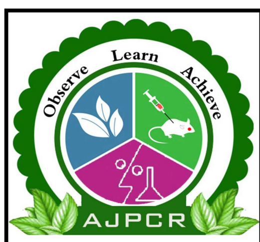
Research Article CODEN: AJPCFF ISSN: 2321- 0915



Asian Journal of Phytomedicine and Clinical Research

Journal home page: www.ajpcrjournal.com

HYPERLIPAEMIC EFFECTS OF A NEUROPEPTIDE HORMONE ISOLATED FROM INSECTS ON VERTEBRATE LIVER

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ABSTRACT

Peptides of the adipokinetic hormone (AKH)/red pigment concentrating hormone (RPCH) family are released from the brain-retro cerebral complexes of insects. They have been reported to mobilize lipids and carbohydrates from their fat body. In the present study, the fat depleting effect of the brain-retro cerebral complex extract of the mango leaf *webber Orthaga exvinacea* (Pyralidae: Lepidoptera) were demonstrated *in vivo* and the results showed that they have a pivotal role in lipid release. We purified the insect adipokinetic hormone by High pressure liquid chromatography and examined its activity on lipid mobilization and release from the liver tissues of two vertebrates- chicken and rat by conducting *in vitro* experiments. The study revealed that hormone extract produced significant increase in lipid mobilization compared to the controls in which insect saline was used instead of hormone extract. There was an increase by 41% (P<0.05) in the case of chicken liver and an increase by 8% (P<0.05) in the case of rat liver. The study suggests the possibility of exploiting the fat depleting effect of insect neuropeptide hormones for therapeutic purposes for human.

KEYWORDS

Peptide hormone, HPLC, Fat body and Hyperlipaemia.

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INTRODUCTION

Neuropeptides play major role in cellular communication in invertebrates and in vertebrates. In arthropods, the energy metabolism is controlled by small (8-10 amino acid residues) peptides of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family[1] present in the neurohaemal tissues (brain-retrocerebral complexes). These peptides are amino terminally blocked by a pyroglutamate residue and end in a

carboxy terminal amide. The peptide sequence contains at least two aromatic amino acids and usually a tryptophan residue will be present in position 4. The AKH peptides are multifunctional in nature[2]. They acts on insect fat body to mobilize stored lipids and carbohydrates, activate glycogen phosphorylase, and inhibit the synthesis of proteins[3], lipids[4].

The present investigation was carried out to study the effect of brain-retrocerebral extract of *Orthaga exvinacea* on lipid metabolism using *in vivo* bioassay and to purify the adipokinetic neuropeptide hormone (AKH) by employing HPLC analysis. Furthermore, the activity of insect AKH on the mobilization and release of lipids from the liver tissues of two vertebrates- chicken and rat was studied by conducting *in vitro* experiments.

MATERIAL AND METHODS

Experimental insects

The larvae of *O. exvinacea* were collected from their natural habitat, mango trees, and transferred to plastic basins. They were reared in the insectary by feeding mango leaves. Sixth instar larvae were used for the experiments. Adults of the plant bug, *Iphita limbata* were collected from University campus and maintained in the insectary on a diet of germinating seeds of Green gram. Mature adults were used for the experiments.

Preparation of brain-retrocerebral extract

The brain retrocerebral complexes were separated from the larvae using fine forceps under a stereo zoom binocular microscope. The tissues were immediately put in to ice cold 80% methanol. They were sonicated for 1 min on ice using ultrasonicator. The extract was centrifuged at 4°C and 10000 rpm for 10 min. The supernatant was collected in to an eppendorf tube and vacuum dried. The dried supernatant was stored at -4°C until used for analysis.

Preparation of Synthetic Lom-AKH

The synthetic peptide Lom-AKH (GenScript Corp; USA) purchased was dissolved in 80% methanol (HPLC grade). Required concentrations of peptide solutions were prepared from this stock solution.

Detection of biological activity

The dried brain-retrocerebral complex extract prepared was dissolved in insect saline (NaCl, 130 mM; KCl, 5 mM; Na2HPO4, 1.9 mM and K2HPO4, 1.7 mM, pH was adjusted to 7.5) to get a final concentration of one gland pair equivalent (gpe) per 5 µl. Haemolymph (2 µl) samples was collected directly from the cut end of antenna in to the precalibrated capillary tube and transferred in to a small test tube. An aliquot of the extract (5 µl) was then injected in to the acceptor plant bug, *Iphita limbata*. Another haemolymph samples was collected (2 µl) directly from the cut end of the other antenna in to another capillary tube and were then transferred in to the bottom of another test tube. Haemolymph samples taken before injections were taken as controls and 60 min after injection as experimentals. The experiments were repeated using 5 µl of insect saline instead of extract. Haemolymph samples collected in various experiments were used for the quantitation of lipids.

