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Identification and Structural Characterization of a Hyperlipemic Neuropeptide from the Mango Leaf Webber, *Orthaga exvinacea* Hampson

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ABSTRACT

Neuropeptides of the adipokinetic/red pigment-concentrating hormone family (AKH/RPCH) regulate energy metabolism in insects. The present study was carried out to elucidate the primary structure of an adipokinetic neuropeptide in the mango leaf webber, *O. exvinacea* employing bioassay, High Pressure Liquid Chromatography (HPLC) analysis and mass spectrometric studies. The presence of adipokinetic activity in the crude extracts of brain-retrocerebral complexes of *O. exvinacea* was demonstrated by heterologous *in vivo* bioassay in the polyphagous plant bug *Iphita limbata*. The extract was separated by Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) and the purified fractions were tested for their adipokinetic effects on the fat body. The maximum hyperlipemic activity was shown by fraction 10. MALDI-TOF-MS analysis of the fraction indicated that the molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct of peptide with mass 1008.471. This molecular ion peak has similar mass as that of already known AKH/RPCH peptide, *Manduca* adipokinetic hormone (*Manduca* AKH). Tandem MS/MS confirmed that the primary structure of *O. exvinacea* AKH is pE-L-T-F-T-S-S-W-G-NH_2 which is identical with Manse-AKH.

Key words: Adipokinetic hormone, *Orthaga exvinacea*, *Iphita limbata*, HPLC, Manse-AKH, MALDI-TOF-MS, tandem mass spectrometry

INTRODUCTION

Neuropeptides are peptide hormones that regulate major physiological and behavioral functions in arthropods. The Adipokinetic Hormones (AKHs) secreted by insect neuronal tissues (brain-retrocerebral complexes) are among the most extensively characterized peptide hormones of the adipokinetic/red pigment-concentrating hormone (AKH/RPCH) family of arthropods, among which about 50 members from most of the important insect orders have been sequenced, including the Lepidoptera (Gade, 1996; Gade *et al.*, 1997). AKHs are pleiotropic in nature, mediating mobilization of energy substrates (lipids and carbohydrates) from the fat body in insects, at the same time they inhibit the synthesis of proteins (Carlisle and Loughton, 1979; Kodrik, 2008), lipids (Gokuldas *et al.*, 1988; Lorenz, 2001) and RNA (Kodrik and Goldsworthy, 1995). The AKH peptides are characterized by a blocked N-terminus (pyroglutamate), an amidated C-terminus and a chain

of 8-10 amino acids. They contain at least two aromatic amino acids, tryptophan in position 8 and phenylalanine or tyrosine in position 4. Recently, the primary structures of 3 more adipokinetic peptides have been elucidated from *Iphita limbata*, *Aularches miliaris* and *Oryctes rhinoceros* (Ajaykumar and Gokuldas, 2011a, b, c). In lepidopterans, an AKH was first sequenced from the tobacco hornworm moth *Manduca sexta* (Ziegler *et al*., 1985) and this nonapeptide was code named Manse-AKH (pQLTFTSSWGamide). Manse-AKH was also found in the silk moth, *B. mori* (Ishibashi *et al*., 1992), whereas, the noctuid moth, *Heliothis zea*, contains Manse-AKH and a decapeptide called Helze-HrTH (Jaffe *et al*., 1986, 1988).

The aim of the present study was to elucidate the primary structure of an adipokinetic neuropeptide of the mango leaf webber, *Orthaga exvinacea,* a serious pest of mango tree. Peptide identities were deduced from comparative RP-HPLC analysis of the native peptide with synthetic peptide standard, as well as from the data obtained by MALDI-TOF Mass Spectrometry (MS) and tandem MS.

MATERIALS AND METHODS

Experimental insects: Larvae of *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae) were collected from their natural habitat, mango trees in Calicut and Malappuram district, Kerala, India and were transferred to plastic basins kept in the insectary and reared by feeding mango leaves. The colony was maintained at 27±2°C and 70-80% relative humidity. Adults were fed with 50% honey. Sixth instar larvae were separated from the colony and used for experiments. Insects of both sexes were used for hormone extraction and bioassay experiments.

