ELUCIDATION OF PRIMARY STRUCTURES OF ADIPOKINETIC NEUROPEPTIDES OF THE INSECTS, *OXYA NITIDULA, AULARCHES MILIARIS, IPHITA LIMBATA* AND *ORYCTES RHINOCEROS*

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By

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DECLARATION

I, **AJAYKUMAR. A. P.**, do hereby declare that the thesis entitled **"ELUCIDATION OF PRIMARY STRUCTURES OF ADIPOKINETIC NEUROPEPTIDES OF THE INSECTS, *OXYA NITIDULA, AULARCHES MILIARIS, IPHITA LIMBATA* AND *ORYCTES RHINOCEROS*"** is an authentic record of the research work carried out by me in the Department of Zoology, University of Calicut, under the guidance of **Dr. M. GOKULDAS**. I further declare that no part of this thesis has been submitted previously for any other Degree.

Calicut University Campus,
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AJAYKUMAR. A. P.

CERTIFICATE

This is to certify that this dissertation entitled **"ELUCIDATION OF PRIMARY STRUCTURES OF ADIPOKINETIC NEUROPEPTIDES OF THE INSECTS, *OXYA NITIDULA, AULARCHES MILIARIS, IPHITA LIMBATA* AND *ORYCTES RHINOCEROS*"** is a bona fide record of research work done by Sri. Ajaykumar. A. P. in the Laboratory of Insect Physiology and Biochemistry of the department under my supervision and guidance, in partial fulfillment of the requirements of the degree of Doctor of Philosophy under the Faculty of Science of the University of Calicut. I also certify that no part of this thesis has been presented before for any other Degree or Diploma.

Dr. M. Gokuldas.
(Supervising teacher)

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CHAPTER I
INTRODUCTION

Neuropeptides play important roles in cellular communication in vertebrates. This is also true for insects in which many physiological, developmental and behavioral processes are affected by neuropeptides produced in neurosecretory cells of the retrocerebral complex. In insects, the retrocerebral complex comprises the corpora cardiaca (CC) and the corpora allata (CA). The CC act as a neurohaemal organ, storing and releasing neuropeptides produced in the brain. The CC also contain intrinsic glandular cells that produce and release several neuropeptides including adipokinetic hormones (AKHs). AKH/RPCH (red pigment concentrating hormone) family is one of the best known groups of arthropod neuropeptides. This name comes from the first member of the family to be fully characterized, a chromatophorin (RPCH) from prawns (Fernlund and Josefsson, 1972). The known actions of AKHs are, however, broader than their name implies. The peptides have been reported from most of the insect orders. It acts on the fat body to mobilize stored lipids and carbohydrates, activate glycogen phosphorylase, accumulate cAMP (Goldsworthy, 1983) and inhibit the synthesis of lipids (Gokuldas, 1989), proteins (Carlisle and Loughton, 1979) and RNA (Kodrik and Goldsworthy, 1995).

The AKHs comprise 8-10 amino acids except for an unusual AKH of the butterfly, *Vanessa cardui* (Kollisch *et al*., 2000) which is a 11-mer in which the AKH is not processed completely from the prohormone and occur in a C-terminally extended form. Recently a phosphorylated member of AKH/RPCH family was identified in the beetle, *Trichostella fascicularis* (Gaede *et al*., 2006). All AKHs possess a pyroglutamate residue blocking the N-terminus and amide group blocking the C-terminus. The amino acid tryptophan and glycine are at positions 8 and 9 (when present). In addition to tryptophan, the peptide contains at least one more aromatic amino acid, most commonly phenylalanine at position 4.

Need and significance of the study

The human brain contains about 100 billion neurons that use a wide array of neurotransmitters to communicate through trillions of synapses. Neuropeptides make up the largest and the most diverse class of signaling molecules used in nervous system communication. Because these polypeptides are crucial to the regulation of nearly all physiological processes, it is of great interest to characterize this diverse assortment of molecules and to determine what effects they elicit on neural circuitry. Due to the overwhelming complexity of mammalian nervous systems, simpler model organisms are often used to study basic principles of neuronal function. Because arthropod nervous systems contain a manageable number of neurons,

many of which exhibit consistent morphological and physiological properties between animals, these organisms provide excellent model systems for investigating neuromodulation in well-defined networks. Furthermore, these organisms contain a rich repertoire of neuropeptides, which are categorized into superfamilies of structurally related isoforms. Some of these peptide families, such as RFamides, tachykinin-related peptides and kinins, are also present in mammals.

Analysis of primary structures of the AKH peptides has shown that there exists an order or family specificity. Such data have been used as additional information to aid in the construction of phylogenetic trees by means of computer programme and protein parsimony algorithms. Gaede *et al*. (2003 b; 2005) analysed the phylogeny of the insect order Odonata, based on the primary structures of AKH peptides present in that group. This was helpful to obtain more clarity about the phylogeny of Odonata. Recently, Gaede and Marco (2008) elucidated the primary structures of AKH complements in a few members of the superfamily Caelifera. In the present study, the investigator used two more insects in the same superfamily, *Oxya nitidula* Walker (Acrididae, Orthoptera) and *Aularches miliaris* Linnaeus (Pyrgomorphidae, Orthoptera). Structural studies have also been conducted in two other insects, *Iphita limbata* Stål (Pyrrhocoridae, Heteroptera) and *Oryctes rhinoceros* Linnaeus (Dynastidae, Coleoptera). The results of the

present investigation may be helpful for constructing phylogenetic trees of the insects based on the primary structure of AKHs.

Peptide chemists all over the world are already engaged in developing a novel type of chemistry to make nonpeptidic analogues of the active core of peptides. With such molecules they hope to realise a variety of practical applications both in medicine and agriculture. Mimetic analogues are valuable tools for the development of future pest management strategies. Chemical and conformational requirements for neuropeptide-receptor interactions represent a template from which agonist/antagonist peptide mimetics, with a potential to disrupt critical insect processes can be developed. The design of biologically active pseudopeptide analogues in which amino acids were replaced by nonpeptide moieties represented a milestone in the development of non-peptide mimetic analogues (Nachman *et al.*, 1995). The designed pseudopeptides are more hydrophobic than the natural one. Increased hydrophobicity and resistance to peptidase attack are likely to be important characteristics for mimetic agonist and antagonist analogues of insect neuropeptides to be used in pest management research (Nachman *et al.*, 1992).

The protein nature of peptides makes them good candidates for DNA technology and genetic engineering because peptide genes can be inserted into crop plants, viruses and bacteria so that the ingestion of transgenic crops

or bacteria by feeding would result in physiological imbalance, moulting disturbance, malformations, decreased body weight or sclerotisation. A recombinant baculovirus expressing a diuretic hormone gene was created that disrupted the normal physiology of silkworm larvae, and increasing speed of kill roughly by 20% over that produced by wild type virus. Subsequently, at least four biologically active peptide hormones have been expressed using recombinant baculoviruses: eclosion hormone (Eldridge *et al.*, 1991), prothoracicotropic hormone (O'Reilly *et al.*, 1995), pheromone biosynthesis activating neuropeptides (Vakharia *et al.*, 1995) and neuroparsin (Girardie *et al.*, 2001), unfortunately each with little success in improving the insecticidal activity of the baculovirus.

Adipokinetic neuropeptide hormone studies become an important area, particularly in the biology of insect pests of crops and insects that acts as intermediate or vector hosts for parasites and/or pathogens that can affect humans or his livestocks. It is seen that increase in AKH does not only aids flight in insects, but also leads to stronger immune responses in locusts (Goldsworthy *et al.*, 2002, 2005). Mullen and Goldsworthy (2006) revealed that lipid mobilization by AKH play important roles in energy dynamics within insects and may be important in a range of functions that are even related to insect survival. It is now believed that parasites, which infect insects, may stimulate the production of AKH in order to mobilize lipids, which can then be utilized by the parasites. All these interesting data on AKH

make it a better insect neuropeptide candidate for investigation on pest control strategies in the coming years.

Even though AKH/RPCH representatives from most of the insect orders have been elucidated, many of the important pest species are still awaiting for such discoveries. In the present study, four insect species including three pest species, viz., *O. nitidula, A. miliaris* and *O. rhinoceros* which cause serious damages to many economically important crops all over the world have been used. The results of the investigation may be helpful for the development of various control measures against these pests in the coming years.

Objectives of the study

A. **Elucidation of primary structures of adipokinetic neuropeptides of the rice grasshopper, *Oxya nitidula***

1) Separation of adipokinetic neuropeptides from the extracts of retrocerebral complexes of *O. nitidula* by reversed phase high-performance liquid chromatography (RP-HPLC)

2) To study the hyperlipaemic effects of the fractions separated on HPLC by a heterologous bioassay in the plant bug, *I. limbata*, *in vivo*.

3) To investigate the similarity of the active peptides in the extracts of retrocerebral complexes of *O. nitidula* to other orthopteran

adipokinetic neuropeptides such as Locmi-AKH-I and Schgr-AKH-II by comparing their elution profiles in HPLC.

4) Identification and sequencing of adipokinetic neuropeptides from the retrocerebral extracts of *O. nitidula* by MALDI-TOF-MS and MALDI-TOF-MS/MS analysis.

B. **Elucidation of primary structure of adipokinetic neuropeptide of the coffee locust, *Aularches miliaris***

5) To study the presence of hyperlipaemic effects of crude extracts of retrocerebral complexes of *A. miliaris* and Locmi-AKH-I by a homologous *in vivo* bioassay.

6) Separation of peptides from the extracts of retrocerebral complexes of *A. miliaris* by HPLC.

7) To detect hyperlipaemic effects of the fractions separated on HPLC by a homologous *in vivo* bioassay.

8) Identification and further sequencing of adipokinetic neuropeptides from the retrocerebral extracts of *A. miliaris* by MALDI-TOF-MS and MALDI-TOF-MS/MS analysis.

C. **Elucidation of primary structure of adipokinetic neuropeptide of the plant bug, *Iphita limbata***

9) Separation of peptides from the extracts of brain-retrocerebral complexes and retrocerebral complexes alone of the plant bug, *I. limbata* by HPLC.

10) To study the hyperlipaemic effects of the fractions separated on HPLC by a homologous *in vivo* bioassay.

11) To investigate the similarity of the active peptides in the brain retrocerebral complexes and retrocerebral complexes alone of *I. limbata* to another heteropteran peptide, Pyrap-AKH by comparing their elution profiles in HPLC.

12) Identification and further sequencing of adipokinetic neuropeptides from the retrocerebral extracts of *I. limbata* by MALDI-TOF-MS and MALDI-TOF-MS/MS analysis.

13) To study the hyperlipaemic effects of injected as well as topically applied synthetic peptides, Locmi-AKH-I and Pyrap-AKH.

D. Elucidation of primary structure of adipokinetic neuropeptide of the rhinoceros beetle, *Oryctes rhinoceros*

14) To study the hyperlipaemic effects of crude extracts of retrocerebral complexes of *O. rhinoceros* by a heterologous *in vivo* bioassay in *I. limbata*.

15) To study the hyperlipaemic effects of the fractions separated on HPLC by a heterologous bioassay in the plant bug, *I. limbata in vivo*.

16) Identification and further sequencing of adipokinetic neuropeptides from the retrocerebral extracts of *O. rhinoceros* by MALDI-TOF-MS and MALDI-TOF-MS/MS analysis.

CHAPTER II
REVIEW OF LITERATURE

Neuropeptides

Neuropeptides are peptidergic chemical messengers that are synthesized in specialized neurons and are released into the general circulation, which in insects and crustaceans is called haemolymph, to reach their target organ(s). Most neuropeptides are, in fact, hormones, which control a number of physiological processes; hence, the neuroendocrine system represents a form of communication between cells, tissues and organs, other than the classical nervous and endocrine systems. Nervous control mechanisms act rapidly through synapses, releasing neurotransmitters into the synaptic cleft and generating action potentials of short duration; the classical endocrine (hormonal) control is slower acting but of longer duration since the hormones are released into circulation often a long distance away from the target organ and it takes some time before the hormones are degraded.

Neuropeptides are the most numerous and diverse of all known types of chemical messengers of metazoans. This is also true for insects, which constitute by far the largest group of animals. The existence of neuropeptides which regulate physiological, developmental and behavioural events in insects

have been known for a long time. However, it is only during the last two decades, that a great number of neuropeptides have been isolated, purified, and their primary structures completely characterised (Gaede 1997 a; Gaede *et al*., 1997 a). The identified peptides have been grouped into families based on structural similarities. A family does not, however, necessarily indicate similarity of function. There are about 20 such families. Some of the neuropeptides have described functions as hormones (such as PTTH, AKH, eclosion hormone, and diuretic hormone), but it is highly likely that some neuropeptides function as neurotransmitters and as neuromodulators that could modify the input or output from neural connections. There is isolated evidence in insects for the co-localization of neuropeptides and neurotransmitters in the same nerve terminals, and neuropeptides are known in some cases to be released simultaneously with neurotransmitters. If some neuropeptides do indeed work in this way, a single neuron in a network may be able to regulate many variations on a basic behaviour by modulation with neuropeptides. Neuromodulators might alter response characteristics of neurons, including such activities as feed-back, free-forward, motor output, and muscle or gland response to nervous activity. Neuropeptides may have roles in embryonic development and as cytokines in non-self recognition and response.

Adipokinetic Hormones

A major group of these neuropeptides is that which regulates physiological homeostasis. The first members were discovered in the 1960s in the American cockroach and in locusts where they are involved in the control of carbohydrate and lipid breakdown, respectively. It is now known that these peptides are members of a large family of structurally related peptides which are found in crustaceans and insects (Gaede, 1996). Such peptides became known under the acronym AKH/RPCH family peptides on the basis of the first members of this family to be fully characterised, viz., an *adipokinetic hormone* from locusts (Stone *et al*., 1976), now called Locmi-AKH-I according to the nomenclature proposed by Raina and Gaede (1988), and a chromatotropic peptide from prawns (Fernlund and Josefsson 1972), the red pigment-concentrating *hormone* (code name: Panbo-RPCH). These peptides are present in the neurosecretory X-organ/sinus gland complex in the eyestalks of crustaceans and in the intrinsic neurosecretory cells of the corpora cardiaca of insects. Both of these structures are neurohaemal organs, analogous to the vertebrate hypothalamo/hypophyseal system and it can thus be inferred that the peptides can be released from the neurohaemal organs into the circulation and, thus, act as true *hormones*. However, release has been demonstrated only in a few cases, for example, during flight in locusts, blowflies and the moth *Manduca sexta* (Gaede, 1992).

Synthesis and release of AKH

The detailed pathway of the biosynthesis of two adipokinetic hormones from *S. gregaria*, including the characterization of the prohormone, have been elucidated by direct protein chemical methodologies, as well as molecular cloning (Gaede *et al*., 2006). Similar studies have been conducted for AKH family members in other insect species and also for RPCH of crustaceans. There is a distinct mRNA encoding for each AKH precursor (up to three in *L. migratoria*) (Bogerd *et al*., 1995), which is translated into distinct precursor, the prepro-AKHs. The organization of the precursor is basically always the same for all AKH and RPCH peptides: a signal peptide is followed by representative AKH sequence, followed by the Gly residue for amidation of the AKH, the dibasic processing site, and C- terminally, “a tail peptide” or “precursor related peptide”. The latter is very long (more than 70 amino acids) in the crustaceans compared to the 28 - 46 residues in insects. There are almost no structural similarity between “signal peptide” and “tail peptides” of insects and crustaceans. Biological function of any “tail peptide” is not known. Studies on the biosynthesis of AKHs from the desert locust have revealed a unique strategy: after cleaving off the signal peptide, the two independently translated monomers of the pro-Locmi-AKH-I consisting of the sequence for Locmi-AKH-I and Cys containing “tail peptide” respectively, are oxidized to a unique precursor dimer forming a disulphide bond. Thereafter the precursor is processed to the following products: two

monomeric molecules of Locmi-AKH-I extended by Gly-Lys-Arg and one monomeric molecule of precursor related peptide. The extended Locmi-AKH is subsequently cleaved by a carboxy peptide H like enzyme, which removes first the Arg and then the Lys residues (Gaede and Auerswald, 2003).

Immuno-cytochemical studies have suggested that individual neurosecretory cells contain all three AKH peptides and that they are co-localized in the same secretary granules (Diederen *et al.*, 1987; Harthoorn *et al.*, 1999), implying their simultaneous release upon stimulation. The AKH-producing cells (AKH cells) continuously synthesize AKH and the synthesis is not affected by its release during flight (Harthoorn *et al.*, 2001). Flight activity is the only known natural stimulus for the release of AKH. A very small fraction of the AKH stored is released during flight and the granules containing newly synthesized AKH only appear to be available for the release (Sharp *et al.*, 1995). *Locusta* tachykinins and crustacean cardioactive peptide (CCAP) stimulate AKH release from the CC *in vitro*. The most potent AKH in the brain extract of the desert locust, *S. gregaria* has been identified and sequenced as PFCNAFTGC-NH_2 (Veelaert, *et al*., 1997). Trehalose has an inhibitory effect on AKH release (Vullings *et al.*, 1998; Van der Horst *et al.*, 1999). There are indications that *Locusta* myoinhibiting peptide and FMRFamide-related peptides also inhibit AKH release from the CC (Vullings *et al.*, 1998; Harthoorn *et al.*, 2001).

Secretion of AKH by the CC is believed to be controlled by conventional neurons from the brain, since severing the nervous connection between brain and CC prevents the flight induced elevation of haemolymph lipid titers (Goldsworthy *et al.*, 1972), while electrical stimulation of these nerves in isolated CC causes the release of AKH (Orchard and Loughton, 1981). AKHs are transported in the haemolymph without any carrier (Oudejans *et al.*, 1996). To terminate the signal, the peptides of AKH/HrTH family are cleaved by endopeptidases into fragments, which are susceptible to further degradation by exopeptidases (Isaac, 1988; Rayne and O'Shea, 1992; Oudejans *et al.*, 1996).

Inactivation of AKH

Once released into the haemolymph, AKHs are under attack by peptidases/proteases (Isaac *et al.*, 1978). During transport in the haemolymph, locust AKHs are not associated with a carrier protein (Oudejans *et al.*, 1996). Half-lives have been determined for various AKHs with near physiological and sometimes totally non-physiological concentrations of peptides. In locusts, when tritiated AKHs with a high specific radioactivity are used, half-lives for the three AKHs during resting conditions and after flight are short but different for each peptide and are differently affected by flight. Locmi-AKH-III has the shortest half-life and breakdown of Locmi-AKH-II is apparently almost not affected by flight at all (Oudejans *et al.*, 1996). One

active peptidases having similar action and properties to mammalian endopeptidase has been reported, but it is enigmatic how one peptidase can cleave the three AKHs at different rates. The temporal titers of the three forms of AKH may be responsible for various later events, such as activation of phosphorylase and/ or lipase in the fat body.

AKH Structure

The primary structures of Locmi-AKH-I and Panbo-RPCH are strikingly similar. Both peptides are cross active in the reciprocal system and the structural similarity also explains why crude extracts of insect CC (which contain one or the other form of AKH) cause blanching in shrimps. The structural similarity was also the basis for classifying these peptides as members of a peptide family, viz., the AKH/RPCH family of peptides. Thus, the peptide family comprises structurally related peptides, which have diverse functions in the two main arthropod taxa. New developments in the analysis of small peptides have greatly advanced this field. For example, improvements in the techniques of isolation and sequencing have occurred along with the introduction of modern mass spectrometric methods for accurate mass determination and sequencing. Fast atom bombardment (FAB) mode used earlier, is now coupled with matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry. These new techniques, combined with the availability of large

amount of peptidic material stored in the CC, made it relatively easy to determine the primary structure of many different natural analogues in about 100 insect species. Far fewer decapod crustaceans have been investigated but, interestingly, the structure of RPCH is highly conserved among crustaceans. In all species from which it has been sequenced or characterized, only RPCH of the AKH/RPCH family peptides can be found in crustaceans. RPCH is, however, also synthesized in an insect species, the bug *Nezara viridula* (belonging to the Order Heteroptera), where it mobilizes lipids (Gaede *et al*., 2003 a). Representative peptides of the AKH/RPCH family have been found in most orders (Table-II. 1). In a number of taxa, gene duplication has taken place and two or even three AKH peptides are found in one species. In contrast to crustaceans (on RPCH present), insects show a high degree of variability in isoforms of AKH peptides. Common characteristics of the family are: a chain length of 8 to 10 amino acids; the N-terminus blocked by pyroglutamic acid (pGlu); the C-terminus blocked by a carboxyamide; amino acids at positions 8 and 9 (when present) are tryptophan and glycine; most of the peptides are uncharged, but there are a few that have an aspartic acid at position 7; there are at least two aromatic acids, at position 4 mostly phenylalanine (but sometimes tyrosine) and at position 8 tryptophan, and a few peptides have a third aromatic acid either at position 2 (Tyr or Phe) or at position 7 (Trp).

In addition to the post-translational modifications at the terminals, the

hypertrehalosaemic hormone (HrTH-I) of the stick insect, *Carausius morosus* (Carmo-HrTH-I) is glycosylated. The site of glycosylation is not the usual serine and threonine (O-glycosylation) or asparagine (N-glycosylation) but tryptophan. As in human ribonuclease, the hexose in Carmo-HrTH-I is very likely C-glycosidically linked to the C-2 atom of the indole ring of Trp (Gaede *et al.*, 1992). In some insects, notably Lepidoptera (eg., the butterfly *Vanessa cardui*), the AKH is not completely processed from the prohormone and, thus, occur in a C-terminally extended form (Gly-Gly-Lys) together with the fully processed peptide Manse-AKH (Kollisch *et al.*, 2000). Unfortunately, no rigorous tests have been conducted as to whether this compound is biologically active or whether the measured biological activity is the result of a breakdown product. Another, as yet unidentified, modification apparently occurs in a hypertrehalosaemic neuropeptide of cicadas. In a number of species two peaks are always found on HPLC separation, but the material of both peak fractions have the same mass and amino acid sequence. When the peptide was synthesized according to the sequence information, its retention time of HPLC coincided only with one of the two peaks derived from natural material (Raina *et al.*, 1995).

The molecular conformation of a peptide at its active site is an important aspect of peptide recognition. Studies on the folding tendencies of peptides in free solution and correlation with activity has proved to be an important step in this respect. Preliminary structure prediction and molecular

model studies (Stone *et al.*, 1976; Goldsworthy *et al.*, 1990) have indicated that in many of the AKHs there is a potential to adopt β-conformation. Secondary structures of six members of the AKH/RPCH family of arthropod neuropeptides have been studied by circular dichroism (CD) spectroscopy (Cusinato *et al.*, 1998). None of the peptides examined shows a clear ordered conformation in aqueous solution, pH 7.5 at room temperature. At low temperatures in ethanidiol/ aqueous buffer (2:1, pH 7.5), however a PII extended conformation in all of the peptides. The β turn observed in most of the AKH peptides are between residues 4-8, the tightness of the β turn is influenced by the presence of phenylalanine (tight turn), or tyrosine (weaker turn).

Recently, the genome of certain insect species has been elucidated, for example, *Bombyx mori*, *Aedes aegypti* and *Tribolium castaneum*. The search for structural homologs of AKHs in the databases of these species revealed the presence of such peptides. Such kinds of predicted peptides were later elucidated from the same insects (*A. aegypti* and *T. castaneum)* with MALDI-MS analysis (Gaede *et al*., 2008).

Functions of AKHs

Hyperlipaemic activity: Although lipids are the main fuels for long-distance flight in locusts, carbohydrates are used during the initial phase of flight and

still contribute substantially during the later phase (see Beenakkers *et al*., 1985). Similar patterns for the use of fuels during flight have been found in the brown locust, *Locustana pardalina* (Gaede, 2008), and in the pyrgomorphid grasshopper, *Phymateus morbillosus* (Gaede *et al*., 1996). Mobilisation of both substrates is controlled by peptides of the AKH/ RPCH family, which stimulate either glycogen phosphorylase or a lipase (not convincingly shown yet) in the fat body. After a lipase is activated, triacylglycerols are broken down to monoacylglycerols, which are subsequently re-acylated to form stereospecific sn-1, 2-diacylglycerols, and these are released from the fat body into the haemolymph (see Beenakkers *et al*., 1985). Here/In the case, AKH/ RPCH family peptides are responsible for an overall increase in the lipid-carrying capacity. For this, the predominant species of lipoprotein in resting locust haemolymph, high-density lipophorin, is loaded with the lipids released from the fat body and simultaneously associates with an apoprotein, apolipophorin III. The overall result is the creation of a larger but less dense particle, low-density lipophorin (Kanost *et al*., 1990). Direct action of AKH/RPCH family peptides on the utilisation of fuel at the flight muscles had been previously reported (Goldsworthy, 1983).

Similar results have been corroborated for the moth, *Manduca sexta*, which uses lipids as the main fuel for flight muscle contraction (Ziegler, 1995). The peptides characterized from various locusts/grasshoppers and from moths are very closely related.

Lipids stored as TAGs in the fat body provide the major source of energy for flight in many insects. The AKH stimulate the fat body to degrade metabolic stores for the synthesis and release of circulating metabolites. These metabolites serve as the major energy sources for peripheral tissues such as muscles and are usually lipids (as in locusts, Mayer and Candy, 1969) or carbohydrates (as in cockroaches, Steele, 1961). In *M. sexta*, a single AKH regulates the mobilization of carbohydrates in larvae and lipids in adults (Ziegler, 1984). In adults of species, which rely on lipid as a flight fuel, commencement of flight initiates the mobilization of lipid stores from the fat body. In *Tenebrio molitor*, *P. americana*, *Carausius morosus* and *Gryllus bimaculatus*, there were no enhanced lipid release after flight or injection of the hormone extract from their CC. When CC-extract was injected into locusts, it had adipokinetic effect (Goldsworthy, 1983; Beenakkers *et al.*, 1985; Gaede, 1990; Cusinato *et al.*, 1991).

Hyperlipaemia by topically treated AKHs: Topical application of homologous Grybi-AKH (100 pmol in 20% 2-propanol) led to a significant increase in the haemolymph lipids, comparable with the maximal increase caused by injection of the same AKH (3 pmol in water). Topical application of AKH has been found to stimulate locomotor activity in crickets (Lorenz *et al*., 2004). Similar results were also observed when Pyrap-AKH was topically applied to the plant bug *P. apterus.* The lipid mobilisation was maximum when 40-100 pmol of Pyrap-AKH was topically applied and first significant

hyperlipaemic response was noticed 2 h after topical application. Increased locomotor activity was found after topical application, the greatest increase in walking activity required topical application of 300 pmol of Pyrap-AKH. These results suggest that AKHs are hydrophobic in nature, and that they have the ability to penetrate through the cuticle under the wings when they are applied with appropriate solvents like methanol, 2-propanol etc. It is speculated that the solvents could play an active role in the penetration of peptides through the cuticular waxes in *P. apterus*. These findings are promising as leads to strategies using AKHs or analogs thereof as substances for pest control purposes (Gaede and Goldsworthy, 2003).

Hypertrehalosaemic activity: Carbohydrate mobilization is another important function of AKH. These are mobilised mainly from glycogen reserves of fat body resulting in an increased level of soluble carbohydrates in the haemolymph. If an AKH induce this mobilization, it is defined as a hypertrehalosaemic hormone. Such effects were noticed in *T. molitor*, *P. americana*, *C. morosus*, *G. bimaculatus*, and *A. gambae*, when CC extracts were injected into locusts, and these extracts had adipokinetic effects. Anoga-AKH-II lack biological activity, and has high sequence similarity with that of Locust AKH-IV (Siegert *et al*., 1999). This has no known function in locusts, but it induces a weak hypertrehalosaemic effect in cockroach and was named Locmi-HrTH. Since it has been speculated that both of these decapeptides are synthesised in the brain and not in the CC, they are most probably typical

AKHs (Siegert, 1999; Kaufmann and Brown, 2006), and their functions remain unknown.

The sequence information from the cockroach peptides has been utilised to construct possible phylogenetic trees (Gaede, 1989, 1995). The structural data on the hypertrehalosaemic peptides from the woodroach *Cryptocercus punctulatus* and the cockroach *Therea petiveriana* helped to confirm the previous morpho-anatomical data placing the woodroach in the cockroach subfamily Polyphaginae (Gaede *et al*., 1997 b).

