PURIFICATION AND PROPERTIES OF THE LOW MOLECULAR WEIGHT PROTEIN FROM HAEMOLYMPH OF THE TSETSE FLY, GLOSSINA MORSITANS MORSITANS

By

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D E C L A R A T I O N

I, Edward Kinyua Nguu, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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ABBREVIATIONS

Abbreviation	Full name/meaning
ApoLp-I	Apolipophorin-I
ApoLp-II	Apolipophorin-II
ApoLp-III	Apolipophorin-III
APS	Ammonium persulphate
BSA	Bovine serum albumin
$Cacl_2$	Calcium chloride
Con A	Concanavalin A
DAP	Diapause associated Protein
DFP	Diisopropylphosphoro-fluoridate
HDL	High density lipoprotein
HRP	Horse radish peroxidase
hsp	Heat shock protein
i.d.	Internal diameter
IEF	Isoelectrofocusing
JH	Juvenile hormone
JHBP	Juvenile hormone binding protein
K_2PO_4	Dipotassium phosphate
KBr	Potassium bromide
KCl	Potassium chloride
LDL	Low density lipoprotein

LMWP	Low molecular weight protein isolated from adult male *G.m. morsitans*.
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff's stain
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl-fluoride sulphonylflouride
PTU	Phenylthiourea
R.I.	Refractive index
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TB	Tris-buffer
TBS	Tris buffered saline
TCA	Trichloroacetic acid
Temed	N, N, N', N' - Tetramethylenediamine
Tris	Tris-(hydroxy methyl) aminomethane

VHDL	Very high density lipoprotein
V/V	Volume per volume
W/V	Weight per volume

UNIT ABBREVIATION

Ci	Curie
Ci/mMole	Curie/millimole
cm	Centimetre
g	Gravitational constant
gm	Gram
g/ml	Gram/millilitre
hr	Hour
KD	Kilodalton
M	Molar concentration
mA	Milliamphere
mCi	Millicurie
mCi/mmole	Millicurie/millimole
mg	Milligram
min	minute
ml	Millilitre
mM	Millimolar
M_r	Relative molecular mass
nm	Nanometre
pH	$-\text{Log}_{10}$ hydrogen ion concentration
pI	Isoelectric point
Sec	Second
µCi	Microcurie
µg	Microgram
µl	Microlitre

µM	Micromolar
V	Volt
W	Watt

SUMMARY

Tsetse fly is an insect of great economic importance to man because it transmits sleeping sickness to both man and his livestock. The insect feeds on a bloodmeal thereby transmitting the disease from infected to healthy individuals. The tsetse fly, Glossina spp. belongs to the order Diptera and family Glossinidae and 22 species have been identified. The fly is mainly found in humid areas especially along the river valleys and bushes in Sub-Saharan Africa.

Insects are known to posses haemolymph as the circulatory fluid equivalent to blood and lymph in vertebrates. In both cases, the circulating fluids have been shown to contain lipoproteins as the major component. Various haemolymph proteins serving a wide range of functions have been described in many insect species. Whereas some of these proteins have been well studied in some insect species, the same has not been done in the tsetse fly despite its great economic importance.

This study therefore, investigates the properties of a low molecular weight protein isolated from haemolymph of adult male G.m morsitans. The protein was purified from haemolymph by a combination of density gradient ultracentrifugation and gel permeation chromatography. The protein has a molecular weight of 23,000 dalton as determined by electrophoresis on

polyacrylamide gels. The hydrated density of the low molecular weight protein, determined by density gradient equilibration was = 1.29 g/ml. Staining of the protein with Sudan Black confirmed that it is lipidated. Carbohydrate analysis showed the low molecular weight protein to be non-glycosylated since it neither bound onto concanavalin A-Sepharose affinity chromatography column nor stained with periodic acid Schiff's stain on SDS-gels.

Amino acid analysis of the low molecular weight protein showed very high content of acidic amino acids, glutamate (16%) and aspartate (13%). The protein also contained high proportions of serine (11%) and glycine (10%). The content of basic amino acid, lysine (10%) was also high.

De novo synthesis using radiolabelled [^{35}S]-methionine and [^{14}C]- leucine shows the low molecular weight protein is synthesized by the fat body and then released into the haemolymph.

An investigation was carried out to determine whether there was immunological cross-reactivity between other haemolymph proteins from eleven insect species representing six orders. From the results obtained by both double radial immunodiffusions and immunoblots, the low molecular weight protein was not detected in any species other than the order Diptera, family Glossinidae to which the tsetse fly belongs.

Thus, no cross-reactivity was observed with other insect haemolymph proteins, suggesting this protein could be unique to tsetse fly.

CHAPTER 1

1. INTRODUCTION:

1:1 Tsetse fly as a medical vector:

The trypanosomes of the brucei subgroup are digenetic (Hoare, 1972). They have a complicated life cycle, part of which occurs in the tsetse fly and the rest in the mammalian host. While in the tsetse fly, the parasites spend part of their early life in the gut and the later stages in the salivary glands. It is the trypanosomes in the salivary glands that are infective to man and his livestock. By nature of their feeding habits, haematophagous insect, such as the Glossina are vectors of many insect borne diseases. This is the mode by which tsetse fly transmits sleeping sickness (Buxton, 1955). Many other insect species transmits other types of diseases (Mattingly and Crosskey, 1983).

1:2 The tsetse fly : General features and life cycle:

The tsetse fly belongs to the order Diptera, family Glossinidae and 22 species have been identified. Their colour is non-descriptive but ranges from yellowish or greyish to dark or blackish brown with the upper side of the thorax marked with greyish brown strips (Buxton, 1955; Liard, 1977). In resting posture, their wings lie closed flat over one another like blades of a pair scissors while the proboscis project horizontally

infront of the head (Nash, 1969; Liard, 1977). The length of the fly together with the palp and proboscis is between 6 and 14 mm (Liard, 1977). The distinction between the sexes is the hypopygium, a cushion like projection on the underside of the tip of male abdomen, this is the male genitalia which is absent in females (Buxton, 1955).

For the female, mating takes place about 3 days after emergence which is normally after the first bloodmeal (Jordan, 1974). The female mate only once during the course of its reproductive life (Pollock, 1970). The female gives birth to live offsprings that are nourished within the uterus (Engelman, 1970). A fully grown larva is produced every 9-10 days(Saunders and Dodd, 1972). Once larviposited, the larva does not feed and usually pupariates within 1-2 hours. During pupariation, the cuticle darkens and hardens to provide a resistant covering capable of preventing dessication (Nash, 1969). The duration of pupal stage, which begins with immobilisation of the larva and lasts until the emergence of the adult fly, varies with species, sex and external conditions particularly temperature. Normally pupal stage has been found to last for 25-35 days (Shah-Ficher and Say, 1989) but durations as long as 50 days (Pollock, 1989) is possible. The teneral flies emerges from the pupal shell by breaking it using its bladder-like ptilinum (Nash, 1969). The duration

of the insect life span varies inversely with maximum temperature in the environment (Nash, 1969; Liard, 1977). It is also species variant.

1.3 Insect Haemolymph Proteins:

1.3.1 Overview:

Haemolymph serves as the circulatory fluid that baths tissue cells and is equivalent to blood and lymph in vertebrates (Beenakkers et al., 1985). Lipoproteins which makes up the major components of the insect plasma, serves various functions (Wyatt and Pan, 1978; Shapiro et al., 1988). The proportion of some haemolymph proteins vary, a factor that can be related to developmental cycle or adaptations. Some proteins such as the lipophorins are present throughout life cycle (Gellissen and Wyatt 1981; Chapman, 1980). Other haemolymph proteins for example vitellogenins (Engelman, 1973; Hagedorn and Kunkel, 1979), storage proteins (Levenbook, 1985) and arylphorins (Ryan et al., 1984b) undergo fluctuations that can be related to specific functions during development. At times of adversity, most insects undergo diapause. Diapause is characterised by the appearance of new proteins, diapause associated proteins, in haemolymph which are absent in non-diapausing ones (Denlinger, 1985).

Several high density lipoproteins (HDL) and very high density lipoproteins (VHDL) have now been purified

from insect haemolymph. Among these proteins are high molecular weight lipoproteins while others are low molecular weight.

1:3:2 High Molecular Weight Lipoproteins:

A variety of high molecular weight lipoproteins of varying densities have been isolated and characterised from haemolymph of many insect species. Table I summarises the properties of these proteins.

1:3:3 Low Molecular Weight Proteins:

Several low molecular weight haemolymph proteins have been purified and their properties examined. Among them are;

1:3:3:1 Low molecular weight Diapause Associated Protein (DAP):

Diapause can occur at any stage of insect life and the stage is characteristic in each species (Denlinger, 1985). Among the notable biochemical changes in haemolymph of diapausing insect is the appearance of protein fraction of disctinct electrophoretic mobility that is absent in non-diapausing counterparts. The accumulation of a low molecular weight DAP in the fat body of diapausing southwestern corn borer, Diatraea grandiosella, has been reported (Brown and Chippendale, 1978). The protein accumulates in fat body of pre-diapausing larvae reaching a peak in newly diapaused larvae then gradually dissappear during diapause (Brown and Chippendale, 1978; Dillwith et al., 1985). The

Table 1 : Properties of major high molecular weight haemolymph proteins

Key:

B	-	Both sexes
F	-	Female
*	-	Common
?	-	Varied or Unknown
✓	-	Present
x	-	Absent
CHO	-	Carbohydrate
P	-	Phosphorous

References

(a) - Chino (1985)

(b) - Riddiford and Law (1983)

(c) - Engelman (1979)

(d) - Levenbook (1985)

(e) - Yeaton (1981)

(f) - Haunerland and Bowers (1986)

(g) - Ryan et al. (1986, 1988)

(h) - Osir et al. (1989)

PROTEIN	SEX	MOLECULAR WEIGHT (RANGE)	NUMBER OF APOPROTEINS	NUMBER OF SUB-UNITS	MOEITY			DENSITY (RANGE)	STAGE FOUND	SPECIAL PROPERTY	MAJOR FUNCTIONS
					LIPID	CHO	P				
LIPOPHORINS (a)	B	500*KD	2*/3	-	✓	✓	x	LOW/HIGH	ALL INCLUDING EGG	HIGH LIPID CONTENT	LIPID TRANSPORT
ARYLPHORINS (b)	B	500KD	-	6	✓	✓	x	VERY HIGH	LARVAL	EXCEPTIONALLY HIGH AROMATIC AMINO CONTENT	STORAGE
VITELLOGENINS (c)	F	500KD	2*	-	✓	✓	✓	HIGH/VERY HIGH	ADULT FEMALE	FEMALE SPECIFIC	EMBRYO DEVELOPMENT
LARVAL STORAGE (d) PROTEINS	B	500KD	-	6	✓	✓	x	HIGH	LARVAL INSTARS	HIGH % AROMATIC AMINO ACIDS	STORAGE
LECTINS (e)	B	UPTO 1500KD	-	?	?	?			ALL	ARE CHO SPECIFIC	PROTECTION AGAINST PATHOGEN
CHROMOPROTEINS (e)	B	500KD	-	4	✓	x	x	VERY HIGH	ALL (LARVAL*)	HAVE PORPHYRIN (BOUND)	CAMOUFLAGE
LIPID TRANSFER PROTEINS (g)	B	400KD	3	-	✓	x	x	HIGH	ALL		CATALYSES LIPID EXCHANGE FROM LIPOPHORINS
HIGH MOLECULAR WEIGHT DIAPAUSE PROTEIN (h)	B	400KD	-	2	✓	✓	x	VERY HIGH	ANY BUT DIAPAUSING	APPEAR ONLY DURING	ASSOCIATES WITH

protein was only found in larval fat body and haemolymph (Brown and Chippendale, 1978; Dillwith and Chippendale, 1984; Dillwith et al., 1985). A protein with immunochemical identity to D. grandiosella DAP was also detected in fat bodies of diapausing larvae of D. Crambidoides and D. Saccharalis (Dillwith et al., 1985).

DAP was found to be of M_r=35,000 (Turunen and Chippendale, 1979). The proteins with immunochemical identity to D. grandiosella DAP identified in D. crambidoides and D. saccharalis are of molecular weights 33,000 and 36,000 respectively (Dillwith et al., 1985). DAP contains 12% aromatic amino acids (Dillwith and Chippendale, 1984). Juvenile hormone was shown to control synthesis and storage of DAP in fat body of diapausing D. grandiosella (Brown and Chippendale, 1978).

Several functions of DAP including that of a storage protein, an enzyme or pro-enzyme, a carrier or procarrier and a protectant were proposed (Brown and Chippendale, 1978; Turunen and Chippendale, 1979; Dillwith and Chippendale, 1984). However, the exact role of DAP in maintenance of diapause remains poorly understood.

1:3:3:2 Apolipophorin-III:

In some adult insect species apolipophorin-III has

been identified as the third apoprotein associating with lipophorin (Shapiro et al., 1988). The protein was first identified as C_L protein (Wheeler and Goldsworthy, 1983 a,b) and C_2 protein (Van der Horst et al., 1984) from the haemolymph of Locusta. The protein has now been shown to be present in M. sexta (Kawooya et al., 1984a), Thasus acutangulus (Wells et al., 1985), Locusta migratoria (Goldsworthy et al., 1985) and Gastrimargus africanus (Haunerland et al., 1986). In both L. migratoria and M. sexta, low levels of apolipophorin-III was also present in larval haemolymph (Kawooya et al., 1984).