Adults of the polyphagous bug, *Iphita limbata* Stål (Pyrrhocoridae: Heteroptera) were collected from Calicut University campus and maintained in cages in the insectary on a diet of germinating seeds of green gram (*Phaseolus radiatus*) and pieces of various tropical fruits. Mature adults were used for the experiments.

Preparation of brain retrocerebral complex extract: The brain-retrocerebral complexes of *Orthaga exvinacea* were removed with the help of fine forceps under a stereozoom binocular microscope (ZEISS, Germany). The tissues were immediately put in to ice cold 80% methanol (HPLC grade) and stored at -4°C until extraction. They were sonicated for 1 min on ice using ultrasonicator (Sonics and Materials, USA). The tissue homogenate was centrifuged at 4°C and 10000 rpm for 10 min. The supernatant was collected in to an eppendorf tube and vacuum dried (Savant, USA). The dried supernatant was stored at -4°C until used for analysis.

Detection of biological activity: The heterologous *in vivo* bioassay for hyperlipemic activity was done in the plant bug, *Iphita limbata*, in which increase of vanillin-positive material (= total lipids) in the hemolymph, was measured. The dried methanolic brain retrocerebral complex extract prepared as mentioned above was dissolved in insect saline (the buffer used for bioassays and fat body incubation contained NaCl, 130 mM; KCl, 5 mM; Na_2HPO_4, 1.9 mM and K_2HPO_4, 1.7 mM, the pH was adjusted to 7.5) to get a final concentration of one gland pair equivalent (gpe) per 5 µL. A sample of hemolymph (2 µL) was collected directly from the cut end of antenna in to a precalibrated capillary tube. The sample was transferred in to the bottom of a small test tube (5 mL capacity). An aliquot of the extract (5 µL) was then injected in to the acceptor plant bug, *Iphita limbata*, through the intersegmental membrane between thoracic and first abdominal segment. The needle was kept

in position for a while to allow mixing of the material with hemolymph and to avoid loss through oozing hemolymph droplet. After 60 min, another sample of hemolymph was collected (2 μL) directly from the cut end of the other antenna in to another capillary tube and was then transferred in to the bottom of another test tube. Hemolymph samples taken before injections were taken as controls and 60 min after injection as experimental.

Measurement of hemolymph lipid levels: The total concentration of lipids in the hemolymph samples were measured using phosphovanillin method (Frings *et al*., 1972). To the hemolymph samples collected in various experiments and kept at the bottom of small test tubes, concentrated sulphuric acid (50 μL) were added, heated in a boiling water bath for 10 min, cooled to room temperature and 2 mL each of phosphovanillin reagent [a 3:2 (v/v) mixture of O-phosphoric acid and aqueous vanillin 0.525% (w/v)] were added. The tubes were thoroughly shaken to mix the content. Optical densities of the pink complex formed were measured within 5 min using UV-vis spectrophotometer at 540 nm against a reagent blank.

High Performance liquid chromatography (HPLC) analysis: The dried extract made from the retrocerebral complexes from *O. exvinacea* was resuspended in 80% methanol (HPLC grade). The extract was filtered using a sample filtration unit (Millipore, USA) with a filter of pore size 0.45 μm. A sample of 20 μL (2 gpe μL^{-1}) of the filtered brain-retrocerebral extract was directly injected into the instrument using a Hamilton micro-syringe. HPLC separations were carried out using Shimadzu system (SPD M 10AVP, LC 10 ATVP, LC-10 ATVP) with a reversed phase column (C_{18}) of 250 mm long and 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^{-1}. 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 pore size Millipore filter. The eluents were monitored at 210 nm using UV-VIS detector. One minute fractions starting from 1 min up to 20 min were collected with a fraction collector for testing their biological activities. The HPLC profiles were exported into Microsoft Word file and were used for further analysis of the data.

The synthetic peptide (100 pmol μL^{-1}) Locmi-AKH-I (GenScript Corp; USA) was injected (20 μL) into the HPLC instrument maintained in the same set up as before for the retrocerebral extracts of *O. exvinacea*. Similarity of retention time of any materials (appearing as peaks) of the extract of *O. exvinacea* with that of the synthetic locust AKH was confirmed by overlaying this profile with that obtained for synthetic Locmi-AKH.