Hyperprolinaemic activity: The amino acid proline can also be used as a substrate for flight. There are, however, major differences between insect species in the quantitative participation of proline in flight metabolism. Only a little proline is metabolised during the onset of flight in the blowfly, *Phormia regina*, to provide tricarboxylic acid intermediates necessary for maximal oxidation of pyruvate (sparker function) (Sacktor and Childress, 1967). In the tsetse fly, *Glossina morsitans*, however, proline is present in impressively high concentration in the flight muscles and is thought to be the exclusive fuel during flight (Bursell, 1981). Proline is only partially oxidised and the alanine formed is transported to the fat body for re-synthesis of proline (Bursell, 1981). In certain beetles varying degrees of oxidation of carbohydrates and proline have been found. In the blister beetle, *Decapotoma lunata*, for example, proline is an important substrate for flight, although its role is

secondary to that of carbohydrates (Auerswald and Gaede, 1995). In the Colorado potato beetle, *Leptinotarsa decemlineata*, as well as in the African fruit beetle, *Pachnoda sinuata*, proline is the major flight substrate and carbohydrates play only a minor role (Weeda *et al*., 1979; Zebe and Gaede 1993; Lopata and Gaede, 1994). It was shown that proline is the exclusive substrate for endothermic warm-up during flight preparation in *P. sinuata* (Auerswald *et al*., 1998).

It was suggested (Weeda, 1981) that proline metabolism in the Colorado beetle is under hormonal control and AKH/RPCH family peptides were the likely candidates, but no endogenous peptide from the Colorado beetle was known at that time. It was shown that injection of crude corpora cardiaca extract decreased haemolymph alanine concentration in the beetle *in vivo*. Furthermore, proline synthesis was stimulated *in vitro* by corpus cardiacum extracts from various insects as well as by synthetic Locmi-AKH-I (Weeda, 1981). In 1989, Gaede and Kellner reported the primary structures of the endogenous AKH/RPCH family peptides from the Colorado potato beetle, which were identical to those purified earlier from the American cockroach.

In beetles of the genera *Scarabaeus* and *Onitis* respectively, two octapeptides each (one common to both groups) have been identified and fully characterised from the corpus cardiacum (Gaede, 1997 b, c). Conspecific injections of synthetic peptide material in low concentration, elicited in all

cases, significant proline increases in the haemolymph (Gaede 1997 b, c). This hyperprolinaemic effect, in conjunction with the demonstrated utilization of proline during flight in these beetles (see above), strongly supports the idea of a hormonal function for the peptides. Similar results have been obtained for the African fruit beetle, *P. sinuata*. Hyperprolinaemia as well as a decrease of the alanine concentration in the haemolymph, upon injection of synthetic Melme-CC, was shown (Auerswald, 1997). Furthermore, it was demonstrated that this response is dose- and time-dependent.

In addition, the injection of closely related bioanalogues of Melme-CC revealed that the receptor in the fruit beetle does not recognize peptides with tryptophan, serine or valine at position 7, but tolerates changes from aspartate (as in the endogenous Melme-CC) to asparagine or glycine (Auerswald, 1997). Somewhat in contrast to these observations are earlier results, which suggest that position 4 is most important for receptor recognition for the mobilization of carbohydrates from the fat body of the same beetle (Gaede *et al*., 1992). Taking these different results into account, it seems likely that two different receptors for Melme-CC mediate the mobilisation of carbohydrates and the stimulation of proline synthesis in the fat body of *P. sinuata*.

AKH and reproduction: It is known that AKHs inhibit RNA and protein synthesis. This may be a general action of AKHs; it has been shown specifically, however, that Locmi-AKH-I represses the synthesis of

vitellogenin *in vitro* at the end of the vitellogenic cycle in *L. migratoria* when the oocytes are fully mature (Moshitzky and Applebaum, 1990). In addition, synthesis of vitellogenin can be reactivated *in vitro* by washing pieces of fat bodies (dissected from locust females shortly before oviposition) in incubation medium. This method of reactivating vitellogenin synthesis fails when Locmi-AKH-I is added. It is believed that the juvenile hormone III (JH-III) activates a transcription factor that subsequently participates in the regulation of JH-dependent genes, such as the vitellogenin gene. AKH may play a role in inactivating this transcription factor, thereby repressing the transcription of vitellogenin (Glinka and Wyatt, 1996). In the cockroach *Blattella germanica,* transcription of the vitellogenin gene in fat bodies from cardioallatectomized females is activated by JH III *in vitro*, and the addition of the endogenous AKH at 10^{-8}M profoundly inhibits the response to JH (Comas *et al*., 2001). *In vivo* experiments with the cricket, *Gryllus bimaculatus* show that repeated injections of the endogenous AKH (3 times per day for 4 days) into females immediately after the adult molt result in (a) 50% difference in the wet weight of the ovary between ringer-injected controls and AKH-treated crickets, the former being heavier; (b) smaller number of ripe eggs in AKH-injected insects; and (c) dramatic decrease in the concentration of vitellogenin in fat bodies of AKH-injected females (Lorenz, 2002).

Alternative functions: Although the mobilization of energy substrates for

flight activity is, without doubt, a major and “classical” function of AKH peptides, they are true multifunctional and pleiotropic hormones that have a large number of well-described actions, such as myo-stimulation including especially cardio-stimulation; inhibition of the synthesis of RNA, fatty acids, and proteins in the fat body; stimulation of biosynthesis of mitochondrial cytoheme a + b, including the induction of gene expression for a cytochrome P-450 enzyme; and stimulation of oxidation of substrates by the flight muscles (Gaede *et al.*, 1997 b).

Because we find up to three different AKH peptides in one species, it is speculated that these isoforms have different tasks or, at least, that one is more potent than the other in a given effect. In locusts, for example, preferential tasks are suggested for the AKHs (Van der Horst *et al.*, 1999), but this is not supported by rigorous experiments for any species.

The other effect of AKH is in connection with the immune response in locusts. Insects lack antibodies, which are so characteristic of the defense system in vertebrates. Injections of laminarin, a major component of fungal cell walls, into the haemolymph of the migratory locust activate the prophenoloxidase cascade, generating quinines that are toxic to microbes (Lavine and Strand, 2002). Activation of the enzyme cascade is prolonged by co-injection of Locmi-AKH-I together with laminarin (Goldsworthy *et al.*, 2003). Interestingly, lipopolysaccharides from gram-negative bacteria activate

the prophenoloxidase system only when coinjected with Locmi-AKH. The effect of AKH on the locust immune system may be partly explained through its effect on apolipophorin III, which is a component of the lipid mobilization system.

Structure activity studies

Attempts at indirect characterizations of the receptor(s) for AKHs have made use of both the variety of naturally occurring analogues, which are known, and the chemical synthesis of analogues (Ford *et al*., 1988; Gaede, 1990; Hayes and Keeley, 1990; Ziegler *et al.*, 1991; Lee and Goldsworthy, 1995 a). It is clear from such studies that the $pGlu^{1}$, Phe^{4} and Trp^{8} residues are the most important for biological activity of the adipokinetic hormones. For example, a study on *Blaberus* HrTH has shown that replacement of Phe^{4} or Trp^{8} either with alanine or D-amino acids results in biologically inactive analogues (Ford *et al.*, 1988), although D-Phe^{4} in HrTH is tolerated, but with lower potency (Hayes *et al.*, 1994). The relative tolerance of the receptor to modifications of the hormone at these positions is not unexpected when the natural variability of the AKHs discovered is considered (Goldsworthy *et al.*, 1997; Gaede *et al.*, 1994); the $pGlu^{1}$ and Trp^{8} residues of AKHs are invariate, and all but three of 32 structural variants have Phe^{4}.

N-terminal modifications to AKH-I: While pGlu[1] is present in all AKHs found to date, it is not itself essential for biological activity; its deletion from AKH-I results in a peptide (des-pGlu-AKH-I) which is essentially inactive (Gaede, 1990), but acetylation of the N-terminal leucine restores some activity in the lipid mobilization and acetate uptake assays (Lee *et al.*, 1997). In AKH-I, pGlu[1] can also be replaced by hydroxyphenyl propionate (HPP), with only a minor reduction in potency, particularly in the assay *in vitro* (Lee *et al.*, 1997), but HPP-[Pro[1]]-*Blaberus* HrTH is virtually inactive (Hayes *et al.*, 1994). Unblocked amino acids may also be substituted at this position in *Locusta*-AKH-I, so [Ala[1]]-, [Pro[1]]- or [Glu[1]]- AKH-I do have some activity in the lipid mobilization assay *in vivo* and the acetate uptake assay *in vitro*. Thus, while [Ala[1]]-AKH-I is only very weakly active *in vitro* perhaps due to rapid breakdown (via an amino peptidase) and removal rather than poor receptor binding per se, [AcAla[1]]-AKH-I is very much more potent *in vivo* than is [Ala[-1]]- AKH-I (Lee *et al.*, 1997). Ziegler *et al.* (1991) found that [Gly[1]] and [Tyr[1]]-*Manduca* AKH were unable to activate glycogen phosphorylase in fat body from *Manduca sexta* larvae, but as is the case with locust AKH-I, the acetylated peptides [AcGly[1]] *Manduca*-AKH (Ziegler *et al.*, 1991) and [AcAla[1]]-*Blaberus*-HrTh (Ford *et al.*, 1988) retain activity.

C-terminal modifications: (Modifications of Thr[10] in AKH-I). The importance of the C-terminal carboxyamide of AKH-I in influencing potency has been investigated (Lee *et al.,* 1996). Replacement of this amide by the free acid causes a severe loss in potency in both lipid mobilization and acetate uptake assays (100-fold *in vivo* and 230-fold *in vitro*), as noted for other AKHs (Ford *et al.,* 1988; Gaede, 1990). Replacement of the C-terminal carboxyamide with a methyl ester was tolerated best of all the carboxyamide modifications studied, with only a 12-fold loss of potency in the lipid mobilization assay, and only a five-fold loss in the acetate uptake assay. Replacement of one carboxyamide hydrogen by a methyl or a phenyl group, or both carboxyamide hydrogens by methyl groups results in very dramatic reductions of potency, although these modifications are better tolerated in the acetate uptake assay (60-160-fold reduction in potency) than the lipid mobilization assay (580-1050-fold reduction in potency. It appears that the C-terminal carboxyamide group of AKH-I is important for potency in both the lipid and acetate uptake assays. However, given that the losses in potency are more marked in the lipid mobilization assay *in vivo*, higher rates of removal of the analogues *in vivo* cannot be excluded, perhaps by a mechanism that has a high affinity for peptides which are more hydrophobic; unfortunately, data on the effects of C-terminal modifications on the rate of removal or inactivation of AKHs in the blood are not available to support this hypothesis – the differential rates of breakdown for *Locusta* AKHs themselves were only

known recently (Oudejans *et al.*, 1996).

Why AKH-III is so potent in the acetate uptake assay: AKH-III is the most potent of the three *Locusta* AKH peptides at inhibiting uptake of acetate into *L. migratoria* fat body *in vitro* (Lee and Goldsworthy, 1995 a), inhibiting RNA synthesis (Kodrik and Goldsworthy, 1995), stimulating cyclic AMP production, activating glycogen phosphorylase (Vroemen *et al.*, 1995 a), and inducing Ca^{2+} efflux (Vroemen *et al.*, 1995 b). This high potency is surprising in comparison with its moderate potency in the lipid mobilization assay *in vivo.* An attempt has been made to investigate the basis for these findings by synthesizing novel analogues of AKHs with Trp^{7} either included or removed (Lee and Goldsworthy, 1996), because the 'double-tryptophan' Trp^{7}-Trp^{8} motif of AKH-III appears to be unique amongst the naturally occurring AKHs. Thus, the decapeptide [Trp^{7}]-AKH-I, and the octapeptides [des-Gly^{9}-Thr^{10}]-AKH-III, [Trp^{7}]-AKH-II and [Trp^{7}]-*Acheta*-AKH were tested in both the acetate uptake and the lipid mobilization assays. With the exception of [Trp^{7}]-AKH-I, which has potency similar to that of AKH-I when measured in the acetate uptake assay, each of these analogues is less potent than its respective parent in each assay. However, the acetate uptake assay appears more tolerant of AKH analogues with the sequence Trp^{7}-Trp^{8}, whereas inclusion of this sequence markedly reduces potency in the lipid mobilization assay. The apparent differences in potencies of the peptides between assays may be consequent on different binding preferences of distinct AKH

receptors, or alternatively, they may be due to different rates of breakdown or removal; AKH-III is inactivated very much more rapidly *in vivo* than either AKH-I or AKH-II (Oudejans *et al.*, 1996). Presumably, rates of breakdown or removal of any particular peptide would be higher in the intact insect, but will vary also between peptides, depending on their primary structure. Thus, apparent changes in potency could be due to differential rates of breakdown or removal rather than to qualitative variations in the peptide-receptor interaction.

Isolation and structural characterization

Bioassays: The first step in the elucidation of the hormonal regulation of a particular metabolic pathway is to bring about changes in this pathway as a response to extracts of the tissue containing putative hormone, and to establish a reliable bioassay. For adipokinetic hormone, such bioassays measure the increase in concentration of haemolymph diacylglycerols (Mayer and Candy, 1969), trehalose (Steele, 1961) and proline (Gaede and Auerswald, 2002). Very sensitive assays can be developed by monitoring the enzyme activities in the target tissues. Nerve tissues other than corpora cardiaca contain biologically active peptides. It is thus necessary to establish the distribution of hormonal activity throughout the nervous system and to ascertain whether these activities from different sources are linked to the same molecules.

Extraction of biologically active material: After the identification of the tissues containing biological activities, an optimum extraction procedure is required. Extraction with 0.1 M acetic acid or 0.1 N hydrochloric acid have been used for the extraction of invertebrate peptides. AKHs and HGHs are very hydrophobic and therefore organic solvents such as 80% aqueous methanol are used. Sufficient biological activities have been obtained from corpora cardiaca using salt solutions (Steele, 1961) or distilled water (Ziegler, 1979). It may prove necessary to introduce peptidase inhibitors into aqueous extraction media to prevent enzymatic breakdown of certain peptides such as diuretic hormone which are rather unstable. Homogenization of tissues can be performed with tissue grinders of the potter-Elvejhem type, glass homogenizer and with ultra sonicators to disrupt tissues. Three to four sonications are sufficient to extract AKH-I and AKH-II from a single pair of locust corpora cardiaca (Siegert and Mordue, 1986).

HPLC Analysis: The introduction of HPLC has greatly facilitated the isolation of insect peptide hormones, and several workers isolated identical peptides independently. The methodologies used by different authors can be very similar, but the resulting chromatograms may look different. As it is clear from the literature, independent studies on the same peptides always revealed identical primary structures. In RP-HPLC the silica base has been bonded with hydrocarbons, e.g., C-8 (octyl) or C-18 (octadecyl) derivatives. Peptides are injected onto the column in a predominantly aqueous solution

and hydrophobic parts of the molecule bind to the C-8 or C-18 residue present in the column. Then an elution gradient in which the aqueous solvent is gradually replaced by a water-miscible organic modifier (e.g. acetonitrile or methanol) is applied to the column. At a particular percentage of organic modifiers the peptide detaches from the stationary phase, elutes from the column and is usually detected with a UV spectrophotometer at wavelengths around 210 nm (peptide bonds) and 280 nm (aromatic amino acids such as phenylalanine and tryptophan). The peptides elute from the columns, and are detected as peaks depending on the type of gradient and the flow rate applied.

Chromatographic solvents used in HPLC must be chosen very carefully for two reasons: first, the solvent system is partly responsible for the interaction between the molecules to be separated and the stationary phase; second, the eluant should be removed completely prior to the bioassay. Therefore, volatile solvents are recommended. If a low pH is required, 0.1% trifluoroacetic acid (TFA) can be used. If a only a slightly acidic pH is needed, ammonium acetate buffers of appropriate molarity can be employed. Both substances can be removed with the water during freeze-drying. Organic solvents frequently used for the isolation of insect peptides are acetonitrile and methanol, which can be easily removed by gentle heating under reduced pressure.

Analysis of Primary Structures: After the identification and purification of

individual peptides the structure analysis can be attempted; this includes the establishment of the amino acid sequence and whether the N- and/or the C-terminals are modified or blocked.

Amino acid composition: The first step in the analysis of a primary structure of a peptide must be the determination of its amino acid composition. From this, the purity of the preparation can be judged, and the likely molecular mass of the peptide calculated; the composition data are also necessary for the interpretation of the results from the structure analysis. Prior to the identification and quantification of amino acids, the peptides must be hydrolyzed. This is a crucial step because amino acids such as tryptophan or proline can be destroyed or derivatized when the peptide is hydrolyzed in 6 M HCl. Mild hydrolysis in 4 M methane-sulfonic acid containing 0.2% tryptamine protects tryptophan and allows its determination (Ziegler, 1985). But even under these conditions relatively large amounts of the tryptophan can sometimes be lost. This is an important point because the composition data are used to calculate the likely number of amino acids present in the peptide.

After hydrolysis, the amino acids are reacted with eg., O-phthalaldehyde (OPA) (Turnell and Cooper, 1982) or phenylthiohydanation (PTH) both are more or less equally sensitive, but PTH derivatives of amino acids are more stable. Amino acids in the hydrolysate are then identified and

quantified using RP-HPLC and reference standards.

Edman degradation: It describes a convenient way to establish the amino acid sequence of peptides and proteins. The Edman degradation requires a free N-terminus. This is coupled to the matrix of the sequencer using a special coupling agent (First step of the degradation). The conditions are then changed to cleave the first amino acid from the N-terminus of the peptide chain (second step), the released amino acid is converted to a PTH derivative because these are very stable (third step), and the derivatives are subsequently determined using conventional amino acid analysis. The cleavage of amino acid is repeated and the sequence of peptide/ protein determined.

Determination of the N-terminus: Edman degradation cannot be performed on peptides, which contain a blocked N-terminus, in most of the insect neuropeptides like, AKH, RPCH, and HGH-I, are all N-terminally blocked. In such situations pyroglutamyl aminopeptidase enymes are used to cleave

N- terminal modification.

Mass spectrometry for the study of neuropeptides

Fast atom bombardment mass spectrometry (FAB-MS): The structure of peptides can be assigned by different approaches. Fast-atom-bombardment mass spectrometry elucidates the complete primary structure directly. FAB-MS is the predominant technique for the elucidation of peptide containing 8-

10 amino acids. The molecule is ionized and the ions subsequently separated and analyzed according to their mass charge ratio (m/z). FAB-MS elucidates the entire structure of a peptide including any modification to the N- and C-terminals. The primary structures of many AKH/RPCH peptides are elucidated by this technique.

Recent mass spectrometric Techniques: Previously, a combination of antibody-based techniques and Edman degradation was commonly used for the study of the peptides. Unfortunately, antibodies are rarely specific to one neuropeptide and Edman degradation requires large amounts of purified sample. Because neuropeptides exist in complex biological matrices at low concentrations, more specific and sensitive mass spectrometry (MS)-based strategies have become popular in peptidomic research. With the advent of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), it became possible to detect biological entities with molecular specificity in very limited samples such as single organs or single cells (Predel *et al.*, 2004, Redeker, 1998; Neupert and Predel, 2005). This has considerable implications in neurobiological studies because even adjacent neurons can have significantly different neuropeptide profiles.

Sample preparation strategies: For mass spectral analysis, the sample can be prepared by homogenizing the tissue and extracting the analyte of interest or it can be investigated directly by MALDI analysis of freshly dissected and

mounted tissue samples with minimal preparation. An extraction-based strategy results in neuropeptide-rich samples via pooling of many organs/cells. The sample can then be enriched for a specific peptide family via immuno-precipitation, desalted using C_{18} columns, and/or fractionated to reduce sample complexity prior to ionization by ESI or MALDI. Reducing the sample complexity through fractionation decreases ionization suppression, thus enabling more comprehensive peptide profiles to be obtained, as evidenced by studies in honey bee (Audsley and Weaver, 2006), crab (Fu *et al.*, 2005) and fruit fly (Baggerman *et al.*, 2005). However, due to the increased sample handling associated with extraction and sample dilution loss, contamination or chemically induced artifacts may result.

Alternatively, placing the tissue on the MALDI plate, coating with matrix, and irradiating the co-crystallized tissue to cause desorption/ionization of the analyte may enable direct tissue MALDI analysis. Not only does this approach require less sample manipulation, but also the spatial localization of neuropeptides in the tissue can be preserved. However, this technique is complicated by the limited amount of analyte contained in small portions of tissue as well as by the high salt and lipid content of tissue. To decrease the impact of the high salt content associated with biological samples, tissues are commonly rinsed with water (Predel *et al.*, 2001) or matrix solution (Kutz and Schmidt, 2004; Li *et al.*, 2003; Takeuchi *et al.*, 2003). Additionally, rinsing

tissue in acidified methanol prior to direct analysis may lead to more efficient peptide extraction and ionization processes (Kutz and Schmidt, 2004). It is possible to analyse a small piece of a tissue rinsed in dilute 2, 5-dihydroxybenzoic acid matrix and acidified methanol by MALDI-Fourier transform mass spectrometry (FTMS). The high resolving power of FTMS is beneficial for distinguishing unique neuropeptides in complex nervous system tissue. Because sensitivity is a concern, matrix volume must be minimized to avoid dilution of the sample. While the dried droplet method of matrix application is sufficient for decapod crustacean organ and neuron analysis, methods that allow application of nanoliters volumes of matrix are more effective at limiting peptide dilution in insect tissue (Predel *et al*., 2001; Balau *et al.,* 2004) by limiting the applied matrix volume to just 20 nanoliters of solution.

Tandem mass spectrometry: In arthropod species such as fruit fly, mosquito and honey bees for which the genome has been sequenced, an exact mass measurement may be employed in conjunction with database searching to identify the amino acid composition of a given neuropeptide (Audsley and Weaver, 2006). However, even with the genomic sequence in hand, prediction of the final bioactive peptide structure can be challenging due to post translational modifications, tissue-specific prohormone processing and unusual processing sites. Therefore, fragmentation techniques such as post source decay (PSD) and collissionally induced dissociation (CID) are often

required to obtain peptide fingerprint or *de novo* sequence information. Accurate mass measurements and fragmentation information can be used in conjunction with web-based tools to facilitate the process of *de novo* sequencing (Eisenacher *et al.*, 2006) and aid in the discovery of novel neuropeptides. Much recent neuropeptide sequencing work has been performed on ESI-quadrupole time-of-flight (QTOF) mass spectrometers (Baggerman *et al.*, 2005; Fu *et al.*, 2005; Predel *et al.*, 2005; Tukeuchi *et al.*, 2003). This instrumental configuration is especially effective for tandem MS because ESI commonly results in multiply charged peptides, which more readily produce, detectable fragments than singly charged ions. In addition, CID coupled with TOF mass analysis encourages the production and detection of amino acid-specific immonium ions, which are valuable for determining peptide composition. As evidenced by the recent identification of almost 60 neuropeptides, 23 of which were *de novo* sequenced in a *Cancer productus* neuroendocrine organ extract (Fu *et al.*, 2005), ESI-QTOF MS/MS is a powerful tool for peptidomic characterization.

For the analysis of small tissue samples, direct tandem MS may be performed with MALDI. For example, direct single-organ neuropeptide fragmentation has been performed by MALDI-TOF-PSD on the cockroach and fleshfly (Predel *et al.*, 2005), MALDI-CID of neuropeptides directly from single organs has been performed on cockroach using Q-TOF and lobsters and crabs by FTMS (Kurtz and Schmidt, 2004). To increase the sensitivity of

in situ peptide sequencing analysis, an in-cell accumulation technique was employed in the MALDI FTMS studies. MALDI-TOF PSD of putative SIFamide peptide in a single *Drosophila melanogaster* neuron produced sufficient sequence information for this peak to be confidently assigned (Predel *et al.*, 2004). Furthermore, Neupert *et al*. (2005) recently used a combination of PSD and CID fragmentation to *de novo* sequence a novel insect periviscerokinin peptide with the sequence PALIPFPRV-NH_2 from direct analysis of a single cell. High-energy CID uniquely available for a MALDI-TOF/TOF instrument produced the side chain-specific w-fragment ion necessary to distinguish between the isobaric amino acids Leu/Ile (Nachman *et al.*, 2005). This is the first reported demonstration of *de novo* sequencing of a peptide from a single-cell preparation of arthropod.

Increasingly, researchers are exploiting the unique advantages of a multifaceted mass spectral approach using direct MALDI analysis to determine neuropeptide localization coupled with pooling and extraction for neuropeptide sequencing. Furthermore, mass spectrometry offers information complementary to that obtained through immunocytochemistry, light microscopy, single-cell molecular biology and electrophysiology. It has even been demonstrated that immuno-cytochemical characterization and MALDI-TOF MS profiling can be performed on the same cell. Also, the elucidation of novel neuropeptides through tandem MS has the potential to lead to the

discovery of previously uncharacterized genes through single-cell reverse transcriptase polymerase chain reaction (RT-PCR). In order to gain the most complete understanding of neuronal signaling, mass spectrometry should be employed as one facet of a multidisciplinary approach.

Koenig *et al.* (2005) analysed several AKHs from different insect species and their particular ionization behaviour with respect to their sequence and they observed following results. AKHs exhibit characteristic ion pairs, $(M+Na)^+/(M+H)^+$ in MALDI-MS and $(M+H+K)^{2+}$, $(M-17+H)^+$, $(M+H)^+$, $(M+Na)^+$ and $(M+K)^+$ in ESI-MS, their high affinity for Na^+ and K^+ alkali cation is observed after reversed phase purification. AKHs rarely form doubly charged ions with protons or sodium while the $(M+H+K)^2$ ion is often abundant suggesting a special conformation of larger metal ion complex possibly related to its size.

Areas of intensive research that will undoubtedly impact the study or arthropod neuropeptidomics of the single-cell level are itemized as follows. MS/MS Labeling reactive groups of neuropeptides can simplify peptide-sequencing efforts. Furthermore, these reactions may be performed *in situ* and therefore show potential for improving neuropeptide sequencing from single-cell preparations. In addition to the more conventional CID fragmentation techniques, two recently developed electron-based tandem MS fragmentation methods, electron-capture dissociation and electron-transfer dissociation

techniques, have shown great promise for sequencing large peptides and small proteins, while preserving labile post-translational modifications. These newer tandem MS techniques, coupled with improvements in instrumentation, should benefit arthropod peptidomic studies.

Signal transduction in AKH activity

Recently AKH receptors have been cloned from the fruitfly, *Drosophila melanogaster* and the silkworm, *Bombyx mori* (Park *et al.*, 2002; Staubli *et al.*, 2002). The receptors are G-protein-coupled and are structurally and evolutionarily related to the gonadotropin releasing hormone receptors from vertebrates. More than one such AKH receptor type can be present in an insect (Staubli *et al.*, 2002). AKH peptides are believed to act by binding to the receptor, which changes conformation and interacts with a G-protein. This subsequently transduces the signal to an enzyme, which produces a second messenger in the cytoplasm. Signal transduction of AKH peptides has only been studied in a few insects and many details remain far from clear, nevertheless a general picture has been deduced (Van der Horst *et al.*, 1999; Gaede and Auerswald, 2003). In general, in hypertrehalosemia, peptides bind to the G-protein-coupled-receptor and activate phospholipase C (PLC) increasing inositol 1, 4, 5-trisphosphate (IP_3) levels. This induces the release of Ca^{2+} from intracellular Ca^{2+} stores, which leads to the initiation of the capacitative Ca^{2+} entry into the cytosol. The increased Ca^{2+} concentration results in the phosphorylation and activation of glycogen phosphorylase by

phoyphorylase kinase. AKH further enhances the efflux of Ca^{2+} from the cytosol to reach the normal basal level. In *P. americana*, production of DAG along with IP_3 has been proposed. DAG in conjunction with Ca^{2+} then activates protein kinase C (PKC), which, in turn, activates glycogen phosphorylase by phosphorylation (Sun and Steele, 2001, 2002). In hyperlipaemia on the other hand, binding of AKH leads to a conformational change in a G protein, which, in turn, activates an adenylate cyclase, resulting in an increase of intracellular cAMP levels. Cyclic AMP stimulates lipase activity, most likely via activation of a protein kinase A (PKA). The influx of extracellular Ca^{2+} is also essential for the adipokinetic effect. In moths, release of Ca^{2+} from IP_3-insensitive intracellular stores causes an increase in the haemolymph lipid titers (Arrese *et al.*, 1999).