Most lipophorins studied are composed of two apoproteins as integral constituents (Ryan et al., 1986). Free apolipophorin-III has been shown to be present in haemolymph with only a small fraction associating reversibly with lipophorin (Kawooya et al., 1984). From species examined, apolipophorin-III was found to be a single polypeptide of M_r=17,000 - 20,000 (Van der Horst et al., 1981; 1984; Wheeler and Goldsworthy, 1983a,b). Apolipophorin-III from M.sexta (M_r=17,000) (Kawooya et al., 1984) and Thasus acutangulus (M_r=20,000) are non-glycosylated while in L. migratoria and G. africanus, the polypeptide (M_r=20,000) is glycosylated. Fat body was shown to be the synthetic site for apolipophorin-III in Locusta migratoria (Izumi et al., 1987; Kanost et al., 1987).

In the species that have apolipophorin-III as the third apoprotein of lipophorin, considerable content of the protein has been found associating with low density lipophorin (Wheeler and Goldsworthy, 1983a,b; Shapiro and Law, 1983). The nature of association remains poorly understood. Kawooya et al., (1986b) has demostrated that apolipophorin-III exhibiits a high affinity for lipid-water interfaces, known to exist on surface of low density lipophorin. It is proposed that apolipophorin-III could be serving as possible recognition signal or activator of the flight muscle lipoprotein lipase that hydrolyses diacylglycerols (Van der Horst et al., 1987).

1:3:3:3 Juvenille Hormone Binding Protein (JHBP)

Juvenille hormones (JHs) have been shown to be present in haemolymph of insects (Gilbert, 1972; Goodman and Chang, 1985; Koeppe and Kovalick, 1986). Kramer et al. (1976) had earlier indicated that JHs are relatively insoluble in polar environment such as insect haemolymph. The JH transport is mediated by carrier proteins called the Juvenile hormone binding proteins (Goodman, 1983).

Since they were first reported by Whitmore and Gilbert (1972), from Hyalophora cecropia, JHBP have subsequently been identified in many insect orders (Goodman and Chang, 1985). The JHBPs vary in molecular weight. A JHBP of molecular weight as high as 680,000

daltons has been identified in a Dictyoptera, Diploptera punctata (King and Tobe, 1988). The protein was found to have a high affinity for JH-III. Many lepidopterans have low molecular weight (20-40 KD) JHBP (Goodman and Chang, 1985). Other insects have their JHBPs of intermediate molecular weight between those of lepidopterans and the highest reported from D. punctata (Goodman and Chang, 1985; Koeppe and Kovalick, 1986).

Apart from size variations, JHBPs also exhibits variations in specificities and affinities for their JHs. Based on molecular weight, JH binding capacity, specificity and affinity, two types of JHBPs are distinguished (De Kort and Granger, 1981; Goodman and Chang, 1985; Koeppe and Kovalick, 1986). One group has low affinity, low specificity but high capacity for JH while the other group exhibits a relatively high affinity, high specificity but low capacity for JH (Goodman and Chang, 1985). High molecular weight JHBPs are normally identified in the first category while generally low molecular weight JHBPs fit in the second category (De Kort and Granger, 1981; Schooley et al., 1984). A third class of JHBPs comprising of high molecular weight proteins with high affinity and specificity for JH-III have been reported (De Kort et al., 1984).

The major role of JHBPs in haemolymph of insects has been identified as that of JH transport and

protection. The binding of JH to haemolymph proteins has been demonstrated in several orders (Whitmore and Gilbert, 1972; Emmerich and Hartman, 1973; Bassi et al., 1977; Kramer and De Kort, 1987). The other important role of these proteins is the protection of JH from degradation by esterases which are abundant in haemolymph (De Kort and Granger, 1981; Hammock, 1985). These esterases hydrolyses the methyl ester of JH to yield biologically inactive acid. There is evidence that JH that is not bound to JHBP is hydrolysed by DFP sensitive esterases (Sanburg et al., 1975a,b). The role of JHBPs in maitaining diapause in D. grandiosella (Turunen and Chippendale, 1979) remains poorly understood.

1:3:3:4 Microvitellogenin:

Other than vitellogenins, microvitellogenins constitutes the second class of female-specific haemolymph proteins (Kawooya et al., 1987). The protein has been purified from haemolymph of female M. sexta (Kawooya and Law, 1983). The protein was initially observed by Telfer et al. (1981) from egg of H. cecropia. By use of antibodies against M. sexta microvitellogenin, the presence of a similar protein was demostrated in T. mollitor haemolymph (Kawooya et al., 1987).

Although microvitellogenin is a female-specific protein present in mature eggs and haemolymph (Kawooya

and Law, 1983; Kawooya et al., 1986a), it was shown to have no physical, chemical or immunological identity to vitellogenins (Kawooya et al., 1987). Unlike vitellogenins, which in most cases is composed of two apoproteins and is of high molecular weight, microvitellogenin is present as a single polypeptide M_r=31,000. Furthermore, the protein has no conjugates viz: lipids, carbohydrates and phosphorous which are normal constituents of vitellogenins (Kawooya et al., 1987). Synthesis of microvitellogenin takes place in fat body where it is released into haemolymph and sequestered into the egg with no further modifications (Kawooya et al., 1986a; 1987). The synthesis and uptake process is controlled by JH (Kawooya and Law, 1983).

Microvitellogenin has been purified and characterised but its biological function remains unknown. But, like vitellogenin, its accumulation in the egg certainly makes it an important component of mature egg.

1:3:3:5 Insect Immune Protein:

Insects have been shown to have a potent humoral immune response system that can be induced by injection with live non-pathogenic bacteria and injuries (Gotz and Boman, 1985). Notable change in haemolymph of insects injected with such organisms and injuries is the appearance of new proteins with antibacterial

properties (Gotz and Boman, 1985; Boman and Hultmark, 1987). The phenomenon of insect immunity has been well studied in diapausing Hyalophora cecropia pupae. When the insect is injected with the bacteria Enterobacter cloacae, 15-20 new proteins appear in haemolymph (Hultmark et al., 1980). The enzyme, lysozyme [EC:3:2:1:17], known for its bacteriolytic activity has been identified as one of the bacterial inducible proteins in haemolymph. The enzyme hydrolyses glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in cell wall peptidoglycan. Lysozyme was first isolated from H. cecropia (Hultmark, 1980) and was initially designated P7 protein (M_r=15,000). The enzyme has now been identified in many other insect orders primarily lepidopterans.

Another category of immune proteins induced by bacterial infections are the cecropins (Hultmark et al., 1980). The proteins were first identified from H. cecropia and were originally designated P9 proteins (Hultmark et al., 1980) but are now re-named cecropins (Steiner et al., 1981). Three main cecropins A, B and D were identified in H. cecropia haemolymph (Steiner et al., 1981; Hultmark et al., 1982). Cecropins makes up a family of basic proteins with molecular weight about 4000 daltons and having broad antibacterial properties (Steiner et al., 1981). Inducible antibacterial proteins with properties similar to cecropins from H.

cecropia have also been identified from several insect orders including the dipteran, Glossina morsitans morsitans (Kaaya et al., 1987).

There are other immune proteins present in haemolymph of immunized insects but showing no direct antibacterial activity. Such is the protein designated P4 (M_r=48,000) that appears in haemolymph of diapausing pupal H. cecropia (Rasmuson and Boman, 1979). A similar protein was reported from M. sexta (Hulbert et al., 1985).

In vitro synthesis studies shows fat body as the synthetic site of immune proteins. This was demostrated in several lepidopterans including H. cecropia (Faye and Wyatt, 1980), Galleria mellonella (De Verno et al., 1984) and M. sexta (Dunn et al., 1985).

1:4 RATIONALE FOR THIS STUDY:

The haemolymph of tsetse fly has been demonstrated to play a crucial role in energy metabolism in that;

(i) it transports proline, the chief metabolic fuel (Bursell et al., 1974), from gut and fat body to the flight muscle where it is oxidised for energy release.

(ii) It acts as a "proline buffer" in that its level remains fairly constant even when

flight muscle level is depleted (Bursell, 1963).

(iii) In females the haemolymph transfers lipids from the fat body to uterine gland for "tsetse milk" production (Langley et al., 1981). The milk is necessary for the nourishment of the larva in utero.

In recent studies, it has been demostrated that the tsetse gut is quite permeable and can allow relatively large molecules such as Fab and Fc fragments of immunoglobulins (Nogge and Gianetti, 1979, 1980; Nogge, 1982) to cross into haemolymph. Flies that were previously fed on human blood died within two hours after feeding on albumin-free antiserum raised against the human albumin (Nogge, 1982). In an earlier investigation, it was observed that when antibodies raised against muscle or nerve tissues of the flesh fly, Sarcophaga falculata, were fed to the flies they were found to be attached specifically to the tissue that had served as antigen (Schlein et al., 1976). These antibodies were found to interfere with the growth and functions of these tissues (Schlein and Lewis, 1976). This observation shows that haemolymph proteins of haematophagous insect such as the Glossina raises the possibility of being targeted as potential insecticides once specific antibodies are raised against such proteins in the host animal. The impact

of such an approach would depend on the importance of the haemolymph protein in question. Therefore, before embarking on such an approach it is important to carry out a thorough study of the properties and functions of such haemolymph proteins.

1.5 AIMS OF THE STUDY:

Like in other insects studied, tsetse haemolymph contains a variety of lipoproteins among them lipophorin (Ochanda et al., Unpublished observation). Present among the other haemolymph proteins is a low molecular weight protein. Little effort has been directed towards the study of Glossina haemolymph proteins despite its great economic importance.

The aim of this project was:

(1) to isolate the low molecular weight protein from the haemolymph of adult male tsetse fly, Glossina morsitans morsitans.

(2) carry out physical and chemical characterisation of the isolated protein and

(3) raise antibodies against the isolated protein so as to test for cross-reactivity among haemolymph proteins from other insect species.

CHAPTER 2

2. MATERIALS AND METHODS:

2:1 Animals: (Insects):

The insects used in this study were supplied by Insect Mass Rearing Technology of the International Centre for Insect Physiology and Ecology (ICIPE). The insects were fed on rabbit blood at least two hours before haemolymph collection.

2:2 Collection of Haemolymph:

Insects were immobilised by chilling at 4°C for 15 minutes. A hind leg was cut at the coxa and then the thorax and abdomen were squeezed. The exuding haemolymph was collected using a microcapillary tube. After bleeding about ten insects the haemolymph was transfered into an Eppendorf tube that contained the bleeding solution [50 mM glutathione, 5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM aprotinin and a few crystals of phenylthiourea (PTU) in phosphate buffered saline (PBS), pH 7.0]. Haemolymph from mature third instar larvae was collected by puncturing the tegument with a sharp needle and exudate collected in a microcapillary tube. Pupal haemolymph was released by piercing the tegument with a sharp needle then squeezing the pupal case gently and the clear exudate collected into a micro-capillary tube.

The pooled haemolymph was centrifuged (5000 x g,

10 minutes, 4°C) in a Microfuge (Beckman) to remove hemocytes.

2:3 PURIFICATION:

2:3:1 Ultracentrifugation:

Ultracentrifugation was performed on a potassium bromide (KBr) density gradient (Haunerland et al., 1987). The haemolymph sample was dissolved into a 44%(w/v) KBr in PBS. After transferring to a 39 ml QuickSeal tube (Beckman), the sample was carefully overlayed with 33%(w/v) KBr in PBS and centrifuged (206,000 x g, 4 hr, 10°C) in a model L8-70 Beckman ultracentrifuge using a VTi-50 vertical rotor. After centrifugation, aliquots (2.0 ml) were carefully siphoned from top of the tube. From each fraction, 100 µl sample was drawn and dialysed against PBS. The samples were then run on a gradient (4-15%) SDS-PAGE. Fractions that contained the low molecular weight proteins were pooled and dialysed, then concentrated by using polyethylene glycol (PEG) at 4°C.

2:3:2 Gel permeation chromatography:

The low molecular weight protein was further purified by gel permeation chromatography. Sephadex G-75 was swelled for 24 hours at 4°C then packed into a column (1.6 cm, i.d, x 100 cm). The column was equilibrated with four bed volumes of PBS, pH 7.0 before sample loading. Fractions (2.0 ml) were collected and the absorbances measured at 280 nm using

a Unicam SP-1800 spectrophotometer (Beckman). Fractions that contained the low molecular weight protein were determined after SDS-PAGE on a 4-20% gradient. The purified low molecular weight protein obtained from adult male G. morsitans haemolymph will subsequently be abbreviated LMWP.

2:4 PHYSICAL AND CHEMICAL CHARACTERISATION:

2:4:1 Density determination:

The LMWP obtained as described under section 2:3 was used for density determination as described under section 2:3:1. In this experiment, centrifugation was carried out at 206,000 x g for 6 hours at 10°C. Fractions (1.0 ml) were obtained from top of the tube and the absorbances read at 280 nm.

Refractive indices of the fractions were determined using a refratometer (Zeiss) and densities calculated from the equation.

$$D = 5.7888\ R.I. - 6.7177$$

(where D = density in g/ml and R.I. is the refractive index) (Pattnaik et al., 1979).

The density of the LMWP was estimated from a plot of protein and density profile versus fraction curve.

2:4:2 Electrophoresis:

2:4:2:1 Sodium dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was carried out according to Laemli (1970). Gradients were cast using a gradient maker

(BRL). The gels were layered with butanol/water (1:1). After polymerisation, butanol was washed off with distilled water and a stacking gel (3% acrylamide) cast on top of the resolving gels. All samples for SDS-PAGE were mixed with an equal volume of SDS-PAGE sample buffer [0.13 M Tris-HCl, pH 6.8 containing; 40%(w/v) SDS, 1%(w/v) β-mercapto-ethanol, 20%(w/v) glycerol and 0.002%(w/v) BromoPhenol Blue]. Samples were boiled in a water bath for 5 minutes then loaded onto the gel. Running buffer [25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS, pH 8.3] was used for separation of the proteins at 30 mA until the tracker dye reached the bottom of the gel.