***In vitro* bioassay for testing the effects of different HPLC fractions on lipid metabolism:** The extracts of retrocerebral complexes of *O. exvinacea* were fractionated on HPLC and the fractions were collected with a fraction collector in 20 separate eppendorf tubes. These one minute fractions of HPLC eluents were dried in a vacuum concentrator. The dried fractions were resuspended in 200 μL of double distilled water and stored until use. Samples of these fractions (2 gpe/20 μL) were tested separately for their hyperlipemic effects on lipid release from the fat body of *O. exvinacea* by conducting *in vitro* bioassays. For this, fat body from individual insects were removed, washed in saline and blotted, chopped and divided in to two halves. One half served as experimental and the other half as control. These halves were then put in to preweighed incubation vials containing 200 μL of standard HEPES buffer and 20 μL of either the hormone of appropriate

concentration (experimental) or 20 μL of distilled water (control) and the fat body weights were determined. Incubations were carried out for 30 min in a shaker water bath set at 37°C. After *in vitro* incubation, all samples of the incubation medium were drawn and the experimental and control samples were quantitated using phosphovanillin reagents and analyzed by spectrophotometric methods.

Matrix assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS): The dried extract of neurohaemal tissues of *O. exvinacea* purified on HPLC present in fraction 10 was collected (2 gpe/μL) and subjected to 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 monitored in Na^+ mode. The system utilized 50 Hz pulsed voltage laser, emitting at 337 nm. The ion source and the flight tube were kept at a pressure of about 7×10^{-7} mbar by turbo molecular pump.

The samples were prepared by mixing equal volumes of peptide solution (fraction 10) (1 mL) and a saturated solution of the matrix, dihydroxybenzoic acid in 1:1 (v/v) acetonitrile: water mixture and applied on a multisample target. The samples were measured in the reflectron mode within the mass range m/z 850-1250 Da. The results of 10-20 shots were averaged to obtain the final spectrum. A standard peptide mixture was used for external calibration.

Tandem-MS/MS: The amino acid sequence information of the peptide was obtained by employing MALDI-MS/MS analysis. During MS/MS or tandem mass spectrometry, fragment ions are generated from a selected precursor ion. The amino acid sequence is determined by calculating the m/z difference (which corresponds to the mass of an amino acid) between the adjacent y-ion peaks and/or b-ion peaks. The tandem mass spectra were acquired by selecting the precursor mass 1030.471 with a 10 Da window and fragments were generated in Post Source Decay (PSD) mode. The molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct $[M+Na^+]$ of peptide with mass 1008.471. The measured monoisotopic masses $[M+Na^+]$ of known or conceptually derived peptides were compared to the calculated masses. Mass spectra were analyzed by using Flex-analysis software.

The MS/MS data were interpreted by using Peptide Fragmentation Ion Analyser-II (PFIA-II) software.

Statistical analysis and data presentation: Values obtained from various bioassay experiments were subjected to statistical analysis for significance and the values are expressed as Mean±Standard deviation as well as percentage difference of the experimental over controls (E/C%). The analyses were performed using SPSS Software (version 10). The graphical representation of change in lipid mobilization was plotted using Origin software and Microsoft Excel program. The data were obtained from the research work conducted in the Department of Zoology, University of Calicut, Kerala, India and Indian Institute of Science, Bangalore, India.

RESULTS

HPLC separation and activity of different fractions on lipid release: The HPLC profile of the brain retrocerebral complex extract of *O. exvinacea* exhibited a cluster of a number of UV absorbing peaks in the initial stages of the run. An isolated large peak was seen eluted at 10 min