The mode of action of AKH during hyperprolinemia appears to be similar to that during hyperlipaemia. It seems that AKH binds to the receptor to cause a conformational change of a Gs-protein, which, in turn, activates an adenylate cyclase. The increase in cAMP levels might activate triacylglycerol lipase (TGL) and consequently TAG breakdown to release FAs. AKH seems to activate Ca^{2+} release from intracellular stores and also the capacitative Ca^{2+} entry into the cytosol. Free FAs produced can undergo β-oxidation, the resulting acetyl-CoA, together with alanine, are used for re-synthesis of proline. Administration of melatonin into the isolated locust corpora cardiaca stimulated the

release of adipokinetic hormone precursor related peptides (APRP 1 and APRP 2), neuroparsins (NPA1, NPA2 and NPB) and diuretic peptides (Huybrechts *et al.*, 2002). The injection of cAMP analogue (which penetrate the cell membrane), causes a stimulation of proline synthesis but not the mobilization of carbohydrate. Cyclic GMP seems to be involved neither in the synthesis of proline nor carbohydrate release in the cetoniid beetle, *Pachnoda sinuata* (Auerswald and Gaede, 2000).

Synthesis and release of AKHs

The corpora cardiaca of the locust, *S. gregaria*, provides a model system to study neuropeptide biosynthesis and processing. Hekimi and O'Shea (1985) using an *in vitro* organ culture studied the synthesis of AKHs in *S. gregaria*. Moshitzky *et al.* (1987 a) found that AKHs are synthesized in the brain of locusts. Noyes and Schaffer (1990) reported that AKH-I and AKH-II are synthesized from very small mRNAs of 550 bases in *S. nitrans*. O'Shea and Rayne (1992) demonstrated that the biosynthesis of AKH-I and II involves the enzymatic processing of larger precursor polypeptides. This bigger peptide molecule is called Pro-AKH. Two other small polypeptides (P1 and P2), which, showed immunoreactivity to anti-AKH and contained (H^3) tryptophan were proposed to be the precursors of AKHs. Prior to processing the AKH prohormones form dimers in the endoplasmic reticulum, which are the direct precursors of AKHs (Hekimi *et al.*, 1989). The glandular lobes of the CC contain approximately 6000-10000 intrinsic neurosecretory

cells which are devoted to the biosynthesis of the AKHs (Hekimi *et al.*, 1989). Oudejans *et al*. (1996) reported that in *L. migratoria* total time necessary for biosynthesis and processing of the bioactive AKHs is only 75 minutes.

Flight is the primary stimulus for the release of AKHs in adult locusts. Release of AKHs was found to be under the control of secretomotor centers in the lateral areas of the protocerebrum (Rademakers, 1977) and this control is probably by octopaminergic neurons (Orchard and Loughton, 1981). Jutsum and Goldsworthy (1976) have suggested that the concentrations of metabolic fuels, such as trehalose and diacylglycerols in the haemolymph, control the release of AKH. Houben and Beenakkers (1973) have demonstrated that the release of hormone is signaled by a decrease in haemolymph trehalose, which becomes depleted after a few minutes of flight. However, sucrose injections caused a delay in lipid mobilization (Cheeseman *et al*., 1976) indicating that the drop in trehalose level during the first few minutes of flight cannot be the primary stimulus for AKH release (Goldsworthy, 1983). Elevated levels of intracellular Ca^{2+} are found to be one of the critical factors, which initiate the process of AKH release. Pannabecker and Orchard (1988) reported that an influx of extracellular Ca^{2+} into the neurosecretory cells provide an essential trigger for initiating AKH release. Pannabecker *et al*. (1987) showed that electrical stimulation of the nerve supplying the CC (nervi corpori cardiaci, NCC-II) results in hormone release. octopamine mimicked this activity

accompanied by an elevation of cAMP and requires extracellular Ca^{2+}. The decline of octopamine levels after 15-20 min of flight is accompanied by a decline in haemolymph trehalose levels, which in turn may contribute to the continued release of AKHs.

Although originally described as adult hormones, AKHs have been shown to serve functions in larval insects. The presence of AKHs have been reported in the larval stages of the *L. migratoria* and *S. gregaria* (Goldsworthy *et al.*, 1972; Gaede and Beenakkers, 1977; Hekimi and O'Shea, 1985; Siegert and Mordue, 1986). However, larval AKH is less active than adults (Mwangi and Goldsworthy, 1977) either due to insufficient cAMP level to activate the lipase system (Gaede and Beenakkers, 1977) or due to lack of C-proteins needed in the lipoprotein conversion (Mwangi and Goldsworthy, 1977). Materials with adipokinetic activity are released *in vitro* from neurosecretory cells of the CC in larval locusts (Pannabecker *et al*., 1987).

Quantitation of AKH

Though the modes of action of AKHs have been studied in detail (See Mordue and Stone, 1981; Beenakkers *et al.*, 1981), very little information is available on the hormone titre in the CC. Stone *et al.* (1976) estimated the AKH content of CC to be between 400-700 pmol in *S. gregaria* and 200-500 pmol in *L. migratoria* on the basis of amino acid analysis. Siegert and

Mordue (1986) reported that 6 weeks old adults contain an amount of 900 pmol AKH-I in *S. gregaria* and more than 540 pmol in *L. migratoria.* They also noticed that in both insects, AKH-I and II are synthesized for more than ten days after the adult moult. The CC of *S. gregaria* contained more peptides than *L. migratoria*. In most stages, female contained more hormones than males. In *S. gregaria* AKH-I increases from 190 pmol (early 5th instar) to 920 pmol (in adult males, 6 weeks old) and to 1200 pmol (in females, 6 weeks old). Likewise, AKH-II increased from about 50 pmol to 180 pmol in males and 260 pmol in females. The maximum amount of AKH-I found in adult *L. migratoria* was approximately 550 pmol in males and about 750 pmol in females. Adipokinetic hormone-II was around 100 pmol and 125 pmol in males and females respectively. At any given time during the period studied, the CC contained more AKH-I than AKH-II: the molar ratio, AKH-I: AKH-II ranged from 2:1 to 6:1. Moshitzky *et al*. (1987 b), using radioimmunoassay quantified AKH-I from the locust *L. migratoria* CC and reported a value between 175 and 440 pmol per CC which corresponds to the values determined using bioassay techniques (Stone *et al.*, 1976). Quantification with RP-HPLC also indicates that the total content of both AKH-I and II changes between stages.

Ziegler (1984) suggested that the *Manduca* CC contained 10-20 times less adipokinetic peptide than that in the locust. The content of AKH in the CC of *M. sexta* was estimated by radioimmunoassay to be 33 pmol per CC

pair in adults, and 35 pmol per CC pair in larvae (Fox and Reynolds, 1990). The smaller quantity of hormone in adult *Manduca* compared with the quantity of AKH-I in the adult locust reflect difference between the two species in the time spent in sustained flight as well as the greater longevity of the adult locusts (several weeks compared with the adult moth, one week). A similarity with the locusts is that a considerable increase in the peptide content of the CC occurs during metamorphosis. Evidently the demands of flight in the adult stage require large store of hormone. Orchard *et al.* (1991) reported that the CC of *Pseudaletia unipuncta* males contains approximately 17.6 pmol of *Manduca*-AKH equivalents. This is almost similar to the amounts observed in other Lepidoptera (Ziegler *et al.*, 1985; Ziegler *et al*., 1990) but considerably lower than the 500-1000 pmol reported for the locusts (Orchard, 1987). Using a RIA procedure, Candy (2002) had estimated the AKH titer of *S. gregaria* haemolymph.

Expanding the knowledge on structure and distribution of neuropeptides is the first step in studying the role and interaction of these molecules in controlling physiological processes. Even though many investigations have been conducted (as mentioned earlier) in the area of structural characterisation of AKHs in insects, there still remains lacunae. Thus it is presumed that this study will yield reliable facts on the structural features of AKHs in insect pests especially from India.

Table II.1: Primary structures of peptides of the AKH/RPCH family

Sl. No.	Code name	Species	Amino acid sequence											Molecular weight (Da)	References
			1	2	3	4	5	6	7	8	9	10	11		
1	Venca-AKH	*Venessa cardui*	pE	K	T	F	T	S	S	W	G	G	K	1208.5	Köllisch *et al*. (2000)
2	Bommo-AKH	*Bombyx mori*	pE	I	T	F	S	R	D	W	S	G	NH_2	1177.55	Gaede *et al*. (2008, Article in press)
3	Locmi-AKH-I	*Locusta migratoria*	pE	L	N	F	T	P	N	W	G	T	NH_2	1158.54	Stone *et al*. (1976)
4	Phymo-AKH-I	*Phymateus morbilosus*	pE	L	N	F	T	P	N	W	G	S	NH_2	1144.53	Gaede *et al*. (1996)
5	Declu-CC	*Decaptoma lunata*	pE	L	N	F	S	P	N	W	G	N	NH_2	1157.52	Gaede (1995)
6	Carmo-HrTH-I	*Carausius morosus*	pE	L	T	F	T	P	N	W	G	T	NH_2	1146.53	Gaede *et al*. (1992)
7	Carmo-HrTH-II	*Carasius morosus*	pE	L	T	F	T	P	N	W	G	T	NH_2	1146.53	Gaede and Rinehart (1987 a)
8	Phyle-CC	*Phymateus leprosis*	pE	L	T	F	T	P	N	W	G	S	NH_2	1137.53	Gaede and Kellner (1995)
9	Tabat-HOTH	*Tabanus attratus*	pE	L	T	F	T	P	G	W	G	Y	NH_2	1150.54	Jaffe *et al*. (1989)
10	Helize-HrTH	*Heliothis zea*	pE	L	T	F	S	S	G	W	G	N	NH_2	1077.48	Jaffe *et al*. (1988)
11	Rommi-CC	*Romalea microptera*	pE	V	N	F	T	P	N	W	G	T	NH_2	1144.5	Gaede *et al*. (1988)
12	Bladi-HrTH	*Blaberus discoidalis*	pE	V	N	F	S	P	G	W	G	T	NH_2	1073.49	Hayes *et al*. (1986)
13	Placa-HrTH-I & II	*Platipura capensis*	pE	V	N	F	S	P	S	W	G	N	NH_2	1116.49	Gaede and Janssens (1994)
14	Locmi-HrTH	*Locusta migratoria*	pE	V	T	F	S	R	N	W	S	P	NH_2	1202.58	Siegert (1999)
15	Anoga –AKH-II	*Anopheles gambiae*	pE	V	T	F	S	R	D	W	N	A	NH_2	1204.56	Kauffman and Brown, (2006)
16	Trica-AKH-II	*Tribolium castaneum*	pE	V	T	F	S	R	D	W	N	P	NH_2	1230.57	Gaede *et al*. (2008, Article in press)
17	Melcin-AKH	*Melittea cinxia*	pE	L	T	F	S	S	G	W	G	NH_2		906.42	Gaede *et al*., unpublished data
18	Manse-AKH	*Manduca sexta*	pE	L	T	F	T	S	S	W	G	NH_2		1007.47	Ziegler *et al*. (1985)
19	Anoga-HrTH	*Anopheles gambiae*	pE	L	T	F	T	P	A	W	NH_2			944.47	Kauffman and Brown, (2006)

(Contd...)

20	Aedae-AKH-I	*Aedes aegypti*	pE	L	T	F	T	P	S	W	NH_2	960.47	Kauffman and Brown, (2008).
21	Psein-AKH	*Pseudogrion inconspicuum*	pE	V	N	F	T	P	G	W	NH_2	929.43	Janssens *et al*. (1994)
22	Libau-AKH	*Libellula auripenis*	pE	V	N	F	T	P	S	W	NH_2	959.45	Gaede (1990)
23	Emppe-AKH	*Empusa pennata*	pE	V	N	F	T	P	N	W	NH_2	986.46	Gaede (1991)
24	Manto-AKH	Mantophasmatodea (Order)	pE	V	N	F	S	P	G	W	NH_2	915.42	Gaede *et al*. (2005)
25	Anaim-AKH	*Anax imperator*	pE	V	N	F	S	P	S	W	NH_2	945.43	Gaede *et al*. (1994)
26	Peram-CAH-I	*Periplaneta americana*	pE	V	N	F	S	P	N	W	NH_2	972.44	Witten *et al*. (1984)
27	Letin-AKH	*Lethocerus indicus*	pE	V	N	F	S	P	Y	W	NH_2	1021.46	Gaede *et al*. (2007 b)
28	Grybi-AKH	*Gryllus bimaculatus*	pE	V	N	F	S	T	G	W	NH_2	919.41	Gaede and Rinehart (1987 b)
29	Tenmo-AKH	*Tenebrio molitor*	pE	L	N	F	S	P	N	W	NH_2	986.46	Gaede and Rosinski (1990)
30	Corpu-AKH	*Corixa punctuata*	pE	L	N	F	S	P	S	W	NH_2	959.45	Gaede *et al*. (2007 a)
31	Panbo-RPCH	*Pandalus borealis*	pE	L	N	F	S	P	G	W	NH_2	929.43	Fernlund and Josefsson (1972)
32	Locmi-AKH-II	*Locusta migratoria*	pE	L	N	F	S	A	G	W	NH_2	903.42	Siegert *et al*. (1985)
33	Schgr-AKH-II	*Schistocerca gregraria*	pE	L	N	F	S	T	G	W	NH_2	933.43	Siegert *et al*. (1985)
34	Trica-AKH	*Tribolium castaneum*	pE	L	N	F	S	T	D	W	NH_2	991.43	Gaede *et al*., (2008, Article in press)
35	Nepce-AKH	*Nepa cenerea*	pE	L	N	F	S	S	G	W	NH_2	919.41	Gaede *et al*. (2007 b)
36	Melme-CC	*Melolontha melolontha*	pE	L	N	Y	S	P	N	W	NH_2	1003.45	Gaede (1991 b)
37	Oniay-CC-I	*Onitis aygulus*	pE	Y	N	F	S	T	G	W	NH_2	983.41	Gaede (1997 a)
38	Scade-I	*Scarabeus deludens*	pE	F	N	Y	S	P	N	W	NH_2	1036.44	Gaede (1997 a)
39	Scade-II	*Scarabeus deludens*	pE	F	N	Y	S	P	V	W	NH_2	1021.46	Gaede (1997 a)
40	Locmi-AKH-III	*Locusta migratoria*	pE	L	N	F	T	P	W	W	NH_2	1072.5	Oudejans *et al*. (1991)
41	Pyrap-AKH	*Pyrrhocoris apterus*	pE	L	N	F	T	P	N	W	NH_2	1000.4	Kodrik *et al*. (2000)
42	Micvi-CC	*Microhodotermis viator*	pE	I	N	F	T	P	N	W	NH_2	1000.4	Liebrich *et al*. (1995)
43	Phymo-AKH-III	*Phymateus morbilous*	pE	I	N	F	T	P	W	W	NH_2	1072.5	Siegert *et al*. (2000)

44	Polae-HrTH	*Polyphaga aegyptica*	pE I T F T P N W NH_2	987.48	Gaede and Kellner (1992)
45	Peram-CAH-II	*Periplaneta americana*	pE L T F T P N W NH_2	987.48	Witten *et al*. (1984)
46	Tabat-AKH	*Tabanus attratus*	pE L T F T P G W NH_2	930.45	Jaffe *et al*. (1989)
47	Phote-HrTH	*Phormia terraenovae*	pE L T F S P N W NH_2	973.46	Gaede *et al*. (1990 b)
48	Erysi-AKH	*Erythmis simplicolis*	pE K N F T P S W NH_2	988.47	Gaede and Kellner (1999)

CHAPTER III
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Experimental insects.

The rice grasshopper, *O. nitidula* (Plate I. a) were collected locally from the paddy fields in different parts of Kerala. After collection they were kept in plastic containers with perforated lids. In the laboratory, they were immediately used for hormone extraction.

Adult insects of both sexes and unspecified age of the coffee locust, *A. miliaris* (Plate I. b) were collected from coffee plantations of Kodak district of Karnataka, India. They were brought to Calicut University campus and maintained in the insectary on a diet of *Terminalia cattapa* leaves. Food and faecal matters were removed daily. The insects for bioassay were used after one week.

Adults of the plant bug, *Iphita limbata* (Plate II. a), were collected locally from the fields. A stock culture of insect was maintained in the laboratory on a diet of sprouted green gram (*Phaseolus aureus*) and ripe banana. The insects of both sexes were used for hormone extraction. Adult female insects, after keeping at least one week in the insectary, were used for

bioassays experiments.

Adult insects of both sexes and unspecified age of the rhinoceros beetle, *Oryctes rhinoceros* (Plate II. b) were collected using a pheromone (aggregation pheromone) trap. After collection, they were kept in plastic containers with perforated lids. The insects were brought to the laboratory and immediately used for hormone extraction.

3.1.2. Equipment _____

1. Binocular stereozoom microscope	(ZEISS, Germany)
2. Deep freezer	(Labline, India)
3. Fine forceps	
4. High-speed refrigerated centrifuge	(Plastocraft, India)
5. HPLC	(Shimadzu, Japan)
6. HPLC Solvent filtration unit.	(Millipore, U.S.A)
7. MALDI-TOF/ TOF	(Bruker Daltonics, Germany)
8. Microliter syringe. (10 µl and 20 µl)	(Hamilton, Switzerland)
9. Micropipettes	(Accupipet, Finnpipet)
10. Refrigerator	(LG, India)
11. Sample filtration unit.	(Millipore, U.S.A)
12. Ultrasonicator	(Sonics & Materials, USA)
13. Vacuum desiccators	(Borosil, India)
14. Vacuum concentrator	(Savant, USA)
15. Vacuum pump	(Rivotek, India)

3.1.3 Chemicals

1. Acetonitrile	(HPLC grade, SRL and Merck)_
2. Dihydroxybenzoic acid	(Sigma)
3. Glycerol trioleate	(Merck)
4. HPLC water	(Merck)
5. Hydrochloric acid	(SRL)
6. Methanol	(HPLC grade, SRL and Merck)
7. Oryctalure (Pheromone)	(Chem Tica International, Consta Rica)
8. Orthophosphoric acid	(SRL)
9. Phosphovanillin	(SRL)
10. Synthetic Locmi-AKH-I	(GenScript Corp., USA)
11. Synthetic Pyrap-AKH	(Gift from Dr. D. Kodrik, Czech)
12. Synthetic Schgr-AKH-II	(Gift from Dr. D.J. Candy, U.K)
13. Trifluoroacetic acid	(SRL)
14. Vanillin	(SRL)

3.2. Methods

3.2.1 Preparation of insect saline

The saline solution used for bioassays and topical application studies contained NaCl, 130 mM; KCl, 5mM; Na_2HPO_4, 1.9 mM and K_2HPO_4, 1.7 mM, the pH of the solution was adjusted to 7.5

3.2.2 Preparation of Phosphovanillin reagent

The phosphovanillin reagent used for spectrophotometric quantitation of lipids was prepared by mixing orthphosphoric acid and 0.525% (w/v) aqueous vanillin in the ratio 3:2 (v/v).

3.2.3 Preparation of lipid standard solution.

The lipid standard solution was prepared by dissolving 500 mg of chromatographically pure glycerol trioleate in 50 ml of chloroform. A working standard solution was prepared by diluting 1.0 ml of stock solution to 10 ml with chloroform so as to get 1.0 µg lipid /1.0 µl. Appropriate volumes of this solution were taken for calibration and for quantification of lipid samples in various experiments.

3.2.4 Preparation of synthetic Locmi-AKH-1 solution

Synthetic Locmi-AKH-1 was purchased from GenScript Corp., USA. A stock solution of 100 µmol of hormone was prepared by dissolving 1.0 mg of hormone in 8.625 ml of 80% methanol (HPLC grade). Solutions with required concentrations of hormone were prepared in 80% methanol (in distilled water) for HPLC analysis and in 20% methanol (in insect saline) for bioassay.

3.2.5 Preparation of synthetic Schgr-AKH-II solution.

The synthetic peptide, Schgr-AKH-II was a gift from Dr. D. J. Candy, School of Biochemistry, University of Birmingham, U. K. The synthetic peptide (200 µmol) was dissolved in 209 µl of 80% methanol (HPLC grade) to make a final concentration of 100 nmol/µl. A solution of 50 pmol/20 µl concentration was prepared in 80% methanol for HPLC analysis.

3.2.6 Preparation of synthetic Pyrap-AKH solution.

Synthetic Pyrap-AKH was a gift from Dr. D. Kodrik, Institute of Entomology, Academy of Sciences, Brinisovsca, Czech Republic. The peptide (1.0 mg) was dissolved in 10 ml of 80% methanol to make a final concentration of 100 µmol/ml. Solutions of other required concentrations were prepared in 80% methanol (for HPLC injection) or in insect saline (for bioassay studies).

3.2.7 Preparation of Corpora Cardiaca extracts of the insects

3.2.7.1 *Oxya nitidula* and *Aularches miliaris*

Corpora cardiaca collected from both sexes of adult insects, *O. nitidula* and *A. miliaris* were used as the sources of the hormone. Heads from the insects were removed and placed with dorsal side up and mouth parts away from us, were cut longitudinally between the eyes into two unequal halves. The smaller part was discarded and from the larger part, retrocerebral

complexes were removed with the help of a pair of fine forceps under a stereozoom binocular microscope. The tissues were immediately put into ice cold 80% methanol (HPLC grade) and stored at -4°C until extraction. Tissues were sonicated for 1 min on ice with an ultrasonicator. The extracts were centrifuged at 4°C and 10,000 rpm for 10 min. The supernatants were collected into an eppendorf tube and vacuum dried. The dried supernatants were stored at -4°C until used for HPLC separations, bioassay studies and mass spectrometric analyses.

3.2.7.2 *Iphita limbata*

Adults of both sexes *I. limbata* were used for collecting corpora cardiaca for hormone extraction. Brain retrocerebral complexes as well as retrocerebral complexes alone were used as the sources of hormones. Heads were removed; the dorsal part of the head capsules was removed using a razor blade. The CC-CA complexes lateral to dorsal aorta were carefully removed with the help of a pair of fine forceps under a stereozoom binocular microscope. The brain-CC-CA complexes were similarly dissected out. All other extraction procedures were same as mentioned earlier (See section. 3.2.7.1). These extracts were individually used for HPLC separations, bioassay studies and mass spectrometric analyses.

3.2.7.3 *Oryctes rhinoceros*

Adult *Oryctes rhinoceros* of both sexes were used for collecting the retrocerebral complexes for hormone extraction. Heads were removed; the dorsal part of the head capsules were removed using a pair of surgical scissors. The CC-CA complexes were carefully dissected out with the help of a pair of fine forceps under a stereozoom binocular microscope. The tissues were immediately put into ice cold 80% methanol (HPLC grade) and stored at -4°C until extraction. Extraction procedures were same as mentioned above (3.2.7.1.). These extracts were used for bioassays, HPLC and mass spectrometric analyses.

3.2.8 Detection of biological activity in the CC extracts

3.2.8.1 *Aularches miliaris*

The dried methanolic extract prepared as mentioned earlier (section 3.2.7.1.) was dissolved in insect saline to get a final concentration of one gland pair equivalent (gpe) per 5 µl. A sample of the extract (5 µl) was injected using a microsyringe (10 µl) into the haemolymph of the accepter pyrgomorphid grasshopper, *A. miliaris*. The injection needle was thrust in the intersegmental membrane between thorax and first abdominal segment, ventrally. The hyperlipaemic effects of the extracts were measured by estimating change in the lipid content of the haemolymph after the injection

of extract using phosphovanillin method. Haemolymph samples (2 µl) were collected directly from the cut end of the antenna into precalibrated capillary tubes, and were then transferred into the bottom of test tubes (5 ml capacity). Haemolymph samples taken before injections (from one antenna) were represented as controls and 60 min after injection (from the other antenna) as experimentals. Similar experiments were carried out using 5 µl of insect saline instead of retrocerebral extract. Haemolymph samples thus collected were used for quantification of lipids (See section. 3.2.12).

3.2.8.2 *Oryctes rhinoceros*

The hyperlipaemic effects of the extracts of retrocerebral complexes of *O. rhinoceros* were studied in the female *I. limbata*. The dried extract was redissolved in insect saline to get a final concentration of 5 gpe per 5 µl. The sample was injected (5 µl) into the haemolymph of the accepter insect. Haemolymph samples were taken from the cut end of one antenna before (control) and 60 min after injection (experimental) from the other antenna. The haemolymph samples were used for the determination of total lipids using the method mentioned elsewhere (See section. 3.2.12).

3.2.9 High Performance Liquid Chromatography (HPLC) of extracts of retrocerebral complexes of insects

3.2.9.1 *Oxya nitidula*

The dried extract made from the retrocerebral complexes from *O.*

nitidula was resuspended in 20 µl of 80% methanol (HPLC grade). The extract was filtered using a sample filtration unit with 0.45 µm filter paper. The samples were directly injected into the instrument by a microsyringe (20 µl). HPLC separations were carried out using Shimadzu system with a reversed phase column (C_{18}) 250 mm long, 4.6 mm i.d. The separation was done in a binary gradient from 43% to 53% solvent B in 20 min and then to 70% within a further 6 min with a flow rate of 1 ml/min. Trifluoroacetic acid (TFA) 0.01% in water (HPLC grade) was used as solvent A, solvent B was 60% acetonitrile in solvent A. All the solvents were filtered through 0.45 µm filter paper. The eluants were monitored simultaneously at 210 and 280 nm using a photo diode array (PDA) detector. One minute fractions starting from 4 min up to 20 min and that at 25 min were collected manually, dried by vacuum concentrator, and were used for testing their hyperlipaemic activity. The HPLC profiles were exported into Microsoft Word file and were used for further analysis of the data.

The presence of already known AKH peptides, Locmi-AKH-I and Schgr-AKH-II in the retrocerebral extracts of *O. nitidula* were investigated by analysing their elution pattern in HPLC. The synthetic peptide, Locmi-AKH-I (100 pmol) was injected into the HPLC instrument maintained in the same set up as before for the retrocerebral extracts of *O. nitidula*. Similar analyses were carried out using 50 pmol of synthetic Schgr-AKH-II. The HPLC

profiles of the peptides were overlaid with that of the retrocerebral extract of *O. nitidula* for comparison.

3.2.9.2 *Aularches miliaris*

The extracts of retrocerebral complexes of *A. miliaris* were separated on HPLC with same instrumental set up as that for *O. nitidula*. However, after a linear gradient from 43 to 53% solvent B, 70% B was achieved within a further period of 10 min. Eluants were monitored both at 210 and 280 nm. The one minute fractions starting from 6 to 19 min and that at 23 min were collected manually and freeze dried. These fractions were used for testing their hyperlipaemic activity.

3.2.9.3 *Iphita limbata*

The HPLC analyses of extracts of brain–retrocerebral complexes of *I. limbata* were carried out using the same instrumental set up as before. However, the analyses were carried out with a linear gradient from 43 to 53 % B within 20 min and the eluants were monitored only at 210 nm. The HPLC fractions starting from 6 to 13 min were manually collected, freeze dried and used for analysing their hyperlipaemic activities. Similar HPLC analysis was carried out with extract made of retrocerebral complexes alone. The HPLC fractions 6 to 12 were manually collected, freeze dried and used for bioassays of hyperlipaemic activities.

The presence of already known AKH peptide, Pyrap-AKH in the retrocerebral extract of *I. limbata* was studied by HPLC analysis. The synthetic peptide Pyrap-AKH (200 pmol) was injected into the HPLC with same instrumental conditions used for extracts of retrocerebral complexes of *I. limbata*. The HPLC profiles of the two were overlaid for comparison.

3.2.9.4 *Oryctes rhinoceros*

The HPLC analysis of extracts of retrocerebral complexes of *O. rhinoceros* were carried out using the same methods mentioned for *I. limbata*. The HPLC fractions 5 to 7 were manually collected, freeze dried and used for determining their hyperlipaemic activity.