After electrophoresis, gels were stained for proteins with Coomassie Brilliant Blue G-250 [0.6%(w/v) in glacial acetic acid/methanol/distilled water (9.2:50:40.8)]. Destaining was done by soaking gels in acetic acid/methanol/distilled water (9.2:50:40.8). The gels were preserved in 7%(v/v) acetic acid.

2:4:2:2 Non-Denaturing PAGE:

Electrophoresis under non-denaturing conditions was carried out as described for SDS-PAGE (section 2:4:2:1) except that buffers did not contain SDS and β-mercaptoethanol. The samples were also not heated.

2:4:3 Molecular weight estimation:

Both SDS-PAGE and native-PAGE were used to determine the molecular weight of the LMWP. For SDS-

PAGE molecular weight estimation, the Pharmacia markers used were : Phosphorylase (M_r=94,000), Albumin (M_r=67,000), Ovalbumin (M_r=43,000), Carbonic anyhydrase (M_r=30,000), Trypsin inhibitor (M_r=20,000) and α-lactalbumin (M_r=14,400). For native molecular weight estimation, Pharmacia high molecular weight markers were used: Thyroglobulin (M_r=669,000), Ferritin (M_r=440,000), Catalase (M_r=232,000), Lactate dehydrogenase (M_r=140,000) and BSA (M_r=67,000).

Marker proteins were loaded in the wells adjacent to the LMWP. To estimate the molecular weights, standard curves for both SDS and native-PAGE were constructed from plots of log molecular weight versus relative mobilities of the protein markers. The molecular weight of the LMWP was read from the standard curve.

2:4:4 Determination of Covalently Bound Carbohydrate in Protein:

2.4.4.1 Staining for carbohydrates in gels:

Staining of gels for covalently-bound carbohydrates was carried out according to Kapitany and Zebrowski (1973).

Samples were first separated by SDS-PAGE. Before staining, gels were fixed in 12.5%(w/v) TCA for 1 hour followed by soaking in 1%(w/v) periodate for 2 hours in dark and then washed extensively with 15%(v/v) acetic acid. Destaining was carried out in 7%(v/v) acetic

acid.

2.4.4.2 Affinity chromatography of Concanavalin A-Sepharose (Con A-Sepharose) Column:

Affinity chromatography was performed using Con A-Sepharose (Pharmcia) column (1.0 cm, i.d, x 10 cm). The column was equilibrated with Con A buffer [0.01 M Tris-HCl, pH 7.5 containing 1 mM $MgCl_2$, 1 mM $CaCl_2$] and elution carried out first using Con A-buffer then 500 mM α-methyl-D-mannopyranoside in Con A-buffer. The column was run at room temperature at a flow rate of 0.14 ml/min. Fractions (1.0 ml) were collected and absorbances measured at 280 nm.

2.4.5 Staining for lipid in gel:

Staining for lipoproteins in gels was carried out according to Narayan (1975). Protein samples were separated by SDS-PAGE (4-15%) then stained by soaking in Sudan Black B solution overnight. Destaining was carried out in acetone/acetic acid/distilled water (3:4:13).

2.4.6 Protein determination:

Protein determination was carried out according to Lowry et al. (1951). To 0.6 ml protein sample appropriately diluted, 3.0 ml of solution A (prepared by mixing 0.5 ml of 1% $CuSO_4$, 0.5 ml 2% Na-K tartarate with 49 ml 2% Na_2CO_3 in 0.1 M NaOH) was added. The solution was allowed to stand for 10 minutes. Then 0.3 ml of solution B [Folin Phenol reagent diluted 1:3 with

distilled water] was added and mixed. The solutions were stored in the dark for 30 minutes and the absorbances measured at 750 nm in a Unicam SP-1800 spectrophotometer (Beckman). Blanks were prepared using 0.6 ml of distilled water run through the same procedure.

From the values obtained using BSA solutions ranging from 0-100 µg a protein standard curve was constructed. Protein concentration in the sample was determined from the standard curve.

2.4.7 Isoelectric point determination:

The isoelectric point of the LMWP was determined on a Pharmacia PhastSystem using commercially prepared PhastGel (3-9%). Pharmacia broad pI calibration kit (pH range 3.50-9.3) was used.

The process involved three steps:

(i) prefocusing: Carried out at 2000 V, 2.5 mA, 3.5 W at 15°C for 75 volt hours. In this step, the Pharmalyte carrier ampholytes in the Phast IEF media were focused to generate a stable linear pH gradient (3.50-9.30).

(ii) Sample application and running:

Sample application was carried out at 200 V, 2.5 mA, 3.5 W at 15°C for 15 volt hours to avoid streaking of the protein while running was carried out at 2000 V, 2.5 mA, 3.5 W at 15°C for 410 volt hours.

During this step sample protein migrated to their isoelectric points on the gel.

(iii) Development:

In this step, the gel was stained using PhastGel Blue R and destained for visualization. The gel was then air dried. To estimate the pI of the LMWP, a standard curve of pH versus migration from cathode of the marker proteins was plotted. The pI of the protein was read from the curve.

2:5 AMINO ACID ANALYSIS:

LMWP sample (1.5 mg) was dialysed extensively in distilled water, then lyophilized. The lyophilized sample was used for amino acid analysis. Hydrolysis of the protein sample was carried out in vacuo in 6 N HCl for 12, 24 and 48 hours after when it was analysed by ion exchange system in a Beckman model 7300 amino acid analyser according to manufacturers' instructions.

2.6 IN VITRO PROTEIN SYNTHESIS IN TSETSE FLY FAT BODY:

2:6:1 Isolation of fat body:

Before dissection, insects were immobilised by chilling them at $4^{o}C$ for 30 minutes. The legs and wings were cut off using fine pair of scissors and the insect was placed in a dissection dish containing lepidopteran saline [0.05 M K_2HPO_4; 0.1 M KCl; 0.004 M NaCl; 0.015 M $MgCl_2$; 0.004 M $CaCl_2$, pH 6.5 containing 1%(w/v) sucrose] (Jungries et al., 1973).

The abdomens were opened with a single median ventral incision. After separating the gut and glands, the fat body was removed and rinsed twice in ice-cold saline, then pooled in an Epperdorf tube with saline maintained on ice. A total of 50 flies were dissected for this study. The fat body was then transferred into another Eppendorf tube containing 200 µl of saline for labelling purpose.

2:6:2 In Vitro labelling :

To the fat body in the incubation vial, 300 µCi L-[^{35}S] methionine (specific activity = 800 Ci/mmole, Amersham, England, U.K.) and 1.1 µCi L-[^{14}C] leucine (specific activity 348 mCi/mmole, Amersham, England, U.K.) were added. Incubation was carried out for 14 hours at room temperature with constant shaking. The incubation mixture was then transferred into an ice cold glass homogenizer containing 3 mM PMSF and homogenized with a teflon pestle. The homogenate was transferred into a clean Epperdorf tube and centrifuged (10,000 x g, 15min, 4^{o}C). The supernatant was carefully siphoned out and re-centrifuged three times until a clear supernatant solution devoid of fat was obtained.

The supernatant solution obtained was concentrated to 50 µl, mixed with an equal volume of SDS-PAGE sample buffer then boiled for 2 minutes. SDS-PAGE of the sample was performed as described in section 2:4:2:1.

LMWP sample was loaded adjacent to the fat body extract.

After staining and destaining the gel, radioactivity signal was enhanced by incubating the gel in EN^{3}HANCE (Amersham) for 30 minutes at room temperature. The gel was then dried in a GSD-4 slab gel drier (Pharmacia) under vacuum for 2½ hours at 24 volts constant heating. The dried gel was then placed in an x-ray cassette with an intensifying screen and exposed to an X-ray film (Fuji-Rx, 18 cm x 24 cm) for 14 days at -70°C. The film was developed by soaking it for five minutes in developer (Kodak) then washing it in water (2 min) followed by soaking in fixer (Kodak) for 5 minutes. The film was then washed in water (2 min) and dried in air for visualization.

2:7 IMMUNOLOGICAL STUDIES:

2:7:1 Raising antibodies against the LMWP:

Antibodies against the LMWP were raised in a young male New Zealand White rabbit. A primer dose (1.0 mg protein) in 1 ml PBS was emulsified in an equal volume of Freund's Complete adjuvant was administered subcutaneously at different sites. A booster injection (200 µg protein) in 0.5 ml PBS in incomplete Freund's adjuvant was administered intramascularly four weeks later. The rabbit was bled after 2 weeks through the main ear artery. To obtain the antiserum, the blood was left to stand at room temperature for 1 hour and

then kept overnight at 4°C. The serum was then separated from the clots by centrifugation (1000 x g, 30 min) and 0.1%(w/v) sodium azide added to the supernatant as an antibacterial agent. The antiserum was stored at -20°C.

2:7:2 Double radial immunodiffusions:

Double radial immunodiffusion was carried out as described by Ouchterlony (1968). Molten agarose (1% (w/v) in PBS) was carefully poured onto a glass plate (10 cm x 10 cm) on a level stand and left to set. Six wells were punched peripherally around a central one. To check for the presence of antibodies against LMWP (the antigen), antiserum was placed in the central well and the antigen in the peripheral wells. Diffusion was allowed to take place in a humid chamber at room temperature for at least 24 hours. The plates were washed extensively with PBS to remove unprecipitated proteins on gel, then dried by blotting with 3 MM filter papers. The gel was then stained and destained as described in section 2:4:2:1.

2:7:3 Immunoblots:

To check for immunological reactivity of the antiserum raised against the LMWP, haemolymph from various insect species were used in immunoblotting experiments (Towbin et al., 1979; Burnette, 1981).

Samples were separated by SDS-PAGE and electrophoretically transferred (50 V, 4 hr, 4°C) onto

a nitrocellulose paper (Schleicher and Schuell) in transfer buffer [20 mM Tris, 150 mM Glycine, 20%(v/v) methanol]. The success of the transfer was ascertained by staining with Rouge Ponceau [0.05%(w/v) in 3%(w/v) TCA]. The paper was destained by washing in distilled water. Non-specific binding sites on the paper were blocked by using 5%(w/v) fat-free milk powder in Tris buffered saline (TBS) [20 mM Tris, 500 mM NaCl, pH 7.5]. The nitrocellulose paper was then incubated with antiserum (diluted x 200 with TBS-Milk-1%) overnight. To wash off the unbound antibodies on the nitrocellulose paper, TBS-Milk-5% followed by TBS was used. Horse radish peroxidase-labelled goat anti-IgG (Sigma) (diluted x 1000 in TBS-Milk-1%) was used to locate the bound antibody. Unbound secondary antibody was washed off with TBS-Milk-5% and Tris-HCl buffer (TB) [1 mM Tris, pH 6.8]. The nitrocellulose paper was then incubated in 0.3%(w/v) 4-chloro-1-napthol in methanol (diluted x 5 with TB) and colour developed by addition of 2 µl aliqouts of H_2O_2 into the solution until satisfactory colour development was achieved. The paper was then dried and preserved by keeping it between filter papers in the dark.

CHAPTER 3

RESULTS:

3:1 PURIFICATION OF THE LOW MOLECULAR WEIGHT PROTEIN:

The purification of the low molecular weight protein from haemolymph of tsetse fly G.m. morsitans was achieved by a two step procedure.

The initial step involved potassium bromide (KBr) density gradient ultracentrifugation in a vertical tube rotor using a Beckman model L8-70 ultracentrifuge. A density gradient 1.21-1.30 g/ml was achieved. Under these conditions, the low molecular weight fractions remained in the sub-phase while lipophorin floated at the top (Fig 1 a,b). The subphase fractions were then applied to a gel chromatography column. Two major peaks (A and B) resulted (Fig 2). On SDS-PAGE, the low molecular weight fraction was in the last half of peak B. Peak A fractions contained high molecular weight proteins.

3:2 PHYSICAL AND CHEMICAL PROPERTIES OF THE LOW MOLECULAR WEIGHT PROTEIN:

3:2:1 Molecular weight and density estimation:

On SDS-PAGE (4-20%), the LMWP was estimated at 23,000 daltons against low molecular weight markers (Pharmacia) standard curve (Fig. 3a,b).

The density of the LMWP was determined in a KBr density gradient (1.22 - 1.30 g/ml). In this system, the protein had a density of 1.29 g/ml (Fig. 4).

3:2:2 Determination of Carbohydrate and lipid moeities in the LMWP

The LMWP neither bound Concanavalin A-Sepharose affinity column (Fig. 8) nor stained for carbohydrate with periodic acid Schiff's stain (Fig. 6). However, the presence of lipids in the protein was confirmed by staining with Sudan Black (Fig. 7). The light staining observed for the LMWP suggested a low lipid content.

3:2:3 Determination of Isoelectric point and amino acid composition

The isoelectric point of LMWP was estimated to be 4.6 on a PhastSystem using PhastGel IEF medium (Fig. 5 a,b). The amino acid composition of the LMWP (Table 2) reveals a predorminace of acidic amino acids, aspartate (13%) and glutamate (16%). A high proportion of neutral amino acids, glycine (10%) and alanine (7%) was observed. The protein contained relatively high levels of the aromatic amino acids phenylalanine (5%), tyrosine (2.43%) and histidine (3.14%) but only trace levels of methionine (0.75%). The absence of cysteine indicated the absence of disulphide linkages in the protein.