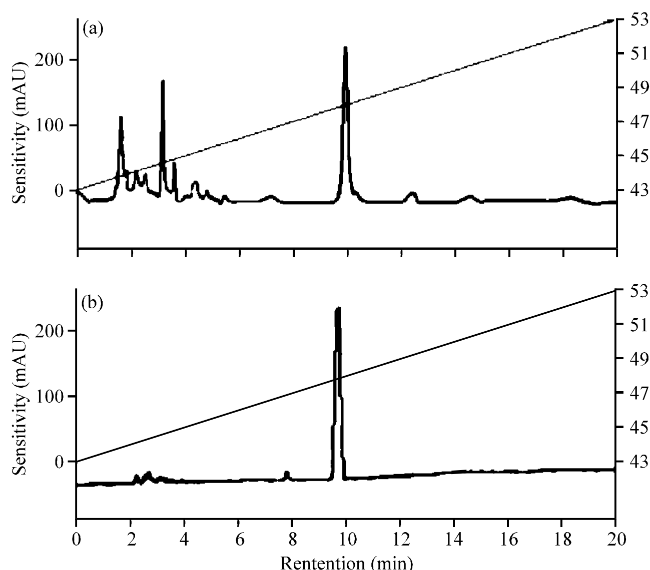

Fig. 1(a,b): The HPLC profiles of brain-retrocerebral complex extract of *O. exvinacea* (a) and synthetic Locmi-AKH (b). The extract (40 gpe) run with a gradient of 43-53% solvent B in 20 min with a flow rate of 1 mL min^{-1} (solvent A = 0.01% Trifluoroacetic acid (TFA) in water, solvent B = 60% acetonitrile in solvent A). The quantity of synthetic AKH loaded was 2 nanomoles

of the run and there were no major peaks observed afterwards except for a few minor peaks at 13, 15 and 18 min of the run (Fig. 1). The similarity of retention time of any materials of the extract of *O. exvinacea* with that of an already reported peptide, the synthetic locust AKH was tested by overlaying this profile with that obtained for synthetic AKH in a similar HPLC run. As seen in the chromatogram, the large peak that had the retention time of 9.7 min was similar to that of the synthetic Locmi-AKH. The peaks obtained at 10 min with the crude brain-retrocerebral complex extract and synthetic peptide had almost identical size.

The materials in all the 20 fractions were collected and tested for their hyperlipemic activity. Results showed that fractions 8, 10, 13, 17 and 19 have significant hyperlipemic activity. Highest hyperlipemic activity was shown by fraction 10 which induced lipid release by 35% ($p<0.001$) above the controls. The materials in the fractions 8, 13, 17 and 19 min showed significant adipokinetic activities with increase of lipids by 28% ($p<0.001$), 20% ($p<0.05$), 16% ($p<0.05$) and 20% ($p<0.001$) over the controls, respectively. Though the fractions 7 and 16 also induced lipid release by 22 and 29%, the results were not found to be statistically significant. Fractions 2 and 18 were found to be slightly hypolipemic, but the effects were not significant. None of the other fractions showed any significant adipokinetic activity (Fig. 2).

MALDI-TOF-MS analysis: The molecular masses of peptides belonging to AKH/RPCH family present in the brain-retrocerebral complex extract prepared from *O. exvinacea* were determined by MALDI-TOF-MS analysis (Fig. 3). The molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct [M+Na^+] of peptide with mass 1008.471. This molecular ion

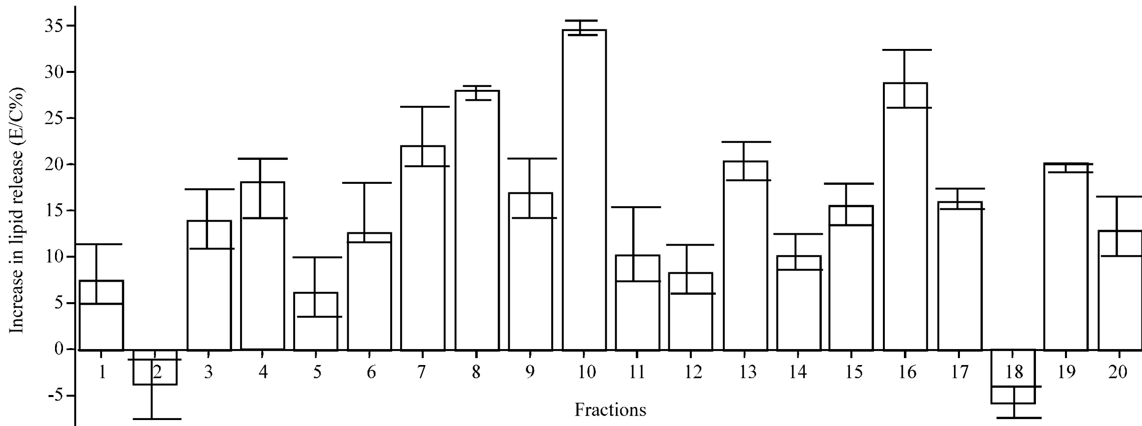

Fig. 2: Hyperlipemic activity of HPLC fractions tested *in vitro* on the fat body of *O. exvinacea*. The increase in total hemolymph lipid is represented as E/C% (Mean±SD)