Quantitation of haemolymph lipids

Total lipids in the haemolymph samples were estimated using phosphovanillin reagent[5]. To the haemolymph samples collected in various experiments, concentrated sulphuric acid (50 µl) were added, heated in a boiling water bath for 10 min and cooled to room temperature. To the tubes 2 ml phosphovanillin reagent 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. The tubes containing haemolymph samples collected from insects injected with insect saline instead of the hormone extract were processed similarly and the change in lipid level was measured which served as controls.

Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) analysis

The dried extract prepared from the brain-retrocerebral complexes of *O. exvinacea* was resuspended in 20 µl of 80% methanol (HPLC grade). The extract was filtered using a sample filtration unit (Millipore, USA) with pore size 0.45 µm. A sample of 20 µl of the extract was directly

injected into the instrument by 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 (C18) 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 pore size Millipore filter. The eluants were monitored at 210 nm using a UV-VIS detector. The retention time of any materials of the extract of *O. exvinacea* with that of already reported peptide, the synthetic locust AKH was tested by overlaying this profile with that obtained for Lom-AKH in a similar HPLC run.

Effect of insect adipokinetic peptides on vertebrate liver

The effect of insect adipokinetic hormone on vertebrate liver was studied using fresh liver tissue from chicken and rat. Samples of fresh chicken liver (10 mg) were weighed out, dipped in 0.9% saline and blotted dry on a filter paper. The tissue was chopped well on a polyvinyl disc with a sharp razor blade. The chopped tissue samples were put in to the bottom of incubation vials (5 ml capacity) with bake lite screw caps containing 200 µl of phosphate buffered saline (PBS) and considered as experimental. Similarly, another set of tubes were processed in the same manner and taken as control. To the medium in the experimental tube, 20 µl of the HPLC purified hormone extract was added whereas the control tube contained 20 µl of distilled water instead of the extract. The whole process was completed as quickly as possible (within 5-6 min). The incubations were carried out for 30 min in a shaker water bath preset at 37°C. After incubation, the samples were brought to room temperature and lipids released in to the medium were extracted[6] procedure. Using a micropipette, 150 µl of the incubated medium was drawn out from the incubation vials. The drawn out samples were deproteinised with 1 ml of chloroform-methanol (1:2 v/v) mixture and were allowed to stand for at least 15 min. To the samples, sodium chloride (1.0 M, 1.0 ml) and chloroform (1.0 ml) were added. The mixtures were shaken well and centrifuged for 3 min at 1000 rpm to separate the organic and aqueous phases. The lower chloroform layer contained the extracted lipids. Lipid samples (100 µl each) were drawn from the lower organic phase using micropipette and transferred to test tubes for quantitation of lipids.

The effect of brain-retrocerebral complex extract in lipid mobilization was also studied using mammalian (rat) liver. A fresh sample of rat liver tissue was weighed out and the lipid release is measured as mentioned above. From the values thus obtained from controls and experimentals, change in the lipid release due to hormone action was estimated.

Quantitation of lipid release from the liver tissue

From the lipid samples thus obtained from chicken and rat liver tissues, chloroform was evaporated off by keeping them at room temperature. The amount of lipids in the samples was measured colorimetrically using phosphovanillin reagent as described previously. The optical densities were measured using UV-vis spectrophotometer at 540 nm against a reagent blank. From the values obtained, absorbance per mg of liver tissue was calculated using the absorbance value of standard lipid (glycerol trioleate) solution.

Statistical analysis and data presentation

Values obtained were subjected to statistical analysis for significance and the values are expressed as mean $\pm$ 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 programme.

RESULTS

Detection of biological activity of hormones *in vivo*

The hyperlipaemic effects of the extracts were measured by estimating change in the lipid content of the haemolymph after the injection of brain-retrocerebral complex extract. Lipids in the

experimental and control samples were estimated by spectrophotometry methods (Table No.1). It was found that the hormone extract induced significant hyperlipaemia. There was an increase in the lipid content by 15% (115.41 $\pm$ 0.11, $P<0.001$). The change in lipid concentration when injected with the synthetic peptide was estimated in the same way. It was observed that the synthetic peptide also produced significant ($P<0.05$) hyperlipaemic effects. The lipid levels increased to 116.11 $\pm$ 0.18 per cent of controls. In the control, the injection of 5 µl of insect saline did not evoke any significant hyperlipaemic activity. On the other hand, there was a small hypolipaemic response (-8%).

Values obtained from the bioassay experiments were subjected to statistical analysis and expressed as mean $\pm$ standard deviation. The values are also expressed as percentage difference of the experimental over controls (E/C%). The analysis shows that the extract of *O. exvinacea* as well as the synthetic hormone has significant hyperlipaemic effects on *I. limbata* fat body to release lipids in to the haemolymph.