Fig 1 : Protein profile:

After density gradient ultracentrifugation fractions (2.0 ml) were collected from top and protein samples from each aliquot run on SDS-PAGE (4-15%). Lipophorin (Lane 1) floated on top of the gradient, as revealed by the presence of the two apoproteins I and II. The low molecular weight proteins appears in the lower phase of the tube (Fig. 1, b).

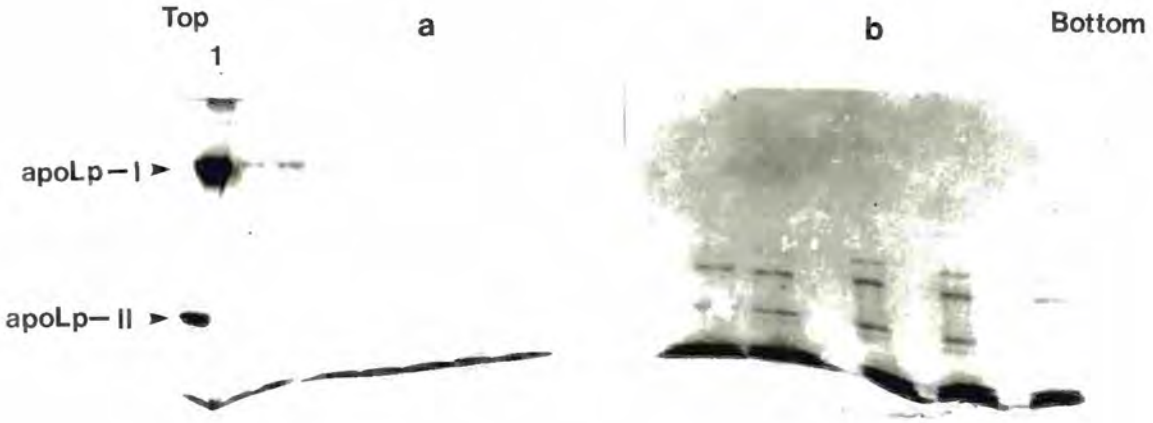

Top
a
b
Bottom
1
apoLp–I
apoLp–II

Fig 2 : Gel permeation chromatography:

The subphase obtained from ultracentrifugation was dialysed against PBS and concentrated to a volume of 3.0 ml. The sample was applied onto a Sephadex G-75 gel column (1.6 cm, i.d, x 100 cm), equilibrated and eluted with PBS. The LMWP eluted in peak B.

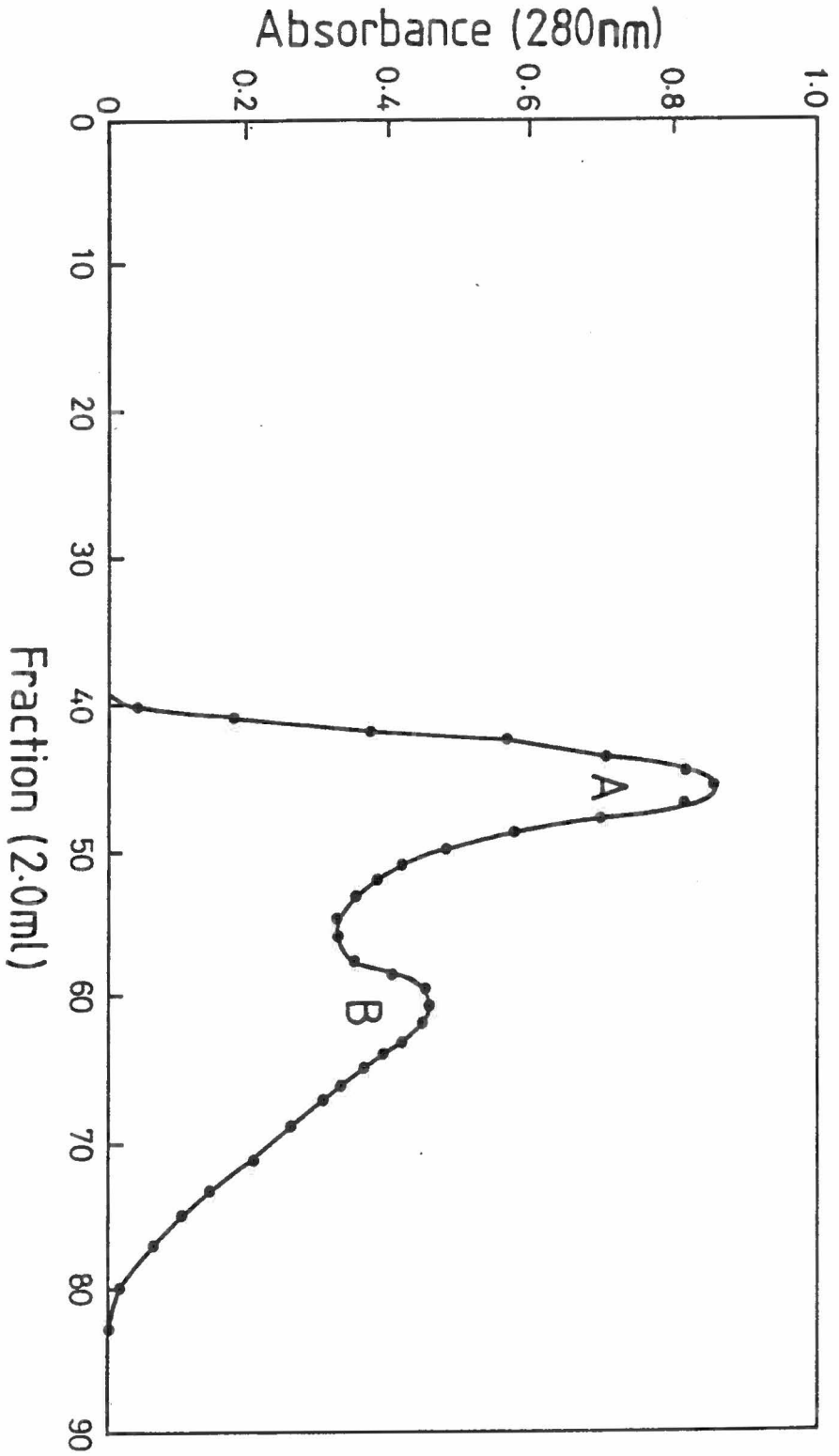

Absorbance (280nm)
0
0.2
0.4
0.6
0.8
1.0
Fraction (2.0ml)
0
10
20
30
40
50
60
70
80
90
A
B

Fig 3: Molecular weight estimation on SDS-PAGE:

(a) Samples were subjected to SDS-PAGE (4-20%) until the tracker dye reached the bottom of gel.

Lane 1. Molecular weight markers (Pharmacia).

2. Purified low molecular weight protein (35 μg)

3. Adult male G.m morsitans haemolymph (35 μg)

(b) SDS-PAGE standard curve molecular weight markers were:

1. Phosphorylase (M_r= 94,000)
2. Albumin (67,000)
3. Ovalbumin (43,000)
4. Carbonic anyhydrase (30,000)
5. Trypsin inhibitor (20,100)
6. α-lactalbumin (14,400)

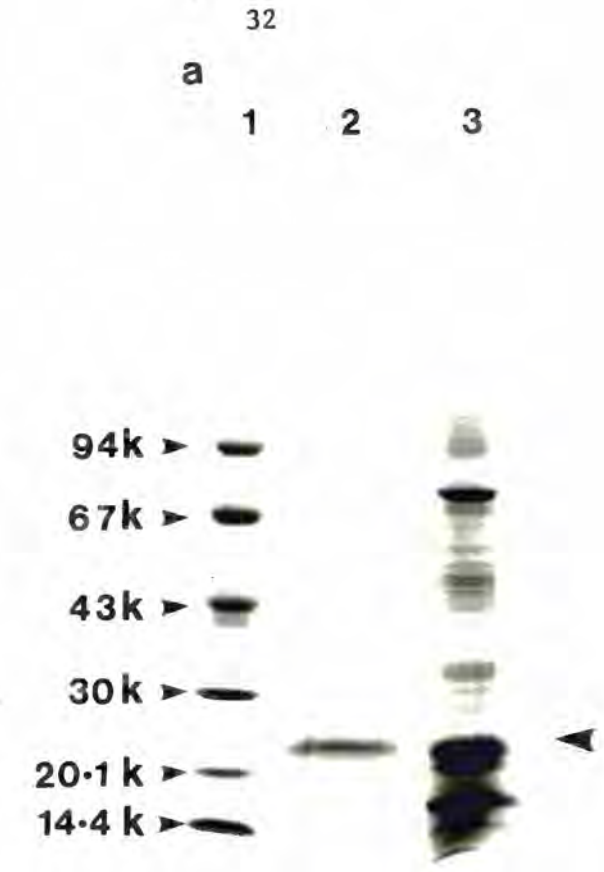

a
1
2
3
94k
67k
43k
30k
20·1 k
14·4 k
b
Log molecular weight
Relative mobility
5·0
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
4·0
0
0·2
0·4
0·6
0·8
1·0

Fig. 4 : Density gradient equilibration:

The LMWP isolated by gel permeation chromatography was centrfuged (206,000 x g, 6 hr, 10°C) in KBr density gradient. Aliquots (1.0 ml) were removed from top of the tube. The absorbance of each fraction was read at 280 nm and density calculated from their refractive index. The density of of the LMWP was estimated to be 1.29 g/ml by extrapolation.

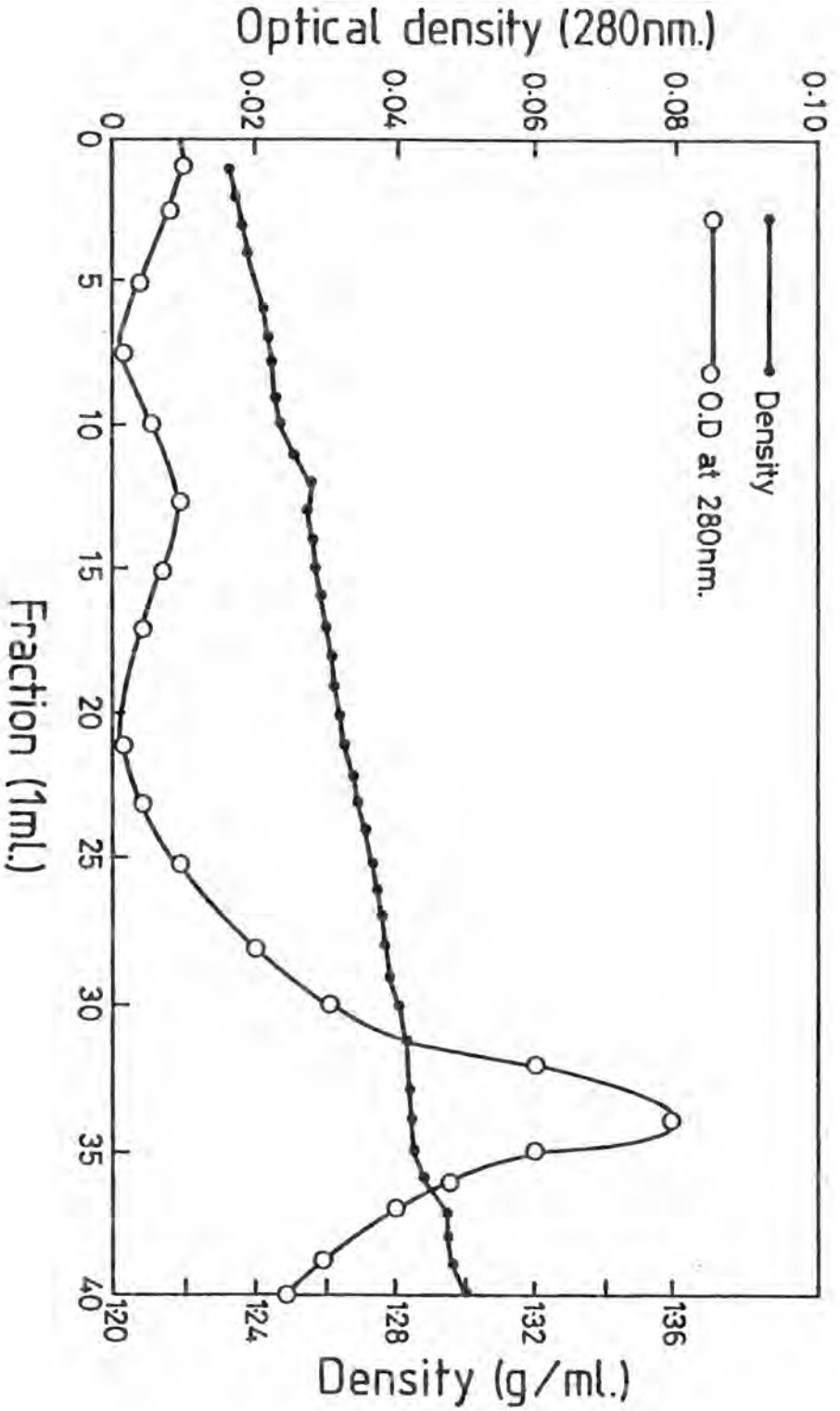

Optical density (280nm.)
0·10
0·08
0·06
0·04
0·02
0
Density
O.D at 280nm.
0
5
10
15
20
25
30
35
40
Fraction (1ml.)
120
124
128
132
136
Density (g/ml.)

Fig. 5 : Isoelectric point determination:

a) The pI of the low molecular weight protein was determined using a PhastSystem (Pharmacia) using commercially prepared PhastGel IEF medium (3-9%). Determination was done according to manufacturers instructions. Broad pI calibration kit (Pharmacia) containing 10 markers of pI range 3.5-9.3 was used.

Lane 1 and 4, pI markers.