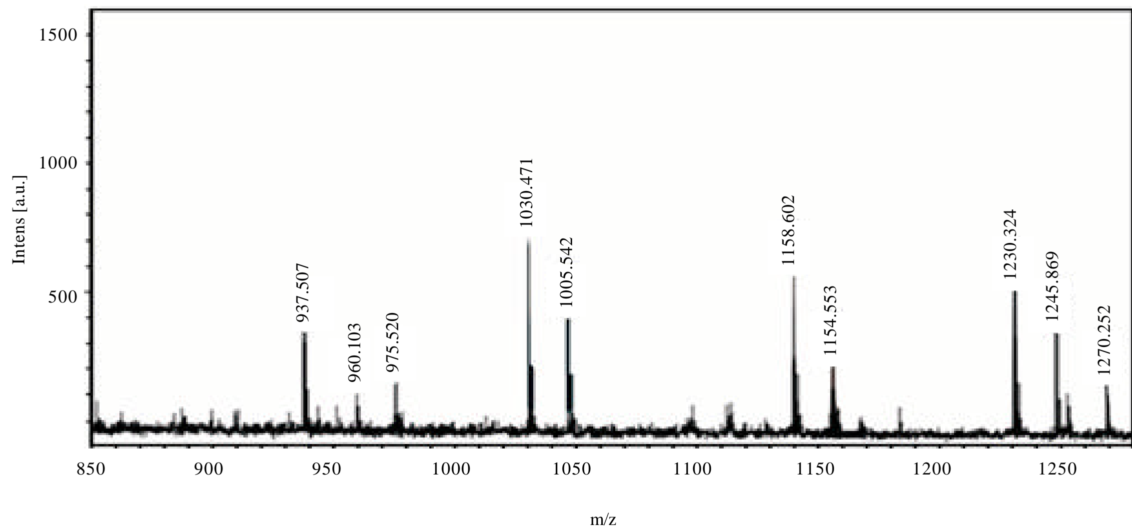

Fig. 3: MALDI-MS spectrum of extract of brain-CC of *O. exvinacea*. The analysis was carried out in reflector positive (Na^+) mode with an acceleration voltage of 50 Hz pulsed N_2 laser, emitting at 337 nm. Dihydroxybenzoic acid was used as matrix

peak has a similar mass as that of an already known AKH/RPCH peptide, *Manduca* AKH. The amino acid sequence information of the peptide was obtained by employing MALDI-MS/MS analysis of the precursor ion.

MALDI MS/MS of the precursor ion 1030.471 Da: The tandem mass spectra (MS/MS) was acquired by selecting the precursor mass (sodium adduct) 1030.471 Da with a 10 Da window and fragments were generated in Post Source Decay (PSD) mode. The MALDI-TOF-MS/MS spectrum