3.2.10 Hyperlipaemic bioassays of fractions separated on HPLC

3.2.10.1 *Oxya nitidula*

Adult female *I. limbata* were used for *in vivo* hyperlipaemic bioassay of fractions collected (See section 3.2.9.1.). The dried fractions were redissolved in 75 µl each of insect saline. Samples of these fractions (5 µl each) were injected using a microsyringe (10 µl) into the haemolymph of the experimental insect. Haemolymph samples were taken before (control) and 60 min after injection (experimental). The samples were used for the determination of lipids by spectrophotometric method (See section 3.2.12).

3.2.10.2 *Aularches miliaris*

The extracts of retrocerebral complexes of *A. miliaris* were fractionated on HPLC. The hyperlipaemic effects of these fractions were determined by homologous bioassays. The dried fractions were redissolved in 150 µl each of insect saline and fractions containing approximately 2 gpe were injected (5 µl) into the haemolymph of *A. miliaris*. Haemolymph samples were collected before (control) and 60 min after (experimental) the injection and were used for quantitation of lipids (See section 3.2.12).

3.2.10.3 *Iphita limbata*

The hyperlipaemic activities of extracts of brain-retrocerebral complexes of *I. limbata* separated on HPLC were tested in female *I. limbata*. Each fraction was redissolved in 50 µl insect saline. Aliquots of 5 gpe (5 µl) were injected into the haemolymph of the insect. Haemolymph samples were collected before (control) and 60 min after (experimental) the injection. Fractions separated from extracts of retrocerebral complexes (alone) using HPLC were similarly reconstituted and analysed in the same way as mentioned above. The hyperlipaemic effects of both sets of the fractions (brain-retrocerebral complexes and retrocerebral complexes alone) were similarly determined separately (See section. 3.2.12).

3.2.10.4 *Oryctes rhinoceros*

The hyperlipaemic activities of extracts of brain-retrocerebral complexes of *O. rhinoceros*, separated on HPLC were tested in female *I.*

limbata. Each fraction was redissolved in 50 µl of insect saline. Fractions containing 5 gpe were injected (5 µl) into the haemolymph of the insect. Haemolymph samples were collected before (control) and 60 min (experimental) after the injection. The samples from experiments and controls were used for analysing total lipid contents by the method mentioned elsewhere (See section 3.2.12).

3.2.11 Hyperlipaemic effects of injected as well as topically applied synthetic peptides, Pyrap-AKH and Locmi-AKH-I

3.2.11.1 Injection studies

The hyperlipaemic effects of various concentrations of synthetic Pyrap-AKH were studied in female *I. limbata*. The hormone was dissolved in insect saline to get the desire quantities (2.5, 5, 10, 25 and 50 pmol respectively) in 5 µl and was injected using a microsyringe (10 µl) into the haemolymph of the insects. Haemolymph samples (2 µl) were drawn before (control) and 60 min after (experimental) the injection. These samples were used for quantitation of lipids.

Similar investigations were carried out with various quantities (2, 4, 6 and 8 pmol respectively) of Locmi-AKH-I using the same methodology as for Pyrap-AKH.

In similar experiment, Locmi-AKH-I (50 pmol/5 µl) was injected into *A. miliaris* to find out hyperlipaemic effect of the peptide *in vivo*.

3.2.11.2 Topical application studies

The hyperlipaemic effects of different concentrations of topically applied synthetic peptide Pyrap-AKH were studied in female *I. limbata*. The hormone was dissolved in 20% methanol (HPLC grade) in Ringer saline to get desired concentrations (10, 25, 50 and 75 pmol respectively) per 5 µl solution. Various concentrations of the Pyrap-AKH were applied (5 µl) on the abdomen under the wing of experimental insects using a micropipette. Haemolymph samples were collected before (control) and 2 h after application of hormone (experimental).

Similar experiments were carried out using various quantities (5, 10, 15, 20, 25 and 30 pmol respectively) of Locmi-AKH-I. Haemolymph samples were collected and lipids estimated using the method described in section 3.2.12.

3.2.12 Quantitation of haemolymph lipids

Total lipids in the haemolymph samples were determined using phosphovanillin reagent (Frings *et al*., 1972). Haemolymph samples collected (2 µl each) in various experiments were deposited into the bottom of test tubes. Concentrated sulphuric acid (50 µl) were added to these samples, heated in a boiling water bath for 10 min, cooled to room temperature and 2 ml of each phosphovanillin reagent were added. The tubes were thoroughly shaken to mix the content. Optical densities were measured within 5 min

using UV-vis spectrophotometer at 540 nm against a reagent blank.

3.2.13 Construction of lipid standard calibration graph

Glycerol trioleate was used for preparing calibration graph. Known quantities of glycerol trioleate were taken in the bottom of test tubes and chloroform content was evaporated off. Concentrated H_2SO_4 (50 µl) were added to each tube and were mixed thoroughly. These mixtures were heated in a boiling water bath for 10 min, brought to room temperature and phosphovanillin reagent (2 ml) were added. The tubes were thoroughly shaken to mix the contents. The optical densities of the samples were measured by the same procedure as above. The values obtained for different concentrations were used for constructing a calibration graph.

3.2.14 Calculations

From the absorbance values obtained for various sample of haemolymph, concentrations of lipids were calculated by applying the formula.

$$\text{Concentration of sample} = \frac{\text{Concentration of standard x Absorbance of sample}}{\text{Absorbance of standard}}$$

From these values, the amounts of lipid released into the haemolymph of insects in various experiments were determined.

3.2.15 Mass Spectrometric analyses

3.2.15.1 Matrix Assisted Laser Desorption Ionisation- Time Of Flight-Mass Spectrometry (MALDI-TOF-MS)

The dried extracts of neurohaemal tissues of the insects, *O. nitidula*, *A. miliaris*, *I. limbata* and *O. rhinoceros* were used for mass spectrometric analysis. Mass spectrometric analysis were performed on an Ultra Flex mass spectrometer in reflectron ion mode, using a 90 ns time delay and a 25 kV accelerating voltage in the positive ion mode. The extracts of retrocerebral complexes of the insects, *O. nitidula* and *O. rhinoceros* were monitored in Na^+ mode and *I. limbata* were monitored in H^+ mode. The retrocerebral extracts of *A. miliaris* were monitored at H^+ and Na^+ modes. The system utilized 50 Hz pulsed voltage laser, emitting at 337 nm. The ion source and the flight tube were kept at pressure of about $7x10^{-7}$ mbar by turbo molecular pump. The samples were prepared by mixing equal volumes of peptide solution and a saturated solution of the matrix, dihydroxybenzoic acid in 1:1 (v/v) acetonitrile: water mixture. A standard peptide mixture was used for external calibration.

3.2.15.2 Tandem- MS/MS

Tandem mass spectra (MS/MS) were acquired by selecting the precursor masses (1181.39 Da, 956.31 Da, 1095.57 Da, 1001.4 Da, 1167.16 Da, and 1026.70 Da) with a 10 Da window and fragments were generated in

Post Source Decay (PSD) mode. A single acquisition was a sum of 360 added shots to generate the MS/MS spectra. Mass spectra were analysed by using Flex-analysis software.

3.2.15.3 Interpretation of MALDI-MS/MS data.

The MS/MS data were interpreted by the aid of Peptide Fragmentation Ion Analyser-II (PFIA-II) software (**http://hodgkin.mbu.IIsc.ernet.in/~pfia/PFIA-11.html**). PFIA-II is a web tool for evaluating possible sequence specific product ion types for *de novo* sequencing of novel peptides. It also provides the side chain d-, v- and w- ions for Leu/IIe arising from high energy Collision Induced Dissociation (CID). Acyclic and cyclic peptide sequences with a maximal length of 25 residues can be queried. The system provides fragmentation pattern and/or fragmentation diagram (s) and the list of all sequence specific product ions (a, b, c, x, y, z and immonium ions) for the protonated adducts of queried sequence.

In the present investigation all the precursor ions selected have similar molecular masses (in $M+Na^+$ mode) as that of already known AKH/RPCH peptides, 1181.39 Da (Locmi-AKH-I), 956.31 Da (Schgr-AKH-II), 1095.57 Da (Phymo-AKH-III/ Locmi-AKH-III), 1167.16 Da (Phymo-AKH-I) and 1026.70 Da (Melme-CC) and that of 1001.4 Da (Pyrap-AKH) in H^+ mode. The sequences of each peptide were entered into the PFIA-II web tool as queried sequence. The fragment ions a, b, y and immonium ions obtained were used for determining the structure of the precursor molecule.

3.2.16 Statistical analysis and data presentation

Results obtained from various hyperlipaemic bioassay experiments were expressed as mean ± standard error values as well as percentage

difference of the experimentals over controls (E/C%). The paired t-test and results of adipokinetic responses were performed with the use of SPSS Software (version 10). The graphical representation of change in lipid mobilization was plotted by Microsoft Excel programme.

CHAPTER IV
RESULTS

4.1 Elucidation of primary structures of adipokinetic neuropeptides of the paddy pest, *O. nitidula*

4.1.1 Purification and biological activity of *O. nitidula* CC extract

Analysis of the extract of retrocerebral complexes of *O. nitidula* using HPLC showed three prominent absorption peaks designated as (1), (2) and (3) with retention times 9.94, 11.81 and 22.9 min respectively monitored simultaneously at 210 and 280 nm with a PDA detector (Figure IV. 1).

The hyperlipaemic effects of materials in the fractions were tested by injecting the reconstituted fractions into acceptor insects, *I. limbata*. The change in lipid release were analysed by a procedure mentioned in materials and methods section (3.2.10.1). Table IV. 1 and Figure IV. 2 represent the hyperlipaemic activity of HPLC fractions. The materials in the fractions 9.94, 11.81 and 22.9 min showed significant adipokinetic activities with increase of lipids by 43 (P<0.05), 25 (P<0.05) and 29% (P<0.05) over the controls respectively. The hyperlipaemia induced by materials in other fractions were 19 (5 min), 24 (6 min), 4.0 (7 min), 14 (8 min), 1.0 (9 min), 13 (11 min), 13 (13 min), 12 (14 min), 12 (15 min), 24 (16 min), 6.0 (17 min) and 3% (19 min) respectively, but none of these values were statistically significant.

4.1.2 Characterisation of AKH peptides from *O. nitidula*

4.1.2.1 By HPLC

The presence of Locmi-AKH-I in the retrocerebral extracts of *O. nitidula* was studied by injecting 100 pmol of synthetic Locmi-AKH-I into the HPLC instrument with identical instrumental condition as that for retrocerebral extract. The data were compared with the HPLC profile of retrocerebral extract and the results are summarized in the Figure IV. 3. As seen in the chromatogram, Locmi-AKH-1 was eluted at same retention time as that of the material having the retention time 9.94 min in the retrocerebral extract of *O. nitidula*. Similar analysis was carried out with 50 pmol of the synthetic peptide, Schgr-AKH-II and compared with the profile of *O. nitidula* retrocerebral extract (Figure IV. 4). The data showed that the Schgr-AKH-II had identical elution time as that for the materials with retention time 11.8 min of retrocerebral extract of *O. nitidula*. These results indicate the presence of Locmi-AKH-I and Schgr-AKH-II in the retrocerebral extract of *O. nitidula*.

4.1.2.2 By MALDI-TOF-MS

The molecular masses of peptides belonging to AKH/RPCH present in the retrocerebral extract were determined by MALDI-TOF-MS analysis. It was carried out in a reflector positive ion (Na^+) mode with an acceleration

voltage of 25 kV and 50 Hz pulsed N_2 laser, emitting at 337 nm. Figure IV. 5 and Table IV. 2 represent MALDI-MS data of extract of retrocerebral complexes of *O. nitidula*. The molecular ion peaks m/z values 1181.39, 956.31 and 1095.5 are indicative of sodium adducts $(M+Na)^+$ of peptides with masses 1158.39 Da, 933.31 Da, and 1073 Da respectively. These molecular ion peaks have similar masses as that of already known AKH/RPCH peptides, Locmi-AKH-I, Schgr-AKH-II, and Locmi-AKH-III/Phymo-AKH-III respectively.

The amino acid sequence information of the peptides were obtained by employing MALDI-MS/MS analysis. The tandem mass spectra (MS/MS) was acquired by selecting the precursor masses (sodium adducts) 1181.39 Da, 956.31 Da, and 1096.00 Da respectively with a 10 Da window and fragments were generated in post source decay (PSD) mode.

4.1.2.3 MALDI MS/MS of precursor ion m/z 1181.39. The MALDI-TOF-MS/MS spectrum of the precursor ion $(M+Na)^+$ =1181.39 Da is shown in the Figure IV. 6. Since the precursor peptide and Locmi-AKH-I has similar molecular mass, the PFIA-II software analyzed the theoretical fragment ions of Locmi-AKH-I. This data was compared with the observed MS/MS fragment ions. The theoretical fragment ions (a, b, y and x, y and z and immonium ions) are shown in the Table IV. 3. The fragment ions a, b and y types were identified from the MALDI-PSD data (Figure IV. 6). The

identified N- terminal ‘b’ type fragment ions are b_2 (m/z 225.1), b_3 (m/z 339.1), b_4 (m/z 486.2), b_5 (m/z 587.2), b_7 (m/z 789.3) and b_8 (m/z 984.4) and the ‘a’ type fragment ions are a_1 (m/z 84.0), a_4 (m/z 458.2) and a_8 (m/z 956.4) respectively. The fragment ions y_1 (m/z 119.0), y_3 (m/z 362.1), y_4 (m/z 476.2), y_5 (m/z 573.2), y_7 (m/z 821.3) and y_9 (m/z 1048) represent the identified C-terminal ‘y’ type ions. All these evidences obtained from HPLC, bioassay and mass spectral analysis clearly indicate that the precursor peptide is biologically active with a primary structure, pE-L-N-F-T-P-N-W-G-T-NH_2.

4.1.2.4 MALDI MS/MS of precursor ion at m/z 956.31. Figure IV.7 represents the results of MALDI-TOF MS/MS analysis of precursor ion $(M+Na)^+$=956 Da in PSD mode. Since the precursor peptide and the Schgr-AKH-II has similar molecular mass, the theoretical ion fragments (a, b, and y) of the latter was found out by PFIA-II programme (Table IV. 4). These ion fragments were compared with the observed MS/MS data (Figure IV. 7). As indicated in the inset of Figure IV. 7, some of the ion fragments, a, b, y and a few immonium ions were identified. The identified N-terminal ‘b’ type fragment ions of the precursor peptide are b_1 (m/z 112.03), b_2 (m/z 225.1), b_3 (m/z 339.1), b_4 (m/z 486.2), b_7 (m/z 731.3) and b_8 (m/z 917.4). The fragment ions at m/z 197.1, 311.2 and 458.2 are the N-terminal a_2, a_3 and a_4 fragment ions respectively. The identified C-terminal ‘y’ type ions are y_2 (m/z 261.1),

y_3 (m/z 362.2 Da), y_4 (m/z 449.2), y_5 (m/z 596.2) and y_6 (m/z 710.2). Two immonium ions of the amino acids threonine (m/z 74.06) and leucine (m/z 86.09) were also identified. These evidences indicate that the precursor peptide has a primary structure, pE-L-N-F-S-T-G-W-NH_2.

4.1.2.5 MALDI MS/MS of precursor ion at m/z 1096.00. The molecular mass of the precursor peptide at m/z 1096.00 is similar to that of an AKH/RPCH peptide, Locmi-AKH-III. The theoretical fragmentation pattern of the peptide was deduced by using PFIA-II software and the result is provided in the Table IV. 5. The MALDI-MS/MS data and the identified peptide fragment ions are represented in Figure IV. 8. The molecular masses of N-terminal 'a' and 'b' type fragment ions are a_3 (m/z 311.1), a_4 (m/z 458.2), b_3 (m/z 339.1) and b_6 (m/z 684.3). The molecular masses of C-terminal 'y' type fragment ions are y_1 (m/z 204.1), y_3 (m/z 487.2), y_4 (m/z 588.2), y_5 (m/z 735.36) and y_7 (m/z 962.4) respectively. The immonium ions of the amino acids asparagine (m/z 87.05) and tryptophan (m/z 159.09) were also identified. From these evidences it is clear that the amino acid sequence of the precursor peptide is similar to that of Locmi-AKH-III/Phymo-AKH-III (pE-L-N-F-T-P-W-W-NH_2/pE-I-N-F-T-P-W-W-NH_2) respectively.

4.2 Elucidation of primary structure of adipokinetic neuropeptide of the coffee locust, *A. miliaris*

4.2.1 Presence of biological activity in the retrocerebral complexes of *A. miliaris*

The dried methanolic extract (see section 3.2.8.1) was dissolved in insect saline to get a final concentration of one gland pair equivalent (gpe). An aliquot of the extract containing 1 gpe (5 µl) was injected into the accepter pyrgomorphid grasshoppers, *A. miliaris*. The haemolymph lipids were measured before (control) and 60 min after injection (experiment) by a procedure mentioned in the materials and methods section. The result of the analysis is summarized in Table IV. 6. The CC extracts induced significant hyperlipaemic effects (up to 10%) whereas in the control, the injection of 5µl of insect saline (solvent) did not evoke any hyperlipaemic activity (-7%).

4.2.2 Adipokinetic response of synthetic peptide, Locmi-AKH-I in *A. miliaris*

The hyperlipaemic effect of the synthetic peptide, Locmi-AKH-1 was studied by injecting the peptide (50 pmol/5 µl) into the coffee locust, *A. miliaris*. The change in total haemolymph lipid concentration was measured before and 60 min after the injection by a procedure mentioned earlier (see section 3.2.11.1). The results of hyperlipaemic bioassay are shown in Table IV. 6. The synthetic peptide Locmi-AKH-I elicited significant hyperlipaemia ($p<0.05$).

4.2.3 HPLC separations and biological activity of fractions

HPLC analysis of the methanolic extract of fifty CC from *A. miliaris* was carried out mentioned in the methodology section (3.2.10.2). The result is given in Figure IV. 9. There were three major absorbance peaks with retention times 8.75, 10 and 24 min respectively (designated as 1, 2 and 3 in Figure IV. 9). One min fractions from 8 to 20 min and twenty fourth min fractions were collected manually in eppendorf tubes and freeze dried. The dried fractions were dissolved in insect saline. Aliquots containing two gland pair equivalent (2 gpe/5 µl) were injected into the coffee locust, *A. miliaris.* The haemolymph lipids were collected before (control) and 60 min after (experiment) injection. The results of the analyses are shown in Figure IV. 10 and Table IV. 7. The materials in the fractions collected during 9 min, 10 min and 24 min showed significant hyperlipaemic effects, giving increases up to 38 ($p<0.05$), 19 ($p<0.05$) and 17% ($p<0.05$) respectively over the controls. The hyperlipaemic activities exhibited by the other fractions, viz., 8, 11, 12, 13, 14, 15, 16, 18, 19 and 20 min were not statistically significant (Table IV. 7).

4.2.4 Characterization of AKH peptides from *A. miliaris*

4.2.4.1 MALDI-MS analysis

Figure IV. 11 and Table IV. 8 represent the MALDI-MS spectra of extracts of retrocerebral complexes of *A. miliaris* in H^+ mode. Similar analysis

was carried out in Na^+ mode, the results of which are given in Figure IV. 12 and Table IV. 9. The results show that the molecular ion peaks with m/z at 1145.62 and 1167.16 (H^+ and Na^+ modes respectively) obtained represent a peptide with molecular mass 1144.53 Da. A similar peptide has been reported from other pyrgomorphid grasshoppers with code name, Phymo-AKH-I. The primary structure of the molecule was further elucidated by MALDI-MS/MS analysis in PSD mode.

4.2.4.2 MALDI MS/MS of precursor ion at m/z 1167.16: Figure IV. 13 represents the MALDI-MS/MS fragmentation spectrum of precursor ion at m/z 1167.16. The theoretical fragmentation pattern was deduced from PFIA-II software and the data is presented in Table IV. 10. The N-terminal 'b' type ion fragments are b_1 (m/z 112), b_3 (m/z 339), b_4 (m/z 486), b_5 (m/z 587), b_8 (m/z 1007.4) and b_9 (m/z 1041). The 'a' type ions are a_2 (m/z 197) and a_5 (m/z 559). The identified C-terminal y type fragment ions are y_1 (m/z 105), y_3 (m/z 348), y_4 (m/z 462), y_5 (m/z 559), y_6 (m/z 660) and y_9 (m/z 1034). The immonium ions of amino acids identified were glycine (m/z 30.03), proline (m/z 70.06), threonine (m/z 74.06), leucine (m/z 80.09) and phenylalanine (m/z 120.01). Thus the primary structure of the precursor ion is derived as pE-L-N-F-T-P-N-W-G-S-NH_2.

4.3 Elucidation of primary structure of adipokinetic neuropeptide of the plant bug, *Iphita limbata*

Extracts of brain-retrocerebral complexes as well as retrocerebral complexes (alone) of *I. limbata* were used as the sources of AKH neuropeptides. The HPLC separation, bioassay and mass spectrometric analyses were carried out using these extracts separately.

4.3.1 HPLC separations and biological activity of fractions

Figure IV. 14 represents the elution profile of extracts of fifty brain-CC –CA complexes of *I. limbata* obtained by RP- HPLC. The chromatogram showed three prominent absorption peaks (represented as 1, 2 and 4) along with a small peak (represented as 3) with retention times 7.01, 11.3, 12.52 and 13.8 min monitored at 210 nm. One min fractions from 6 to 13 min were collected and dried as mentioned earlier. Aliquots of these fractions representing five gland pair equivalent (5 gpe) were injected into the plant bug, *I. limbata* to test the hyperlipaemic effects *in vivo.* The results of the analyses are summarized in Table IV. 11 and is represented as a histogram (Figure IV. 15). As depicted in the histogram, the materials in the HPLC fractions with retention times 6 and 12 min induced significant adipokinetic response up to 53 and 43% over control respectively. The materials in the fractions 7, 9 and 10 min induced hyperlipaemic responses with increases up to 21, 30 and 34% over the controls. However, these effects were not

statistically significant.

Similar analyses were carried out with fifty CC-CA complexes (alone) collected from *I. limbata*. The result of the HPLC analysis is provided in Figure IV. 16. The data showed six prominent absorption peaks (designated as 1, 2, 3, 4, 5 and 6) with retention times 7.2, 8.02, 8.85, 9.75, 12.11 and 13.48 min respectively. Each one min fraction from 8 to 14 min were manually collected, freeze dried and reconstituted in insect saline as mentioned in the materials and methods section (3.2.10.3). An *in vivo* bioassay was carried out to study the hyperlipaemic effects of materials in the collected fractions, and results are represented as histogram in Figure IV. 17 and Table IV. 12. The materials in the HPLC fractions 12 and 14 min induced significant adipokinetic activities and increases by 65% and 45% ($P<0.05$) over control respectively. The materials present in the other fractions 8, 9, 10, 11 and 13 also showed slight hyperlipaemic activities, viz., increases up to 10, 20, 29, 22 and 16% respectively, which were not statistically significant.

4.3.2 Hyperlipaemic effects of the synthetic peptides, Pyrap-AKH and Locmi-AKH-I injected as well as applied topically

4.3.2.1 *In vivo* injection experiments

Various concentrations of the synthetic adipokinetic hormone, Pyrap-AKH-I were tested for their effects on the amount of lipids in the haemolymph of *I. limbata in vivo*. The hormone was injected into the

haemolymph of the insects; the total lipids present in the haemolymph were measured in controls and experimentals as mentioned in materials and methods section (3.2.11.1). The results are presented in Figure IV. 18 and Table IV. 13. The data show that in the haemolymph lipid increase induced by Pyrap-AKH is dose dependent. The maximal hyperlipaemic response was noticed with a concentration 10 pmol (27%). The hyperlipaemic effects of other concentrations are 13 (2.5 pmol), 16 (5 pmol), 16 (25 pmol) and 9% (50 pmol) respectively.

Similar analyses were carried out with synthetic Locmi-AKH-I, the results of which are presented in Figure IV. 19 and Table IV. 14. The concentration, 6 pmol/5 µl of synthetic peptide induced maximal hyperlipaemic response up to 46% over control. The lipids released with other concentrations of Locmi-AKH-I were 34 (2 pmol), 37 (4 pmol) and 30% (8 pmol) over control respectively. All the values were statistically significant ($p<0.05$).

4.3.2.2 Topical application experiments

The hyperlipaemic effects of various quantities of topically applied synthetic peptides, Pyrap-AKH and Locmi-AKH-I were studied by a method described in materials and methods section (3.2.11.2). The results of the analyses using Pyrap-AKH are given in Figure IV. 20 and Table IV. 15. The data indicate that the haemolymph lipid induced by topically applied Pyrap-

AKH is dose dependent. The peptide with a concentration of 25 pmol/5 µl induced maximal hyperlipaemic response, 19%. A gradual decrease in lipid release was noticed with the peptide above 25 pmol. The hyperlipaemic effects induced by other concentrations were 15 (10 pmol), 16 (50 pmol), 13 (75 pmol), and 12% (100 pmol) respectively.

Similar analyses were carried out with synthetic Locmi-AKH-I. Figure IV. 21 and Table IV. 16 represent the hyperlipaemic effects of topically applied Locmi-AKH-I. As indicated by the results, there was a dose dependent hyperlipaemic effect. Locmi-AKH-I, 15 pmol induced a maximal hyperlipaemic activity of 41% ($p<0.05$) over the control values with a gradual decline with higher concentrations. The activities of other concentrations were 15 (5 pmol), 27 (10 pmol), 30 (20 pmol), 37 (25 pmol) and 35% (30 pmol) respectively over the control values. All the values were statistically significant ($p<0.05$).

4.3.3 Characterization of AKH/RPCH peptides from *I. limbata*

4.3.3.1 By HPLC

The presence of Pyrap-AKH in the extracts of brain-retrocerebral complexes of *I. limbata* was tested by injecting 500 pmol of the synthetic peptide into the HPLC instrument with identical instrumental conditions as before. The results are shown in the Figure IV. 22. As seen in the Figure, the Pyrap-AKH was eluted with a retention time 12 min which was exactly same

as that of the biologically active peak detected in the previous run for the extract.

Similar analysis was carried out with crude CC-CA extract and synthetic Pyrap-AKH. The data show that the active material in the fraction 12.11 min of the retrocerebral extract also had the same retention time as that of the synthetic pyrap-AKH (Figure IV. 23).

4.3.3.2 By MALDI-MS analysis

Aliquots of extracts of brain-CC-CA complexes of *I. limbata* were analysed by MALDI-MS and the results are summarized in Figure IV. 24 and Table IV. 17. Among the abundant peptide ion peaks, two weak molecular ion peaks with m/z values at 1001.4 and 986.82 were detected which are the representatives of hydrogen adducts (H^+) of peptides with molecular mass 1000.4 Da and 985.82 Da respectively. The peptide mass of one of these is similar to that of Pyrap-AKH (1000.4 Da). The other is very close to that of the Peram-CAH-II (987.4 Da).

Similar investigations were carried out using extracts of retrocerebral complexes alone of *I. limbata*. The results are shown in the Figure IV. 25 and Table IV. 18. The MALDI-MS spectra have given a few ion signals, among which two prominent hydrogen adducts $(M+H)^+$ of molecular ion peaks with m/z values at 1001.4 and 986.82 were identified, the peptide with mass 1000.4 Da was selected for further sequencing of the peptide in MALDI-PSD

mode.

4.3.3.3 MALDI MS/MS of precursor peptide at m/z 1001.4 $(M+H)^+$: Figure IV. 26 represents the fragmentation pattern of the precursor ion 1001.4 Da $(M+H)^+$. The theoretical fragmentation pattern of Pyrap-AKH was deduced from web tool, PFIA-II, the results of which are provided in Table IV. 19. The identified C-terminal 'y' type ions are; y_3 (m/z 415.2) and y_6 (m/z 777.36). The N- terminal a type ions are a_2 (m/z 197.12), a_4 (m/z 458.24), a_6 (m/z 656.34), a_7 (m/z 770.38) and a_8 (m/z 956.4) and 'b' type ions are; b_1 (m/z 112.03), b_3 (m/z 339.16), b_4 (m/z 486.2), b_5 (m/z 587.28), b_6 (m/z 684.33) and b_7 (m/z 798.37). In addition to these, immonium ions of amino acids proline (m/z 70.06), threonine (m/z 74.06), leucine (86.09) and phenyl alanine (m/z 120.08) were also identified. From these informations, the primary structure of the precursor ion is derived as pE-L-N-F-T-P-N-W-NH_2, which is the same as that of the already known AKH/ RPCH peptide Pyrap-AKH.

