contact dermatitis, prurigo and erythema are common **after** treatment with pediculocides. In animals lice may be controlled by pour-on spraying or bathing infested animals with organophosphates, chlorinated hydrocarbons, carbamates and pyrethroids or subcutaneous injection of ivermectin. Some anti-lice preparations have caused toxic side effects in animals and care must be **taken** to avoid residual insecticides in meat and milk products.

Resistance of head lice to organochlorines such as DDT was described by Maunder (1971). Body lice strains have been found to be resistant to DDT, malathion (Graatz, 1977b). Cross resistance has been demonstrated between lindane and cyclodiene compounds such as dieldrin (Busvine,1967). In Israel malathion carbaryl, pyretnrin or bioallethrin are used as active ingredients in about 15 formulations. Clinical trials have shown that some of them are not effective (Armoni *et al.*, 1988).

The emergence of insecticide-resistant louse strains, the growing government controls limiting the use of pesticides, widening awareness in the community and the harmful consequences to the environment and the hazards of pesticide residues to farm products have stimulated research into the development of alternative control measures (Elvin and Kemp, 1994)

1.7.2 Bio-Control.

The use of bacteria and fungi as bio-control agents of insects has attracted great interest in recent years. *Bacillus thuringiensis* serotype H-14 has been found to be toxic to the larval stages of mosquitoes and black flies and field trials have proved to be successful (Goldberg et al., 1977;). *Bacterium mathini* and *Bacillus sphaericus*

both isolated in from *G. morsitans* were found to be lethal to the adult tsetse following ingestion. However, biological control such as use of bacteria or fungus would not be feasible for the control of body louse as these ectoparasites permanently feed on their host, and this would mean exposing the host to secondary infections.

1.7.3 Immunological **Control.**

A degree of protection against blood-feeding ectoparasites such as ticks and mosquitoes has been achieved by immunizing hosts with antigenic material from the parasite itself. Ectoparasites which had been fed on immunized hosts took smaller bloodmeals, exhibited decreased fecundity and increased mortality (Wikel, 1982; Licliman & Gossing, 1988; Ramasamey *et al,* 1988; Wikel, 1988; Kemp *et al,* 1989; Willadsen & Mckenna, 1991; Ben-Yakir *et al,* 1994).

In some case a host may acquire resistance to ectoparasites after natural exposure. This is usually due to cutaneous hypersensitivity reaction to antigen in parasite saliva. Recent reports indicate that naturally acquired and induced immunity against blood feeding arthropods acted at least in part-by causing direct damage to the midgut tissue (Walker and Fletcher, 1987; Kemp *et al.,* 1989). The damage to the gut caused leakage of the bloodmeal components to the haemocel. This leakage may have enhanced immunological damage to internal organs (Chinzei and Minoura, 1988) or may have caused the death of the parasite (Kemp *et al.,* 1986; Ben-Yakir and Mumcouglu, 1988). Histological studies showed that the gut cells were destroyed, their proliferation inhibited and digestion was blocked in the parasites that fed on resistant hosts (Walker and Fletcher, 1987; Willadsen *et al,* 1988,1989). It should be

oted that the damage to the gut surface can also affect the vectoring capacity of blood feeding ectoparasites. The inner membrane of the gut is the site of recognition, attachment and penetration of vector-borne pathogens e.g. Plasmodia, Trypanosoma and Leishmania. Both naturally acquired and induced resistance have been shown to interfere with pathogen transmission by arthropod vectors (Wikel, 1982) thus making it an attractive site for immune mediated damage. There are other features which make the gut of blood feeding arthropods an attractive target for immune mediated damage. Fresh whole bloodmeal mixed with salivary anti-coagulants is taken into the gut, cellular components and large proteins e.g. antibodies are usually concentrated further in the gut by filtration and excretion of excess fluid. Blood feeding arthropods take large bloodmeal and as a result digestion is slow. Potent antibodies can be found in the sut lumen several days after feeding (Hatfield, 1988; Lackie and Gavin, 1989).

Vaccine induced immunity to ticks acts, at least in part, by causing a direct damage to the parasite's gut (Agbede & Kemp; 1986 Kemp *et al,* 1989). This damage causes leakage of blood components into the haemocel and may lead to death. Naturally-induced resistance to *Rhipicephalus appercdiculutus* ticks is caused partly by failure of the gut to grow to full functional capacity (Walker & Fletcher, 1987).

Induced resistance to lice was observed in mice infected with *Polyplax serratta* (Ratzlaff *et al,* 1982). and in rats infested with *Polyplax spinulosa* (Volf & Grubhoffer,1991).

A high degree of resistance to lice was induced by immunizing rabbits with an extract of p. *humanus* midgut (Ben-Yakir & Mumcouglu, 1988; Ben-Yakir *et al,* 1994)). Lice fed on immunized rabbits took smaller bloodmeals, had higher

mortality, layed fewer eggs and took longer to develop, compared with lice fed on control animals (Ben Yakir *et al.*, 1994). The main cause of death was leakage of the bloodmeal into the louse haemocel due to gut rapture by antibodies directed against gut proteins. The louse resistance persisted for several months post immunisation along with persistent high anti-louse antibody (IgG) titres in the serum.

Polyclonal antibodies raised against a whole extract of rat louse recognised at least eleven antigenic components by immunoblotting technique (Volf & Grubhoffer, 1991), whereas in sera raised against body lice upto nine immunogenic antigens were observed (Ben-Yakir & Mumcouglu,1989; Ochanda *et al*1995).

The successful immunisation of mice against *P.serrata* and the positive results on preliminary experiments on the immunisation against *P.h.humanus* indicate that immune protection against body louse has a high chance of success.

%

The idea that one day blood sucking arthropods may be controlled by vaccinating host animals is an appealing one. An ideal approach is therefore to target the appropriate molecule within the gut, one which is both accessible to antibody and is critical to the successful processing of the bloodmeal (Lehane, 1994).

Proteins are the most abundant nutrients of the bloodmeal. Hence the major digestive enzymes in blood sucking insects are protease. Blood feeding insects utilise a range of peptidases, glycosidases and other enzymes to process the bloodmeal (Billingsley, 1994). In the horn fly gut, significantly high proteinase activity have been reported. The proteinase were also found to be pH sensitive (Hori *et al.,* 1981). In tsetse fly *Glossina morsitans morsitans,* the presence of six alkaline proteases have been demonstrated. These include aminopeptidase; carboxypeptidase A;

carboxypeptidase B; trypsin; protease IV (trypsin-like); and protease VII (chymotrypsin-like). The combined activity of these proteases is entirely sufficient to account for complete hydrolysis of blood proteins (Gooding & Rolseth, 1976).

Even though stimulation, release and activity of digestive enzymes are both predictable and relatively easy to manipulate, their potential as target sites for insect control has received very little attention (Hon *et al.,* 1981). However several new approaches to vector control which might target their exploitation have emerged (Crampton, 1994). Targeting proteases for vaccine development require their thorough study and understanding of the group molecules involved.

1.8 JUSTIFICATION AND RATIONALE

Sucking lice are (Anoplura) permanent, obligatory ectoparasites of man and animals. They are very host specific and feed exclusively on blood. Man is infested with the head louse (*Pediculus humanus capitis*), the body louse *{Pediculus humanus humanus)* and the pubic louse (*Phthirius pubis*) which have a cosmopolitan distribution.

The number of cases of head, body and pubic lice infestation in man throughout the world is estimated as being hundreds of millions (Taplin & Meinkin, 1987). Infestation by head lice has increased world-wide since the middle of the sixties (Graatz, 1977a). Today, head lice infestation is more prevalent among children than all other childhood communicable diseases combined (Anonymous, 1986). About 6-12 million people, mainly children, are affected annually with lice in the USA (Atkinson *et al*, 1986). IN Israel, the past **20-25** years, **15-25%** of all children

between 4-13 years are infested with head lice, another 20-30% showed signs of previous infestation (Tamir et al., 1984; Mumcouglu *et al*, 1990. Head lice infestation are also very common in Kenya (Chunge, 1986).

The most important foci of louse infestation are in Africa with most of the cases being reported from Burundi, Rwanda and Ethiopia. Louse-borne relapsing fever is reported principally from Ethiopia and the Sudan (Tarizzo, 1973). The two countries have common borders with Kenya. In 1974 an epidemic of relapsing fever was reported from these areas with several thousand cases occurring in the Sudan a^d several hundred in Ethiopia (Sholdt *et al,* 1979). ,U,VFRS/TY *of* * ~ GHIR°M0 1

Resistance of head lice to organochlorines such as DDT and lindane was described by Maunder (1971). Recently it was reported that strains of head lice in Israel have also become r^istant to permethrin (Mumcouglu *et al.,* 1995)

There is therefore an urgent need for the development of new methods for the control of ectoparasites, because of the emergence of strains resistant to pesticides and restrictions in the use of chemical pesticides due to laws governing health and environmental protection. In addition most insecticides used today are effective for a short period of time and very few of them penetrate the egg shell and kill the embryo.

The immunisation of hosts with antigenic material from blood-feeding arthropods is a method for the control of ectoparasites which started approximately 60 years ago and which recently gained much interest due to advances in the in-vitro synthesis of animal proteins by genetic engineering.

Since the midgut of body louse contains several immunogenic proteins that can be targeted as candidate immunogens, there is therefore need to further

characterise these proteins and determine their physiological functions in body louse. In this thesis, body louse midgut aminopeptidase enzyme was characterised and its properties discussed.

1.9 AIMS AIND OBJECTIVES

1.9.1 Overall Aim.

The overall aim of this study is to characterise digestive enzymes within body louse midgut.

1.9.2 Specific Objectives

1) To determine the aminopeptidase activity in the body louse midgut

2) To analyse the effect of blood meal on aminopeptidase activity

3) To purify the body louse midgut aminopeptidase enzyme

4) To analyse the enzymatic properties of the body louse midgut aminopeptidase enzyme.

CHAPTER 2

MATERIALS AND METHODS

2.1.1 Reagents.

The laboratory chemicals used in the experiments were of analytical grade obtained from Serva, Sigma, Pierce, Pharmcia-LKB and Bio-Rad.