Lane 2 and 3, Low molecular weight protein from tsetse fly haemolymph.

a) pI standard curve. pI markers were:

1, Lentil lectin (basic) (pI=8.65)

2, Lentil lectin (middle) (8.45)

3, Lentil lectin (acidic) (8.15)

4, Horse myoglobin (basic) (7.35)

5, Horse myoglobin (6.85)

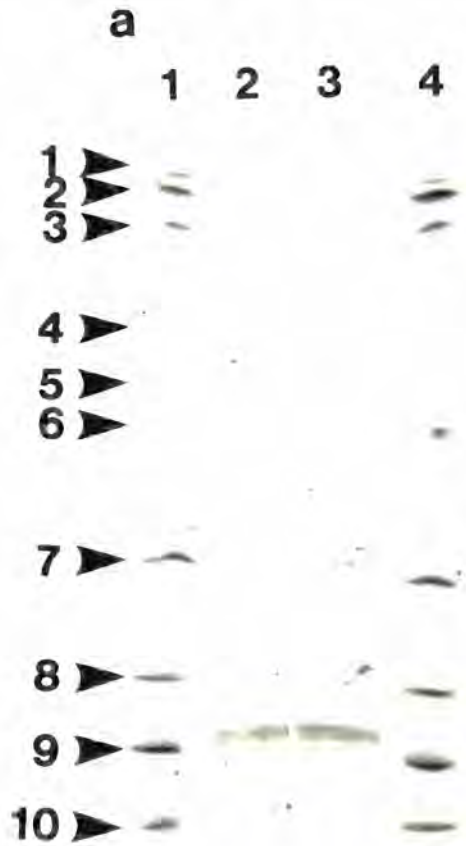
6, Human carbonic anyhydrase B (6.55)

7, Bovine carbonic anyhydrase (5.85)

8, α-lactoglobulin-A (5.20)

9, Soyabean trypsin inhibitor (4.55)

10, Amyloglucosidase (3.50).


a
1 2 3 4
1
2
3
4
5
6
7
8
9
10

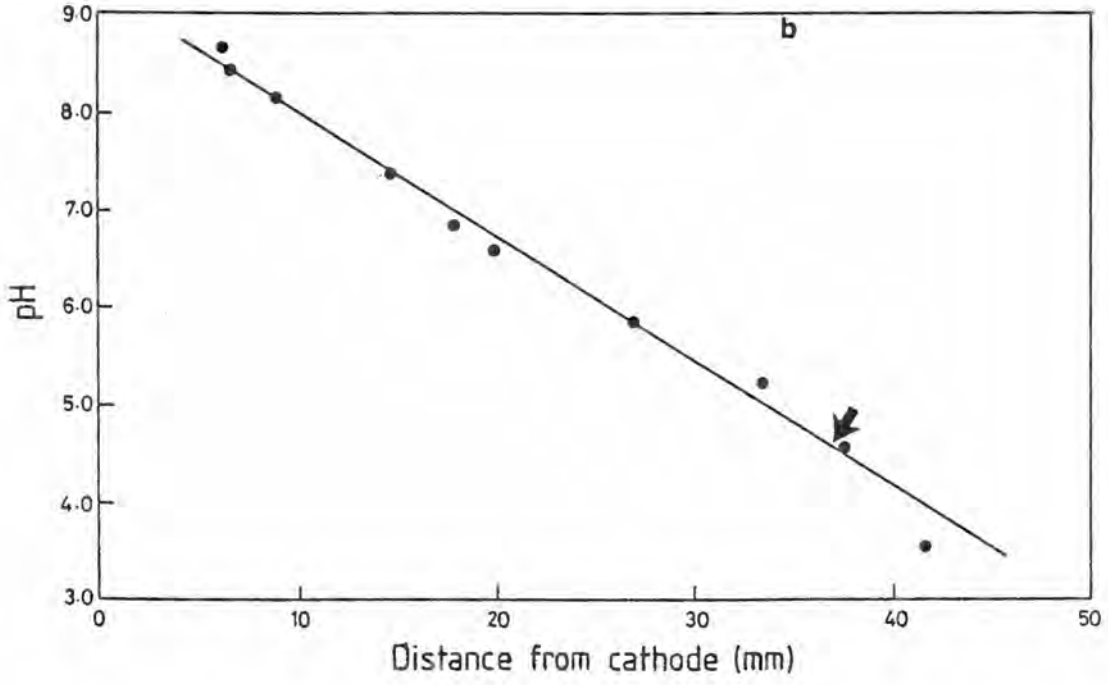

b
pH
9·0
8·0
7·0
6·0
5·0
4·0
3·0
0
10
20
30
40
50
Distance from cathode (mm)

TABLE 2

AMINO ACID COMPOSITION OF THE LOW MOLECULAR WEIGHT PROTEIN FROM HEMOLYMPH OF TSETSE FLY GLOSSINA MORSITANS MORSITANS

Amino acid	Mole %
Aspartic acid[b]	13.43
Glutamic acid[b]	16.14
Serine	11.26
Glycine	10.26
Histidine	3.14
Argininine	1.24
Threonine	4.25
Alanine	6.82
Proline	3.62
Tyrosine	2.43
Valine	2.96
Methionine	0.75
Isoleucine	2.13
Leucine	5.62
Phenylanine	5.09
Lysine	10.53
Total	99.97

Samples were hydrolysed for 12, 24 and 48 hours then analysed by ion exchange in a Beckman model 7300 amino acid analyser.

[b]Includes acid and amide

a - Taken from Dilwith and Chippendale (1984). Protein hydrolysed in 6 N HCl than subjected to cation exchange chromatography. Aspartate and glutamate may be present as their amides. Tryptophan was not determined.

b - Taken from Kawooya and Law (1983) Duplicate samples were hydrolysed in 6 N HCl at 110°C *in Vacuo* for 24, 48 and 72 hr. Cysteine and cystine were determined as cysteic acid after perfomic acid oxidation. Tryptophan was determined by amino acid analysis preceded by mild hydrolysis in 3 N mercaptoethane sulfonic acid (22 hr, 110°).

c - Taken from Wells *et al* (1985). Duplicate samples hydrolysed for 24, 48 and 72 hr in 6 N HCl *in vacuo*, 110°C.

d - Taken from Kawooya *et al* (1984). Duplicate samples hydrolysed for 24 hr in 3 N mercaptoethane sulfonic acid *in vacuo*, 110°C.

TABLE 3

AMINO ACID COMPOSITION OF SOME LOW MOLECULAR WEIGHT HAEMOLYMPH PROTEINS (FOR COMPARISON PURPPOSES)

NB : THE DATA IS GIVEN (OR WORKED OUT) AS RESIDUES PER MOLECULE

AMINO ACID	DAP FROM FAT BODY OF *D. grandiosella*[a]	MICROVITELLOGENIN FROM *M. SEXTA*[b] HAEMOLYMPH	APOLIPOPHORIN-III FROM HAEMOLYMPH OF		THE LMWP FROM *G.M. MORSITANS*
			T.acutangulus[c]	*M.Sexta*[d]	
	M_r = 35,000	M_r = 32,000	M_r = 20,000	M_r =17,000	M_r = 23,000
Aspartate	40	43	24	19	27
Threonine	20	11	7	8	10
Serine	12	20	10	13	30
Glutamate	32	26	30	31	29
Proline	8	8	4	2	9
Glycine	12	23	9	6	41
Alanine	12	23	17	24	22
Cysteine	4	1	0	0	0
Valine	16	20	15	10	7
Methionine	4	7	2	2	1
Isoleucine	12	17	9	2	4
Leucine	32	21	16	11	11
Tyrosine	24	12	2	1	3
Phenylalanine	12	11	9	8	8
Lysine	32	15	29	22	19
Histidine	12	7	4	4	5
Arginine	12	20	4	2	2
Tryptophan	ND	5	0	0	0
Total	296	290	191	165	231

Fig 6: Staining for Carbohydrates:

SDS-PAGE (4-15%)
Stained with Periodic Acid
Schiff Stain.

Lane 1. G.m. morsitans haemolymph (50 µg)

2. Low molecular weight purified from G.m. morsitans (50 µg) (position shown by arrow).

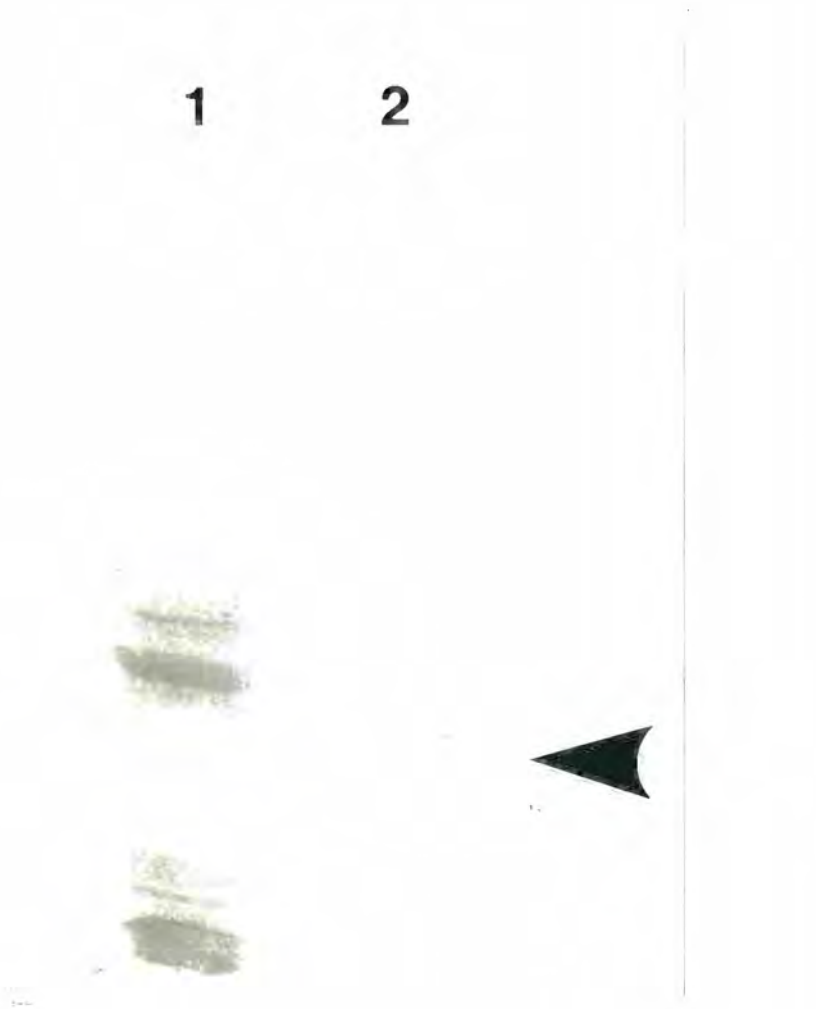

1
2

Fig 7 : Staining for Lipids:

SDS-PAGE (4-15%)

Stained with Sudan Black B stain.

Lane 1. G.m. morsitans hemolymph (20 µl)