4.4 Elucidation of primary structure of adipokinetic neuropeptide of the rhinoceros beetle, *Oryctes rhinoceros*

4.4.1 Hyperlipaemic activity of extracts of retrocerebral complexes

The crude retrocerebral extract of *O. rhinoceros* was used for bioassays. The dried extract prepared as mentioned earlier (section 3.2.8.2) were reconstituted into a final concentration of 5 gpe/5 µl. Aliquots of the

sample were injected (5 µl) into the acceptor bugs, *I. limbata*. The hyperlipaemic bioassays of the fractions were carried out and the results of the analyses are summarised in the Table IV. 20. The data showed that the extracts of retrocerebral complexes of *O. rhinoceros* induced significant hyperlipaemia up to 10% over the control.

4.4.2 HPLC separation and bioassays of fractions

An extract of fifty retrocerebral complexes of *O. rhinoceros* was subjected to HPLC analysis with the instrumental conditions as mentioned earlier (see section 3.2.9.4). The result of the analysis is given in Figure IV. 27. The HPLC profile indicates the presence of a few absorption peaks with retention times 4.91, 5.39 and 6.18 min at 210 nm. One min fractions from 4 to 7 min were manually collected and used for determining their hyperlipaemic activities. The hyperlipaemic bioassays of the fractions were carried out using the method mentioned earlier (section 3.2.10.4). The results are provided in the Table IV. 21. From the table it is clear that the materials in the fractions 6 and 7 min showed significant hyperlipaemic effects 12 and 5% increase respectively over the controls. The materials in the 4 min fraction also showed hyperlipaemia (4%) but it was not statistically significant.

4.4.3 Characterisation of AKH peptide from *O. rhinoceros*

4.4.3.1 MALDI-MS analysis

The extracts of retrocerebral complexes of *O. rhinoceros* were used for the MALDI-MS analysis. The analysis was carried out in Na^+ mode as mentioned in the materials and methods section (3.2.15.1). The result of the analysis is provided in Figure IV. 29 and Table IV. 22. The data show molecular masses of only a very few peptides. The molecular ion peak at m/z 1026.70 is the representative of sodium adducts $(M+Na)^+$ of the peptide with molecular mass 1003.70 Da. The mass of this peptide is similar to that of already identified adipokinetic peptide, Melme-CC (1003.45 Da). This peptide was used as a precursor molecule for the elucidation of primary structure.

4.4.3.2 Precursor ion at m/z 1026.70: The primary structure of the precursor peptide was elucidated by MALDI-MS/MS analysis in PSD mode as mentioned earlier. The result of the analysis is given in the Figure IV. 30. The theoretical fragment ions of the AKH peptide Melme-CC was analysed with PFIA-II. The result of the analysis is presented in the Table IV. 23. The identified N-terminal 'b' type fragment ions are, b_1 (m/z 112.03), b_2 (m/z 225.1), b_4 (m/z 502.23), b_5 (m/z 589.26) and b_7 (m/z 801.34) and the 'a' type fragment ions are a_2 (m/z 197.12) and a_8 (m/z 959.42) respectively. The fragment ions y_3 (m/z 362.1) and y_4 (m/z 476.2) represent the identified C-terminal 'y' types ions. From these inferences it is confirmed that the precursor peptide is the already known Melme-CC with amino acid sequence,

pE-L-N-Y-S-P-D-W-NH_2.

Table IV. 1. Amount of lipid released into the haemolymph of *I. limbata* injected with fractions of the retrocerebral complexes of *O. nitidula* separated on HPLC

Sl. No.	Fractions (min)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
1	4	3.15	2.99	94.92±12.8
2	5	3.19	3.78	118.49±7.6
3	6	6.81	8.42	123.64±12.8
4	7	4.24	4.42	104.24±5.82
5	8	5.12	5.81	113.47±15.41
6	9	9.05	9.22	101.87±6.31
7	10	5.72	8.17	142.90±8.98 *
8	11	6.89	7.78	112.91±11.4
9	12	6.46	8.12	125.85±4.3 *
10	13	6.88	7.77	112.93±9.7
11	14	3.09	3.45	113.03±5.61
12	15	3.06	3.46	113.07±9.9
13	16	6.10	7.43	121.80±11.9
14	17	5.5	5.84	106.18±9.6
15	18	3.45	3.35	97.12±5.41
16	19	3.37	3.45	102.37±8.9
17	23	4.36	5.63	129.12±4.56 *

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after (experiment) injection of (3 gpe) fractions separated on HPLC. (*) Indicates $p< 0.05$.

Table IV. 3. A snapshot of web tool showing the output of PFIA-II for the queried sequence of qLNFTPNWGT (m/z=1158.54). The data show the theoretical fragment ions a,b,c and x, y, z and immonium ions of the amino acids

Sequence Entered	:	qLNFTPNWGT (q = Pyroglutamate)
Sequence Length	:	10 Amino acids
Molecular Mass	:	1158.54578 Da
Proton adduct (m/z) (z = +1)	:	1159.55378
Sequence Type	:	Linear Peptide
N-terminus modification	:	None (H)
C-terminus modification	:	Amidated (NH2)
Cysteine modification (C*)	:	None
Sulfation modification	:	None
Phosphorylation modification	:	None

a1 : 84.04493	a2 : 197.12899	a3 : 311.17192	a4 : 458.24033	a5 : 559.28801	a6 : 656.34077	a7 : 770.38370	a8 : 956.46301	a9 : 1013.48447	a10 : 1114.53215
b1 : 112.03985	b2 : 225.12391	b3 : 339.16684	b4 : 486.23525	b5 : 587.28293	b6 : 684.33569	b7 : 798.37862	b8 : 984.45793	b9 : 1041.47939	b10 : 1142.52707
c1 : 129.06701	c2 : 242.15107	c3 : 356.19400	c4 : 503.26241	c5 : 604.31009	c6 : 701.36285	c7 : 815.40578	c8 : 1001.48509	c9 : 1058.50655	
-- q --	-- L --	-- N --	-- F --	-- T --	-- P --	-- N --	-- W --	-- G --	-- T --
	x9 : 074.50085	x8 : 961.41679	x7 : 847.37386	x6 : 700.30545	x5 : 599.25777	x4 : 502.20501	x3 : 388.16208	x2 : 202.08277	x1 : 145.06131
	y9 : 048.52157	y8 : 935.43751	y7 : 821.39458	y6 : 674.32617	y5 : 573.27849	y4 : 476.22573	y3 : 362.18280	y2 : 176.10349	y1 : 119.08203
z10 : 142.52707	z9 :1031.49504	z8 : 918.41098	z7 : 804.36805	z6 : 657.29964	z5 : 556.25196	z4 : 459.19920	z3 : 345.15627	z2 : 159.07696	z1 : 102.05550

Immonium Ions:

G	:	30.03436	P	:	70.06566
T	:	74.06058	q	:	84.04493
L	:	86.09696	N	:	87.05583
F	:	120.08131	W	:	159.09221

Table IV. 4. A snapshot of web tool showing the output of PFIA-II for the queried sequence of qLNFSTGW (m/z=933.43). The data show the theoretical fragment ions a,b,c and x, y, z and immonium ions of the amino acids

Sequence Entered	:	qLNFSTGW (q = Pyroglutamate)
Sequence Length	:	8 Amino acids
Molecular Mass	:	933.43444 Da
Proton adduct (m/z) (z = +1)	:	934.44244
Sequence Type	:	Linear Peptide
N-terminus modification	:	None (H)
C-terminus modification	:	Amidated (NH2)
Cysteine modification (C*)	:	None
Sulfation modification	:	None
Phosphorylation modification	:	None

a1 : 84.04493	a2 : 197.12899	a3 : 311.17192	a4 : 458.24033	a5 : 545.27236	a6 : 646.32004	a7 : 703.34150	a8 : 889.42081
b1 : 112.03985	b2 : 225.12391	b3 : 339.16684	b4 : 486.23525	b5 : 573.26728	b6 : 674.31496	b7 : 731.33642	b8 : 917.41573
c1 : 129.06701	c2 : 242.15107	c3 : 356.19400	c4 : 503.26241	c5 : 590.29444	c6 : 691.34212	c7 : 748.36358	
-- q --	-- L --	-- N --	-- F --	-- S --	-- T --	-- G --	-- W --
	x7 : 849.38951	x6 : 736.30545	x5 : 622.26252	x4 : 475.19411	x3 : 388.16208	x2 : 287.11440	x1 : 230.09294
	y7 : 823.41023	y6 : 710.32617	y5 : 596.28324	y4 : 449.21483	y3 : 362.18280	y2 : 261.13512	y1 : 204.11366
z8 : 917.41573	z7 : 806.38370	z6 : 693.29964	z5 : 579.25671	z4 : 432.18830	z3 : 345.15627	z2 : 244.10859	z1 : 187.08713

Immonium Ions :

G	:	30.03436	S	:	60.04493
T	:	74.06058	q	:	84.04493
N	:	87.05583	L	:	86.09696
F	:	120.08131	W	:	159.09221

Table IV. 5. A snapshot of web tool showing the output of PFIA-II for the queried sequence of qLNFTPWW (m/z=1072.51). The data show the theoretical fragment ions a,b,c and x, y, z and immonium ions of the amino acids

Sequence Entered	:	qLNFTPWW (q = pyroglutamate)
Sequence Length	:	8 Amino acids
Molecular Mass	:	1072.51302 Da
Proton adduct (m/z) (z = +1)	:	1073.52102
Sequence Type	:	Linear Peptide
N-terminus modification	:	None (H)
C-terminus modification	:	Amidated (NH2)
Cysteine modification (C*)	:	None
Sulfation modification	:	None
Phosphorylation modification	:	None

a1 : 84.04493	a2 : 197.12899	a3 : 311.17192	a4 : 458.24033	a5 : 559.28801	a6 : 656.34077	a7 : 842.42008	a8 : 1028.49939
b1 : 112.03985	b2 : 225.12391	b3 : 339.16684	b4 : 486.23525	b5 : 587.28293	b6 : 684.33569	b7 : 870.41500	b8 : 1056.49431
c1 : 129.06701	c2 : 242.15107	c3 : 356.19400	c4 : 503.26241	c5 : 604.31009	c6 : 701.36285	c7 : 887.44216	
-- q --	-- L --	-- N --	-- F --	-- T --	-- P --	-- W --	-- W --
	x7 : 988.46809	x6 : 875.38403	x5 : 761.34110	x4 : 614.27269	x3 : 513.22501	x2 : 416.17225	x1 : 230.09294
	y7 : 962.48881	y6 : 849.40475	y5 : 735.36182	y4 : 588.29341	y3 : 487.24573	y2 : 390.19297	y1 : 204.11366
z8 : 1056.49431	z7 : 945.46228	z6 : 832.37822	z5 : 718.33529	z4 : 571.26688	z3 : 470.21920	z2 : 373.16644	z1 : 187.08713

Immonium Ions :

P:70.06566 T:74.06058
q :84.04493 L:86.09696
N :87.05583 F:120.08131
W :159.09221

Table IV. 6. The amount of lipid released into the haemolymph of *A. miliaris.*

Source	(n)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
Insect saline	5	2.42	2.26	93.38±2.51
Aularches CC extract	5	2.85	3.13	110.13±0.81 *
Locmi-AKH-I 50 pmol/5 µl	7	3.20	3.51	109.83 ± 0.26 *

Values are expressed as means ± SEMs. The haemolymph lipids were measured before (control) and 60 min after (experiment) the injection of insect saline, CC-CA extract of *A. miliaris* and Locmi-AKH-I. (*) Indicates $p < 0.05$

Table IV. 7. Amount of lipid released into the haemolymph of *A. miliaris* injected with fractions of extracts of its retrocerebral complexes separated on HPLC.

Sl. No	Fractions (min)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
1	8	2.75	2.98	108.36±5.31
2	9	2.03	2.80	137.93±7.65*
3	10	2.62	3.10	118.32±6.10*
4	11	2.64	2.94	111.26±11.62
5	12	3.04	3.38	111.17±8.48
6	13	3.52	3.87	112.10±15.68
7	14	2.9	2.91	100.34±5.57
8	15	2.52	2.76	109.52±19.20
9	16	3.12	3.51	112.50±4.50
10	17	3.55	3.09	-87.04±9.20
11	18	2.45	2.53	103.26±5.10
12	19	2.10	2.15	102.38±11.19
13	20	2.61	2.73	104.59±1.99
14	24	1.9	2.22	116.84±3.15 *

Values are expressed as means ± SEMs (n=5). The haemolymph lipids were measured before (control) and 60 min after (experiment) injection of (2 gpe) fractions separated on HPLC. (*) Indicates $p< 0.05$.

Table IV. 10. A snapshot of web tool showing the output of PFIA-II for the queried sequence of qLNFTPNWGS (m/z=1144.53). The data show the theoretical fragment ions a,b,c and x, y, z and immonium ions of the amino acids

Sequence Entered	:	qLNFTPNWGS (q= Pyroglutamate)
Sequence Length	:	10 Amino acids
Molecular Mass	:	1144.53013 Da
Proton adduct (m/z) (z = +1)	:	1145.53813
Sequence Type	:	Linear Peptide
N-terminus modification	:	None (H)
C-terminus modification	:	Amidated (NH2)
Cysteine modification (C*)	:	None
Sulfation modification	:	None
Phosphorylation modification	:	None

a1 : 84.04493	a2 : 197.12899	a3 : 311.17192	a4 : 458.24033	a5 : 559.28801	a6 : 656.34077	a7 : 770.38370	a8 : 956.46301	a9 : 1013.48447	a10 : 1100.51650
b1 : 112.03985	b2 : 225.12391	b3 : 339.16684	b4 : 486.23525	b5 : 587.28293	b6 : 684.33569	b7 : 798.37862	b8 : 984.45793	b9 : 1041.47939	b10 : 1128.51142
c1 : 129.06701	c2 : 242.15107	c3 : 356.19400	c4 : 503.26241	c5 : 604.31009	c6 : 701.36285	c7 : 815.40578	c8 : 1001.48509	c9 : 1058.50655	
-- q --	-- L --	-- N --	-- F --	-- T --	-- P --	-- N --	-- W --	-- G --	-- S --
	x9 : 1060.48520	x8 : 947.40114	x7 : 833.35821	x6 : 686.28980	x5 : 585.24212	x4 : 488.18936	x3 : 374.14643	x2 : 188.06712	x1 : 131.04566
	y9 : 1034.50592	y8 : 921.42186	y7 : 807.37893	y6 : 660.31052	y5 : 559.26284	y4 : 462.21008	y3 : 348.16715	y2 : 162.08784	y1 : 105.06638
z10:1128.51142	z9 : 1017.47939	z8 : 904.39533	z7 : 790.35240	z6 : 643.28399	z5 : 542.23631	z4 : 445.18355	z3 : 331.14062	z2 : 145.06131	z1 : 88.03985

Immonium Ions :

G	:	30.03436	S	:	60.04493
P	:	70.06566	T	:	74.06058
q	:	84.04493	L	:	86.09696
N	:	87.05583	F	:	120.08131
W	:	159.09221			

Table IV. 11. Amount of lipid released into the haemolymph of *I. limbata* injected with fractions of its brain-retrocerebral complexes separated on HPLC

SL. No.	Fractions (min)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
1	6	24.94	38.08	152.68±3.51*
2	7	53.03	64.26	121.17±10.12
3	8	29.36	29.37	100.01±8.9
4	9	34.08	44.13	129.48±14.3
5	10	29.2	39.16	134.11±16.2*
6	11	41.22	51.10	123.98±6.12*
7	12	29.69	42.31	142.51±15.92*
8	13	74.26	79.9	107.59±3.11

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after (experiment) injection of 5 gpe of fractions separated on HPLC. (*) Indicates $p< 0.05$.

Table IV. 12. Amount of lipid released into the haemolymph of *I. limbata* injected with its retrocerebral complexes separated on HPLC

Sl. No.	Fractions (min)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
1	8	22.1	24.48	109.82±5.32
2	9	19.03	22-82	119.15±8.03
3	10	20.9	26.92	128.80±9.12
4	11	21.11	25.33	120.02±10.31
5	12	22.05	36.41	165.15±10.22*
6	13	27.22	31.82	116.91±7.32
7	14	29.11	42.27	145.21±12.12*

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after (experiment) injection of (5 gpe) fractions separated on HPLC. (*) Indicates $p< 0.05$.

Table IV. 13. Increase in the lipid release into the haemolymph of *I. limbata* injected with synthetic Pyrap-AKH

Concentration (pmol)	Lipid release (µg/µl)		E/C %
	Control (C)	Experiment (E)	
2.5	2.31	2.62	113.34±4.01*
5	3.02	3.51	116.23±2.31*
10	2.50	3.18	127.20±1.92*
25	2.72	3.16	116.17±3.31*
50	1.99	2.17	109.05±1.01*

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after the injection (experiment) of different concentrations of synthetic Pyrap-AKH. (*) Indicates $p< 0.05$

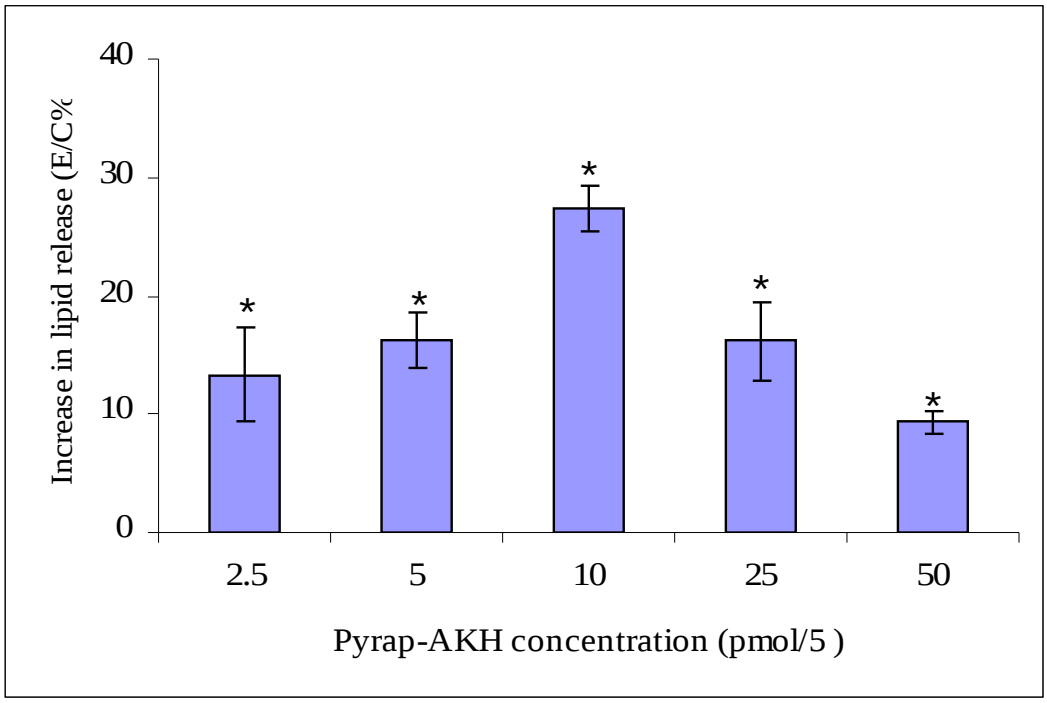

Figure IV. 18: Change in the amount of lipid released into the haemolymph of *I. limbata* injected with synthetic Pyrap-AKH. Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 120 min after the injection (experiment) of different concentrations of synthetic Pyrap-AKH.

Table IV. 14. Amount of lipid released into the haemolymph of *I. limbata* injected with synthetic Locmi-AKH-1

Concentration (pmol)	Lipid release (µg/µl)		E/C %
	Control (C)	Experiment (E)	
2	1.79	2.41	134.63±8.91 *
4	2.02	2.77	137.12±9.38 *
6	1.92	2.79	145.31±7.12 *
8	2.53	3.30	130.43±5.89 *

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after the injection (experiment) of different concentrations of synthetic Locmi-AKH-I. (*) Indicates $p< 0.05$.

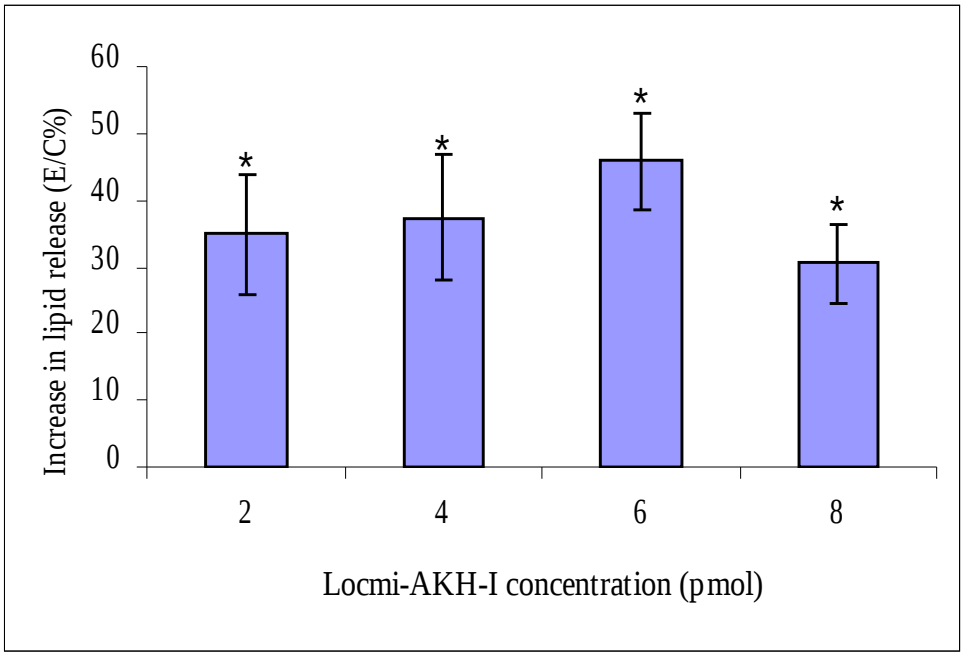

Figure IV. 19: Change in the amount of lipid released into the haemolymph of *I. limbata* injected with synthetic Locmi-AKH-1. Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after the injection (experiment).

Table IV. 15. Amount of lipid released into the haemolymph of *I. limbata* with topically applied synthetic Pyrap-AKH.

Concentration (pmol)	Lipid release (µg/µl)		E/C %
	Control (C)	Experiment (E)	
10	3.01	3.46	114.95±2.10 *
25	2.79	3.33	119.35±3.60 *
50	2.59	3.01	116.21±3.02 *
75	2.88	3.26	113.19±5.91 *
100	3.58	4.02	112.29±6.11 *

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 120 min after the topical application (experiment) of different concentrations of synthetic Pyrap-AKH. (*) Indicates $p< 0.05$

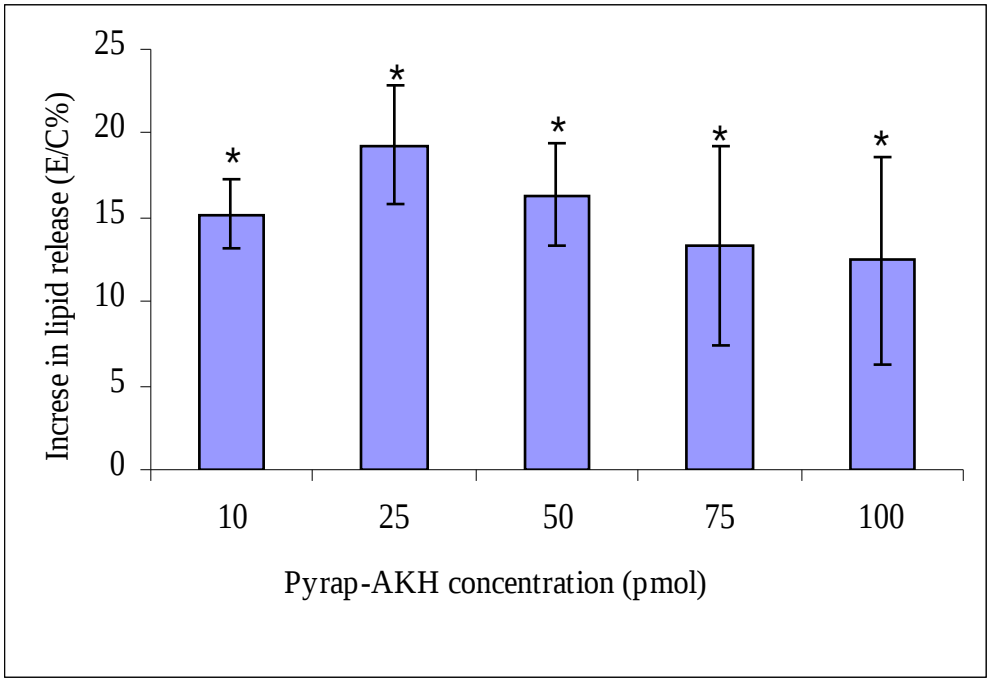

Figure IV. 20: Change in the amount of lipid released into the haemolymph of *I. limbata* with topically applied synthetic Pyrap-AKH. Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 120 min after the topical application (experiment).

Table IV. 16. Amount of lipid released into the haemolymph of *I. limbata* with topically applied synthetic Locmi-AKH-1

Concentration (pmol)	Lipid release (µg/µl)		E/C %
	Control (C)	Experiment (E)	
5	1.95	2.25	115.38±3.25 *****
10	1.80	2.27	126.11±6.12 *****
15	1.59	2.23	140.25±6.19 *****
20	2.69	3.49	129.74±7.38 *****
25	3.02	4.14	137.08±8.19 *****
30	1.98	2.67	134.84±9.32 *****

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 120 min after the topical application (experiment) of different concentrations of synthetic Locmi-AKH-I. (*) Indicates $p < 0.05$.

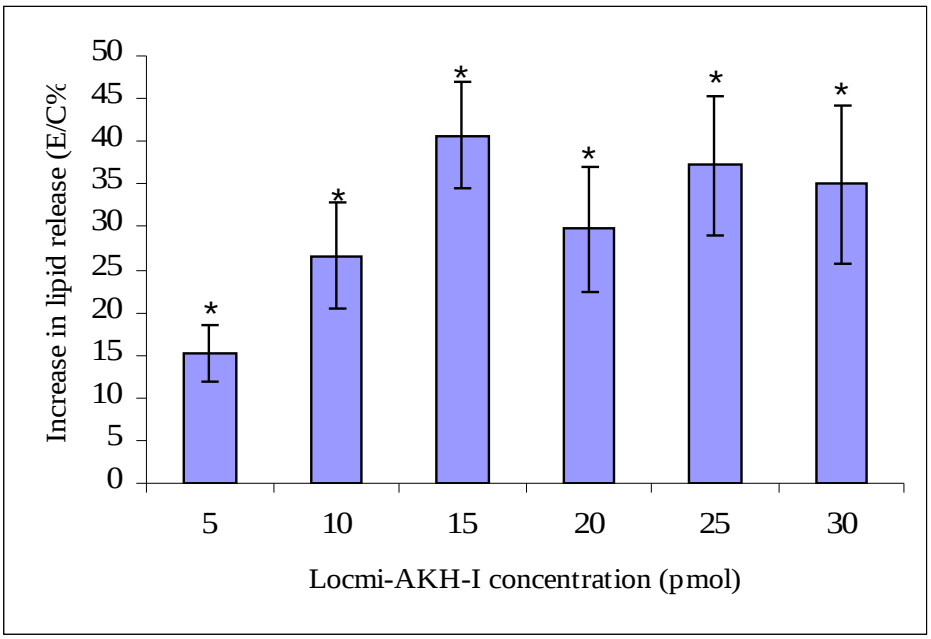

Figure IV. 21: Change in the amount of lipid released into the haemolymph of *I. limbata* with topically applied synthetic Locmi-AKH-I. Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 120 min after the topical application (experiment).