2.1.2 Materials and Equipment.

The following materials and equipment were used in the following experiments Adhesive tape, Cotton wool, Cloth (Soft carpet material), Dissecting kit, FPLC Homogenizer, Incubator, Lice holder, Microscope, Petri-dishes, Rabbit shaver Freezer, Water bath, uv/visible Spectrophotometer.

2.2 Experimental Insects and Animals.

A strain of the human body louse *Pediculus humanus humanus,* which had been adapted to feed on rabbits were obtained from Professor Rachel Galun's laboratory at Kuvin Centre Department of Parasitology Hebrew University-Hadassah Medical School, Jerusalem, Israel. The lice were fed every other day on shaved abdomen of rabbits which had been sterilized with 70% alcohol. When not feeding, iice were kept in an incubator maintained at 30 ±1°C and 70 ±5 % humidity. Under these conditions, egg incubation takes 8-9 days, each of the three nymphal instar stages lasts 4-5 days to molt into the next stage of development, and females live for about 30 days laying

two to three eggs per day after fertilization. The rabbits that were **used** were New Zealand white rabbits reared in the Department of Zoology at the

University of Nairobi.

2 3 Preparation of Midgut Homogenates.

Lice were immobilized on adhesive tape and midguts were dissected in a drop of Saline at 4° C under a microscope. Midguts were washed and rinsed several times in Saline and after removal of all non midgut tissues, stored in eppendorf tubes at -20°C with no significant loss in Leucine Aminopeptidase (LAP) enzyme activity for several months.

For enzyme assays, 1000 midguts were pooled and homogenized as follows

(i) Midguts were homogenized in cold 0.5ml of Saline in a glass/glass homogenizer and then centrifuged at 10,000g for 10 min at 4°C.

(ii) Residue from (i) above was re-homogenized in cold 0.5ml of Saline containing 1% triton X-100 in a glass/glass homogenizer and then centrifuged at 10,000g for 10 min at 4°C.

(iii) Midguts were homogenized in cold 0.5ml of Saline containing 1% triton X-100 in a glass/glass homogenizer and then centrifuged at 10,000g for 10 min at 4°C.

2.3.1 Preparation of Midgut Homogenates for Latency Studies.

Batches of 200 midguts were homogenized in three different buffers as shown below:

a) Saline

b) Saline containing 0.1% Tween 20

c) Saline containing 1% triton X-100

The solutions were centrifuged at 10,000g for 10 min at 4°C and then made up to 400|il using the appropriate buffer then 25(j.l of the homogenate (6.9, 14.7 and 32 *xg* of protein respectively) was used in the enzyme assay (Ferreira *et al.* 1988)

2.4 Aminopeptidase Assay.

For the assay of LAP activity, 25 |il of 0.16M LpNA [Sigma chemical Co, St Louis., MO], in Di-methyl formamide [DMF]) was added to 950 ul of 50mM phosphate buffer pH 8.0 at 30° C. The reaction mixture was equilibrated at 30 ° C for 10-15 min in a water bath. Midgut homogenate (15jil about 3.75 \ig of protein) was added and the reaction was run for 30 min at 30°C and stopped by the addition of 0.3 ml of 30% acetic acid (Billingsley, 1990). The absorbance at 405nm was determined at 2 min interval for 30 min using Beckman model DU 50 spectrophotometer fitted with a thermostat control. The enzyme activity was calculated by using an extinction coefficient of 8,800 mM cm-1 (Erlanger *et al.,* 1961) and the unit of activity was defined as nmoles/min/mg protein.

All assays were run in triplicate, with controls containing substrate and buffer before incubation period (to allow spontaneous breakdown of substrate) and the homogenate added after the acetic acid (to allow for absorbence inherent in the sample).

2.4.1 Optimum pH.

The optimal pH for aminopeptidase activity was determined using buffers with PH ranging from pH 4.0 to pH 9.0 of 0.1M citrate buffer pH 4.0 to 6.5, 0.1M

Phosphate buffer pH 6.0 to pH 8.0 and 0.1M Tris HC1 buffer pH 7.5 to 9.0 substituted **in the** standard assay as described in section 2.4. (Billingsley, 1990).

2.4.2 Thermal **Stability.**

Body louse midgut homogenates supernatant aliquots were incubated at 50°C **in** a waterbath and the assay carried out as described in section 2.4. at 30 min intervals from 30 min to 150 min.(Klinkowstrom *et al.*, 1994). The data obtained was used to determine the thermal stability.

2.5 Induction of Aminopeptidase Activity by Bloodmeal

Freshly hatched nymphs were fed on rabbit blood. Whole nymphs were homogenized at various tinje intervals following a bloodmeal. Hydrolytic activity of aminopeptidase against LpNA was then determined as described in section 2.4. For each time interval about 100 nymphs were used.

Similarly, freshly moulted female adults from third nymphal instar were homogenized at various intervals after bloodmeal and fhe enzyme activity determined, for each time interval, 10 female adult lice were used.

Lice whose midguts had been removed were homogenized in both Saline and buffer containing 1% triton X-100 and then assayed for aminopeptidase activity.

Rabbit serum was also assayed for aminopeptidase as described in section 2.4.

[2]-6 Kinetic Studies.

Aminopeptidase assay was carried out as described in section 2.4 for both

'dsut homosenate prepared in Saline and midgut homogenate prepared in the resence of 1% Triton X-100, at various substrate concentrations ranging from 0 ?$_n$iM- 4 OmM (Billingsley, 1990). In each assay fixed enzyme concentration i.e 15ul (3 75 jig of protein) of homogenates was used.

2.6.1 Inhibition Studies

The following various inhibition studies were carried out

i) The effect of I'lO Phenathroline.

riO Phenathroline was pre-incubated at various concentrations with midgut homogenate at 30° C for 10-15 min, and the assay was started by the subsequent addition of the substrate. Aminopeptidase assay was carried out as described in section 2.4. with **15 JIll** (3.75 jig of protein) of midgut homogenate supernatant.

a) Homogenate was incubated with various concentrations of l'lO Phenanthroline ranging from 0.2mM - l.OmM for 5 min and the reaction initiated by the addition of 1,66mM of the substrate.

b) The effect of l'lO Phenanthroline was also checked in the presence of various concentrations of the substrate ranging from 0.2mM - 1.66mM.

ii) Effect of Mn^{2+}ions

Aminopeptidase assay was carried out as described in section 2.4 with constant substrate concentration (1.66mM), constant enzyme concentration (15|j.l (3.75 jig of protein) of homogenate) at various concentrations of manganese ions (Mn^{2}) ranging from 2mM -20mM.

2.7 Protein Assay.

Protein estimation was carried out by the Pierce Bicinchoninic acid BCA protein method (Pierce Rockfordjll., U.S.A.).

BCA reagents A & B were mixed in the ratio 50:1 respectively. Samples made upto 1 OOjul with water was then added to 2mls of the reagent mixture. For the blank 100f.il of distilled was added. The final mixture was then incubated at 37°C for 30 min and the optical density was read at 562nm using a spectophotometer. Bovine serum albumin (BSA fraction V) was used as the protein standard.

2.8 Partial Purification and Molecular Weight Determination.

For molecular weight determination midguts were homogenized as described in 2.3.1,-%The supernatant ^/as then filtered through a 0.45jim millipore filter before being used for partial purification through a superose 6/12 columns (Pharmacia, Basel, Switzerland) Gel filtration of aminopeptidase. Superose 6/12 column was equilibrated with 50mM phosphate buffer pH 8.0 at 25°C. An aliquot of 200^1 (150 Hg of protein) of sample prepared as described in Section 2.3 was applied to the column and 2ml fractions were collected at a flow rate of lml/min. The protein content of the fractions was monitored by recording the absorbance at 280nm. An aliquot of 100j.il was taken from each of the 2ml fractions and assayed for hydrolytic activity against LpNA as described in 2.4. The fraction that exhibited activity against LpNA were pooled and reapplied to the Superose column under the same conditions. The column was calibrated under identical conditions by using Blue Dextran (Mr=2x 10^6),Thyroglobulin (669 KDa), Bovine gamma globulin (158 KDa), Chicken

ovalbumin (44 KDa), Equine myoglobin (17 KDa), Vitamin B12 (1.3 KDa).

2.9 Polyacrylamide Gel Electrophoresis.

Native polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Gradient gels (3-20%) were cast using gradient marker (BRL) and topped by 3.13% stacking gel.

The running gel was prepared as follows;

Volumes	3% Separating gel	20% Separating gel
Monomer (ml)	1.8	12.0
Buffer (ml)	4.6	4.6
D H_20 (ml)	11.4	1.2
Temed'(iil)	- 6.0	6.0
10%APS (|il)	160.0	90.0

The gel solutions were mixed as above omitting the TEMED and APS. TEMED and APS was then added to the separating gel solution, swirled to mix and poured between the glass plates to within 1 cm of the notch. The gel solution was overlaid with 1-2 ml of butanol saturated with water. The mixture was allowed to polymerize for 45 min at room temperature. Butanol saturated with water overlay was removed and the top of the gel rinsed with lxstacking buffer. 3.13% of stacking gel, 6.1 ml distilled water, 5 ml buffer, 1.02 ml monomer, 0.01 ml TEMED and 0.05 ml APS) was poured to within 2-3 mm of the glass plate. After the gel had polymerized , the top was rinsed with the electrophoresis tank buffer (25mM Tris ,192mM Glycine,

pH 8.3)

Samples were then dissolved in equal volumes of sample buffer (130mM Tris-HCl 20% glycerol, 0.002% bromophenol blue, 1% B-mercaptoethanol, pH(6.8) . An aliquot of 50 [x\ (about 1.25 jig of protein) of the preparation was then loaded on the gel. Electrophoresis was performed at room temperature (25°C) at a constant current of 25mA through the stacking gel until the sample had separated through the stacking gel, then the current was increased 30mA to enable the sample to separate through the separating gel until the bromophenol dye marker reached a few millimetres from the bottom of the gel.

Gels were removed rinsed with water before being stained with Coomassie Brilliant Blue (Weber and Osborn, 1969) in a solution of acetic acid methanol and distilled water (9.2:50:40.8) overnight. The gels were treated with several changes of destaining solution (acetic acid:methanol:distilled water (9.2:50:40.8) respectively for several hours at room temperature (25°C). Destained gels were stored in 7% acetic acid until photographed.

Native-PAGE was used to determine the molecular weight of the unknown sample proteins. The following protein standards from Bio-Rad (U.S.A) were used.