2. LMWP (50 µg), arrow.

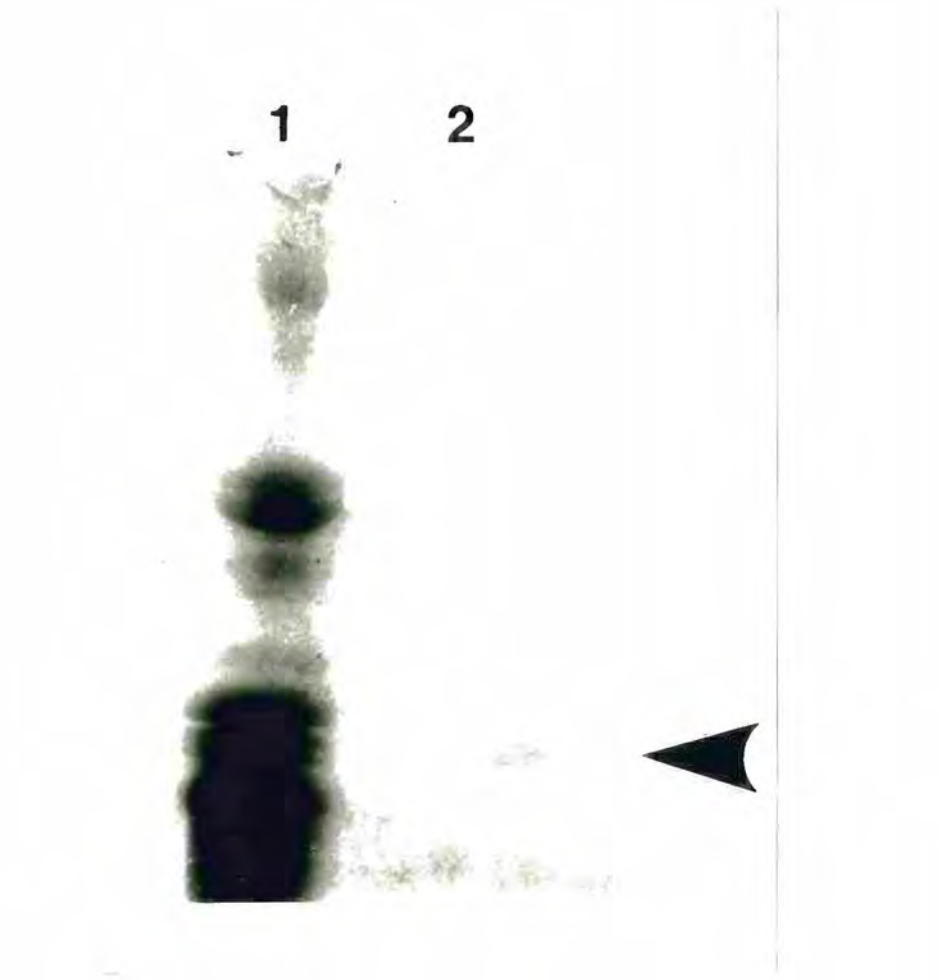
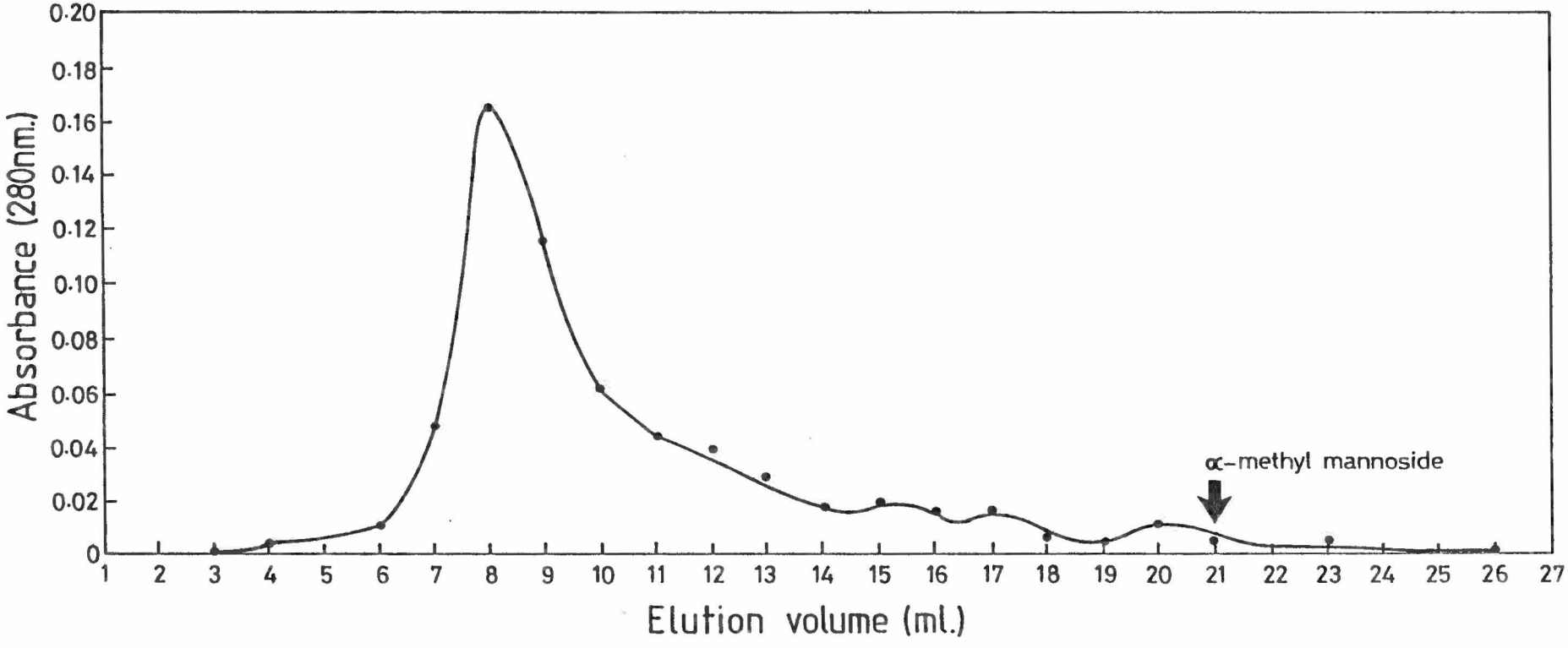

1
2

Fig 8 : Affinity chromatography:

Low molecular weight fraction obtained from gel filtration was concentrated and loaded onto Concanavalin A-Sepharose column (1.0 cm, i.d x 10 cm). The protein was eluted with both Con A-buffer and 500 mM methyl α-D-mannopyranoside in Con A buffer. Fractions (1.0 ml) were collected and O.D. read at 280 nm.


0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
Absorbance (280nm.)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Elution volume (ml.)
α-methyl mannoside

3:3 DE NOVO SYNTHESIS OF LOW MOLECULAR WEIGHT PROTEIN IN THE FAT BODY TISSUE OF G.m MORSITANS

The ability of the fat body tissue of G.m.morsitans to synthesize LMWP was studied by incubating the tissue with radiolabelled amino acids, L- [^{14}C] leucine and [^{35}S]-methionine. After incubation, the tissue was homogenised and centrifuged. The extract (supernatant) was separated by SDS-PAGE and the labelled proteins visualised by fluorography.

The result indicated that the fat body extract contained a number of labelled proteins of varied molecular weights. The LMWP was shown to be one of the proteins present in the extract. The labelled LMWP co-migrated with purified LMWP loaded in an adjacent well (Fig 9, arrow). This result shows that the LMWP is synthesized by the fat body then secreted into haemolymph.

Fig. 9 : Fluorogram of fat body synthesized proteins:

The fat body tissue from adult male G.m. morsitans was incubated in medium containing [^{35}S] methionine (300 µCi, specific activity 800 Ci/mMole) and L-[^{14}C] leucine (1.1 µCi, specific activity 348 mCi/mMole). The tissue was then homogenised and centrifuged to obtain a clear extract. After mixing with an equal volume of SDS-PAGE sample buffer, the sample was separated by SDS-PAGE (4-20%. The gel was dried and exposed to an X-ray film.

Lane 1 and 2, contain the total fat body extract. Amount in 2 is double that in lane 1.

Lane 3, was loaded with non-labelled LMWP from heamolymph. (Arrow shows the position of the protein).


1
2
3

3:4 IMMUNOCHEMISTRY OF THE LOW MOLECULAR WEIGHT PROTEIN:

3:4:1 Double radial immunodiffusion analysis:

The presence of antibodies against the low molecular weight protein was detected by double radial immunodiffusion (Ouchterlony, 1968). A high antibody titre was produced after a booster injection administered four weeks after primary dose (Fig 10A). The presence of a single precipitin band (Fig 10B) observed when haemolymph sample was reacted with antiserum against the LMWP showed the antigen to be pure.

Immunological relationship between LMWP and haemolymph proteins from other insect species was checked by double radial immunodiffusion. There was no precipitin band (Figures 11 and 12) with haemolymph from other species. The larval and pupal stage haemolymph from G.m. morsitans showed some weak precipitin bands (Fig 13).

3:4:2 Immunoblots analysis:

The immunological relationship between LMWP and haemolymph samples from six insect orders was checked by immunoblotting experiments.

The protein samples were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose, then reacted with rabbit sera against the LMWP. Horse radish peroxidase (HRP) conjugated goat anti-IgG

(Sigma) was used to locate the IgG bound proteins. Colour development was done by incubating the blot with 4-chloro-1-napthol and H_2O_2. Figures 14, 15, 16 and 17 shows the cross reactivity trend. A strong cross-reactivity with a protein in the haemolymph of Glossina pallidipes was observed (Fig 17). The protein showing the strong cross-reactivity is of similar electrophoretic mobility to the LMWP.

Figure 16 shows the results obtained when haemolymph samples from larval, pupal and adult female G.m. morsitans were incubated with anti-LMWP serum. The antibody detected protein of mobility very close to that of the LMWP (Fig 16). This corresponds to the deep band observed in female haemolymph and the upper band in larval haemolymph. In the blots where adult male G.m. morsitans haemolymph is analysed (ref. Fig 15 and 17), only one discrete band is observed. However, in blots where larval, pupal and female G.m. morsitans haemolymph is analysed more than one protein is detected. The fact that the protein displays some cross-reactivity means that they share antigenic determinants with the LMWP.

No cross-reactivity was detected in haemolymph of other species used in this study (Fig 15). From these results it is clear that the protein is found only in tsetse flies. The presence of LMWP in female G.m. morsitans (Fig 16) suggests that the protein is not

sex-specific.

The cross-reactivity trend observed in the haemolymph from larval, pupal and adult stages (Fig 16) suggests that the LMWP accumulates in adult tsetse fly and is only present in very low quantity in the other stages. Table 4 summarises the immunological properties of the LMWP.

Fig. 10 : Double radial immunodiffusion

A : Shows the presence of antibodies against LMWP (the antigen). Central well had antiserum (10 µl) while the peripheral wells contained LMWP.

well 1, 10 µg

4, 5 µg

B : Shows cross-reactivity against adult male G.m. morsitans haemolymph. Central well had antiserum (10 µl) while the peripheral wells contained haemolymph in decreasing amount as follows:

wells 1, 10 µl. 2, 7 µl. 3, 5 µl

4, 3 µl. 5, 2 µl. 6, 1 µl

A

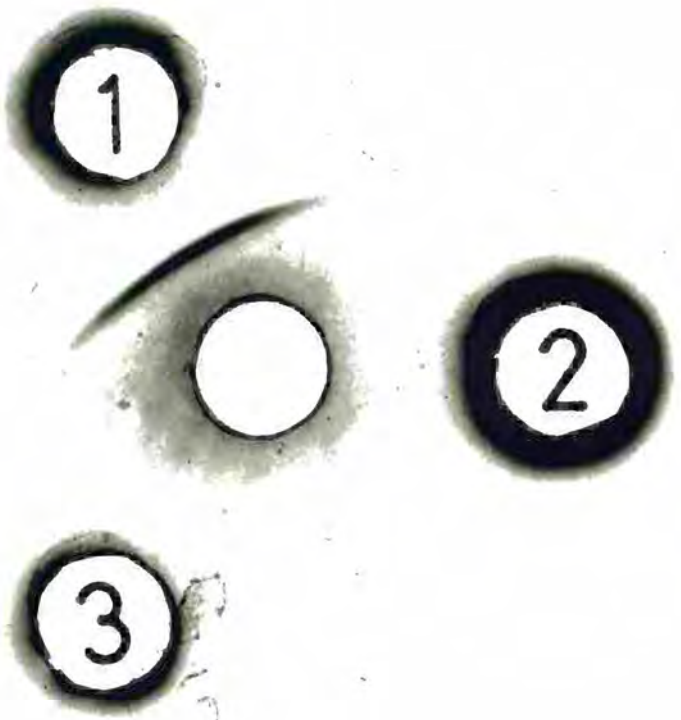
B

Fig. 11 : Double radial immunodiffusion of haemolymph samples from other insect species against anti-LMWP serum

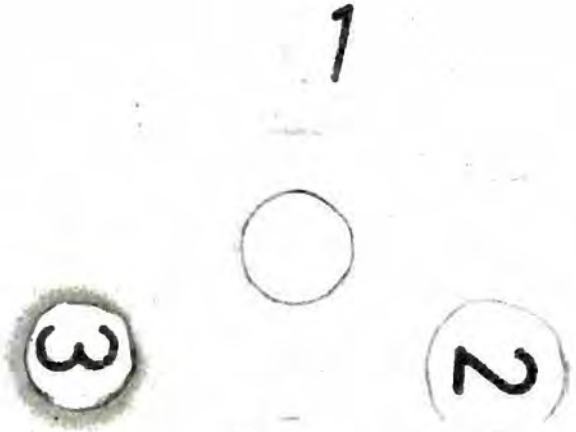
The central wells contained antiserum (10 µl) while the peripheral wells contained haemolymph samples from:

A. 1, Adult male G.m. morsitans (10 µl)
   2, Adult M. domestica (Diptera) (10 µl)
   3, Adult M. domestica (5 µl)

B. 1, Adult male G.m. morsitans (10 µl)
   2, Adult A. mellifera (Hymenoptera) (10 µl)
   3, Adult T. mollitor (Coleoptera) (10 µl)



A



B

Fig. 12 : Double radial immunodiffusion of haemolymph samples from other insect orders against anti-LMWP serum

The central wells contained antiserum (10 µl) while the peripheral wells contained haemolymph samples from:-

1, Adult male G.m. morsitans (Diptera) (10 µl)

2, Adult L. migratoria (Orthoptera) (10 µl)

3, Adult S. gregaria (Orthoptera) (10 µl)

4, Adult P. americana (Dictyoptera (10 µl)

5, 6th instar S. exempta (Lepidoptera) (10 µl)

6, 6th instar G. mellonella (Lepidoptera) (10 µl)

7, 6th instar C. partellus (Lepidoptera) (10 µl)

8, 6th instar B. fusca (Lepidoptera) (10 µl)

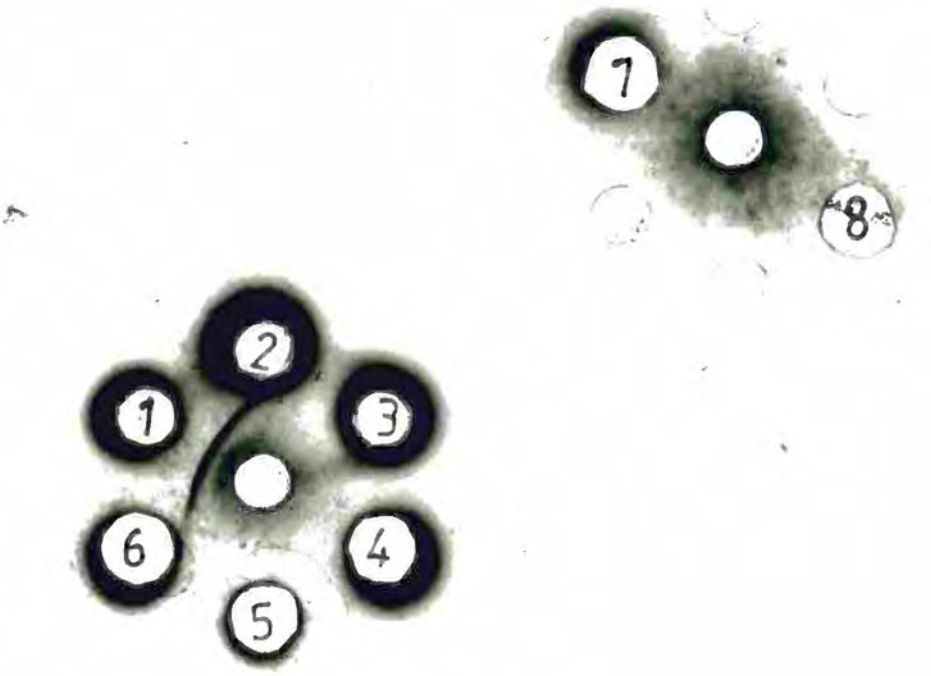

7
8
2
1
3
6
4
5

Fig. 13 : Double radial immunodiffusion of haemolymph samples from various developmental stages of the tsetse fly

The central well contained antiserum (10 µl) while peripheral wells contained haemolymph samples from:-

1, Adult male G.m. morsitans (6 µl
2, 3rd instar larvae G.m. morsitans (6 µl)
3, 2 day old pupae G.m. morsitans (7 µl).