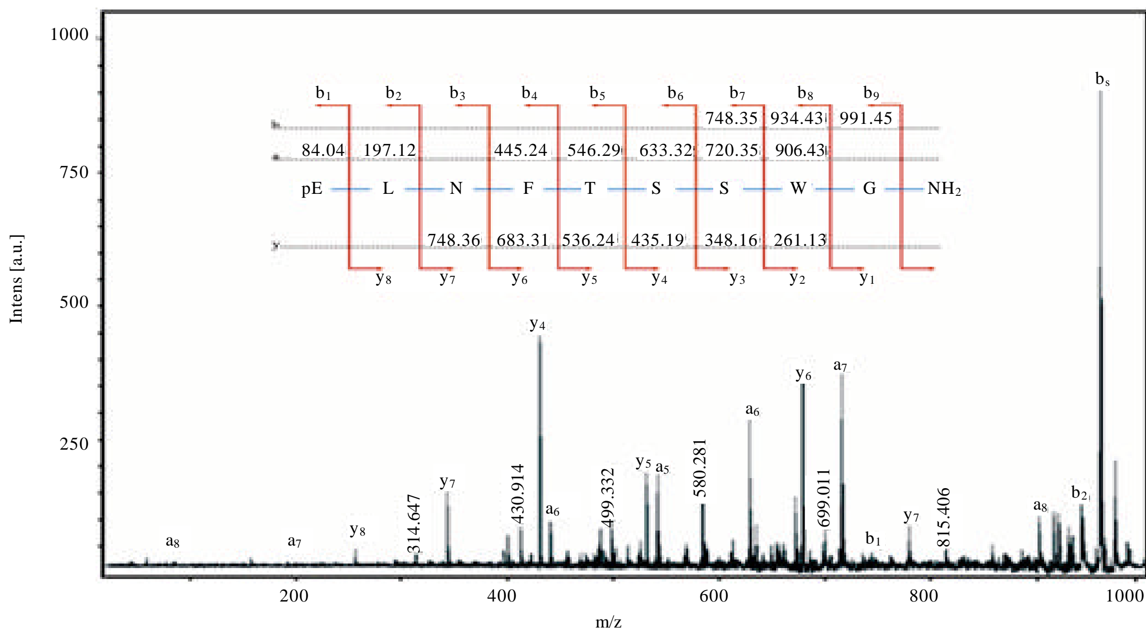

Fig. 4: MALDI-MS/MS spectrum of $(M+Na)^+$ = 1030.471 Da from *O. exvinacea*. Inset shows sequence assignment of the peptide, together with theoretical and calculated masses for "y", "b" and "a" fragment ions obtained in the MS/MS spectrum

of the precursor ion $[M+Na^+]$ = 1030.471 Da is shown in t he Fig. 4. This data was compared with the observed MS/MS fragment ions. The fragment ions a, b and y types were identified from the MALDI-PSD data. The identified N-terminal 'a' type fragment ions are a_1 (m/z 84.04), a_2 (m/z 197.12), a_4 (m/z 445.24), a_5 (m/z 546.29), a_6 (m/z 633.32), a_7 (m/z 720.35) and a_8 (m/z 906.43) and the 'b' type fragment ions are b_7 (m/z 748.35), b_8 (m/z 934.43) and b_9 (m/z 991.45), respectively. The fragment ions y_2 (m/z 261.13), y_3 (m/z 348.16), y_4 (m/z 435.19), y_5 (m/z 536.24), y_6 (m/z 683.31) and y_7 (m/z 784.36) represent the identified C- terminal 'y' type ions. Thus the primary structure of the precursor is derived as pE-L-T-F-T-S-S-W-G-NH_2.

DISCUSSION

Adipokinetic hormones stimulate the fat body to degrade metabolic stores for the mobilization and release of circulating metabolites. It regulates intermediary metabolism in the fat body, resulting in hyperlipaemia. Injections of extracts of brain-retrocerebral complexes have been shown to elicit lipid mobilization in locusts (Goldsworthy, 1983; Beenakkers *et al*., 1985) and butterflies (Kollisch *et al*., 2000). Previous studies also have shown significant hyperlipemic responses by the fat body of *Iphita limbata* injected with brain-retrocerebral complex extracts of *Spodoptera mauritia* (Kumari and Gokuldas, 2001) *I. limbata* (Rasheed and Gokuldas, 2002) and *Hieroglyphus banian* (Gokuldas *et al*., 2012). Similar reports have been corroborated for the moth, *M. sexta* which uses lipids as the main fuel for flight muscle contraction (Ziegler, 1995). In the present study it was observed that, injection of an extract of brain-retrocerebral complex of *O. exvinacea* into the plant bug, *I. limbata* elicits lipid mobilization into the hemolymph.

It is evident that AKHs of same structure can mobilize different energy substrates in different insects which in turn reveals the possibility that these hormones evolved from some common ancestral molecule and that they can attach to a limited number of receptor molecules. Manse-AKH is widely distributed in lepidopteran insects and many of them possess either adipokinetic or hyperglycaemic effects. Manse-AKH mobilizes lipids in the silkmoth, *B. mori* (Ishibashi *et al.*, 1992) and the butterfly, *Vanessa cardui* (Kollisch *et al.*, 2000), carbohydrate in the noctuid moth, *Heliothis zea* (Jaffe *et al.*, 1988) and both lipids and carbohydrates in the tobacco hornworm moth, *Manduca sexta* (Ziegler *et al.*, 1985). These facts suggest that AKHs trigger the particular mobilization pathways leading to the release of stored energy.