The standards used were: Thyroglobulin (669 KDa), Ferratin (440 KDa), Catalase (232 KDa),Lactase Dehydrogenase (140 KDa), BSA (67 KDa). After staining the gels and destaining, the molecular weights were determined from plots of log molecular weight versus relative migration of the protein standard.

2.10 Agarose Gel Electrophoresis

2% Agarose was prepared in 5mJVl phosphate buffer pH 7.0. Samples were dissolved in equal volumes of sample buffer (bromophenol blue and glycerol) and an aliquot of the preparation (40jal (1.0 [ig of protein) was then loaded on the gel. The running buffer was 40mM phosphate buffer pH 7.0. Electrophoresis was performed at room temperature at a constant voltage (50 volts). The electrophoresis was allowed to run until the blue dye marker was a few mm from the bottom of the gel. Gels were removed and rinsed with water before staining. Two sets of gels were run under the same conditions and one was stained with Coomassie blue while the other was stained for LAP activity.

The following molecular weight standards were used to determine molecular weight. Alcohol Dehydrogenase (150 KDa), BSA (66 KDa), Carbonic anhydrase (29 KDa), Cytochrome C (12.4 KDa).

2.11 Staining for LAP activity

The staining solution was prepared in 100ml 0.1M phosphate buffer pH 8.0 containing lOOmg LpNA- Leucine para Nitro anilide, 40 mg L-aminoxidase, 20 mg Horse-raddish peroxidase and lOOmg 3 ethyl 9 methyl carbazole . Gels were immersed in the solution and left to stand overnight. After staining and destaining, gels were then photographed and the molecular weights were determined from log of molecular weight versus relative mobility.

CHAPTER 3

RESULTS

3.1 Optimum pH and Thermal Stability.

Midgut aminopeptidase activity against LpNA was determined from pH 4.0 to 9.0 using 0.1M citrate buffer, 0.1M phosphate buffer and 0.1M Tris-HCL buffer. Maximum activity occurred at pH 8.0 with both phosphate and Tris-HCl buffers. (Fig.l).

Body louse midgut aminopeptidase enzyme was fairly stable after incubation at 50°C for 150 min. The remaining enzyme activity was about 80% after the incubation duration. (Fig.2)

v

3.2 Latency Studies.

Homogenization of body louse midguts in saline resulted in an activity of 9.3 ± 0.2jiM/min/mg protein. However when midguts were homogenized in the presence of 0.1% Tween 20 the resulting activity was 13.4 ±0.4|aM/min/mg protein which reflects approximately 1.5 fold increase in activity. When midguts were homogenized in the presence of 1% triton X-100, the resulting activity was 51 ± 5nM/min/mg protein, which reflects approximately 5.5 fold increase in activity compared to homogenization in saline. (Fig.3) & (Tablel).

Fig 1. Aminopeptidase activity in relation to pH sensitivity

pH 3.0-6.5 = 0.1M Citrate Buffer

pH 6.5-8.0 = (TIM Phosphate Buffer

pH 8.0-9.0 = 0.1M Tris-HCl Buffer

Aminopeptidase activity was carried out as in section 2.4 substituting the buffi accordingly.

Data represents the average values of three determinations.


0.10-
0.08-
0.06-
0.QA-
0.02-
0

Fig 2. Thermal inactivation (50°C) of LAP from Body louse midguts

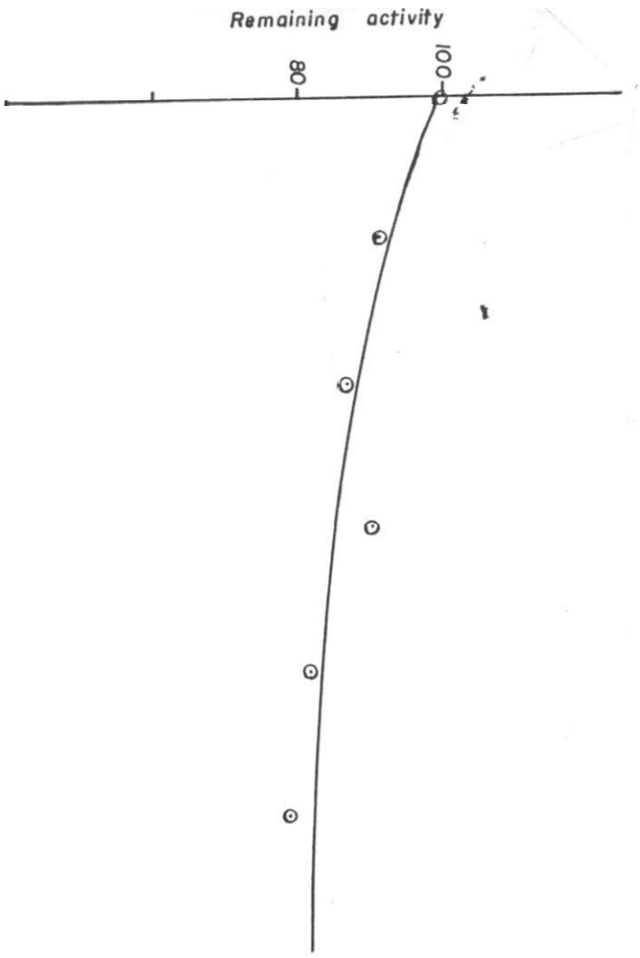

Remaining activity
80
100

Fig 3. Demonstration of membrane-associated LAP activity

A- A Midguts homogenized in Saline

•—• Midguts homogenized in 1% Tween 20

• —• Midguts homogenized in 1% triton X-100

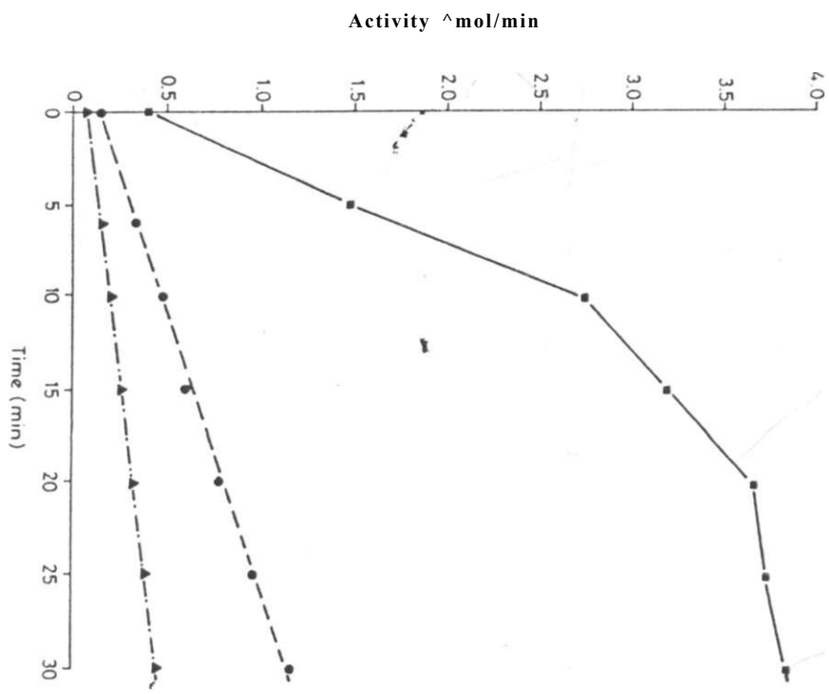

Activity ^mol/min
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Time (min)
0
5
10
15
20
25
30

Table 1. Effect of Homogenisation in Detergent on LAP activity

Homogenization Buffer	Enzyme Activity in umoles/min/ml	Protein concentration in mg/ml	Specific Activity uinoles/min/mg	Increase in Activity
Saline	2.55 ±0.05	0.28	9.3 ±0.2	-
1% Tween 20	6.75 ±0.35	0.5	1 13.5 ±0.4	1.5
1% Triton X-100	53.7 ±5	1.1	51 ±3	5.5

3.3 Stimulation of Aminopeptidase Activity by Bloodmeal.

Body louse was found to have some residual LAP activity before bloodmeal. After a bloodmeal, the aminopeptidase activity in the body louse increased gradually with time. Maximum activity was realized 48 hrs after feeding. Thereafter the activity decreases. By 72 hrs aminopeptidase activity had reached pre feeding levels in adult lice but not in nymphs i.e after 72 hrs activity comes down rapidly in adult lice but gradually in nymphs. Nevertheless, the pattern of activity of aminopeptidase in both female adults and newly hatched nymphs remained fairly the same. (Fig.4A & 4B). Rabbit serum was found to have aminopeptidase activity of about (1%) 0.01 JJI mols/min/ml compared to body louse midgut aminopeptidase which exhibited an activity of 1.36 p. mols/min/ml . Lice whose guts had been removed were found to have aminopeptidase of,.about (7%) 0.09 \yl mols/min/ml compared to midgut aminopeptidase.

3.4 Kinetic Studies.

With fixed enzyme concentration and increasing substrate concentration, a typical Michaelis Menten curve (sigmoid/hyperbola) was obtained. Thus an increase in substrate concentration resulted in a very rapid rise in velocity of reaction in both soluble and detergent soluble enzyme. However, the activity of LAP in the latter was much higher than the former.(Fig.5A). The Lineweaver Burk plot of 1/V vs 1/[S] showed that soluble enzyme and detergent soluble enzyme had the same Km value (0.7uM) and different maximum velocity values (0.2 and 0.57uM/min respectively) (Fig.5B) under the same conditions. Fig 5B also indicates that there is substrate

inhibition when the concentration of the substrate is above 2.0mM.

When the pellet from guts homogenized in saline was rehomogenized in the presence of 1% triton X-100, the Lineweaver Burk plot of 1/V vs 1/[S] showed that the enzyme had a Km value of 0.7fiM and the maximum velocity was 0.61jiM/min.

Fig 4. Aminopeptidase activity in relation to Bloodmeal

[A] Newly hatched nymphs: **[B]** Female adults.

