Primary structure of an adipokinetic neuropeptide from the rhinoceros beetle, *Oryctes rhinoceros* L (Coleoptera: Dynastidae)

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KEY WORDS

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ABSTRACT

Background: Neuropeptides play an important role 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. Small neuropeptides of the adipokinetic hormone/red pigment concentrating hormone family (AKH/RPCH) are one of the important groups of peptides that regulate physiological homeostasis. **Purpose**: The present investigation was carried out to elucidate the primary structure of adipokinetic neuropeptides in the rhinoceros beetle, *O. rhinoceros*. **Methods and Results:** In the present investigation, an adipokinetic neuropeptide from the coconut pest, *Oryctes rhinoceros* was isolated from corpora cardiaca by HPLC; the chromatographic fractions were tested for adipokinetic activity in the plant bug, *Iphita limbata in vivo.* Two UV absorbance peaks were found to be significantly active in elevating haemolymph lipid levels. MALDI-MS analysis of the extract indicated that the molecular mass, 1003.70 Da is similar to the already known AKH from another beetle, *Melolontha melolontha*. MALDI-MS/MS analysis confirmed that its primary structure is exactly similar to the structure reported for the Melme-AKH (pE-L-N-Y-S-P-D-W-NH_2). **Coclusion**: The findings suggest that the distribution of AKH peptides has shown that there exists a taxonomic order or family specificity. This data can be used as additional information to aid in the construction of phylogenetic trees by means of computer programme and protein parsimony algorithms.

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Introduction

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 characterized.[1,2] 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. Adipokinetic hormone /Red pigment concentrating hormone family (AKH/RPCH) is one of the important group of peptides regulates physiological homeostasis. These peptides are synthesized and stored in the intrinsic cells of corpora cardiaca and stored in corpora allata (retrocerebral complex). Common characteristics of the peptides 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. 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 amino acid either at position 2 (Tyr or Phe) or at position-7.[3,4] It acts on the fat body to mobilize stored lipids and carbohydrates, activate glycogen phosphorylase, accumulate cAMP[5,6,7] and inhibit the synthesis of lipids[8,9] proteins[10,11] and RNA.[12]

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.[13] 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. Structural studies of insect neuropeptide hormones also enables to develop biotechnological strategies for insect pest management. Recombinant DNA techniques may be used to bring hormonal imbalance and thereby suicidal effects in pest species of insects. Agonistic and antagonistic hormone mimics could also be developed to influence cellular activities

Even though AKH/RPCH representatives from most of the insect orders have been elucidated, many of the important pest species are still awaiting such discoveries. The present investigation was carried out to elucidate the primary structure of adipokinetic neuropeptides in the rhinoceros beetle, *Oryctes rhinoceros* using HPLC, bioassay and mass spectrometric analyses (MALDI-MS and MS/MS).

Methods

Experimental organism

Adults of both sexes and unspecified age of the rhinoceros beetle, *O. rhinoceros* were collected using a pheromone (aggregation pheromone) trap (Chem Tica International, Consta Rica). After collection, they were kept in plastic containers with perforated lids. The insects were brought to the laboratory and immediately used for hormone extraction.

Preparation of Corpora Cardiaca extract

Adult *O. rhinoceros* of both sexes were used for collecting the retrocerebral complexes for hormone extraction. Heads were removed; the dorsal part of the head capsules was 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. 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.

High Performance Liquid Chromatography (HPLC)

The dried extract made from the retrocerebral complexes from *O. rhinoceros* 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 (SCL 10 AVP, LC 10 ATVP, LC 10 ATVP) 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 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 at 210 nm. One minute fractions starting from 4 to 7 min were collected manually, dried by vacuum concentrator (Savant, USA), and were used for testing their hyperlipaemic activity.

The elution pattern of the fractions of retrocerebral extracts of *O. rhinoceros* was compared with that of an AKH peptide from another beetle, *Melolontha melolontha* (Melme CC) obtained by injecting 100 pmole of the peptide, running with the same instrumental set up as that used for *Oryctes*

Hyperlipaemic bioassays of fractions separated on HPLC

Adult female *Iphita limbata* were used for *in vivo* hyperlipaemic bioassay of fractions collected. 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 ml) 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.