Values are expressed as means $\pm$ standard deviation (n=6). *Indicates the significance of difference between the controls and experimentals.

Characterization of the adipokinetic peptide by HPLC

The elution profile of the chromatography was recorded (Figure No.1). The elution profile clearly showed 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 of the run, and there were no major peaks observed afterwards except for a few minor peaks at 13, 15 and 18 minutes of the run.

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 Lom-AKH. The peaks obtained at 10 min with the crude brain-retrocerebral complex extract and synthetic peptide had almost identical size.

Effect of insect AKH on vertebrate liver

The effect of insect adipokinetic hormone on lipid mobilization and release from the liver of two vertebrates, chicken and rat were studied by conducting *in vitro* experiments (Table No.2). The extract produced significant effects in lipid mobilization from chicken liver [141.46$\pm$9.60 ($P<0.05$)]. But no such effects were observed in similar experiments conducted using insect saline (-5%).

The effect of brain-retrocerebral complex extract in lipid mobilization was also similar on rat liver (Table No.3). In this case, the hormone extract produced significant effects in lipid mobilization 107.78$\pm$1.86 ($P<0.05$), even though the effect was relatively very small. The experiments using insect saline instead of hormone extract did not produce any hyperlipaemic effects (-4%).

Values are expressed as means $\pm$ standard deviation (n=6). *Indicates the significance of difference between the controls and experimentals.

DISCUSSION

In insects, lipids are important fuels for long distance flight. However carbohydrates are used in the initial stage of flight and also contribute substantially in the later phase[7]. Lipids are largely depleted from the glycogen reserves of fat body causing an increased level of soluble carbohydrates in the haemolymph. Injection of locust retrocerebral extract triggers haemolymph sugar (hypertrehalosemic effect) in cockroaches[8] and elevates haemolymph lipid (adipokinetic effect) in locusts[9]. When the brain-CC extract injected into locusts or in to lepidopteran insects, it produced an adipokinetic effect[10]. In *M. sexta*, a single AKH mediate the mobilization of carbohydrates in larvae and lipids in adults[11]. A significant hyperlipaemic response was shown by the fat body of *I. limbata* injected with neurohaemal extracts of *Spodoptera mauritia*[12] and *I. limbata*[13] Comparable results have been reported for the month, *Manduca sexta*, which utilizes lipids as the prime fuel for flight muscle contraction[14]. Our investigation revealed that, injection of this peptide hormone in to the plant bug, *I. limbata* elicits lipid mobilization in the

haemolymph. From these *in vivo* experiments it can also be interpreted that, both native hormone extract and synthetic AKH had almost similar stimulatory effect on lipid release. The RP-HPLC separation of the extract indicated the presence of a single peak exhibiting biological activity. The comparison of HPLC profiles of the Synthetic AKH and that of *O. exvinacea* retrocerebral extract revealed that the extract contained materials having similar retention times.

The fat mobilizing effect of AKH was studied in vertebrate (chicken) liver (Table No.3) as there has been a similarity of this hormone with vertebrate hormone at functional level[15]. AKHs resemble glucagon[16], a peptide hormone from the α-pancreatic islets cells in vertebrates and the vertebrate catecholamine adrenalin[17]. Another vertebrate candidate whose function can be compared with AKHs is vertebrate adiponectin, a hormone discovered recently from vertebrate adipose tissue[18] which increases the oxidation of fat and thereby reducing the intracellular triglyceride content of the liver and muscle and an increase of the cellular sensitivity to insulin[19]. From our present investigation there has been conclusive evidence that AKH regulates intermediary metabolism in the fat body, resulting in hyperlipaemia. It is possible that the activity observed with the crude extract is elicited with the component having similar structure as that of leptin, a vertebrate protein with fat depleting effect. The brain-retrocerebral complex extract have been shown to contain more than one AKH-like compound that are able to elicit hyperlipaemia. The fat depleting effect of insect AKH can be correlated with AKH receptor belongs to the same protein family as the gonadotropin releasing hormone receptor family[20]. Staubli *et al.*[21] pointed out that the AKH receptor is related to the human gonadotropin releasing hormone receptor. Gonadotropin releasing hormone and AKH receptors as well as the corazonin and AKH/corazonin-related peptide (ACP) receptors all belong to a very large receptor family. They are found in vertebrates[22], insects, nematodes, crustaceans, molluscs and in primitive chordates. Ziegler *et al.*[23] compared the *M. sexta* AKH receptor with the known AKH receptors of other insects and with gonadotropin releasing hormone-like receptors of invertebrates. Studies on vertebrate and mammalian systems showed that, this hormone can mobilize fat *in vitro.*

Table No.1: *In vivo* analysis of the lipid released from the haemolymph of *I. limbata* in response to crude and synthetic extract of *O. exvinacea*