• • Homogenized in Saline

• — • Homogenized in 1% Triton X-100

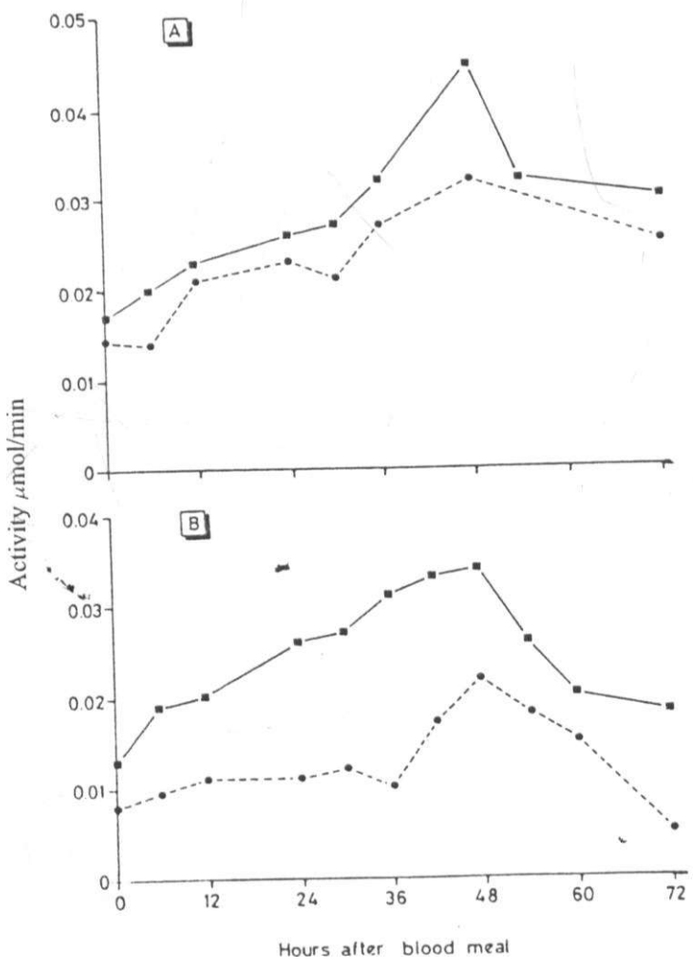
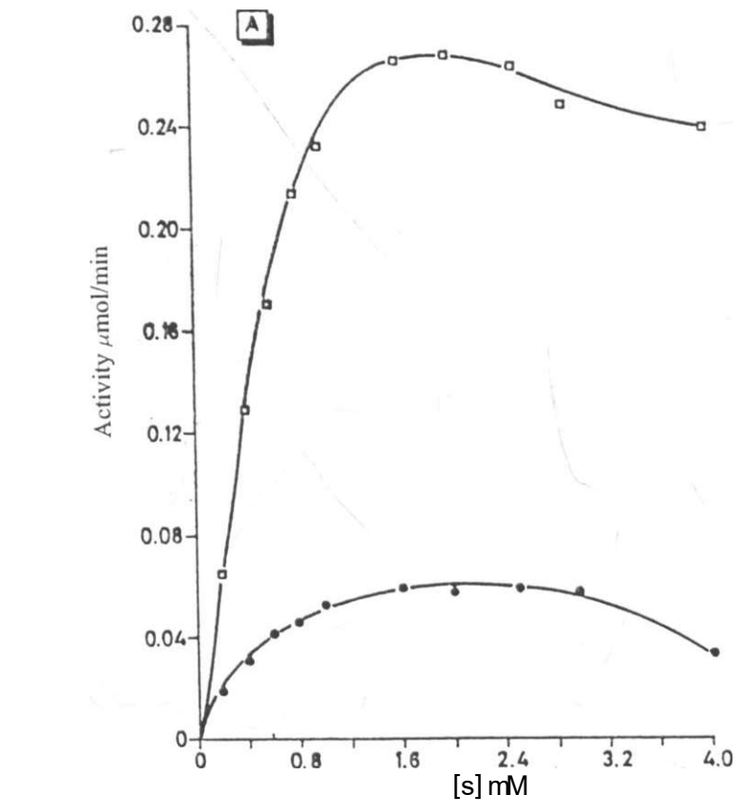
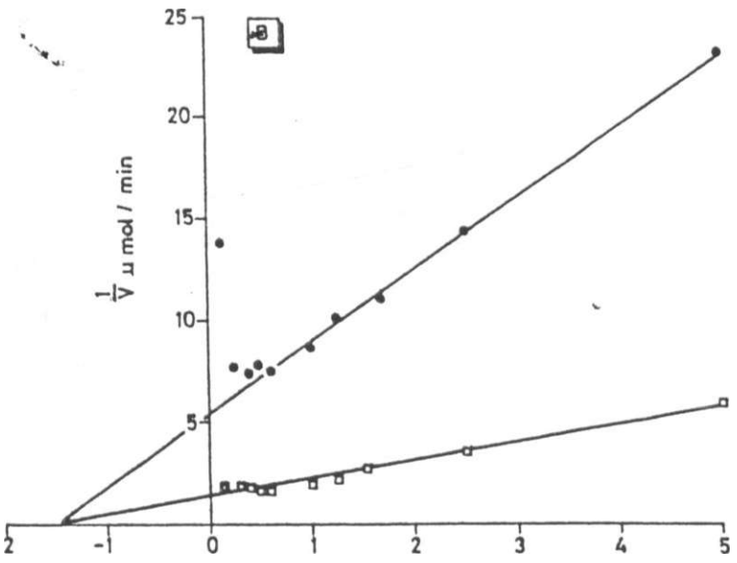

A
B
0.05
0.04
0.03
0.02
0.01
0
0.04
0.03
0.02
0.01
0
0
12
24
36
48
60
72
Activity μmol/min
Hours after blood meal

Fig 5A & **5B.** Michaelis-Menten Curve (A) and Lineweaver-Burk plot **(B)** reflecting the aminopeptidase activity in relation to increasing substrate concentration.

•—• Homogenisation in Saline Buffer. (Aqueous soluble enzyme)

a—a Homogenisation in 1% Triton X-100. (Detergent soluble enzyme)


A
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
Activity μmol/min
0
0.8
1.6
2.4
3.2
4.0
[s] mM


B
25
20
15
10
5
1/V μ mol / min
2
-1
0
1
2
3
4
5
S] mM

3.5 Inhibition Studies.

The Lineweaver Burk plot for the inhibition showed that aminopeptidase is competitively inhibited by the chelating agent l'lO Phenanthroline. For both soluble and membrane bound enzyme LAP, the K_m = 0.71+ 0.04mM and the K, = 0.0534 0.0025mM (Fig.6A & 6B & Table 2

The Dixon plots for both the inhibitor 1' 10 Phenanthroline and sensitivity to manganese ions (Mn^2) showed that the inhibitions were not complete (Fig7&8). respectively.

3.6. Partial purification and Molecular Weight Determination on Superose 6/12.

Initial chromatographs of buffer-homogenized midguts resulted in an absorbance profile consisting several protein peaks. The main peaks are labelled I to VI in the profiles (Fig 9& 10).

Fractions from these runs were assayed for hydrolytic activity of aminopeptidase enzyme against LpNA. In saline midgut extracts fractions eluting between 14ml & 20 ml exhibited activity against LpNA. Maximum activity was restricted within fractions eluting between 14ml & 15ml and this represented aminopeptidase of molecular weight (67± 3 KDa). The Aminopeptidase was not associated with the major eluting protein from the column (Peak V)(Fig 9).

Fig 6A &6B. Lineweaver-Burk plot reflecting the l'lO Phenanthroline inhibition kinetics activity at different inhibitor concentrations.

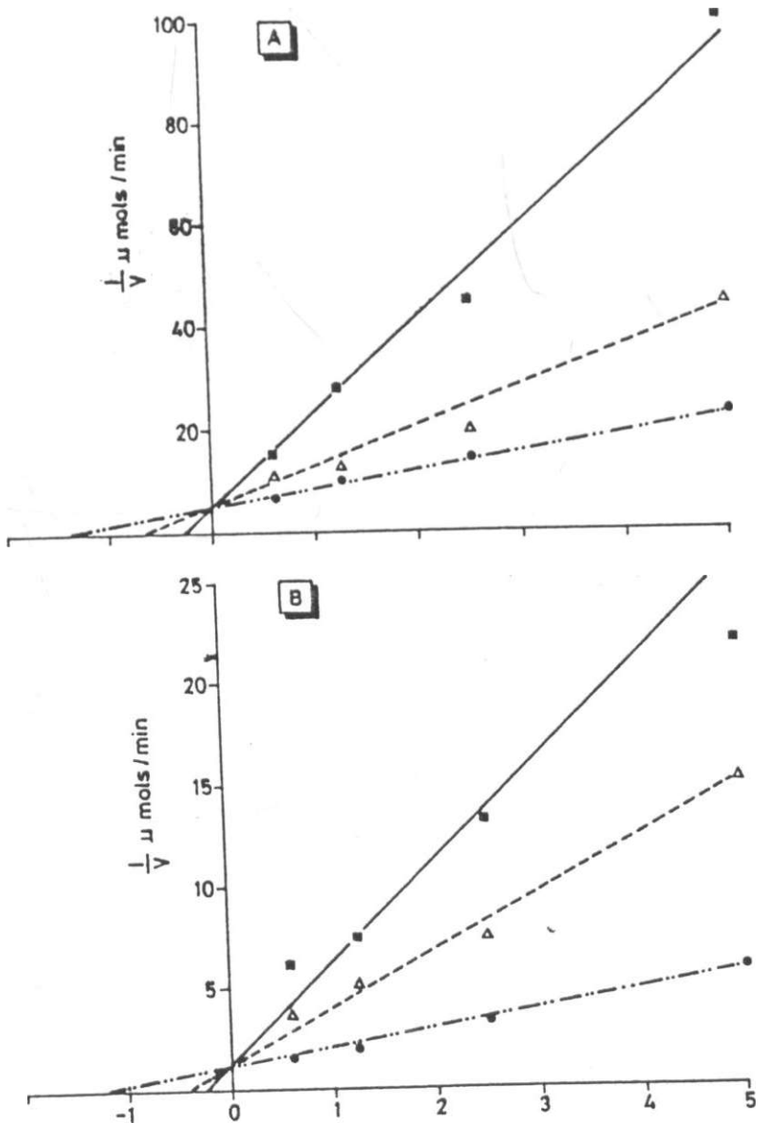
A. Homogenisation in Saline Buffer. (Aqueous soluble enzyme)

B. Homogenisation in 1% Triton X-100. (Detergent soluble enzyme)

$^{V}v.$ No inhibitor

A A O.lmM T10 Phenanthroline

• • 0.2mM l'lO Phenanthroline



V

Table 2. Phenthroline inhibition of LAP

	No Inhibitor	[Phenanthroline] miM			
		0.01		0.02	
	K_{ni}m\l	K'$_m$mM	Kj mg/ml	K'$_m$mM	K< mg/ml
Soluble LAP	0.71±0.04	2.0	0.055±0.002	3.33	0.05410.002
Membrane bound LAP	0.7110.04	2.25	0.05010.003	3.65	0.05310.002

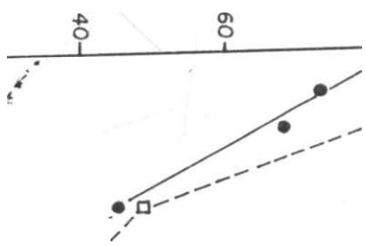
Fig 7 Effect of Inhibition on LAP activity by HO Phenanthroline

Inset is analysis using Dixon plot.