Matrix Assisted Laser Desorption Ionization- Time of Flight- Mass spectrometry (MALDI-TOF-MS)

The dried extract of neurohaemal tissues of the insect was used for mass spectrometric analysis. Mass spectrometric analysis were performed on an Ultra Flex mass spectrometer (Bruker Daltonics, Germany) in reflectron ion mode, using a 90 ns time delay and a 25 kV accelerating voltage in the positive ion (Na^+) mode. 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.

Tandem- MS/MS

Tandem mass spectra (MS/MS) were acquired by selecting the precursor mass (1003.70 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.

Quantitation of haemolymph lipids

Total lipids in the haemolymph samples were determined using phosphovanillin reagent.[14] 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.

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.

Results and Discussion

An extract of fifty retrocerebral complexes of *O. rhinoceros* was subjected to HPLC analysis with the instrumental conditions as mentioned earlier. The result of the analysis is given in Fig. 1. The HPLC profile indicates the presence of a few absorption peaks with retention times 4.91, 5.39 and 6.18 min (represented as 1, 2 and 3 respectively) at 210 nm. One min fractions from 4 to 7 min were manually collected and used for determining their hyperlipaemic activities. The results are provided in the Fig. 2. From the data it is clear that the materials in the fractions 6 and 7 min showed significant hyperlipaemic effects 12 and 5% in-

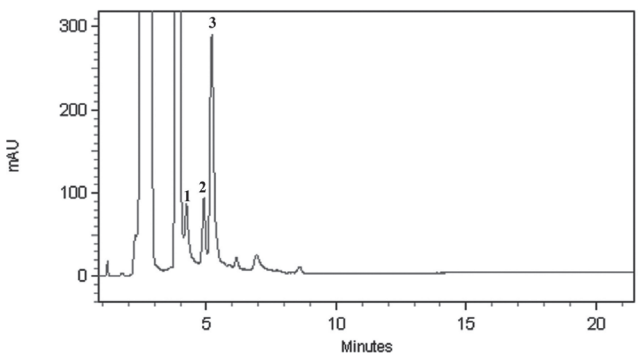

Fig. 1: The HPLC profile of extracts of retrocerebral complexes of *O. rhinoceros*. The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A=0.01% trifluoro acetic acid in water, solvent B = 60% acetontrile in solvent A). The eluants were monitored at 210 nm.

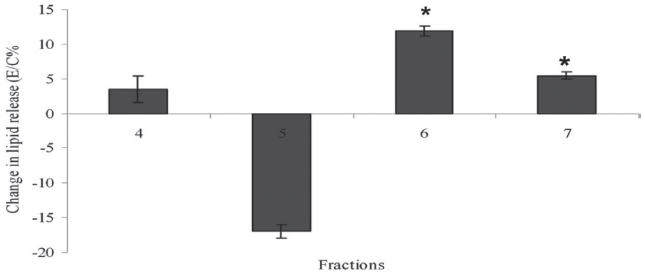

Fig. 2: The hyperlipaemic effect of fractions separated on HPLC. The change in total haemolymph lipid is represented in histogram as E/C%. *Indicates $P<0.05$.