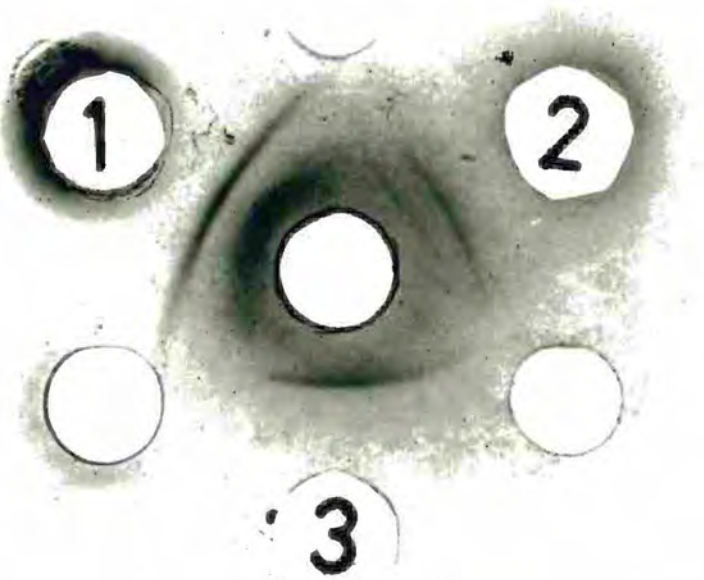

1
2
3

Fig. 14 : Immunoblot analysis of haemolymph samples from male and female tsetse fly against the anti-LMWP serum

Lanes 1, Haemolymph sample from adult male *G.m. morsitans* (30 µg)

2, LMWP (30 µg)

3, Haemolymph sample from adult female *G.m. morsitans* (30 µg)

4, Low molecular weight markers (Pharmacia).

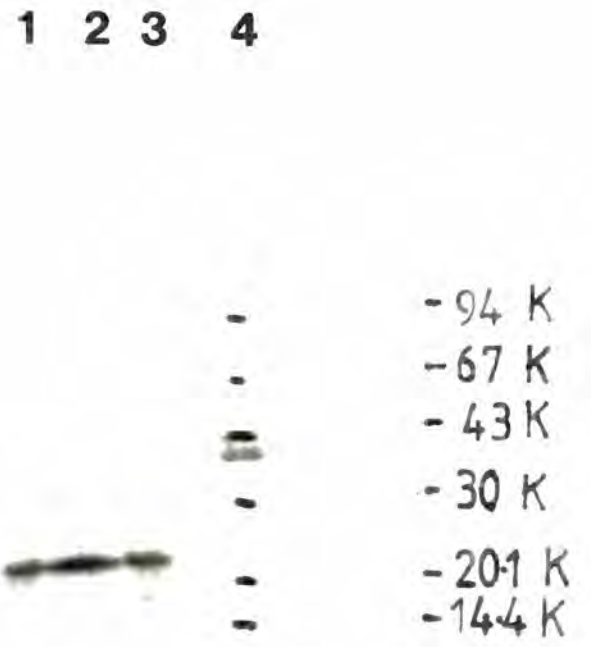

1 2 3 4
- 94 K
- 67 K
- 43 K
- 30 K
- 20·1 K
- 14·4 K

Fig. 15 : Immunoblot analysis of haemolymph samples from various insect orders against the anti-LMWP serum.

lanes contained haemolymph samples (40 µg protein) from:-

1, 6th instar larvae B. fusca (Lepidoptera)

2, 6th instar larvae G. mellonella (Lepidoptera)

3, 6th instar larvae C. partellus (Lepidoptera)

4, 6th instar larvae S. exempta (Lepidoptera)

5, Adult T. mollitor (Coleoptera)

6, Adult A. Mellifera (Hymenoptera)

7, Adult P. americana (Dictyoptera)

8, Adult S. gregaria (Orthoptera)

9, Adult L. migratoria (Orthoptera)

10, Adult M. domestica (Diptera)

11, Adult male G. m. morsitans and

12, Low molecular weight markers (Pharmacia).

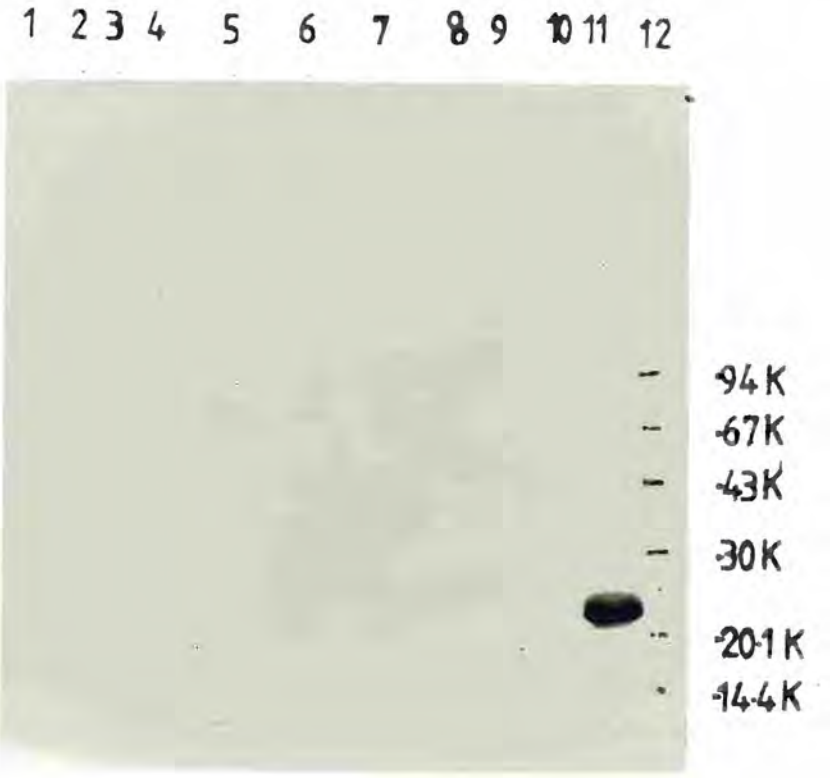

1 2 3 4 5 6 7 8 9 10 11 12
94K
67K
43K
30K
20·1K
14·4K

Fig. 16 : Immunoblot analysis of haemolymph samples from various developmental stages of the tsetse fly against the anti-LMWP serum

Lane contained haemolymph samples from:

1, Adult female G.m. morsitans (40 µg)

2, 2 day old pupal G.m morsitans (25 µg)

3, 3rd instar larval G.m. morsitans (25 µg)

4, LMWP (30 µg)

5, Low molecular weight markers (Pharmacia).

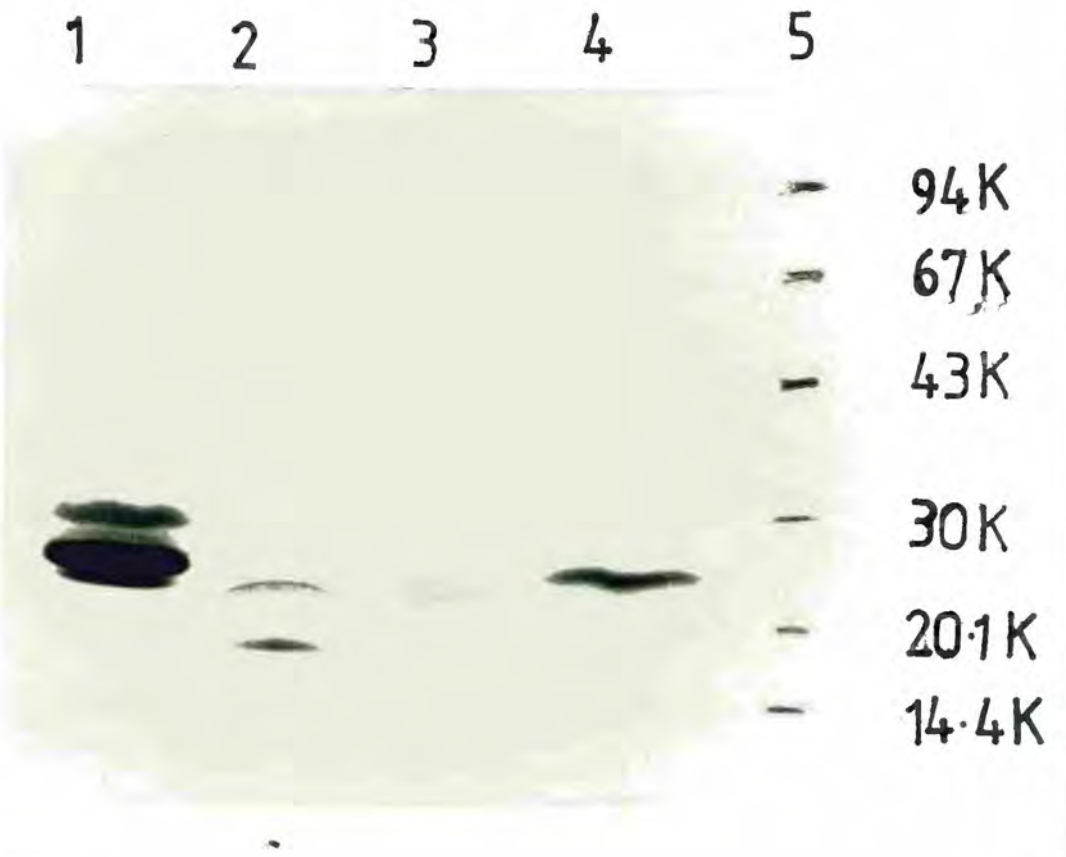

1
2
3
4
5
94K
67K
43K
30K
20·1K
14·4K

Fig. 17 : Immunoblot analysis of haemolymph sample from other tsetse fly species.

Lane

1, Adult male G.m. morsitans haemolymph (30 µg)

2, Adult Glossina pallidipes haemolymph (30 µg).


1
2

TABLE 4

CROSS REACTIVITY DETERMINATION BETWEEN ANTISERUM AGAINST LOW MOLECULAR-WEIGHT PROTEIN AND OTHER INSECT HAEMOLYPH PROTEINS

	Insect Species	Insect Order	Immunodiffusion test	Immunoblot test
1.	Purified low molecular weight protein from adult male Glossina Morsitans morsitans	Diptera	+	+
2.	Adult Male Glossina Morsitans morsitans	Diptera	+	+
3.	Adult female Glossina Morsitans morsitans	Diptera	ND	+
4.	Adult Locusta Migratoria	Orthoptera	-	-
5.	Adult Schistocerca gregaria	Orthoptera	-	-
6.	Busseola fusca (6th Instar larvae)	Lepidoptera	-	-
7.	Spodoptera exempta (6th instar larvae)	Lepidoptera	-	-
8.	Chillo partellus (6th instar larvae)	Lepidoptera	-	-
9.	Gallera mellonella (6th instar larvae)	Lepidoptera	-	-
10.	Adult Periplaneta americana	Dictyoptera	-	-
11.	Adult Tenebrio mollitor	Coleoptera	-	-
12.	Adult Apis mellifera	Hymenoptera	-	-
13.	Adult Glossina pallidipes	Diptera	ND	+
14.	Adult Musa domestica	Diptera	-	-
15.	Larval Glossina morsitans morsitans	Diptera	$+^{f}$	$+^{f}$
16.	Pupal Glossina morsitans morsitans	Diptera	$+^{f}$	$+^{f}$

\+ means strong precipitin line in immunodiffusion and strong band in immunoblot
\- Means negative
ND Not determined
$+^{f}$ means the precipitin band was faint

CHAPTER 4

DISCUSSION:

Lipoproteins constitute important components of insect haemolymph. The properties of haemolymph lipoproteins has been previously described (Wyatt and Pan, 1978; Engelman, 1979; Riddiford and Law, 1983; Chino, 1985).

To facilitate the study of the properties and functions of haemolymph proteins, purification is a necessary step. This was emphasised earlier by Wyatt and Pan (1978). To date various methods have been employed in purification of haemolymph lipoproteins notably the lipophorins. This has involved one or a combination of more than one method. Affinity chromatography was used by Gellissen and Emmerich (1980) in the purification of lipphorins from the haemolymph of locusts and Silkworms. Repeated gel filtration alone was used to purify lipophorin from L. migratoria haemolymph (Mwangi and Goldsworthy, 1977; Van der Horst et al., 1979). Peled and Tietz (1975) used a combination of cold ethanol precipitation and sucrose density gradient ultracentrifugation to isolate lipophorin from haemolymph of locust, L. migratoria. A technique involving specific precipitation and DEAE-Cellulose column chromatography has also been used in purification of insect lipophorins (Chino et al., 1969, 1981; Chino and Kitazawa, 1981). Density gradient

ultracentrifugation method has been utilized mostly in separation of high density lipoproteins (Pattnaik et al., 1979; Thomas, 1979).