• — • Homogenisation in Saline. (Aqueous enzyme)

•— • Homogenisation in 1% Triton X-100 (Soluble enzyme)

% *Original* **activity**



umois I

Fig 8 Sensitivity of LAP activity to Manganese (Mn^{2+}) ions

Inset is analysis using Dixon plot.

•—• Homogenisation in Saline. (Aqueous enzyme)

•—• Hgmogenisation in 1% Triton X-100 (Soluble enzyme)

% Original activity

± u mois / mm

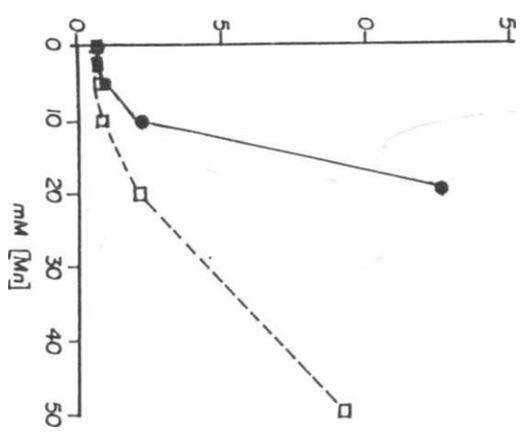


Fig 9 Superose 6 FPLC profile

Separation of samples of midgut extracts homogenized in:

A. Saline buffer

B. 1% Triton X-100

— - Protein detected by measuring absorbance at 280nm

— Aminopetidase activity against LpNA

Main peaks are labelled I to VI

Peak II represents Aminopeptidase enzyme protein elution point.

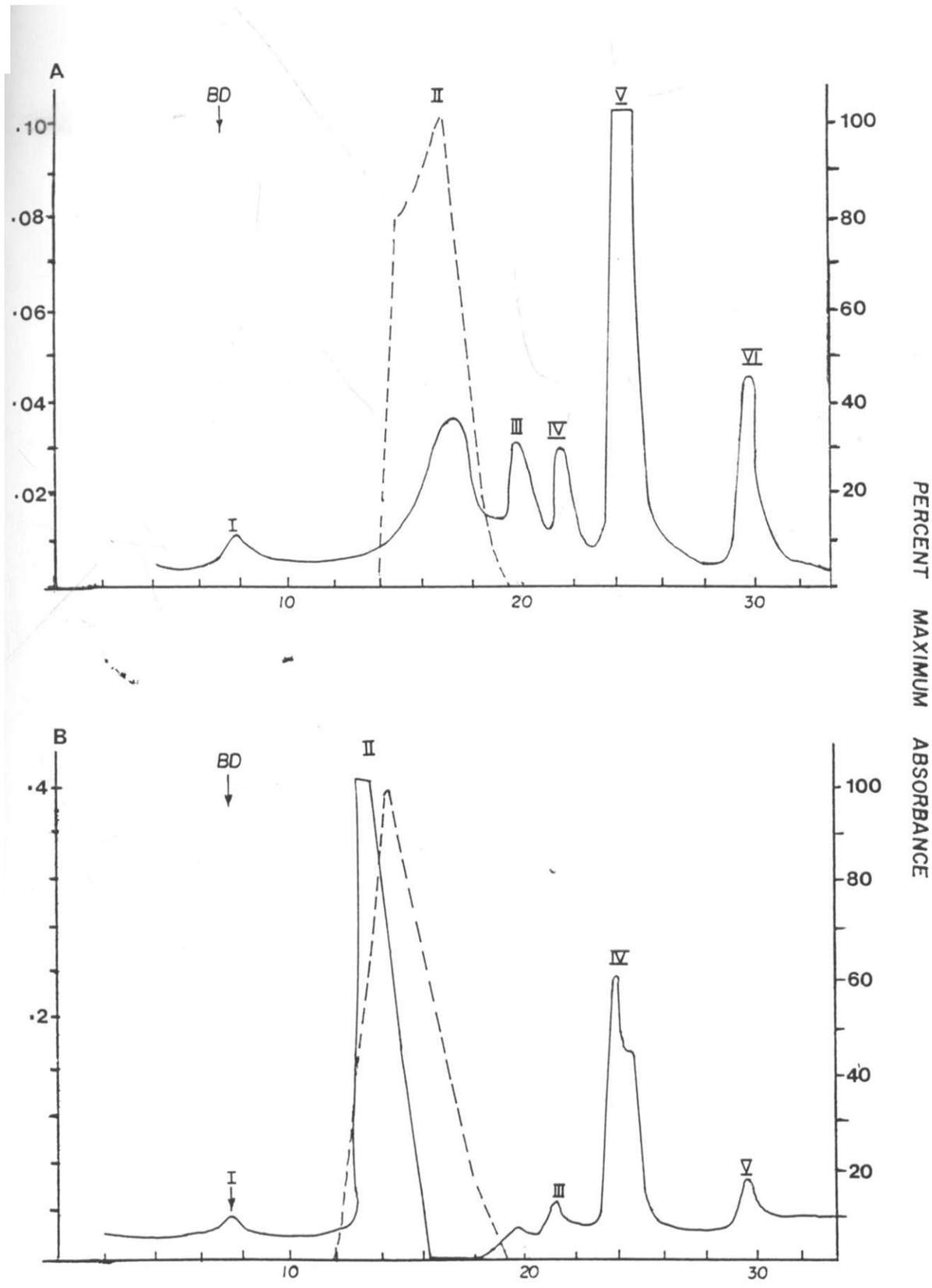

A
BD
II
V
VI
III
IV
I
·10
·08
·06
·04
·02
100
80
60
40
20
10
20
30
B
BD
II
IV
I
III
V
·4
·2
100
80
60
40
20
10
20
30
PERCENT MAXIMUM ABSORBANCE
ELUTION VOLUME (ML)

Fig 10. Superose 12 FPLC profile

Separation of samples of midgut extracts homogenized in:

A. Saline buffer

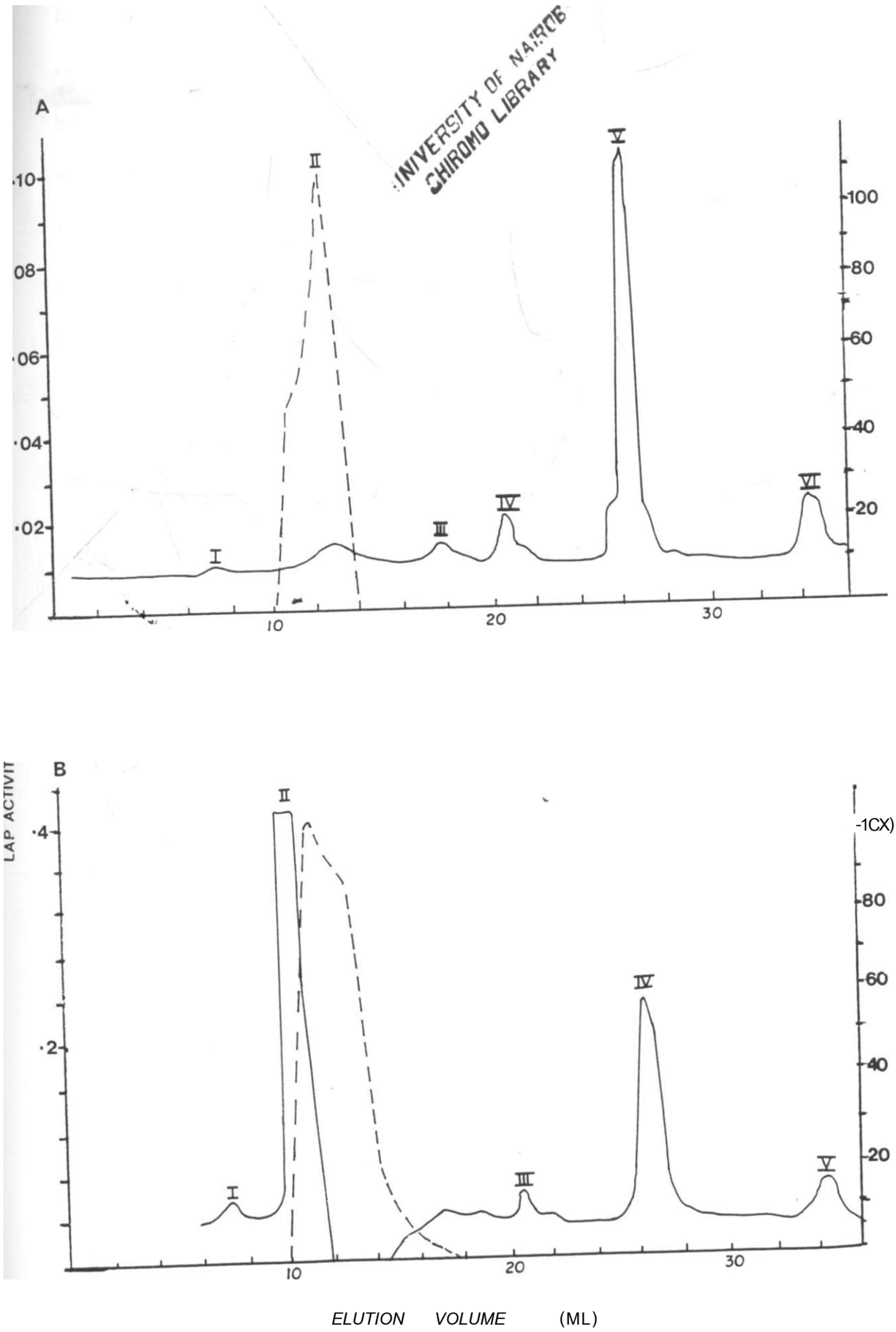
B. 1% Triton X-100

— " Protein detected by measuring absorbance at 280nm

— Aminopeptidase activity against LpNA

Main peaks are labelled I to VI

Peak II represents Aminopeptidase enzyme protein elution point.