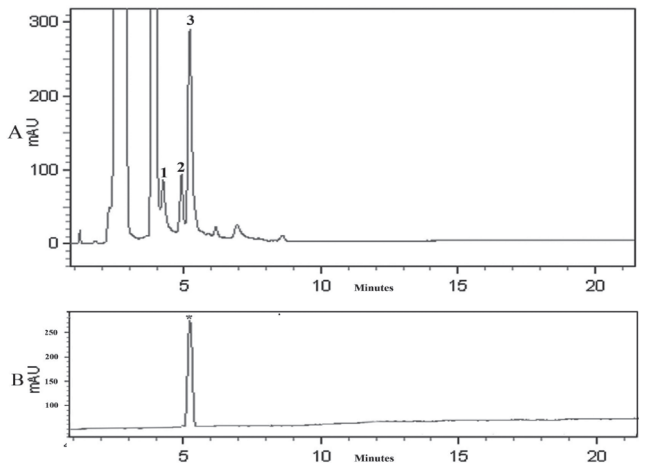

Fig. 3: The HPLC profiles of extracts of retrocerebral complexes of *O. rhinoceros* (A) and synthetic Melme-CC (B). The analysis was carried out on a C_{18} Hibar column. The extract was run with a gradient of 43-53% B in 20 min (solvent A = 0.01% trifluoro acetic acid in water, solvent B = 60% acetontrile in solvent A). The eluants were monitored at 210 nm.

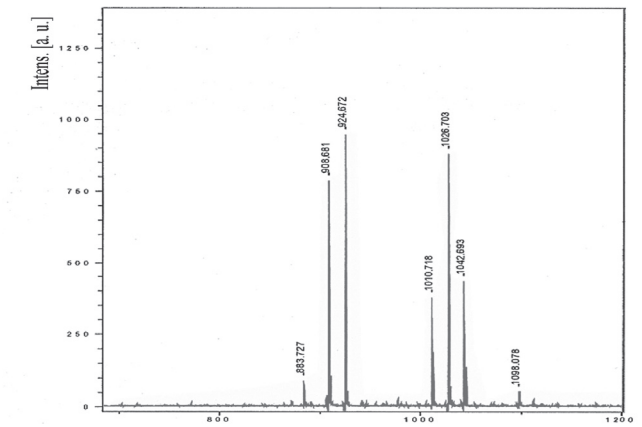

Fig. 4: MALDI-MS spectrum of extract of corpora cardiaca of *O. rhinoceros*. The analysis was carried out in reflector positive mode with an accelaration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Diriydroxybenzoic acid was used as matrix.

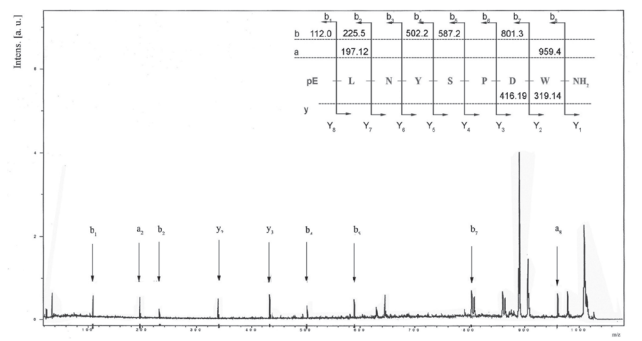

Fig. 5: MALDI MS/MS spectrum of the ion $(M+Na)^+ = 1003.70$ Da from *0. rhinoceros*, inset shows the sequence assignment of the peptide, together with theoretical and calculated masses for "b", "y" and "a" type fragment ions, obtained in the MS/MS spectrum.

crease respectively over the controls. The materials in the 4 min fraction also showed hyperlipaemia (4%) but it was not statistically significant. The comparison of HPLC profiles of retrocerebral extracts and synthetic Melme-CC are presented in the Fig.3. As seen in the chromatogram the Melme-CC was eluted with same retention time as that of materials having retention time 5.39 min in the retrocerebral extract.

MALDI-MS analysis

The result of the MALDI-MS analysis is provided in Fig. 4. 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.

MALDI-MS/MS of 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 Fig.5. The theoretical fragment ions of the AKH peptide Melme-CC was analysed with PFIA-II. 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 a8 (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.

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. rhinoceros*, 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*[14] *I. limbata*[16,17] and *O. nitidula.*[18]

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.*[19] 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.*[17,18] 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.[19,20] 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.**[21,22] 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 1). Further investigations are necessary to draw conclusion about the evolution of AKHs in beetles. Recent investigations revealed that AKHs not only aid in insect flight but also many other essential metabolic activities in them[6,11] 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. 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.

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Table 1: 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

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