Table IV. 17. Molecular ion peak characteristics of MALDI-MS profile of extracts of brain and retrocerebral complexes of *I. limbata* in H^+ mode

m/z	Resolution	Intensity
450.289	2933	5773.58
485.429	3461	229.26
506.237	3025	1726.42
679.045	1648	21429.08
825.353	3626	631.47
905.573	2795	8223.49
986.821	2428	4941.73
1001.420	4349	4377.64
1165.857	3324	277.00
1258.901	2905	814.57
1400.003	3066	404.65
1533.971	3007	400.56

The analysis was carried out in reflector positive mode with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

Table IV. 19. A snapshot of web tool showing the output of PFIA-II for the queried sequence of qLNFTPNW (m/z=1000.47). The data show the theoretical fragment ions a,b,c and x, y, z and immonium ions of the amino acids

Sequence Entered	:	qLNFTPNW (q = Pyroglutamate)
Sequence Length	:	8 Amino acids
Molecular Mass	:	1000.47664 Da
Proton adduct (m/z) (z = +1)	:	1001.48464
Sequence Type	:	Linear Peptide
N-terminus modification	:	None (H)
C-terminus modification	:	Amidated (NH2)
Cysteine modification (C*)	:	None
Sulfation modification	:	None
Phosphorylation modification	:	None

a1 : 84.04493	a2 : 197.12899	a3 : 311.17192	a4 : 458.24033	a5 : 559.28801	a6 : 656.34077	a7 : 770.38370	a8 : 956.46301
b1 : 112.03985	b2 : 225.12391	b3 : 339.16684	b4 : 486.23525	b5 : 587.28293	b6 : 684.33569	b7 : 798.37862	b8 : 984.45793
c1 : 129.06701	c2 : 242.15107	c3 : 356.19400	c4 : 503.26241	c5 : 604.31009	c6 : 701.36285	c7 : 815.40578	
-- q --	-- L --	-- N --	-- F --	-- T --	-- P --	-- N --	-- W --
	x7 : 916.43171	x6 : 803.34765	x5 : 689.30472	x4 : 542.23631	x3 : 441.18863	x2 : 344.13587	x1 : 230.09294
	y7 : 890.45243	y6 : 777.36837	y5 : 663.32544	y4 : 516.25703	y3 : 415.20935	y2 : 318.15659	y1 : 204.11366
z8 : 984.45793	z7 : 873.42590	z6 : 760.34184	z5 : 646.29891	z4 : 499.23050	z3 : 398.18282	z2 : 301.13006	z1 : 187.08713

Immonium Ions:

P	:	70.06566	T	:	74.06058
q	:	84.04493	L	:	86.09696
N	:	87.05583	F	:	120.08131
W	:	159.09221			

Table IV. 20. Amount of lipid released into the haemolymph of *I. limbata* injected with the extracts of retrocerebral complexes of *O. rhinoceros*

Source	(n)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
Extract of retrocerebral complexes	6	3.03	3.32	109.57±0.53

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after injection of 5 gpe of fractions separated on HPLC (experiment). (*) Indicates $p< 0.05$

Table IV. 21. Amount of lipid released into the haemolymph of *I. limbata* injected with the fractions of extracts of retrocerebral complexes of *O. rhinoceros* separated on HPLC

Sl.No.	Fractions (min)	Lipid release (µg/µl)		E/C %
		Control (C)	Experiment (E)	
1	4	3.38	3.50	103.55±1.92
2	5	2.42	2.01	83.05±0.92
3	6	2.79	3.12	111.82±0.76*
4	7	2.39	2.52	105.43±0.51*

Values are expressed as means ± SEMs (n=6). The haemolymph lipids were measured before (control) and 60 min after injection of 5 gpe of fractions separated on HPLC (experiment). (*) Indicates $p< 0.05$

Table IV. 22. Molecular ion peak characteristics of MALDI-MS profile of extracts of retrocerebral complexes of *O. rhinoceros* in Na^+ mode.

m/z	Resolution	Intensity
883.727	3639	93.23
908.681	3441	789.41
924.672	3556	947.35
1010.718	3904	380.48
1026.703	3961	882.38
1042.693	3894	436.13
1098.078	3985	53.70
1258.156	4360	390.76
1280.156	4624	89.79
1296.158	4439	72.84
1415.245	4032	31.85
1785.570	5426	41.13

The analysis was carried out in reflector positive mode with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

Table IV. 23. A snapshot of web tool showing the output of PFIA-II for the queried sequence of qLNYSPDW (m/z=1003.43). The data show the theoretical fragment ions a,b,c and x, y, z and immonium ions of the amino acids

Sequence Entered : qLNYSPDW. (q = Pyroglutamate)
Sequence Length : 8 Amino acids
Molecular Mass : 1003.43992 Da
Proton adduct (m/z) (z = +1) : 1004.44792
Sequence Type : Linear Peptide
N-terminus modification : None (H)
C-terminus modification : Amidated (NH2)
Cysteine modification (C*) : None
Sulfation modification : None
Phosphorylation modification : None

a1 : 84.04493	a2 : 197.12899	a3 : 311.17192	a4 : 474.23525	a5 : 561.26728	a6 : 658.32004	a7 : 773.34698	a8 : 959.42629
b1 : 112.03985	b2 : 225.12391	b3 : 339.16684	b4 : 502.23017	b5 : 589.26220	b6 : 686.31496	b7 : 801.34190	b8 : 987.42121
c1 : 129.06701	c2 : 242.15107	c3 : 356.19400	c4 : 519.25733	c5 : 606.28936	c6 : 703.34212	c7 : 818.36906	
-- q --	-- L --	-- N --	-- Y --	-- S --	-- P --	-- D --	-- W --
	x7 : 919.39499	x6 : 806.31093	x5 : 692.26800	x4 : 529.20467	x3 : 442.17264	x2 : 345.11988	x1 : 230.09294
	y7 : 893.41571	y6 : 780.33165	y5 : 666.28872	y4 : 503.22539	y3 : 416.19336	y2 : 319.14060	y1 : 204.11366
z8 : 987.42121	z7 : 876.38918	z6 : 763.30512	z5 : 649.26219	z4 : 486.19886	z3 : 399.16683	z2 : 302.11407	z1 : 187.08713

Immonium Ions :

S : 60.04493		P	: 70.06566
q : 84.04493		L	: 86.09696
N : 87.05583		D	: 88.03984
Y : 136.07623		W	: 159.09221

Table IV. 18. Molecular ion peak characteristics of MALDI-MS profile of extracts of retrocerebral complexes of *I. limbata* in H^+ mode.

m/z	Resolution	Intensity
909.446	4165	72.82
927.551	3472	124.56
986.858	3369	189.76
1001.457	4227	93.15
1023.445	4100	336.76
1039.424	4018	347.58
1124.596	4679	76.90
1165.565	3468	42.73
1199.579	4724	142.64
1270.657	4318	88.61
1279.599	4313	521.41
1312.653	5047	92.99
1340.597	4864	87.14

The analysis was carried out in reflector positive mode with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

Table IV. 2. Molecular ion peak characteristics of MALDI-MS profile of extracts of retrocerebral complexes of *O. nitidula* in Na^+ mode.

m/z	Resolution	Intensity
919.76	3240	59.32
956.315	3231	1942.42
972.349	3248	3470.79
974.417	2821	271.21
1095.570	3348	218.94
1111.546	3698	343.39
1167.636	3465	2214.76
1181.655	3701	2651.15
1197.650	3557	3823.76

The analysis was carried out in reflector positive mode with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

Table IV. 8. Molecular ion peak characteristics of MALDI-MS profile of extracts of retrocerebral complexes of *A. miliaris* in H^+ mode.

m/z	Resolution	Intensity
984.523	4220	46.56
1011.689	4619	51.16
1039.748	4389	472.83
1077.648	4223	995.27
1131.033	3212	68.77
1137.716	4846	29.87
1145.62	4832	62.72
1204.657	4271	1327.80
1243.733	5009	178.32

The analysis was carried out in reflector positive mode with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

Table IV. 9. Molecular ion peak characteristics of MALDI-MS profile of extracts of retrocerebral complexes of *A. miliaris* in Na^+ mode.

m/z	Resolution	Intensity
983.151	136.70	31.00
987.125	4213	95.09
999.124	4574	462.89
1029.127	4838	56.92
1045.099	4669	198.73
1077.229	4699	442.31
1115.172	4803	197.57
1167.167	4877	2124.57
1183.147	4869	5593.96
1204.243	4840	719.11
1242.172	5752	103.47
1305.434	5128	80.03

The analysis was carried out in reflector positive mode (Na^+) with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

CHAPTER V
DISCUSSION

The structural similarity between the hypothalamo-hypophysial system of mammals and the pars intercerebralis-corpora cardiaca-corpora allata complex of insects has been identified about six decades ago (Scharrer and Scharrer, 1944). The neurosecretory materials originating from these tissues control a variety of physiological functions. But the isolation and structure identification of the active materials in these cells were successfully done only recently. Refinements in high-performance liquid chromatography and methods in peptide sequencing greatly contributed to this. Brown and Starratt (1975) for the first time identified an insect peptide called proctolin. The second insect neuropeptide to be identified was the adipokinetic hormone from the locust, *L. migratoria* (Stone *et al*., 1976). This peptide is one of the hormones involved in lipid and carbohydrate mobilisation. No other neuropeptides were identified until 1985 when a second adipokinetic hormone, Schgr-AKH-II was identified (Gaede *et al*., 1986). Since then, there has been a surge of new peptides from several insects from different groups. Structural and functional studies have revealed that many of these hormones have similarities, pointing out to the phylogenetic relationships of insects with regard to the presence of various peptide hormones.

In the present study, the primary structures of AKH/RPCH peptides in a few insects were elucidated by MALDI-MS/MS in PSD mode. Although fragmentation by MALDI-MS/MS in PSD mode did not produce all C-terminal and N-terminal ions of the peptides, bioassays of crude extracts of neurohaemal tissues, HPLC fractionations and subsequent bioassays of the fractions and comparison of HPLC profiles of synthetic AKHs with native peptides, provides substantial evidences for peptide confirmation.

Elucidation of primary structures of AKH neuropeptides of *Oxya nitidula*

For the isolation of biologically active materials, a bioassay that is easy and reliable is paramount in monitoring the success of purification throughout the various steps. Since it was not possible to collect sufficient quantity of haemolymph samples for bioassays from *O. nitidula*, the identification of AKHs were done by hyperlipaemic bioassays conducted in another insect, *I. limbata* (heterologous). Earlier studies in our laboratory demonstrated significant hyperlipaemic response by the fat body of *I. limbata* injected with CC extracts of *Spodoptera mauritia* (Kumari and Gokuldas, 2001), *I. limbata* (Rasheed and Gokuldas, 2002) and *O. nitidula* (Ajaykumar and Gokuldas, unpublished data).

The RP-HPLC separation and further bioassays of fractions of retrocerebral complexes of *O. nitidula* showed the presence of a few UV-

absorbing peaks exhibiting biological activity (Figure IV. 2). The biologically active materials with retention times 9.94 min, 11.81 min and 22.9 min in HPLC (Figure IV.1) showed absorbance peaks at 210 and 280 nm in a PDA detector. Peptide bonds and the aromatic amino acids, give maximum absorbance at 210 and 280 nm respectively. All members of AKH/RPCH family of peptides have been shown to have a tryptophan residue at position 8 (Gaede *et al*., 1997 a). The comparison of HPLC profiles of the synthetic peptides, Locmi-AKH-I and Schgr-AKH-II with that of *O. nitidula* retrocerebral complex extract showed that the extract contained materials having similar retention times 9.94 and 11.81 min respectively (Figure IV. 3 and 4). This data indicate the presence of Locmi-AKH-I and Schgr-AKH-II peptides in the retrocerebral complex extract of *O. nitidula*.

The primary structures of AKH/RPCH peptides were elucidated by MALDI-MS/MS in PSD mode. The MALDI-MS/MS analyses showed the presence of the AKH/RPCH peptides, Locmi-AKH-I (pE-L-N-F-T-P-N-W-G-T-NH_2), Schgr-AKH-II (pE-L-N-F-S-T-G-W-NH_2) and Locmi-AKH-III/Phymo-AKH-III (pE-L-N-F-T-P-W-W-NH_2/pE-I-N-F-T-P-W-W-NH_2) in the retrocerebral complexes of *O. nitidula*. The already identified peptides, Locmi-AKH-III and Phymo-AKH-III have similar molecular mass, 1072.5 Da. Locmi-AKH-III differs from Phymo-AKH-III in a leucine/isoleucine exchange (Siegert *et al*., 2000). Since leucine and isoleucine have same molecular mass the peptides with leucine/isoleucine replacement cannot be

distinguished with MALDI-PSD analysis. Siegert *et al*. (2000) distinguished these peptides using their difference in hydrophobic interaction in RP-HPLC, i.e., Phymo-AKH-III elutes consistently earlier than Locmi-AKH-III. Recently Nachman *et al*. (2005) devised a new methodology to unambiguously distinguish between leucine and isoleucine from their side chain fragmentation by MALDI-MS/MS in CID mode. So it is suggested that the unambiguous determination of the primary structure of peptide with molecular mass 1072.5 Da in the retrocerebral complex extract of *O. nitidula*, could be done by comparing the HPLC profiles of synthetic peptides, Locmi-AKH-III/Phymo-AKH-III with that of crude extract or by fragmenting the peptide in MALDI-MS/MS in CID mode.

In the present investigation, it has been established that the rice grasshopper, *O. nitidula* possesses more than three AKH/RPCH peptides. This result is not unusual, because the orthopterans, *L. migratoria*, *P. morbillosus*, *D. spumens*, *Lamarkiana sparrmani*, *Zonocerus elegans*, *Bullacris discolor* and *P. leprosus* (Gaede and Marco, 2008) have been found to contain more than three AKH peptides (Table V. 1). Siegert (1999) demonstrated the presence of a fourth inactive AKH peptide in *L. migratoria*, which is active in the American cockroach, *P. americana*. Why some insect species should require more than one AKH, especially when many other species appear to have only one structural variant is not completely clear. The potencies of the AKHs tested vary within and between different assays.

Locmi-AKH-I is the most potent in the lipid mobilization assay (Lee and Goldsworthy, 1995 a), while Locmi-AKH-III is the most active in RNA inhibiting bioassay (Kodrik and Goldsworthy, 1995) and the acetate uptake test (Lee and Goldsworthy, 1995 b). On the other hand, release of carbohydrates from the locust fat body is affected by Locmi-AKH-II and not by Locmi-AKH-I (Loughton and Orchard, 1981).

Recently Gaede and Marco (2008) investigated the adipokinetic neuropeptides of the superfamily Caelifera. The data show that the AKHs Locmi-AKH-I and Schgr-AKH-II are present in most of the subfamilies of family Acrididae investigated so far (Table IV. 2). The present investigations in the rice grasshopper, *O. nitidula* substantiate the above results, i. e., the insect has two AKH peptides, Locmi-AKH-I and Schgr-AKH-II (Table V. 2). Thus it is assumed that the genes coding for this two AKHs are highly conserved in the subfamilies of Acrididae during the course of evolution. Moreover, the primary structures of AKHs in the experimental organism are helpful in the construction of phylogenetic tree of the order Orthoptera.

Most of the insect species found to have three or more AKHs, are mostly active fliers (Gaede, 2006). Vroemen *et al*. (1998) pointed out that long distance flight in *L. migratoria* is attributed to the multiplicity of AKH neuropeptides in its CC. The rice grasshopper *O. nitidula* is not a continuous flier, it flies very short distances and the main locomotory activity comprises

hopping. Thus it is suggested that the AKHs in *O. nitidula* may be helpful for comparatively less energy requiring activities like, short distance flight and hopping. Studies in *P. apterus* and *G. bimaculatus* are in agreement with the above suggestion, i.e., in these insects AKHs are useful for activities such as walking (Socha *et al*., 1999, Lorenz *et al*., 2004), the energy demand for which is comparatively less. Gaede and Spring (1989) demonstrated that the romilid grasshopper, *Romalea microptera* does not respond to its endogenous AKH peptides. In the present investigation, the hyperlipaemic effects of *O. nitidula* were tested in another insect *I. limbata*. Due to reasons mentioned elsewhere, homologous bioassays were not been able to be conducted in *O. nitidula* and the sensitivity of the fat body to native hyperlipaemic factors has not therefore been investigated.

Elucidation of primary structure of adipokinetic neuropeptide of the coffee locust, *Aularches miliaris.*

The present study identified the primary structure of one peptide from the corpora cardiaca of the pyrgomorphid grasshopper, *A. miliaris***.** It is a decapeptide of AKH/RPCH family. Similar peptide was first identified from a pyrgomorphid grasshopper *P. morbillosus* and code named as Phymo-AKH-I with amino acid sequence pE-L-N-F-T-P-N-W-G-S-NH_2. Gaede *et al*. (1996) identified two other biologically active AKH peptides, Schgr-AKH-II and Phymo-AKH-III from the same insect. Even though the HPLC profile of retrocerebral extract of *A. miliaris* gave more than one biologically active

peaks both at 210 and 280 nm (Figure IV. 10), we could not identify the molecular masses (933.43 Da;Schgr-AKH-II and 1072.5 Da;Phymo-AKH-III) corresponding to these peptides. Since regulation of intermediary metabolism in grasshoppers appears to be very complicated (Siegert *et al*., 2000), it is suggested that there may be other novel AKH peptides than those already detected that are helpful for mobilizing energy reserves (lipids and carbohydrates) and this may be true with *A. miliaris*. However, the pyrgomorphid grasshopper *P. leprosus*, contains only a single AKH, Phyle-CC. Hence, it will be worth investigating to find out the novel peptides whose biological activities have been confirmed but the structure has not been able to be figured out.

This peptide, Phymo-AKH-I is most similar in structure (90%) to Locmi-AKH-I (threonine is replaced by serine at 10^{th} position) and Phyle-CC (threonine is replaced by asparagine at 3^{rd} position). The amino acid exchange at position 3 has been observed in many AKH/RPCH family peptides and is a conservative modification (Gaede *et al*., 1994). The substitution at position 10, where four amino acid residues (serine, asparagine, threonine and tyrosine) have been found to get exchanged in the AKH/RPCH peptides (Gaede *et al*., 1994) cannot be explained easily by single step point mutation. Phymo-AKH was identified earlier from two other insects, *P. morbillosus* and *D. spumens* coming under the same insect family, Pyrgomorphidae (Table V. 3). These results show that the distribution of Phymo-AKH has some family

specificity. However, two species, *P. morbillosus* and *P. leprosus* coming under the same genus (*Phymateus*) exhibited the presence of two different decapeptides (Phymo-AKH-I and Phyle-CC) in their CC. Since the data regarding AKH complements in different species of these groups are not completely available, further studies are essential to make conclusions regarding the phylogeny of this group.

Characterisation of adipokinetic neuropeptide of the plant bug, *I. limbata*

Pyrap-AKH is the first heteropteran AKH/RPCH peptide isolated from the firebug, *P. apterus*, family Pyrrhocoridae (Kodrik *et al*., 2000). Later, AKH/RPCH peptides of representative species of families Nepidae, Belostomatidae, Corixidae and Notonectidae were identified (Gaede *et al*., 2007 b).

Earlier studies in our laboratory have demonstrated that the crude extracts of CC from *I. limbata* were able to increase the levels of haemolymph lipid in homologous bioassay (Rasheed and Gokuldas, 2002). In the present study, HPLC fractions of the retrocerebral extracts were studied for their effect *in vivo*. It has been shown that some of these fractions had significant hyperlipaemic effect. In addition, the synthetic Pyrap-AKH had identical retention time as that of biologically active fractions of the extracts (brain-retrocerebral complexes and retrocerebral complexes alone) in HPLC (Figure IV. 16 and 19) indicating similarity in the hydrophobic interaction of the

active components in the retrocerebral complex extracts to that of Pyrap-AKH. The MALDI-MS and tandem MS/MS analysis confirmed the presence of already known Pyrap-AKH in *I. limbata*. These results are in close agreement with the studies conducted by Kodrik *et al*. (2000). They demonstrated that the Pyrap-AKH elevated haemolymph lipid level. The aim of the intense lipid mobilization in *P. apterus* is to ensure their enhanced locomotor and dispersal activities even when flight was substituted by running (Socha *et al*., 1999; Kodrik *et al*., 2002 a). The insects, *P. apterus* and *I. limbata* come under the same family and they exhibit similarity in morphology, locomotion, feeding habits etc. Thus it is presumed that the Pyrap-AKH present in *I. limbata* may be helpful for inducing locomotor activity by elevating haemolymph lipid level. Further studies are necessary to establish this proposition with respect to locomotion in *I. limbata*.

Pyrap-AKH is an octapeptide, showing maximal sequence similarity with Locmi-AKH-I and Phymo-AKH-I. The first eight amino acid residues of Pyrap-AKH are identical to these peptides (Kodrik *et al*., 2000). Pyrap-AKH exhibited 87.5% similarity with five octapeptides, Emppe-AKH, Locmi-AKH-III, Micvi-AKH, Peram-CAH-II and Tenmo-HrTH (indicating the change by one amino acid) (Table V. 4). It also exhibits structural similarity with four decapeptides (which means an exchange of one amino acid within the first eight amino acids of the molecule and the addition of two more amino acids at the C-terminus), Carmo-HrTH, Phyle-CC, Declu-CC and

Rommi-CC. It has been observed that Pyrap-AKH has relatively low similarity (62.5%) with the cicada peptides, Placa-HrTH-I and II (Kodrik *et al*., 2000). Recent observations of Gaede *et al*. (2006) that Pyrap-AKH is found in an orthopteran (Family: Pyrgomorphidae) suggests the possibility of the presence of this peptide in other insect groups also (Table V. 5).

Recently, Kodrik *et al*. (2002 b) identified another AKH/RPCH peptide from the CC of firebug, *P. apterus*, the Peram-CAH-II. HPLC separation and further fractionation of extracts of both brain-CC-CA complexes and retrocerebral complexes (alone) indicated the presence of more than one adipokinetic factors in them. However, by MALDI-MS analysis, we could not find any molecular mass corresponding to Peram-CAH-II. This might be due to the fact that these peptides are either absent in *I. limbata* or are present in a concentration below the detection limit of the instrument we used.

Effects of injected as well as topically applied AKHs

When topically applied, Locmi-AKH-I and Pyrap-AKH exhibited significant hyperlipaemic effects with a maximal response at 120 min whereas the AKH peptides induced hyperlipaemia after 60 min of injection. The significant hyperlipaemic effect brought about by topically applied Pyrap-AKH and Locmi-AKH-I clearly show that these peptides have the ability to penetrate the cuticle of *I. limbata*. Similar high hyperlipaemic response

resulted in *G. bimaculatus* (Lorenz *et al*., 2004) and *P. apterus* (Kodrik *et al*., 2002 a) when native peptides were applied topically. In *G. bimaculatus* the time taken for maximal hyperlipaemic response was 120 min whereas in *P. apterus* it was 180 min. These results suggest that the rate of penetration of peptides into the cuticle vary from insect to insect or may be from group to group, although in these cases, the peptides were applied on the insect at more or less morphologically similar locations, i.e., underneath the wings and over the dorsal part of the metathorax. Several factors, including chemistry of the cuticle, that influence the rate of penetration of peptides dissolved in polar organic solvents (such as methanol, propanol etc.) have to be investigated to draw conclusion as to the time, solvents and concentrations to be used to get the desired hyperlipaemic effect.

It has been found that Locmi-AKH-I exhibited relatively higher hyperlipaemic activity than Pyrap AKH in *I. limbata* in both injection as well as topical application experiments. This result is in agreement with the data obtained by Socha *et al*. (1999). Among several AKHs they tested (Placa-HrTH, Manse-AKH, Locmi-AKH-I and *P. apterus* CC extract), Locmi-AKH-I exhibited the highest adipokinetic potency. Moreover, in earlier reports on hyperlipaemic bioassays with other AKHs (Locmi-AKH-I, -II and -III) tested in *L. migratoria* it has been shown that Locmi-AKH-I is the most potent hyperlipaemic hormone (Kodrik and Goldsworthy, 1995).

Better hyperlipaemic response was observed in injection studies compared to topical application. This is suggestive of a stressing factor induced by injection that could play a role in the appearance of complex response to AKHs as reported in *P. apterus* where the hyperlipaemic activities resulting from injection as well as topical application of Pyrap-AKH when compared, was shown to have better activity in injection experiments. They suggested the induction of an injury stressor in *P*. *apterus*. When the injury stressor is absent (as in topical application) the responses are lesser (Kodrik *et al*., 2002). Similar speculations may explain the difference in the hyperlipaemic responses between the two methods of AKH treatments in *I. limbata*. Earlier studies in locusts showed that the insect exhibited hyperlipaemia not only at the time of flight but also when exposed to stressful conditions like poisoning with neurotoxin (insecticides), plant allelochemicals, unsuitable temperatures, injuries and starvation (Steele, 1985).

Characterization of AKH peptides from *Oryctes rhinoceros*

The sequence of the adipokinetic peptide from the CC of the rhinoceros beetle, *O. rhinoceros,* is same as that of the already elucidated AKH peptide, Melme-CC. The amino acid sequence of the peptide is pE-L-N-Y-S-P-D-W-NH_2. Similar peptide has been sequenced earlier from two beetles, *Melolontha melolontha* and *Geotrupes stercorosus* (Gaede, 1991). In

these species, the Melme-CC is responsible for the regulation of lipid as well as carbohydrate metabolism. In our study, we obtained similar results in the case of lipid mobilizing activity of the extracts of retrocerebral complexes in *I. limbata*. Bioassays with synthetic Melme-CC in *P. sinuata* have shown that it induces mobilization of carbohydrates and the stimulation of proline synthesis. Since the effects of native peptide on *O. rhinoceros* have not been carried out it remains to be established what is the exact function(s) of Melme-CC in this insect. One possible physiological role of Melme-CC may be to make available acetyl CoA from triacylglycerol, for the synthesis of proline in their fat body (Gaede, 1991; Weeda, 1981). Homologous bioassays are to be carried out to confirm the hyperlipaemic, hyperprolinemic and hyperglycemic effects of Melme-CC in *O. rhinoceros*.

The Melme-CC exhibits remarkable structural similarity with Scade-CC-I, a peptide isolated from *Scarabeus* sp. Here the non-aromatic amino acid leucine at second position is replaced by an aromatic amino acid phenyl alanine; both the residues are hydrophobic and therefore retention times are fairly similar. Examination of structures of AKHs in fruit and dung beetles' in the context of evolutionary trends suggests that Scade-I and -II, Oniay-CC-II and Melme-CC are closely related and differ from each other by a point mutation at positions 7 or 2. Oniay CC-I is closely related to Schgr-AKH-II which contains a leucine at position 2 instead of tyrosine in Oniay-CC-I (Gaede, 1997 a). Previous studies indicated that Melme-CC is present in four

species of beetles, viz., *M. melolontha, G. stercorosus, P. sinuata* and, *P. marginata* coming under different families of the insect order Coleoptera (Table V. 6). Further investigations are necessary to draw conclusion about the evolution of AKHs in beetles.

The aforementioned discussion on the adipokinetic neuropeptides from the insects, *O. nitidula*, *A. miliaris*, *I. limbata* and *O. rhinoceros* representing three different insect orders (Orthoptera, Heteroptera and Coleoptera) confirms the fact that adipokinetic neuropeptides are present in most of insect groups. Recent investigations revealed that AKHs not only aid in insect flight but also many other essential metabolic activities in them (Kodrik *et al.*, 2008). This may be the reason for presence of AKHs in most of the insect groups. This study also revealed a family or group similarity in the primary structures of adipokinetic neuropeptides. The AKHs identified from *O. nitidula* (Locmi-AKH-I, Schgr-AKH-II and Locmi-AKH-III/Phymo-AKH-III) are seen highly restricted to the insects coming under the same order (Orthoptera). Similar results have been obtained for *A. miliaris, I. limbata* and *O. rhinoceros*. Thus it is presumed that the present investigation may be helpful for tracing the evolutionary history of these groups. Insect pest control using neuropeptides is an emerging branch of agricultural entomology. Knowledge of primary structures of AKHs in insect pests is very much helpful to develop biotechnology based insect pest management strategies which are need of hour to create a pesticide free environment.