In detergent midgut extracts, fractions eluting between fractions 12ml & 20ml exhibited activity against LpNA. Maximum activity was restricted to fractions eluting between 14 ml & 16ml. This peak also represented aminopeptidase of molecular weight 67 ± 3 KDa. The aminopeptidase enzyme was similarly purified on Superose 12 column. The profiles obtained was similar to those of Superose 6 column (Fig 10). Peaks III to VI did not have aminopeptidase enzyme catalytic activity on LpNA.

Fractions exhibiting maximum activity against LpNA were pooled and re-purified on the column(s) after dialysis. The dialysis process seemed to have affected the aminopeptidase catalytic ability as no activity was observed against LpNA this could be due to denaturation of the enzyme protein during dialysis (Fig 11). Fig 12 shows standard protein curve from the Superose profiles used for calculating the molecular weight. \ **

3.6.1 Analysis of midgut proteins by Native Polyacrylamide Gel Electrophoresis.

Fractions purified from Superose 6 revealed one protein band of molecular weight Mr«69,000 on Native PAGE with homogenates extracted in saline (Fig 14) and two protein bands of molecular weights 67 KDa and 69 KDa with homogenates extracted in 1% Triton X-100 (Fig 13). Fig 15 shows the standard protein curve for the Native PAGE used for calculating the molecular weight. Both gels from Fig 13 & 14 were run together for comparative purpose. Hence in Fig 14, no crude midgut extract was applied.

3.7 **Analysis of midgut proteins by Agarose Gel Electrophoresis.**

Agarose Gel electrophoresis for gels stained for LAP activity showed that for homogenates prepared in saline and in the presence of 1% triton X-100, the proteins that showed aminopeptidase activity exhibited the same molecular weight of 70 ± *2* Kda (Fig. 16). Fig 17 shows the standard protein curve for the Agarose gel used for calculating the molecular weight.

Fig 11. Re-Chromatography of Pooled fractions exhibiting LAP activity against LpNA. **J** Superose 6 FPLC Profile. No aminopeptidase activity was detected.

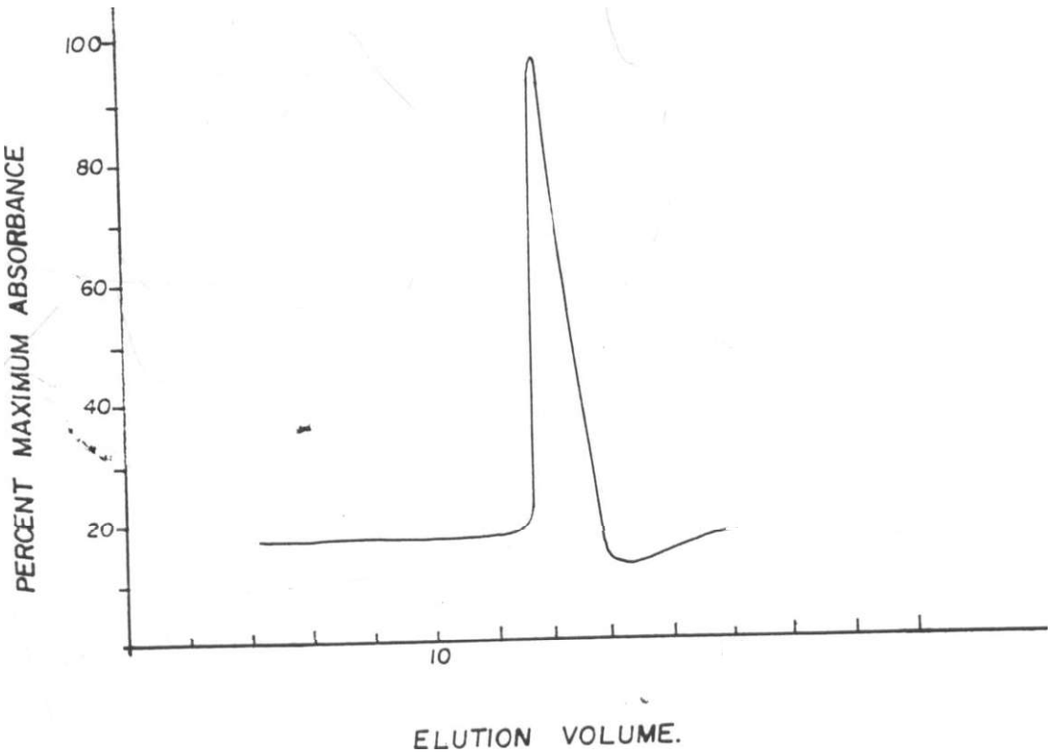
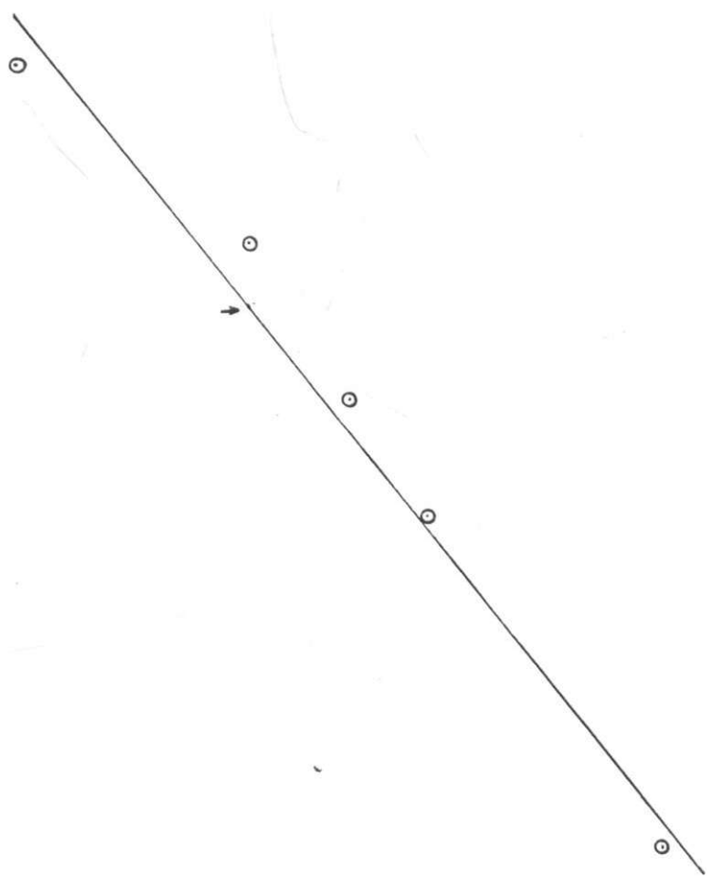

PERCENT MAXIMUM ABSORBANCE
100
80
60
40
20
10
ELUTION VOLUME.

Fig 12. Standard curve of log of molecular weight against relative mobility on Supe 6 column

Standards: Thyroglobulin (669 KDa), Bovine gamma globulin (158 KDa) Chic ovalbumin (44 KDa), Equine myoglobin (17 KDa), Vitamin B12 (1.3 KDa). S molecular weight was obtained for both Superose 6 & 12

The arrow shows the molecular weight point of aminopeptidase enzyme (Log 4.825 ; KDa)

60-



12 14 16 18 20 22

Relative mobility

$

Fig 13. Native PAGE showing LAP protein band after purification of midgut extracts Superose 6 FPLC. 1.25 ug of protein was loaded on each column

Lanes: 1&7 Molecular weight markers, 2.Fractions eluting between 12-20 n 3.Fractions skiting between 20-24 mis, 4.Fractions eluting between 24-30 mis Triton 10), 5.Crude homogenate (extract prepared in 1% Triton X-10) , 6. Crude homogenj (extract prepared in Saline).

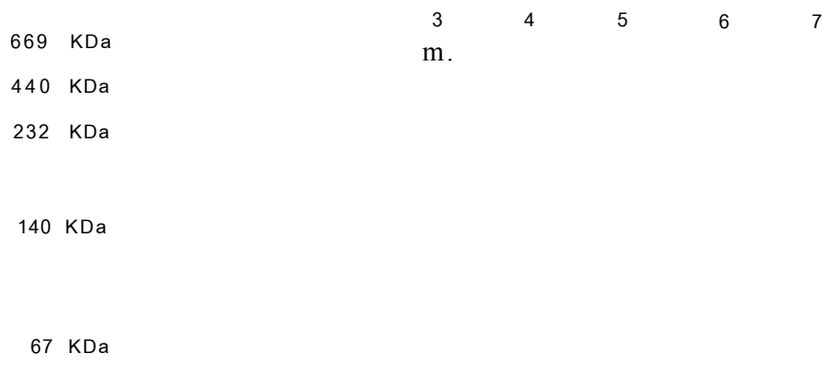

3
4
5
6
7
669 KDa
440 KDa
232 KDa
140 KDa
67 KDa

Fig 14. Native PAGE showing the protein bands on pooled fractions after purificat midgut extracts prepared in on Superose 6.