S.No	Source	Lipid release (µg/µl)		E/C (%)	Significance* (P)
		Control (C)	Experiment (E)		
1	Insect saline	3.53	3.27	92.63±2.77	N.S
2	Brain-retrocerebral extract	2.53	2.92	115.41±0.11	<0.001
3	Synthetic AKH	2.53	2.93	116.11±0.18	<0.05

Table No.2: Effect of insect AKH on lipid release from rat liver *in vitro*

S.No	Source	Lipid release (µg/mg)		E/C (%)	Significance* (P)
		Control (C)	Experiment (E)		
1	Insect saline	94.54	90.94	95.76±1.18	N.S
2	Brain-retrocerebral extract	94.54	101.90	107.78±1.86	<0.05

Table No.3: Effect of insect AKH on lipid release from chicken liver *in vitro*

S.No	Source	Lipid release (µg/mg)		E/C (%)	Significance* (P)
		Control (C)	Experiment (E)		
1	Insect saline	102.08	97.08	95.10±3.12	N.S
2	Brain-retrocerebral extract	99.19	140.32	141.46±9.60	<0.05

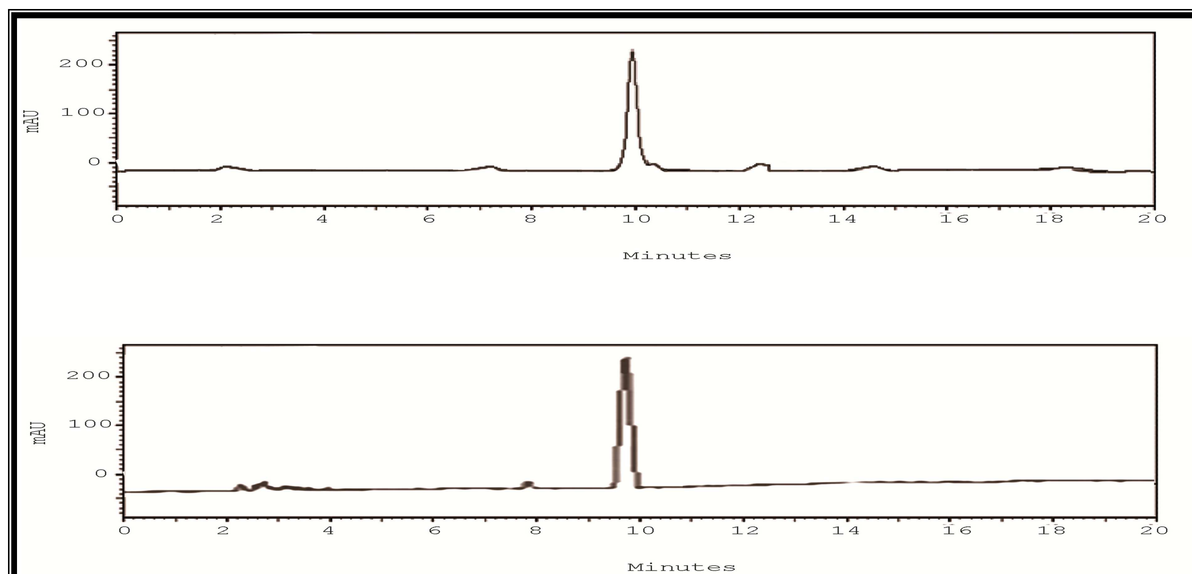

Figure No.1: The HPLC profiles of crude brain-CC extract of *O. exvinacea* (a) and synthetic Lom-AKH (b). The extract was run with a gradient of 43-53% solvent B in 20 min with a flow rate of 1 ml/min (solvent A = 0.01% Trifluoroacetic acid (TFA) in water, solvent B = 60% acetonitrile in solvent A)

CONCLUSION

The *in vivo* studies showed the fat depleting effect of the brain-retrocerebral complex extract of the mango leaf webber Orthaga exvinacea. Investigations on the adipokinetic effect of the HPLC purified hormone extract on the lipid release from vertebrate liver suggests the possibilities of using this hormone effectively for therapeutic purposes for controlling obesity. By using the information available on these lines, in future, we can target this hormone effectively for therapeutic purposes for controlling obesity. However, much more studies is still needed on the physiological role of AKHs during flight of the insect, their signal transduction, the dynamics of release of the hormones from the brain tissue into the haemolymph, or the receptor binding features, possibilities of targeting the fat depleting effect (using animal models) of this nona peptide identified from the lepidopteran insect, *O. exvinacea*.

ACKNOWLEDGEMENT

We thank the University of Calicut, Kerala for providing laboratory facilities and financial assistance for this research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: D. Umadevi *et al.* Hyperlipaemic effects of a neuropeptide hormone isolated from insects on vertebrate liver, *Asian Journal of Phytomedicine and Clinical Research,* 4(2), 2016, 33 - 39.