Table V. 1. Orthopteran insects having more than three adipokinetic neuropeptides.

Sl. No	Species	Adipokinetic neuropeptides	References
1	*Locusta migratoria*	Locmi-AKH-I Locmi-AKH-II	Stone *et al*. (1976) Siegert *et al*. (1985)
2	*Locusta pardalina*	Locmi-AKH-III	Oudejans *et al*. (1991)
3	*Phymateus morbillosus*	Phymo-AKH-I Schgr-AKH-II	Siegert *et al*. (2000) Gaede and Kellner (1995)
4	*Dictyophorus spumens*	Phymo-AKH-III	
5	*Lamarkiana sparrmani*	Grybi-AKH Pyrap-AKH Phymo-AKH-III	Gaede (2006)
6	*Zonocerus elegans*	Schgr-AKH-II Peram-CAH-II Phymo-AKH-III	Gaede (2006)
7	*Phymateus leprosus*	Phyle-CC Schgr-AKH-II Phymo-AKH-III	Gaede and Marco (2008)
8	*Bullacris discolor*	Phyle-CC Schgr-AKH-II Peram-CAH-II	Gaede and Marco (2008)
9	*Oxya nitidula*	Locmi-AKH-I Schgr-AKH-II Locmi-AKH-III or Phymo-AKH-III	Present study

Table V. 2. The insects of Acrididae sub-families which possess the adipokinetic hormones, Locmi-AKH-I and Schgr-AKH-II

Sl. No	Species	Subfamily	References
1 2	*Acrotylus spp.* *Spingonotes spp.*	Oedipodinae	Gaede (1988)
2	*Acrida acuminata*	Acridinae	Gaede and Marco (2008)
3	*Eyprepocnemis plorans*	Eypropocnemidinae	Gaede and Marco (2008)
4 5 6	*Melanoplus sanguinipes* *M. differentialis* *Barytettix psolus*	Melanoplinae	Taub-Montemayor *et al*. (2002)
7 8 9 10	*Acanthacris ruficornis* *Nomadacris septemfasciata* *Anacridium aegypticum* *Schistocerca gregaria*	Cyrtacanthacridinae	Gaede and Marco (2008)
11	*Rachitopsis curvipes*	Euryphyminae	Gaede and Marco (2008)
12	*Oxya nitidula*	Oxyinae	Present study

Table V. 3. Orthopteran insects which possess Phymo-AKH-I in their CC

Sl. No	Species	Family	References
1	*Phymateus morbillosus*	Pyrgomorphidae	Siegert *et al*. (2000)
2	*Dictyophorus spumens*	Pyrgomorphidae	Siegert *et al*. (2000)
3	*Aularches miliaris*	Pyrgomorphidae	Present study

Table V. 4: Primary structures of peptides of the AKH/RPCH family showing high similarity (100% - 87%) with Pyrap-AKH

Sl. No.	Code name	Species	Amino acid sequence											Molecular weight (Da)	References
			1	2	3	4	5	6	7	8	9	10	11		
1	Pyrap-AKH	*Pyrrhocoris apterus*	pE	L	N	F	T	P	N	W	NH_2			1000.4	Kodrik *et al*. (2000)
2	Locmi-AKH-I	*Locusta migratoria*	pE	L	N	F	T	P	N	W	G	T	NH_2	1158.54	Stone *et al*. (1976)
3	Phymo-AKH-I	*Phymateus morbillosus*	pE	L	N	F	T	P	N	W	G	S	NH_2	1144.53	Gaede *et al*. (1996)
4	Emppe-AKH	*Empusa pennata*	pE	V	N	F	T	P	N	W	NH_2			986.46	Gaede (1991 a)
5	Locmi-AKH-II	*Locusta migratoria*	pE	L	N	F	T	P	W	W	NH_2			1072.5	Oudejans *et al*. (1991)
6	Micvi-CC	*Microhodotermis viator*	pE	I	N	F	T	P	N	W	NH_2			1000.4	Liebrich *et al*. (1995)
7	Peram-CAH-II	*Periplaneta americana*	pE	L	T	F	T	P	N	W	NH_2			987.48	Witten *et al*. (1984)
8	Tenmo-HrTH	*Tenebrio molitor*	pE	L	N	F	S	P	N	W	NH_2			986.46	Gaede and Rosinski (1990)
9	Carmo-HrTH-I	*Carasius morosus*	pE	L	T	F	T	P	N	W	G	T	NH_2	1146.53	Gaede *et al*. (1992)
10	Carmo-HrTH-I	*Carasius morosus*	pE	L	T	F	T	P	N	W	G	T	NH_2	1146.53	Gaede and Rinehart (1987 a)
11	Phyle-CC	*Phymateus leprosis*	pE	L	T	F	T	P	N	W	G	S	NH_2	1137.53	Gaede and Kellner (1995)
12	Declu-CC	*Decaptoma lunata*	pE	L	N	F	S	P	N	W	G	N	NH_2	1157.52	Gaede (1995)
13	Rommi-CC	*Romelea microptera*	pE	V	N	F	T	P	N	W	G	T	NH_2	1144.5	Gaede *et al*. (1988)

Table V. 5. Insect species which posses the adipokinetic neuropeptide, Pyrap-AKH in their corpora cardiaca

Sl. No	Species	Family	References
1	*Pyrrhocoris apterus*	Pyrrhocoridae	Kodrik *et al*. (2000)
2	*Lamarkiana sparrmani*	Pyrgomorphidae	Gaede (2006)
3	*Iphita limbata*	Pyrrhocoridae	Present study

Table V. 6. Coleopteran species having the adipokinetic neuropeptide, Melme-CC in their corpora cardiaca

Sl. No	Species	Family	References
1	*Melolontha melolontha*	Melolonthidae	Gaede (1991)
2	*Geotrupes stercorosus*	Geotrupidae	Gaede (1991)
3	*Pachnoda sinuata*	Scarabaeidae	Gaede *et al*. (1992)
4	*Pachnoda marginata*	Scarabaeidae	Gaede *et al*. (1992)
5	*Oryctes rhinoceros*	Dynastidae	Present study

SUMMARY

In the present investigation the adipokinetic neuropeptides of the insects, *O. nitidula*, *A. miliaris*, *I. limbata* and *O. rhinoceros* were carried out and the summary of the results are as follows.

1) HPLC separation of extracts of retrocerebral complexes of *O. nitidula* showed a few UV absorbing peaks when monitored simultaneously at 210 and 280 nm which on analysis were shown to contain compounds exhibiting significant hyperlipaemic activities.

2) Comparison of HPLC profiles obtained with synthetic AKHs, Locmi-AKH-I and Schgr-AKH-II with that of retrocerebral complexes of *O. nitidula* showed the presence of similar peptides in the extract.

3) MALDI-TOF-MS analysis of retrocerebral extracts of *O. nitidula* showed the presence of the AKH/RPCH peptides, Locmi-AKH-I, Schgr-AKH-II and Locmi-AKH-III/Phymo-AKH-III. Further fragmentation of these peptides by MALDI-MS/MS in PSD mode indicated their primary structures as pE-L-N-F-T-P-N-W-G-T-NH_2, pE-L-N-F-S-T-G-W-NH_2 and pE-L-N-F-T-P-W-W-NH_2/pE-I-N-F-T-P-W-W-NH_2 respectively.

4) Extracts of retrocerebral complexes of *A. miliaris* and synthetic Locmi-

AKH-I induced significant hyperlipaemic activities in homologous *in vivo* bioassays.

5) HPLC analysis of the CC-CA extract of *A. miliaris* showed a few UV absorbing peaks when monitored simultaneously at 210 and 280 nm, which on analysis showed significant hyperlipaemic activity.

6) MALDI-TOF-MS and MALDI-TOF-MS/MS analysis confirmed the presence of AKH peptide Phymo-AKH-I in *A. miliaris* having a primary structure of pE-L-N-F-T-P-N-W-G-S-NH_2.

7) Separation of peptides from the extracts of brain-retrocerebral complexes and retrocerebral complexes alone of the plant bug, *I. limbata* by HPLC showed the presence of a few UV absorbing peaks at 210 nm.

8) Homologous *in vivo* bioassays of fractions separated on HPLC showed the presence of adipokinetic factors.

9) Comparison of HPLC profiles of synthetic Pyrap-AKH with that of extracts of brain-retrocerebral complexes and retrocerebral complexes alone of *I. limbata* showed the presence of Pyrap-AKH in both of the tissue extracts.

10) MALDI-TOF-MS analysis showed the presence of Pyrap-AKH in *I. limbata*. Further fragmentation by MALDI-TOF-MS/MS confirmed

the primary structure as pE-L-N-F-T-P-N-W-NH_2.

11) The injected as well as topically applied synthetic peptides Locmi-AKH-I and Pyrap-AKH showed significant hyperlipaemic activities.

12) Heterologous *in vivo* bioassays of retrocerebral complexes of *O. rhinoceros* in *I. limbata* showed adipokinetic factors in the extract.

13) Bioassays of the HPLC fractions showed significant adipokinetic activities in two fractions.

14) MALDI-TOF-MS and MALDI-TOF-MS/MS analysis showed the presence of Melme-CC with a primary structure, pE-L-N-Y-S-P-D-W-NH_2 in *O. rhinoceros*.

Suggestions for further research

Based on the findings of the study, the investigator suggest the following areas in which future researches can be carried out:

1) Study the hyperlipaemic and hypertrehalosemic effects of native AKHs, Locmi-AKH-I, Schgr-AKH-II and Locmi-AKH-III/Phymo-AKH-III in *O. nitidula.*

2) Isolation and unambiguous identification of peptide with molecular mass 1072.5 Da (caused due to leucine isoleucine modification) in the retrocerebral complexes of *O. nitidula*.

3) Identification of more adipokinetic neuropeptides from the coffee locust, *A. miliaris*.

4) Study of biological activities of topically applied synthetic peptides in other insects (pests).

5) Study the hyperlipaemic, hypertrehalosemic and hyperprolinaemic effects of Melme-CC in *O. rhinoceros*.

6) Quantitation of native AKHs in the insects, *O. nitidula*, *A. miliaris*, *I. limbata* and *O. rhinoceros*.

7) Localisation of adipokinetic neurosecretory cells in the retrocerebral complexes of the insects, *O. nitidula*, *A. miliaris*, *I. limbata* and *O. rhinoceros*.

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PLATE III. 1

a. *Oxya nitidula* **Walker**

b. *Aularches miliar*is **Linnaeus**

PLATE III. 2

a. *Iphita limbata* **Stål**

b. *Oryctes rhinoceros* **Linnaeus**

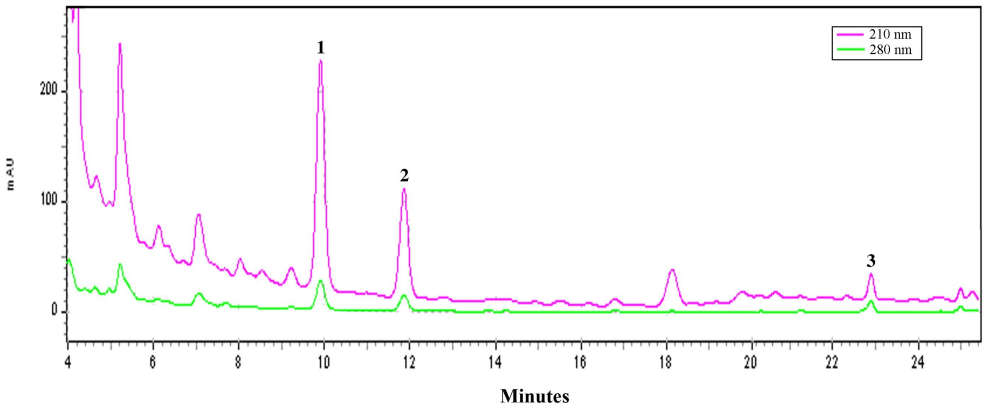

Figure IV. 1: The HPLC profile of extracts of retrocerebral complexes of *O. nitidula.* The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min and then to 70% B within a further 6 min (solvent A=0.01% trifluoro acetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 and 280 nm.

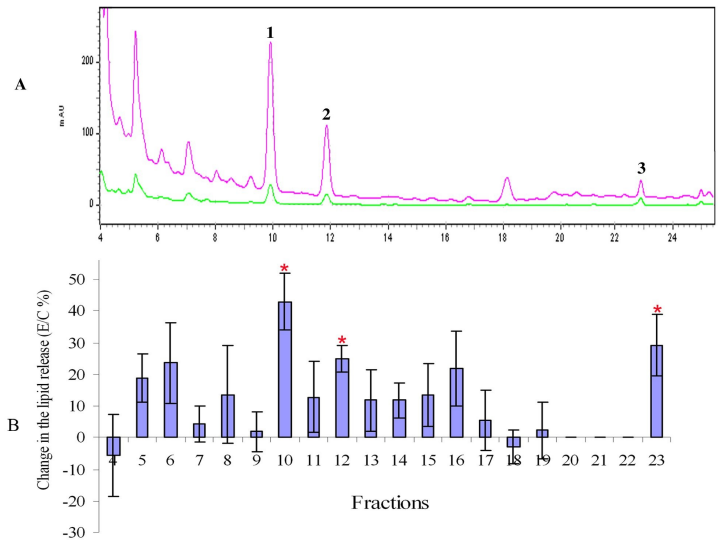

Figure IV. 2: HPLC profile of corpora cardiaca extract of *O. nitidula* (A) monitored at 210 and 280 nm. Fractions were collected and tested for hyperlipaemic activity. The change in total haemolymph lipid is represented in histogram (B) as E/C%. (*) Indicates $P< 0.05$

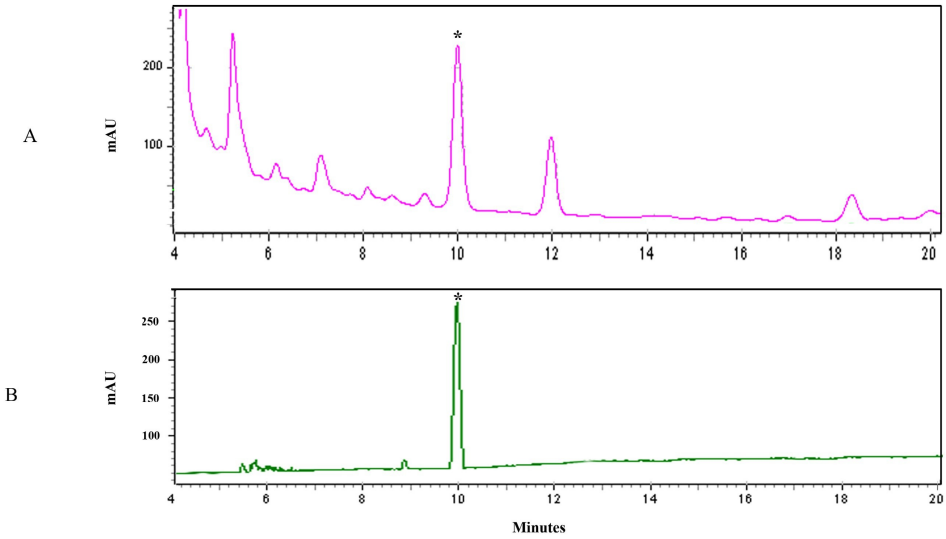

Figure IV. 3: The HPLC profiles of crude corpora cardiaca extract of *O. nitidula* (A) and synthetic Locmi- AKH- I (B) monitored at 210 nm. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 nm. (* Indicates the peaks of interest).

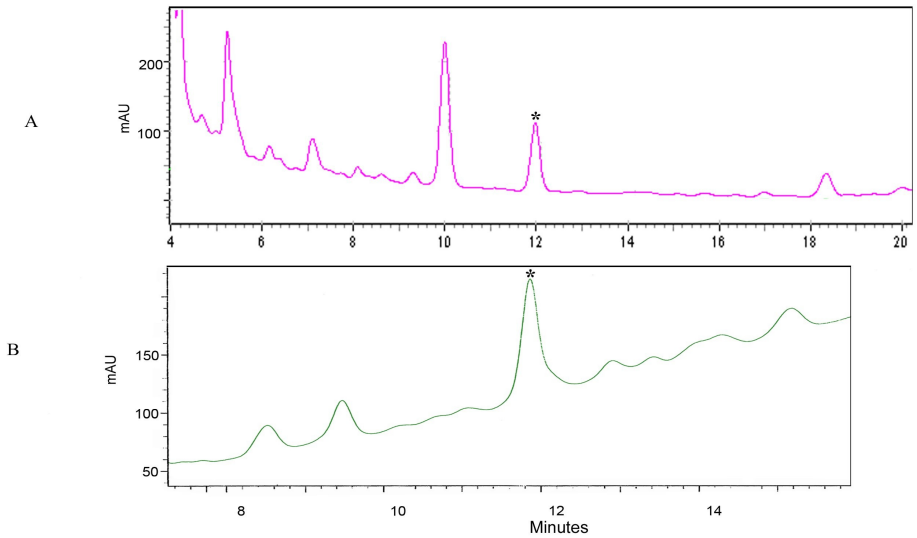

Figure IV. 4: The HPLC profiles of crude corpora cardiaca extract of *O. nitidula* (A) and synthetic Schgr - AKH- II (B). The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 nm. (* Indicates the peaks of interest. Other peaks in the synthetic Schgr-AKH-II profile (B) probably represent impurity/degradation products).

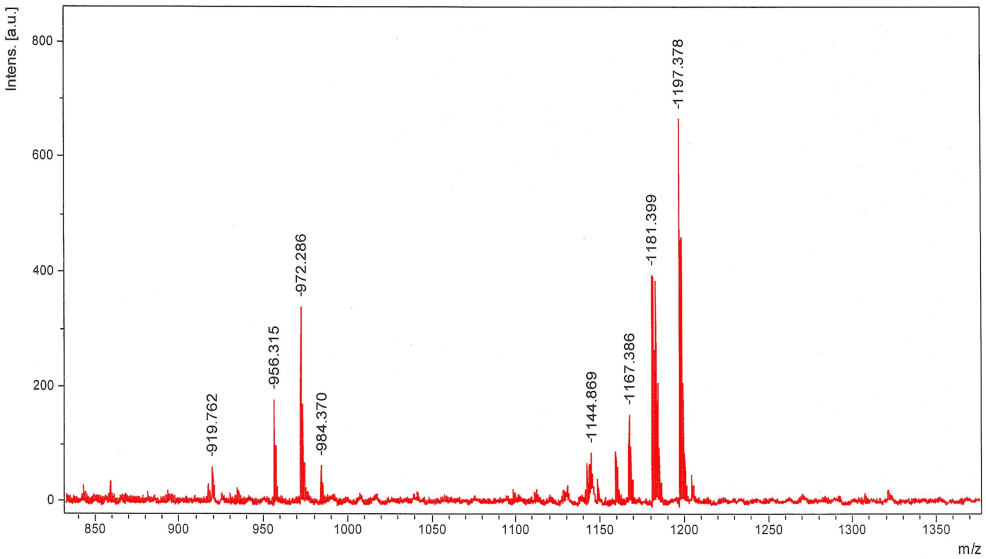

Figure IV. 5: MALDI-MS spectrum of extract of corpora cardiaca of *O. nitidula* . The analysis was carried out in reflector positive (Na^+) mode with an accelaration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix

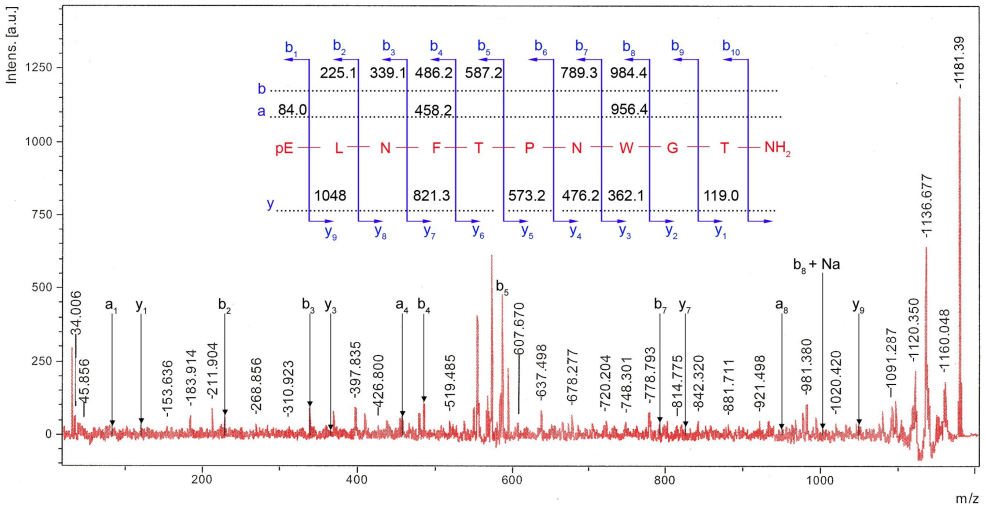

Figure IV. 6: MALDI-MS/MS spectrum of $(M+Na)^+$ =1181.39 Da from *O. nitidula*. Inset shows sequence assignment of the peptide, together with the theoretical and calculated masses for "y", "b" and "a" fragment ions obtained in the MS/MS spectrum.

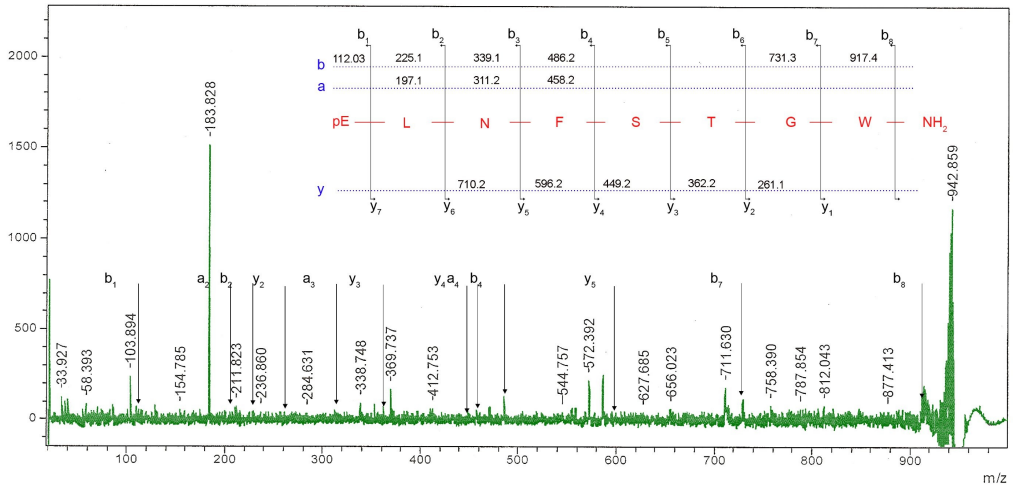

Figure IV. 7: MALDI MS/MS spectrum of $(M+Na)^+ = 956.31$ Da from *O. nitidula*. Inset shows sequence assignment of the peptide, together with the theoretical and calculated masses for "y", "b" and "a" type fragment ions, obtained in the MS/MS spectrum.

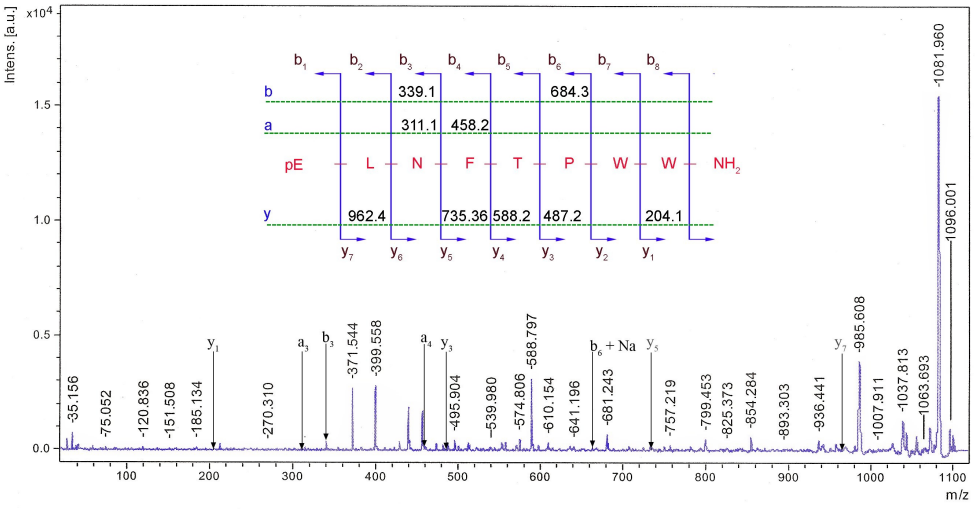

Figure IV. 8: MALDI MS/MS spectrum of the ion $(M+Na)^+ = 1096.001$ Da from *O. nitidula*. Inset shows the sequence assignment of the peptide, together with theoretical and calculated masses for "b", "y" and "a" type fragment ions, obtained in the MS/MS spectrum.

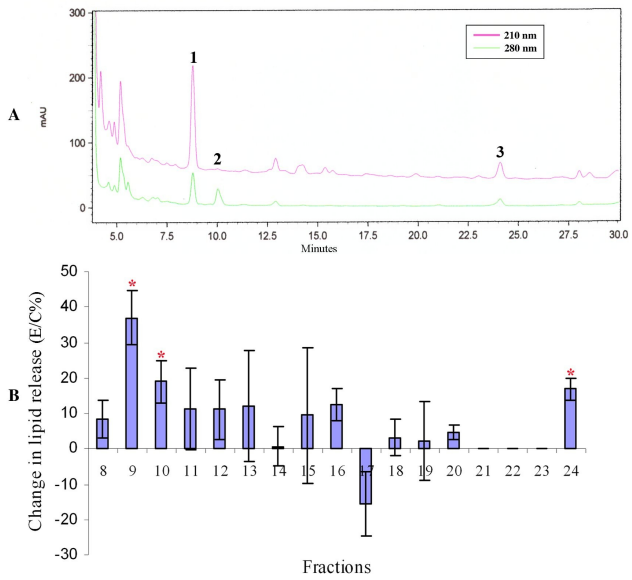

Figure IV. 10: The HPLC profile of extracts of retrocerebral complexes of *A. miliaris* (A). Fractions were collected and tested for hyperlipaemic activity. The change in total haemolymph lipid is represented (B) as E/C% in histogram. (*) Indicates $p<0.05$

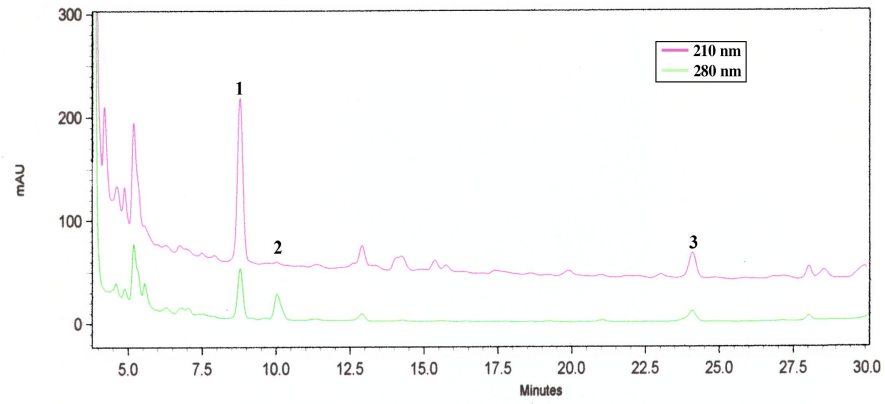

Figure IV. 9: The HPLC profile of extracts of retrocerebral complexes of *A. miliaris*. The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min and then to 70% B within a further 6 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 and 280 nm.