Although several methods have been used in purification of haemolymph lipoproteins, preparative density gradient ultracentrifugation (Haunerland et al., 1987) has emerged as the method of choice. The method has further been boosted by the use of vertical rotors that are effective for density gradient ultracentrifugation, yielding separations in considerably less time than is possible with other rotors (Gregor, 1977; Wells and Brunk, 1979). This is because, compared to other types of rotors of same maximum radii, the vertical tube rotor has the highest minimum radius hence particles inside it experiences the greatest relative centrifugal force.

In this study a low molecular weight protein from haemolymph of tsetse fly, Glossina morsitans morsitans has been purified by a combination of KBr density gradient ultracentrifugation using a vertical tube rotor and gel permeation chromatography (section 3:1). From SDS-PAGE the protein appears homogenous.

The results (section 3:2:1) shows the LMWP to be of M_r=23,000 on SDS-PAGE. The molecular weight compares with that of antibacterial proteins, attacins (20-23 KD) found in the haemolymph of Hyalophora cecropia (Pye and Boman, 1977; Hultmark et al., 1983).

Kaaya et al. (1987) has reported the presence of proteins with properties similar to those of attacins in the haemolymph of the tsetse fly G.m morsitans. Other haemolymph proteins of comparable molecular weight to the LMWP is the heat shock protein (hsp) M_r=23,000 identified from Drosophila melanogaster haemolymph after exposure to high temperatures (Arshburner and Bonner, 1979; Petersen and Mitchell, 1985). A JHBP, M_r=25,000 was also reported from larval stages in plodia interpunctella (Ferkovich et al., 1975) and Galleria mellonella (Rudincka et al., 1979).

On the basis of their lipid content, insect lipoproteins can be classified according to their hydrated densities and this has been the basis of their separation by density gradient ultracentrifugation. The hydrated density of the LMWP was determined as 1.29 g/ml (section 3:2:1) by density gradient ultracentrifugation. This is within the range for very high density lipoprotein (Thomas, 1979). Another protein with comparable density is the chromoprotein (density 1.26 g/ml) isolated from Heliothis zea (Haunerland et al., 1987). A density of 1.29 g/ml has been reported for M. sexta vitellogenin (Osir et al., 1986) and B. fusca high molecular weight diapause associated protein (Osir et al., 1989). However, these are high molecular weight proteins with low lipid

contents. The very high density of the LMWP suggests that the protein has low lipid content. This conclusion is supported by the light staining obtained with Sudan Black (section 3:2:2).

The low molecular weight protein is lipidated but not glycosylated. Most haemolymph proteins have been found to have a lipid moety (Beenakkers et al., 1985; Shapiro et al., 1988). However, the amount of lipid associated with haemolymph proteins varies. Some haemolymph proteins lack lipids. The high molecular weight haemolymph proteins generally have high proportions of lipid making them either high density or low density lipoproteins. A lipid content as high as 50% has been observed with some low density lipophorins (Chino, 1985; Shapiro et al., 1988). A lipid content of 15.7% was reported for vitellogenin from the cockroach, Blatella germanica (Kunkel and Pan, 1976). Some haemolymph proteins exists in non-lipidated forms. A protein P4 (M_r=48,000) isolated from pupal haemolymph of diapausing H. cecropia following injection with non-pathogenic bacteria exists as a lipid-free polypeptide (Rasmuson and Boman, 1979). The low molecular weight female-specific protein, microvitellogenin (M_r=31,000) also lacks lipids. The storage proteins, arylphorins are found to posses non-covalently associated lipid 1-2% (Riddiford and Law, 1983; Ryan et al., 1984b; Kramer et al., 1980).

The LMWP was found to be non-glycosylated. The protein neither bound to Concanavalin A nor stained with PAS (section 3:2:2). Glycosylation is common to those high molecular weight proteins in insect haemolymph. Generally, both apoproteins of lipophorins are glycosylated and the oligosaccharides are of the high mannose type (Ryan et al., 1984a; Nagao et al., 1987). Apolipophorin-III, found in some adult insect species that rely on lipid as flight fuel (Ryan et al., 1984a) has been shown to be glycosylated only in some species. Apolipophorin-III is non-glycosylated in Thasus acutangulus (Wells et al., 1985), M. sexta (Kawooya et al., 1984). However, in G. africanus (Haunerland et al., 1986) and L. migratoria the protein is glycosylated. In L. migratoria the protein associates with very high (12.5%) carbohydrate content (Wheeler and Goldsworthy, 1983a,b; Van der Horst et al., 1984). A similar observation was reported for the P4 protein from immune haemolymph of diapausing H. cecropia. The protein (M_r=48,000) is glycosylated in M. sexta (Hulbert et al., 1985) but not in H. crecropia (Anderson and Steiner, 1987). The presence of high mannose oligosaccharide chains in glycosylated haemolymph proteins has also been exploited for purification purposes (Ryan et al., 1986, 1989; Kawooya and Law, 1983). High mannose oligosaccharides binds to concanavalin A (Baenziger and Fiete, 1979).

The function of carbohydrate moeties found in haemolymph proteins has not been clearly established but a recognition role either at secretory site or at the metabolic site has been suggested (Beenakkers et al., 1985). Osir et al (1986), showed that de-glycosylation of M. sexta vitellogenin did not affect its uptake by oocytes.

Amino acid composition analysis of the LMWP revealed the predorminance of acidic amino acids, glutamate and aspartate (section 3:2:3). The protein also has a high content of the neutral amino acids, glycine and alanine. A high content of basic amino acid, lysine was observed. Although the protein showed a relatively high amount of the aromatic amino acids, the amount is less than that of arylphorins (Telfer et al., 1983) as well as other storage proteins (Levenbook, 1985). Any haemolymph protein found to have exceptionally high content of aromatic amino acid is basically categorised as a storage protein (Tojo et al., 1978; Telfer et al., 1983). Amino acid composition from few other low molecular weight proteins is included (Table 3) for comparison. A general dorminance of acidic amino acids is observed in these proteins. Some of these proteins for example apolipophrorin-III plays a transport role (Shapiro et al., 1988). The high content of acidic amino acid in the LMWP certainly confers the protein with a

relatively high negative charge. It is possible that the high negative charge is important in execution of the role played by the protein. Such a role could involve binding of positively charged molecules in haemolymph. A relatively high proportion of lysine is also observed in the DAP (M_r=35,000) isolated from D. grandiosella as well as in apolipophorin-III from M. sexta and T. acutangulus. This is also the case with the LMWP. The high content of acidic and neutral amino acids present in the LMWP accounts for its low pI = 4.6. The pI reported for microvitellogenin from M. sexta (ref. Table 3) (M_r=31,000) is 7.3 (Kawooya et al., 1987) while that of DAP from D. grandiosella is pI=5.9 (Turunen and Chippendale, 1980).

Synthesis studies using fat body indicated that the low molecular weight protein is one of the proteins synthesized by the fat body of G. m. morsitans then secreted into haemolymph. Virtually all haemolymph proteins examined from a wide range of insects are synthesized by fat body (Dean et al., 1985). Fat body has also been shown to synthesize and store lipids (Beenakkers et al., 1985) and amino acids notably proline in Glossina (Bursell,1977). After the demonstration that haemolymph lipoproteins serves as the source of fat body granules (Tojo et al., 1978), there are further suggestions (Ogawa and Tojo, 1981; Roberts and Brocks, 1981) that fat body degrades

arylphorins for the purpose of tissue and cuticle formation. This however, has not been confirmed.

In some insects, other tissues have been demonstrated to contribute to the haemolymph protein pool. In calpodes ethlius, arylphorin was shown to be synthesized in the midgut (Palli and Locke, 1987a) and epidermal cells (Palli and Locke, 1987b) as well as in the pericardial cells (Fife et al., 1987). The same was demonstrated in Dacus olae (Zongza and Dimitriadis, 1988) and in Leptinotarsa decemlineata (Perferoen and De Loof, 1986). Zacchary and Hoffman (1984) have also explored the possibility that lysozyme, an antibacterial peptide present in haemolymph of insects immunized with bacteria, is also synthesized by the hemocytes.

The use of immunology in identification, comparison and quantitation of haemolymph proteins was earlier stressed by Wyatt and Pan (1978). Since then, many researchers including Chino and Kitazawa (1981), Ryan et al. (1985), Wells et al. (1985) and Kawooya et al. (1987) have used immunological methods to check for proteins with similar antigenic determinants from haemolymph proteins of other species. A positive reactivity is always expected when antigen shares epitopes with the protein under investigation.

In this study, out of the species covering six insect orders tested for cross-reactivity using

antibodies raised against the LMWP, only members of the family Glossinidae showed a positive reactivity (ref. Table 4). This means that the LMWP is only present in the family Glossinidae. Dillwith et al. (1986) using anti-D. grandiosella DAP identified proteins that shared antigenic determinants with DAP in D. crambidoides and D. saccharalis. The proteins were of comparable molecular weights. Using a similar approach, Kawooya et al. (1987) demonstrated presence of a protein similar to microvitellogenin of M. sexta in Tenebrio mollitor. Ogoyi et al. (1989), using anti-Busseola fusca lipophorin showed that lipophorins from insects of same order shared antigenic determinants. However, this was only established in lepidopterans. Ryan et al. (1984a) demonstrated cross-reactivity between anti-M. sexta apolipophorin-II with apolipophorin-IIs from eight species representatives of seven insect orders. This demonstrated cross-reactivity over a very wide genetic background which also revealed conservation of structural integrity of lipophorin in view of its role in insect lipid transport (Chino, 1985). In another experiment, Ryan et al. (1984b) showed that anti-M. sexta arylphorin cross-reacts with arylphorins from Apis mellifera. M. sexta belongs to the order Lepidoptera while A. mellifera is a Hymenoptera. Kawooya et al. (1987) showed that M. sexta vitellogenins and

microvitellogenins bears no immunological relationship although both are female specific and are found to accumulate in eggs. Moreover, anti-M. sexta apolipophorin-III failed to cross-react with Leptoglosus Zonatus (Hemiptera) apolipophorin-III despite the fact that the two play similar role of lipid transport (Ryan et al., 1984a).

The above observations suggests that the LMWP present in haemolymph of both sexes of the tsetse fly G.m. morsitans could be a Glossina protein. From the immunological work carried out earlier, it is clear that so long as epitopes are shared between proteins, cross-reactivity is possible irrespective of their originality. Haemolymph from a dipteran, Musa domestica used in this study did not show any cross-reactivity with the anti-LMWP sera. M. domestica belongs to the family Muscidae which genetically is very close to the family Glossinidae. Indeed, the Glossina was formerly placed in the same family Muscidae until a new family Glossinidae was established.

The tsetse fly feeds solely on blood (Bursell, et al., 1974) from which it derives all its metabolic requirements. The digestion of bloodmeal releases proline which is the chief metabolic fuel. In course of digestion, there is a build up of fats in fat body (Bursell et al., 1974). Such a reserve is important

for energy metabolism in times of proline shortages and hunger cycles (Bursell et al., 1974). For the tsetse fly, albumin ingested with a bloodmeal has been shown to cross, undigested the peritropic membrane into haemolymph (Nogge and Giannetti, 1979). The nutritional significance of albumin in Glossina was investigated by Nogge and Giannetti (1980) who reported that flies that were previously fed on human blood died within 2 hours after feeding on de-albuminated anti-serum raised against the human albumin. Death was shown to be due to osmotic pressure imbalance in haemolymph of the flies (Nogge and Giannetti, 1982). Kabayo (1982) further demonstrated the nutritional significance of albumin in reproduction. Flies that were fed on de-albuminated blood failed to reproduce (Kabayo, 1982). However, in this study the function of the LMWP was not elucidated. The presence of the protein in haemolymph of tsetse fly which is known to be peculiar in its mode of reproduction and feeding could possibly be related to either of the two or both. This is subject to further work.

The development of a safe control measure for tsetse fly has been a challenge for a long period of time. The success of this endeavour will rest on a concerted effort in understanding the Biochemistry and Physiology of the insect. In this regard, there is hope as far as using haemolymph proteins as biological

pesticides is concerned. This is based on the fact that antibodies can cross the gut of haematophagous insects undigested (Schlein et al., 1976; Schlein and Lewis, 1976; Nogge and Giannetti, 1979, 1980). Such antibodies (raised in host animal) would precipitate, in vivo, the specific proteins (antigens) right in haemocoele rendering them functionless possibly leading to death or impairment of reproductive capacity. From literature, haemolymph proteins are known to serve various functions, some of which are very crucial to life of the insect. So haemolymph protein study especially for haematophagous insects, notably Glossina forms a very strong basis in future research geared to developing a safe tsetse control measure.

In this study, the function of the low molecular weight protein from haemolymph of tsetse fly G. morsitans morsitans was not elucidated. In future it is necessary to:

(i) Establish whether the low molecular weight protein described in this work is a property of all known blood sucking insects or just the Glossina.

(ii) Elucidate the role that the protein play in Glossina haemolymph especially in connection to reproduction.

(iii) Know whether the LMWP has any connection in in the transport of proline, which is the

chief metabolic fuel for flight, via haemolymph.

(iv) Establish whether the protein is involved in any hormonal metabolism.

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