The activity of the brain-retrocerebral complex extract was tested *in vivo* in the plant bug, *I. limbata* (heterologous bioassay) showed that the extract and also the synthetic peptide, Locmi-AKH-I can increase lipid (adipokinetic) in the hemolymph. The RP-HPLC separation of the neuronal tissue extract of *O. exvinacea* indicated that the hormone extract contained compounds that elutes on an octadecyl silane column using acetonitrile as the solvent. A comparison of HPLC profiles of the synthetic Locmi-AKH-I with that of the retrocerebral complex extract *O. exvinacea* showed that the hormone extract contained materials having similar retention times as that of synthetic AKH. It was found that the most active fraction, eluted at 9.7 min was having a close retention time as that of the synthetic Locmi-AKH-I, i.e., 10 min. The data obtained from *in vitro* bioassay studies with the HPLC fractions of the hormone extract of *O. exvinacea* revealed that several fractions had hyperlipemic activity. At least five fractions have significant activity among which the highest activity was shown by fraction 10. However, no activity were shown by fractions appeared in the initial stages. Thus from the present study, it is evident that the materials in the fraction 10 showed significant adipokinetic with increase in lipid release.

The present study also included experiments to elucidate the primary structure of the adipokinetic peptide from the brain-retrocerebral complex extract of the mango leaf webber *O. exvinacea* using MALDI-TOF/MS/MS. The molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct [M+Na^+] of peptide with mass 1008.471. This result is in agreement with the studies conducted by Ziegler *et al.* (1985). The molecular ion peak obtained has similar mass as that of already known AKH/RPCH peptide, *Manduca* AKH. The identity of the pE form was fully confirmed by MS/MS analysis. Fragmentation of the precursor ion produced an almost full series C- terminal y- ions (y_2, y_3, y_4, y_5, y_6, y_7) and prominent b- ions corresponding to b_7, b_8 and b_9 and a series of a- ions (a_1, a_2, a_4, a_5, a_6, a_7, a_8) and numerous other masses in confirmation. The characteristic y- and b- type product ions, in conjugation with diagnostic y-NH_3 and b-H_2O ions characterized these peptides as members of the AKH family. Mass spectrometric analysis of the biologically active fraction revealed the chemical composition of the compound is pE-L-T-F-T-S-S-W-G-NH_2. The presence of amino acids phenylalanine at position 4, Leu at position 2, tryptophan and glycine at positions 8 and 9 unambiguously shows that the derived peptide belongs to the AKH family. Thus the results from bioassays using crude extract and HPLC fractions and mass spectrometry analysis show that the biologically active material contains a nonapeptide.

CONCLUSION

The present study revealed the presence of bioactive peptide hormone in brain-retrocerebral complex of the lepidopteran insect *O. exvinacea*. The chemical composition of the peptide identified is pE-L-T-F-T-S-S-W-G-NH_2. The study demonstrated that *O. exvinacea* contained the same AKH as found in several moth species such as *Manduca* AKH, suggesting that there exists a family specificity and also indicates their phylogenetic relationship.

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REFERENCES

Ajaykumar, A.P. and M. Gokuldas, 2011a. Amino acid sequence of an adipokinetic neuropeptide from the plant Bug, *Iphita limbata* stal. Am. J. Biochem. Mol. Biol., 1: 349-358.

Ajaykumar, A.P. and M. Gokuldas, 2011b. Elucidation of primary structure of an adipokinetic neuropeptides from the coffee locust *Aularches miliaris* L. (Orthoptera: Pyrgomorphidae). Int. J. Biol. Chem., 5: 127-135.

Ajaykumar, A.P. and M. Gokuldas, 2011c. Primary structure of an adipokinetic neuropeptide from the rhinoceros beetle, *Oryctes rhinoceros* L. (Coleoptera: Dynastidae). Ann. Neurosci., 18: 100-104.

Beenakkers, A.M., D.J. Van der Horst and W.J.A. Van Marrewijk, 1985. Insect lipids and lipoproteins and their role in physiological processes. Prog. Lipid Res., 24: 19-67.