Lanes: 1&3. Molecular weight markers

2&4. Polled fractions eluting between 12-20ml

1.25 ug of protein was loaded on each well

NB. Crude midgut extract not analysed (see page 47, sec 3.6.1)

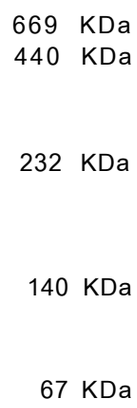

669 KDa
440 KDa
232 KDa
140 KDa
67 KDa

Fig 15. Determination of relative moleular weight of aminopeptidase using Native PAGE

Standard curve log molecular weight against relative mobility

Standards: Ferritin (440 KDa), Catalase (232 KDa), Lactase Dehydrogenase

'(140 KDa), BSA (67 KDa)

The arrows show the molecular weights point of the aminopeptidase

(Log 4.825 = 67 KDa & Log 4.84 69 = KDa)

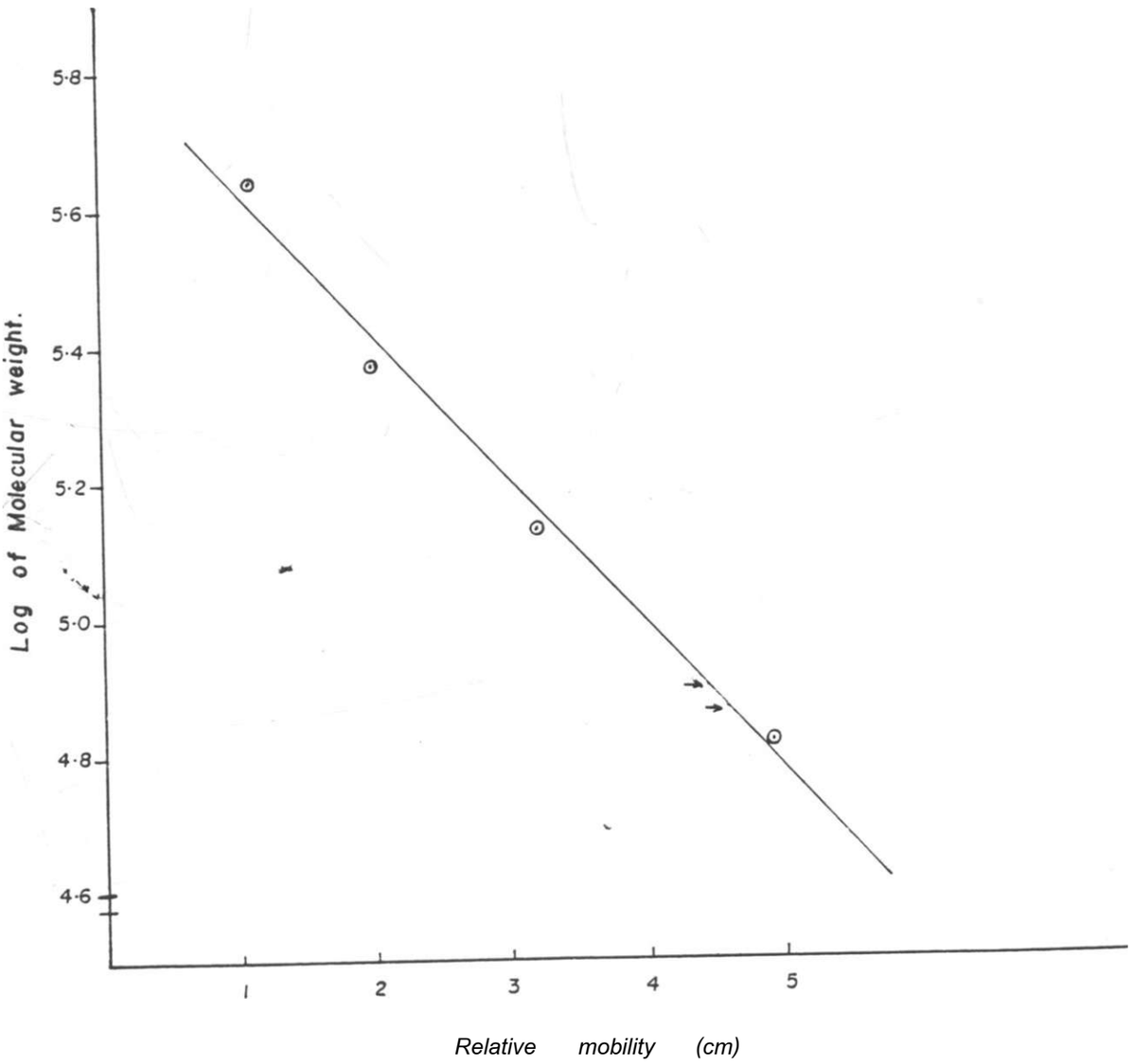
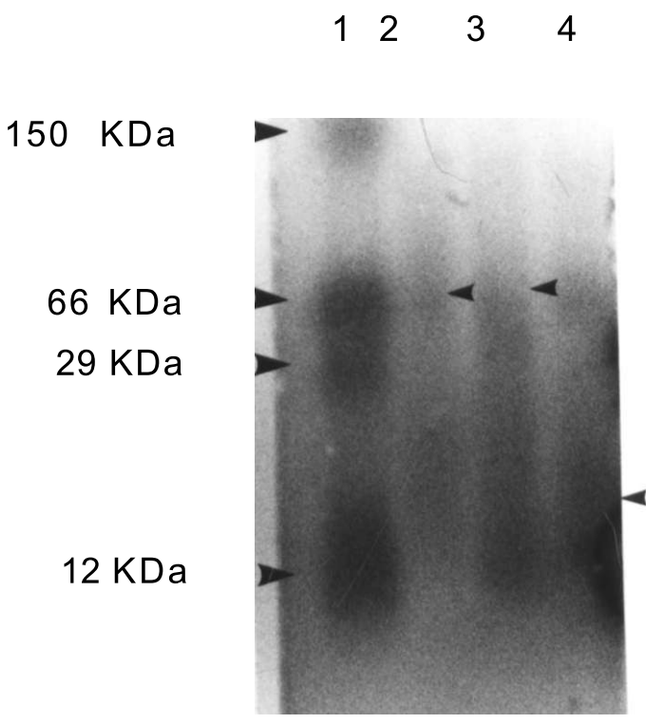

Log of Molecular weight.
5·8
5·6
5·4
5·2
5·0
4·8
4·6
1
2
3
4
5
Relative mobility (cm)

Fig 16A. Agarose gel showing LAP protein band after staining with Coomassie blue.

Lanes: 1. Molecular weight markers, **2.** Crude homogenate (saline extracts)

3. Crude homogenate (Triton X-100 extracts), 4. Crude homogenate (saline extracts)



-f

Fig 16B. Agarose gel showing LAP protein band after staining with for activity with **L-**aminoxidase.

Lanes: 1. Molecular weight markers, **2.** Crude homogenate (saline extracts), 3.Crude homogenate (1% triton X-100 extracts), **4.**Commercial aminopeptidase from pig kidney.

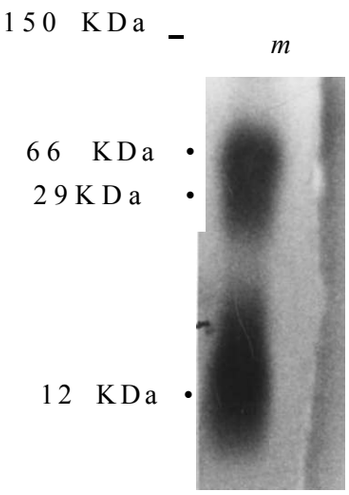
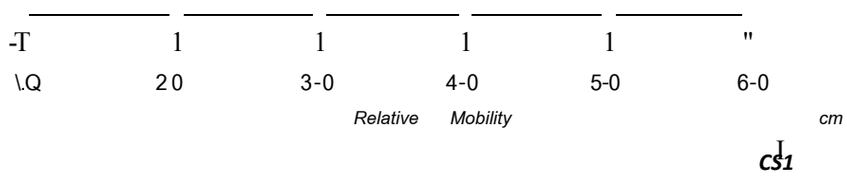

150 KDa
m
66 KDa
29KDa
12 KDa

Fig 17. Determination of relative molecular weight of aminopeptidase using Agaro; Standard Protein curve log molecular weight against relative mobility

Standards: Alcohol Dehydrogenase (150 KDa), **BSA** (66 KDa) Carbonic anh> (29KDa), Cytochrome C (12.4 KDa)

The arrow shows the molecular weight of aminopeptidase (Log 4.85 = 70 KDa).


-T
1
1
1
1
"
\.Q
2 0
3-0
4-0
5-0
6-0
Relative
Mobility
cm
CS1

CHAPTER 4

DISCUSSION.

The body louse belongs to the majority of blood sucking insects which produce a range of alkaline proteases (Lehane, 1994). Recent studies indicate that the body louse midgut contains several proteins with their molecular weights) ranging between 12 KDa and 117 KDa when analysed by SDS- PAGE electrophoresis. Some of the gut proteins are immunogenic and some are glycosylated. Seven of them (12 KDa, 17 KDa, 29 KDa, 35 KDa, 40 KDa, 55 KDa and 97 KDa) were major bands based on their intensity of staining. The immunization of rabbits with midgut extract elicited the production of protective polyclonal antibodies. These antibodies reacted strongly with all major midgut proteins as well as 63 KDa and 117 KDa when tested by the western blotlechnique. Analysis of the proteins revealed that the 12 KDa, 25 KDa, 35 KDa, 45 KDa, 87 KDa and 97 KDs were glycosylated and none of them contained a lipid moiety (Ochanda *et al.,* 1996, Mumcouglu *et al.,* 1996). The gut proteins also include enzymes such as trypsin and chymotrypsin enzyme whose function is to digest the bloodmeal (Borovsky & Schlein, 1988). In this study, results indicate that aminopeptidase is a major proteolytic enzyme of the louse midgut proteins.

The midgut proteins especially the enzymes are released more through detergent solubilization. Maximal release of aminopeptidase enzyme in body louse was realised when midguts were homogenized in the presence of a detergent (1% triton X-100). However there was a residual amount of activity in the midgut membrane residue indicating that the enzyme protein is integrated within the midgut membrane. This tends to imply that the body louse midgut aminopeptidase enzyme is

associated more with the epithelial tissue cell membrane. Immumofluorescence and immunogold techniques have shown that most of the immunogenic proteins of the louse gut are concentrated on the surface of epithelial cells (Mumcouglu *et al.,* 1996). Since aminopeptidase enzyme was extracted both by saline and detergent, solubilization indicates that it forms part of the louse midgut proteins. Midgut membrane bound aminopeptidase enzyme have been found in several insects such as *Glossina morsitans morsitans* Gooding & Rosleth, 1976), *Rynchosiara americana* (Ferreira & Terra, 1985) *Drosophila melanogaster* (Walker *et al.,* 1980*), Aides Egypt* (Graf & Briegel, 1982) *Bombyx mori* (Sumida & Eguchi, 1983), *Erynnis ello* (Santos and Terra, 1984), *Tipula abdominalis* (Sharma *et al.,* 1984), and *Anopheles Stephensi* (Billingsley, 1990). In *Aedes aegypti* and *Anopheles Stephensi* 40% of the aminopeptidase enzyme activity is associated with the epithelium (Billingsley, 1990). In *Rynchosiara americana* 80% of the activity originally present in the midgut caecal membrane is solubilized in Triton X-100 (Ferreira & Terra, 1985). Specificities of those aminopeptidases are unknown, which makes it difficult to speculate on their precise role in the terminal digestion of proteins.