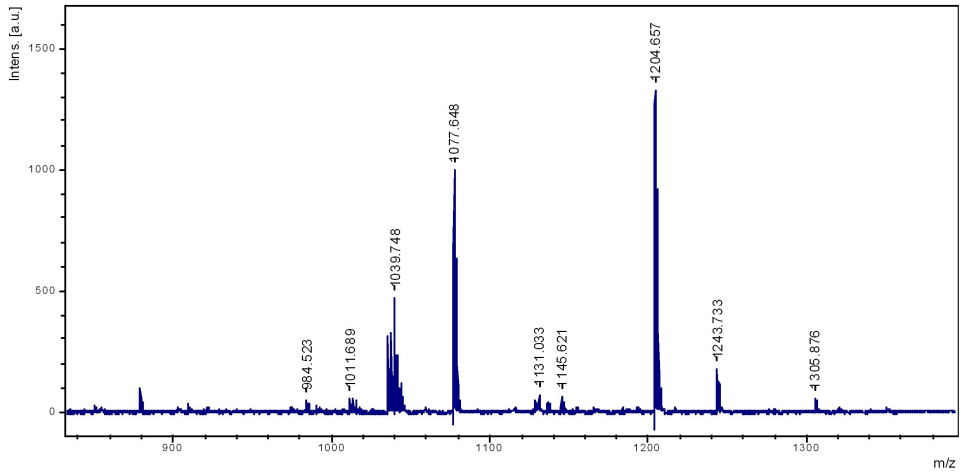

Figure IV. 11: MALDI-MS profile of extracts of retrocerebral complexes of *A. miliaris*. The analysis was carried out in reflector positive mode $(H)^+$ with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix.

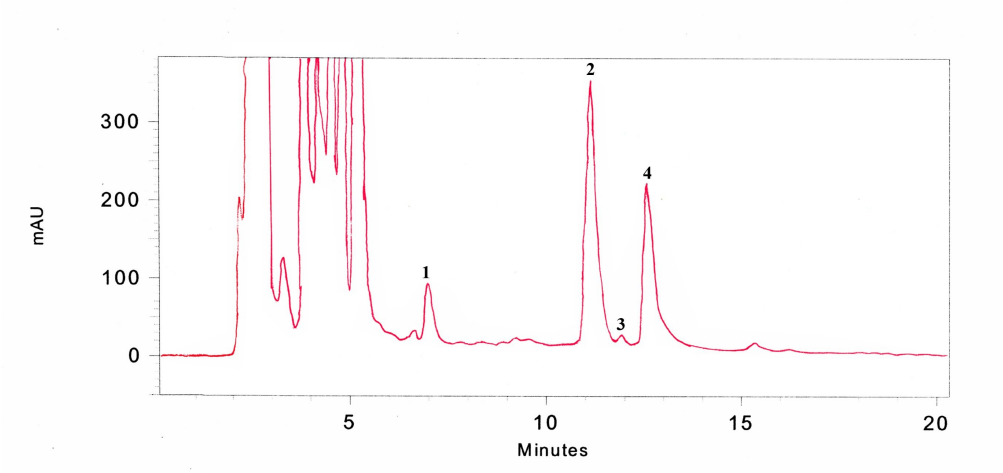

Figure IV. 14: HPLC profile of extracts of retrocerebral complexes of *I. limbata*. The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 nm.

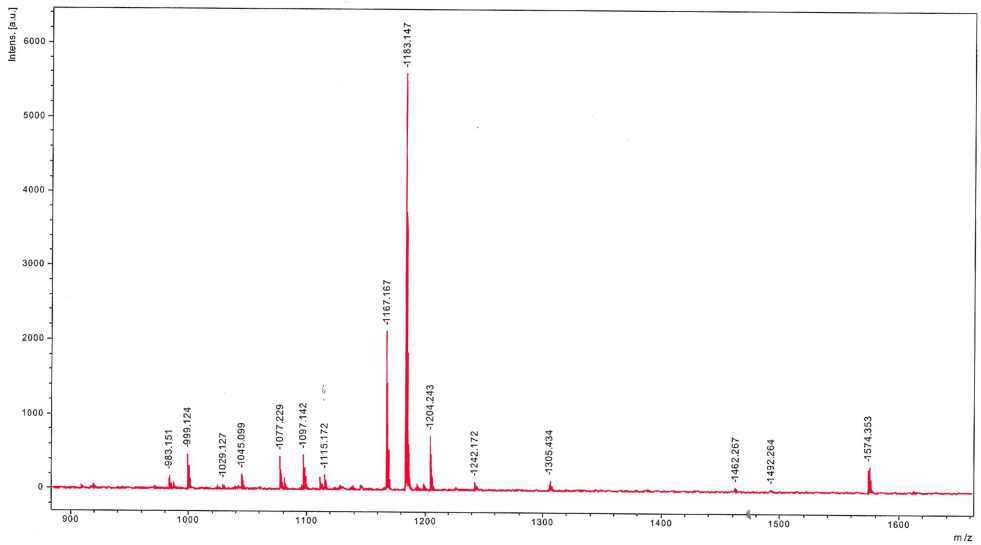

Figure IV. 12: MALDI-MS spectrum of extract of corpora cardiaca of *A. miliaris* The analysis was carried out in reflector positive $(Na)^+$ mode with an accelaration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix

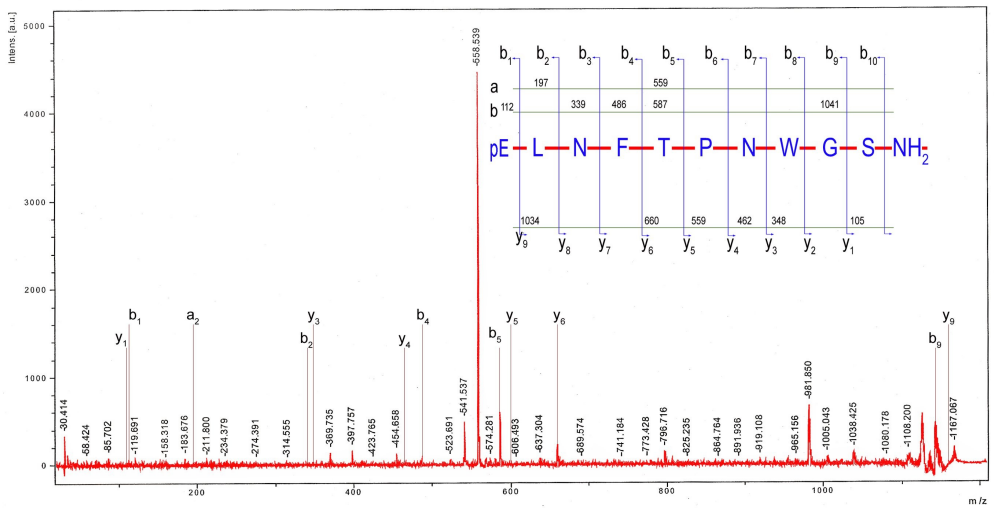

Figure IV. 13: MALDI MS/MS spectrum of the ion (M+Na) =1166.53 Da from *A. miliaris*. Inset shows the sequence assignment of the peptide, together with theoretical and calculated masses for "b", "y" and "a" type fragment ions, obtained in the MS/MS spectrum.

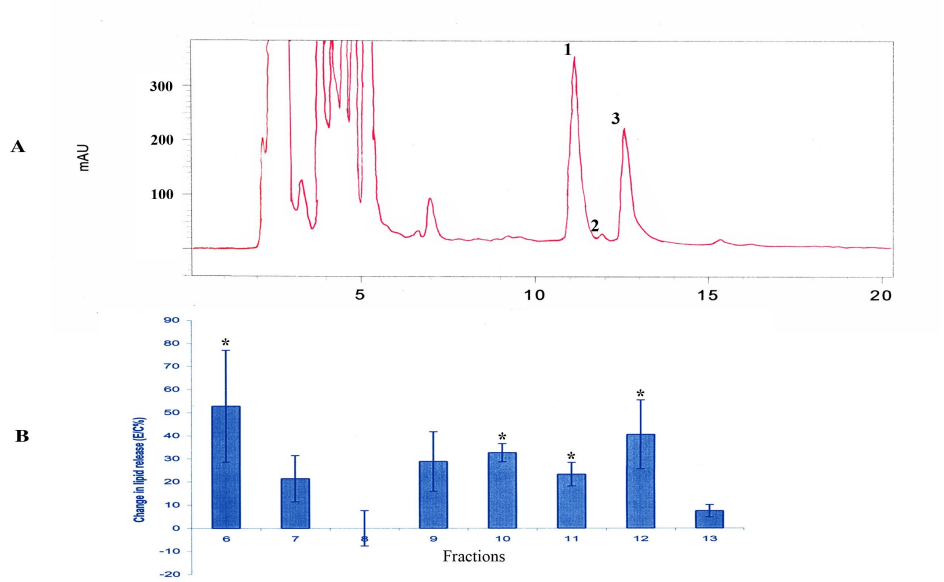

Figure IV. 15: The HPLC profile of extracts of brain retrocerebral complexes of *I. limbata* (A). Fractions were collected and tested for hyperlipaemic activity. The change in total haemolymph lipid is represented in histogram (B) as E/C% . (*) Indicates

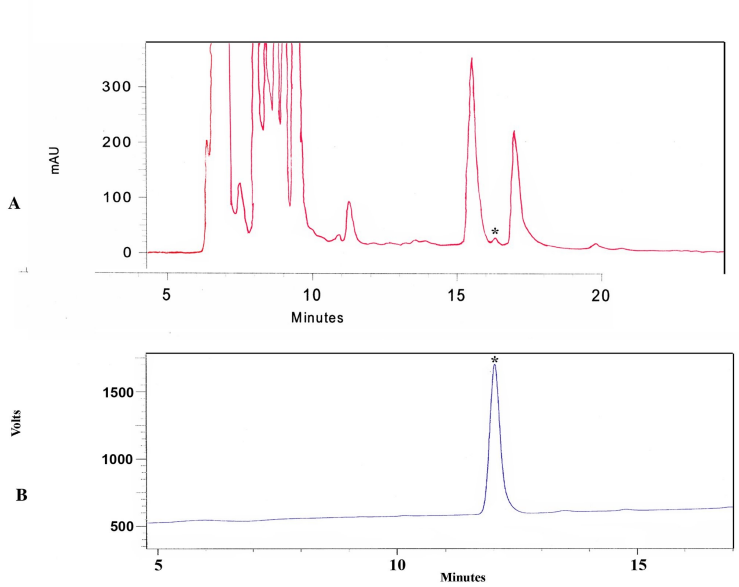

Figure IV. 22: The HPLC profiles of extracts of brain - retrocerebral complexes of *I. limbata* (A) and synthetic Pyrap - AKH (B) monitored at 210 nm. The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 nm. (*) Indicates the peaks of interest.

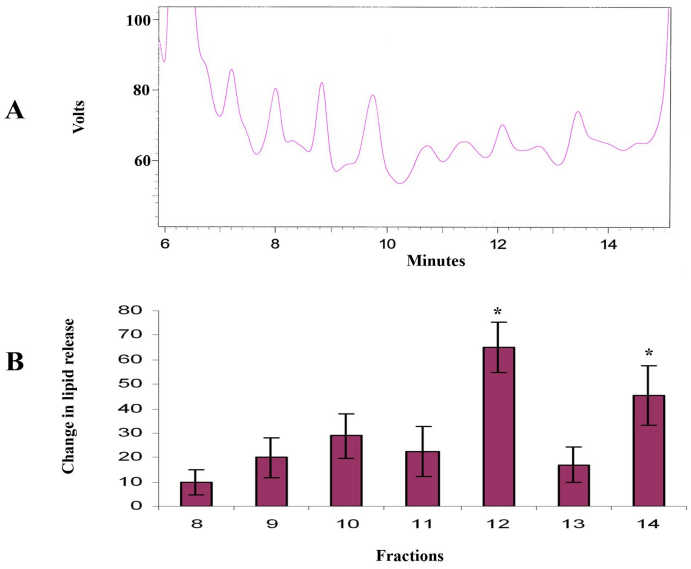

Figure IV. 17: The HPLC profile of extracts of retrocerebral complexes of *I. limbata* (A), peak fractions were collected, tested for hyperlipaemic activity. The change in total haemolymph lipid is represented (B) as E/C % in histogram. (*) Indicates $P<0.05$

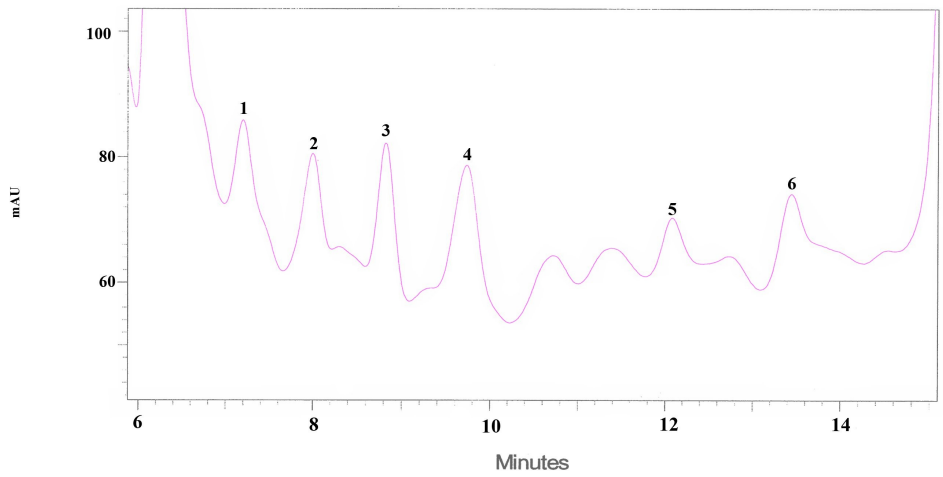

Figure IV. 16: HPLC profile of extracts of retrocerebral complexes of *I. limbata*. The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetonitrile in solvent A). The eluants were monitored at 210 nm. Numbered are the major peaks.

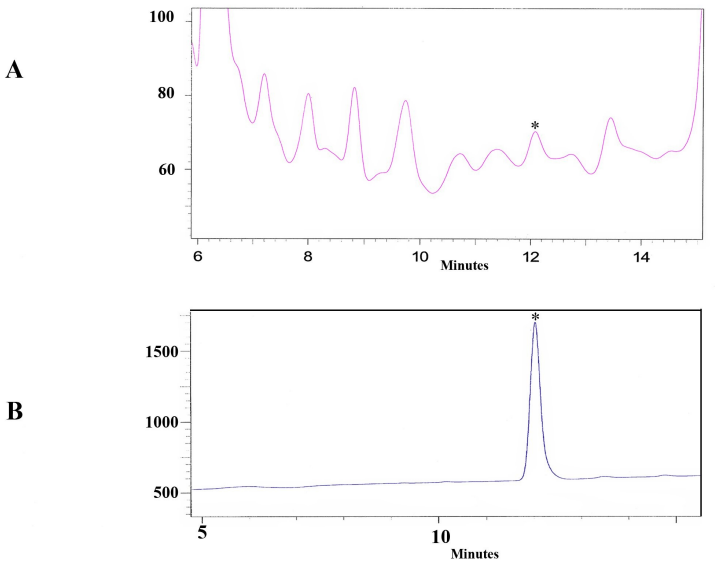

Figure IV. 23: The HPLC profiles of crude corpora cardiaca extract of *I. limbata* (A) and synthetic Pyrap - AKH (B) monitored at 210 nm.The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B=60% acetontrile in solvent A). The eluants were monitored at 210 nm. (*) Indicates peaks of interest.

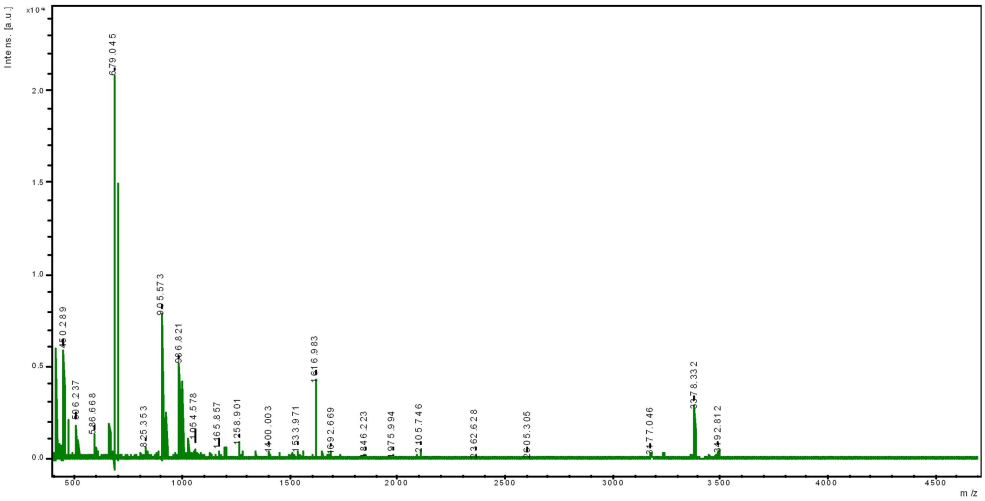

Figure IV. 24. MALDI-MS profile of extracts of brain and retrocerebral complexes of *I.limbata*. The analysis was carried out in reflector positive mode (H^+) with an acceleration voltage of 50Hz pulsed N_2 laser, emitting at 337nm. Dihydroxy benzoic acid was used as matrix.

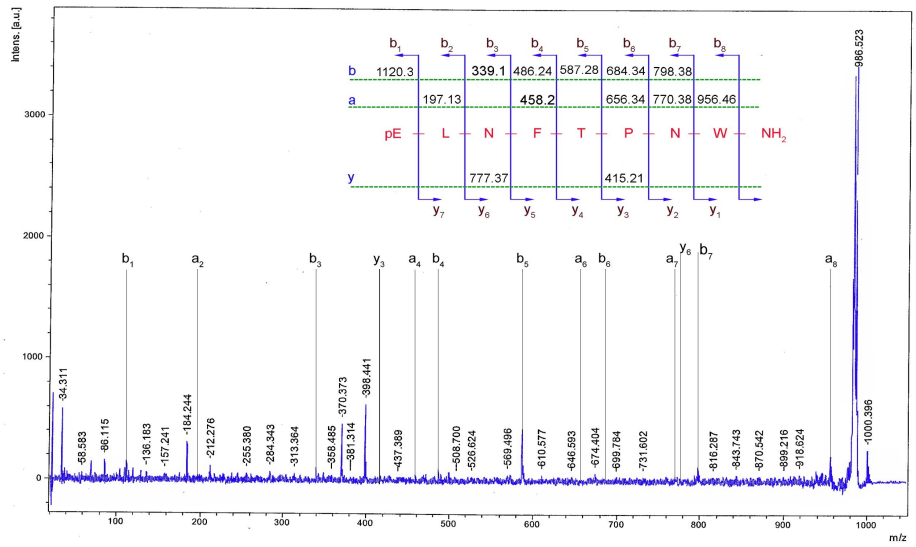

Figure IV. 26: MALDI MS/MS spectrum of the ion $(M+H)^+$ =1000.4 Da from *I. limbata*, inset shows the sequence assignment of the peptide, together with theoretical and calculated masses for "b", "y" and "a" type fragment ions, obtained in the MS/MS spectrum.

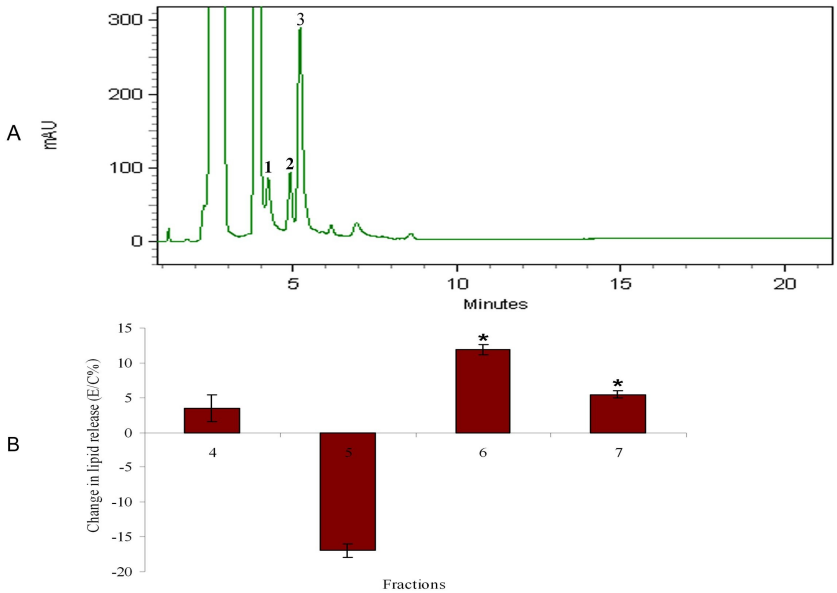

Figure IV. 28: The HPLC profile of extracts of retrocerebral complexes of *O. rhinoceros* (A). Fractions were collected and tested for hyperlipaemic activity. The change in total haemolymph lipid is represented in histogram (B) as E/C% . (*) Indicates $P<0.05$

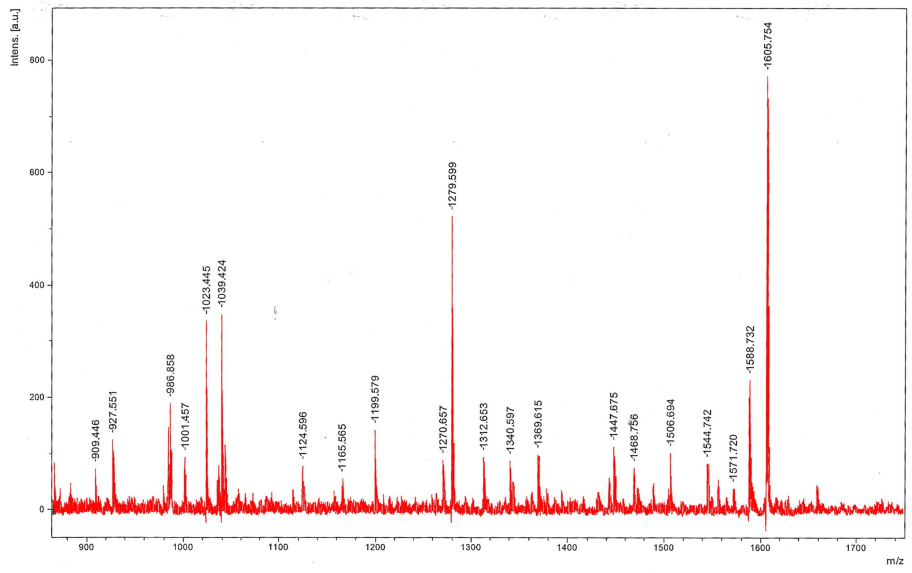

Figure IV. 25: MALDI-MS spectrum of extract of corpora cardiaca of *I. limbata*. The analysis was carried out in reflector positive $(Na)^+$ mode with an accelaration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix

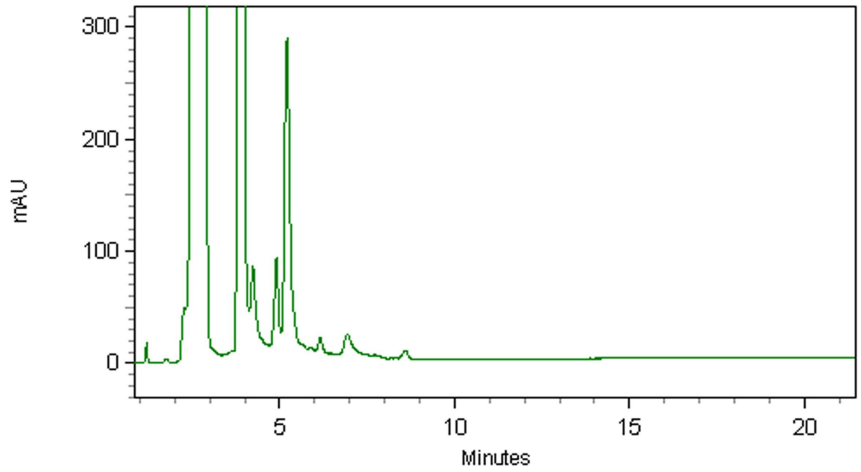

Figure IV. 27: The HPLC profile of extracts of retrocerebral complexes of *O. rhinoceros*. The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoroacetic acid in water, solvent B = 60% acetontrile in solvent A). The eluants were monitored at 210 nm.

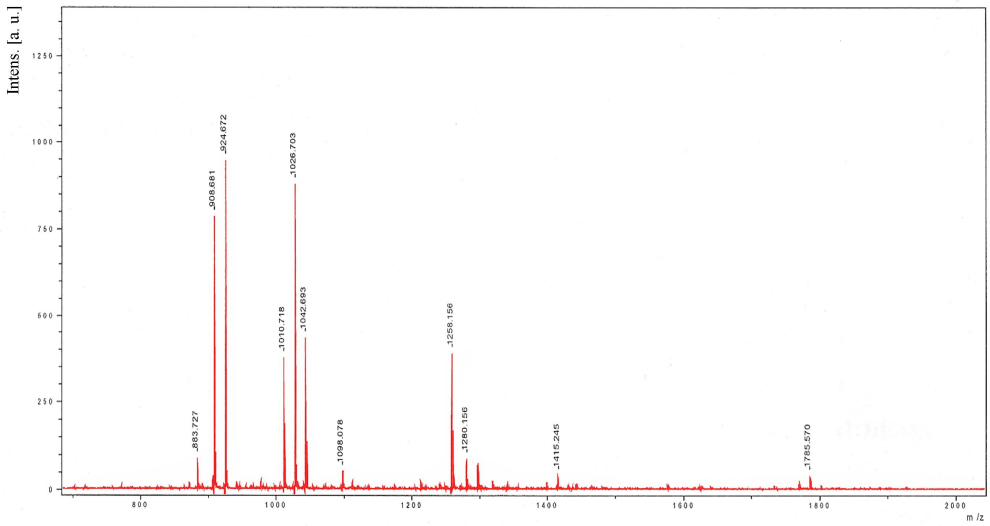

Figure IV. 29: MALDI-MS spectrum of extract of corpora cardiaca of *O. rhinoceros* . The analysis was carried out in reflector positive mode (Na^+) with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix

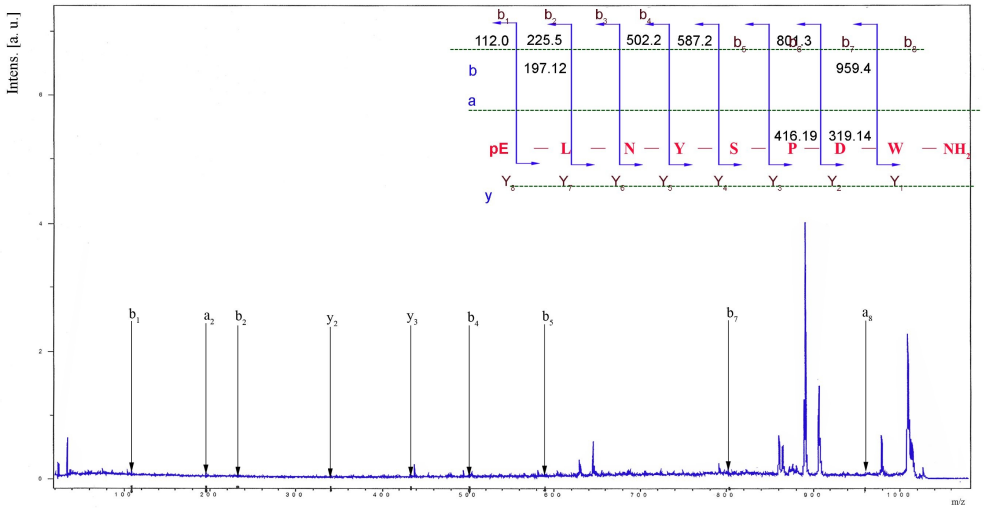

Figure IV. 30: MALDI MS/MS spectrum of the ion $(M+Na)^+$ =1003.70 Da from *O. rhinoceros*, inset shows the sequence assignment of the peptide, together with theoretical and calculated masses for "b", "y" and "a" type fragment ions, obtained in the MS/MS spectrum.