Carlisle, J.A. and B.G. Loughton, 1979. Adipokinetic hormone inhibits protein synthesis in *Locusta*. Nature, 282: 420-421.

Frings, C.S., T.W. Fendly, R.T. Dunn and C.A. Queen, 1972. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin. Chem., 18: 673-674.

Gade, G., 1996. The peptide revolution in insects illustrated by the AKH/RPCH family of peptides. Zeitschrift fur Naturforschung, 51: 607-617.

Gade, G., Hoffmann, K.H. and J.H. Spring, 1997. Hormonal regulation in insects: facts, gaps and future directions. Physiol. Rev., 77: 963-1032.

Gokuldas, M., A.P. Ajaykumar, D. Umadevi, V.S. Binitha, K.M. Ismail and K.A. Rasheed, 2012. Identification of an AKH/RPCH family peptide in the rice grasshopper, *Hieroglyphus banian* (Acrididae: Orthoptera). J. Entomol., (In Press).

Gokuldas, M., P.A. Hunt and D.J. Candy, 1988. Inhibition of lipid synthesis *In vitro* in the locust, *Schistocerca gregaria*, by a factor from *Corpora cardiaca*. Physiol. Entomol., 13: 43-48.

Goldsworthy, G.J., 1983. The Endocrine control of flight metabolism in locusts. In: Advances in Insect Physiology, Berridge, M.J., J.E. Treherne and V.B. Wigglesworth (Eds.). Academic Press, New York, USA., pp: 149-204.

Ishibashi, J., H. Kataoka, H. Nagasawa, A. Isogai and A. Suzuki, 1992. Isolation and identification of adipokinetic hormone of the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem., 56: 66-70.

Jaffe, H., A.K. Raina, C.T. Riley, B.A. Fraser and G.M. Holman *et al*., 1986. Isolation and primary structure of a peptide from the corpora cardiaca of *Heliothis zea* with adipokinetic activity. Biochem. Biophys. Res. Commun., 135: 622-628.

Jaffe, H., A.K. Raina, C.T. Riley, B.A. Fraser and T.G. Bird *et al*., 1988. Isolation and primary structure of a neuropeptide hormone from *Heliothis zea* with hypertrehalosemic and adipokinetic activities. Biochem. Biophys. Res. Commun., 155: 344-350.

Kodrik, D. and G.J. Goldsworthy, 1995. Inhibition of RNA synthesis by adipokinetic hormones and brain factor (s) in adult fat body of *Locusta migratoria*. J. Insect Physiol., 41: 127-133.

Kodrik, D., 2008. Adipokinetic hormone functions that are not associated with insect flight. Physiol. Entomol., 33: 171-180.

Kollisch, G.V., M.W. Lorenz, R. Kellner, P.D. Verhaert and K.H. Hoffman, 2000. Structure elucidation and biological activity of an unusual adipokinetic hormone from corpora cardiaca of the butterfly, *Vanessa cardui*. Eur. J. Biochem., 267: 5502-5508.

Kumari, P.S. and M. Gokuldas, 2001. Hyperglycaemic response of fat body from *Spodoptera mauritia* (Noctuidae: Lepidoptera) to neuropeptide hormones. Entomon, 26: 53-64.

Lorenz, M.W., 2001. Synthesis of lipids in the fat body of *Gryllus bimaculatus*: Age dependency and regulation by adipokinetic hormone. Arch. Insect Biochem. Physiol., 47: 198-214.

Rasheed, K.A. and M. Gokuldas, 2002. Evidences for adipokinetic activity in *Iphita limbata* Stal (Pyrrhocoridae: Heteroptera). Entomon., 27: 125-136.

Ziegler, R., 1995. Adipokinetic Hormone in *Manduca sexta*: Physiological Actions. In: Insects: Chemical, Physiological and Environmental Aspects, Konopinska, D. (Ed.). Wroclaw University Press, Wroclaw, Poland, ISBN-13: 9788322913031, pp: 35-41.

Ziegler, R., K. Eckart, H. Schwarz and R. Keller, 1985. Amino acid sequence of *Manduca sexta* adipokinetic hormone elucidated by combined Fast Atom Bombardment (FAB)/tandem mass spectrometry. Biochem. Biophys. Res. Comm., 133: 337-342.