In this study the midgut of body louse was found to have an aminopeptidase enzyme that is active in the alkaline pH. The aminopeptidase enzyme activity was high in a pH range from 7.0 to 8.5 with an optimum activity at about pH 8.0 (Fig 1). The body louse midgut aminopeptidase enzyme is similar to those of the hornfly (Hori *et al.,* 1981), *Aedes Egypt* (Kung, 1978), *Stomoxys calcitrans* (Champlain & Fisk, 1986), *Glossina morsitans morsitans* and *Anopheles stephensi* with regard to pH optimum and especially the shape of the activity curve was very similar to that of

Anopheles stephensi and *Glossina morsitans morsitans* (Langley, 1966 & Billingsley, 1990)).

The aminopeptidase enzyme in body louse midgut was also found to be thermally stable. Thermal inactivation of the catalytic activity of aminopeptidase enzyme from the body louse midgut results in 20% loss of activity after incubation at 50°C for 150 min. This indicates that the enzyme is fairly stable with regards to temperature variation. These results corroborate those obtained with *Rhynchosiara. americana,* where thermal inactivation of membrane bound aminopeptidase enzyme from midgut caecal cells showed that the denatured enzyme displays 65% activity of the native enzyme after incubation at 50°C for 150 min. (Ferreira & Terra, 1985). Aminopeptidase enzyme isolated from pig kidney was also found to be fairly stable. At 65°C, 1*Q activity is lost and at 75°C 50% activity is lost in 10 min but only 5% is lost in the 1st5min (Wachsmuth *et al.,* 1966).

Aminopeptidase enzyme activity in the body louse is stimulated by a bloodmeal, and maximum activity was realized about 48h after blood meal (Fig4). However the peak of activity appears later as compared to trypsin-like enzyme where maximum activity is realized 24h after blood meal and virtually none is found 48h after bloodmeal (Borovsky & Schlein, 1988). The aminopeptidase enzyme activity persists even 96h after bloodmeal. The blood meal stimulation of aminopeptidase enzyme activity is similar to that found in other insects. In the mosquito the peaks of aminopeptidase enzyme activity correspond to maximum postfeeding trypsin levels in both *Aedes aegypti* (24h) and *Anopheles stephensi* (30h) (Billingsley, 1990). In the hornfly and the stable fly the maximum activity for trypsin-like enzyme appears

earlier than the maximum activity for aminopeptidase enzyme. In the hornfly, maximum activity for trypsin-like enzyme is realised after about 7h and aminopeptidase enzyme is 14h, and in the stablefly maximum activity for trypsin-like enzyme is realised after about 15h and aminopeptidase enzyme is 23h (Hori *et al,* 1982). Although stimulation by a bloodmeal, is evident it is not yet known if aminopeptidase enzyme in haemtophagus insects is under secretory control from peptides rather than proteins in the peritrophic membrane (Billingsley and Downe, 1985). The findings of other workers have somehow tried to shed some light into the mechanism of enzyme bloodmeal stimulation process. In the female *Glossina morsitans*, Gooding (1977b) observed that trypsin-like enzyme appears earlier than aminopeptidase enzyme . This may be due to the fact that aminopeptidase enzyme in these insects is in the epithelium of the posterior midgut. It has been reported that some components in the blood meal ingested enters the posterior midgut to stimulate the secretion or production of trypsin-like enzyme these blood proteins are then hydrolysed to peptides and amino acids by trypsin-like enzyme. Some of the peptides may penetrate into the epithelium to stimulate secretion and production of aminopeptidase enzymeand thus trypsin-like enzyme appears earlier than aminopeptidase enzyme (Langley, 1966; Gooding, 1974a, 1974b; 1977b Lehane, 1977). It is also possible that, like in the mosquitoes, trypsin activity is directly induced by the presence of soluble protein(s) in blood, while aminopeptidase enzyme activity may be stimulated independently from that trypsin (Briegel & Lea, 1975).

In kinetic studies of the aminopeptidase enzyme extracted in saline and detergent, an enzyme activity of same km value but different catalytic activities with

respect to maximum velocity was demonstrated with LpNA as substrate. Studies on aminopeptidase enzyme activity in mosquitoes have revealed variable affinities for different substrates including LpNA (Billingsley, 1990). The aminopeptidase enzyme activity is competitively inhibited by the chelating agent 1'10 Phenanthroline and is sensitive to Mn^{2+} ions, with ImM of Mn^{2+} ions reducing the activity by over 90% (Fig 6,7&8). Competitive inhibition by 1'10 Phenanthroline has also been demonstrated in soluble aminopeptidase enzyme of *Rynchosiara americana* (Ferreira & Terra, 1983). 1'10 Phenanthroline inhibition of aminopeptidase enzyme in *Anopheles stephensi* is almost complete inhibition (Billingsley, 1990). The Phenanthroline inhibition is typical of mammalian type aminopeptidase enzyme E3411 and E3412 (Barman TE, 1974) but are contradicted by effect of Mn^{2+} and low molecular weight of the bo£iy louse enzyme.

1'10 Phenanthroline may bind reversibly to the active site through some metal ions in the region of the active site. Nevertheless it is possible that aminopeptidase enzyme depends more on the ring system of Phenanthroline than in its chelator properties (Ferreira & Terra, 1985). This implies that Aminopeptidase enzyme -Substrate- 1'10 Phenanthroline complex can give rise to products but more slowly than Aminopeptidase enzyme-Substrate complex (Fig 7 inset). Mammalian type aminopeptidase enzyme is activated by Mg^{2}~ and Ca^{2}~ which do not seem to have significant effect on the body louse aminopeptidase enzyme (results not shown). *Rynchosiara americana* midgut soluble aminopeptidase enzyme is also not activated by calcium ions but *Anopheles stephensi* midgut aminopeptidase enzyme is activated by both Mg^{2+} and Ca^{2+} (Klinkowstrom et al., 1994 & Billingsley, 1990).

Results from Fast Protein Liquid Chromatography (FPLC) on Superose 6 show the presence of only one aminopeptidase enzyme activity peak in both saline and 1% triton X-100 midgut extracts (Fig 9&10). Native PAGE electrophoresis analysis also indicate the presence of only one protein band of molecular weight 69 KDa in salinefractions and two protein bands of molecular weights 67 KDa and 69 KDa in 1% triton X-100 fractions. In-gel staining of the enzyme activity revealed one band of similar molecular weight 70 KDa in both saline and 1% triton X-100 fractions.

More than one aminopeptidase enzyme has been noted in all hematophagus insects previously studied in detail. Three aminopeptidases have been described in the stable fly *Stomoxys calcitrans* and at least two aminopeptidases in the hornfly *Haematohla irritans,* (molecular weights not shown) (Hori *et al,* 1983). In *Anopheles stephensi* three aminopeptidases of molecular weights 32 KDa, 123 KDa and 550KDa have been reported (Billingsley, 1990). In *Rhynchosiara americana* larval midguts, three aminopeptidases molecular weights ranging between 100 KDa and 200KDa were reported (Ferreira & Terra, 1985; Klinkowstrom *et al*., 1994). In this study the body louse aminopeptidase enzyme protein was of molecular weight 67 KDa -69KDa and falls within the range of the molecular weight of proteins reported for the body louse midgut, (Ochanda *et al,* (1996). These findings suggest that only one aminopeptidase enzyme is present in the louse midgut. The body louse midgut aminopeptidase enzyme could possibly occur as a family of charged isomers since the saline soluble and the detergent soluble (membrane bound) appear to be the same in molecular weight, km, ki and have the same optimal activity pH value. *Tineola*

bisselaeola also has similar aminopeptidases which occur as families of charge isomers of what seems to be the same enzyme protein (Ward, 1975a,b). In *Rhynchosiara americana* midgut caeca, (Ferreira & Terra, 1985) using semi-preparative gel electrophoresis isolated the two major soluble aminopeptidases active on LpNA. Employing LpNA and ArpNA as substrates they found that all the physical and kinetic properties of the two enzymes, except for pi values, were identical. Due to this they concluded that the enzymes were charge isomers, and the differences observed were thought to result from difference in glycosylation. Nevertheless it has been shown that the two enzymes have different substrate specificity (only one of them hydrolyses AsppNA and PropNA) and that they have different subcellular distribution suggesting that they are different enzymes (Klinkowstrom *et al*., 1994). In this study, LpNA wa? the only substrate used and thus the results reflect aminopeptidase enzyme activity on LpNA only but demonstrate the significance of aminopeptidase enzyme as the major digestive protease in body louse.

Induced resistance to the human body louse in rabbits immunized with an extract of the louse midgut caused lice feeding on these rabbits to take smaller bloodmeals, have higher mortality, lay fewer eggs and take longer to develop than lice feeding on control animals (Ben-Yakir *et al,* 1994). Some of the immunogenic proteins, including proteolytic enzymes are excreted with the louse faeces (Mumcouglu *et al*., 1996). Lice faecal antigens are known to cause intense skin reactions in humans when they come in contact with the site of the bite (Peck *et al,* 1943). Similarly, antigens of the house dust mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* which show a homology to serine proteases

(chymotrypsin and trypsin) are excreted with faeces. When these allergens are inhaled by atopic patients they can cause allergic rhinitis and bronchial asthma in humans (Heyman & Chapman, 1990).

Acquired resistance after infestation of blood-feeding arthropods is thought to result from the injected salivary antigens. However, gut excretions products such as digestive enzymes, antigenic material derived from the gut epithelial cells, peritophic membrane and blood remains from the host animal, are excreted with faeces and cause immune and allergic reactions by entering through the host skin and mucous membrane.

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