Identification of a metabolic peptide hormone from the neurohaemal tissues of *Orthaga exvinacea*

D. Umadevi, K.U.M.A. Rafeeq, M. Gokuldas

Department of Zoology, Division of Insect Biochemistry and Physiology, University of Calicut, Kerala, 673635, India, Email: uma.biochem@gmail.com

ABSTRACT

The presence of biologically active compounds in the neuronal tissues of the mango leaf webber, *O. exvinacea* that can influence lipid, carbohydrate and protein metabolism were investigated. The activity of the brain-retrocerebral complex extract tested *in vivo* in the polyphagous plant bug, *Iphita limbata* (heterologous bioassay) revealed that the extract and also the synthetic peptide, Locmi-AKH-I can increase both lipid (adipokinetic) and carbohydrate (hyperglycaemic) in the haemolymph whereas they showed inhibitory effects on haemolymph protein content. *In vitro* studies using fat body taken from *O. exvinacea* showed similar response of the fat body to the crude extract and the synthetic peptide. The extract was separated by RP-HPLC analysis. Comparison of HPLC profiles of the synthetic peptide with that of the retrocerebral complex extract of *O. exvinacea* showed that the hormone extract contained materials having similar retention times as that of synthetic AKH. The *in vitro* bioassays using purified fractions for lipid and carbohydrate mobilization on the fat body showed a prominent hyperlipaemic and hyperglycaemic effects, but significant inhibition were obtained when tested for the protein turnovers. The present study revealed that the peptide hormone extracted is a stimulator of fat body lipid and carbohydrate release, showing an increase in lipid and carbohydrate titers, whereas they are potent inhibitors of protein metabolites.

Keywords: *Orthaga exvinacea*, RP-HPLC, *Iphita limbata*, hyperlipaemic, hyperglycaemic

INTRODUCTION

Neuropeptides play a major role in cellular communication in invertebrates as well as in vertebrates. The energy metabolism in insects is regulated by the action of small (8-10 amino acid residues) peptides of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family [1] present in the neuronal tissues (brain-retrocerebral complexes). They are amino terminally blocked by a pyroglutamate residue and end in a carboxy terminal amide and contain at least two aromatic amino acids, tryptophan in position 4. Insect AKHs are multifunctional [2]. It acts on the fat body to mobilize stored lipids and carbohydrates, activate glycogen phosphorylase, accumulate cAMP [3,4] and inhibit the synthesis of proteins [5,6], lipids [7], and RNA [8]. Studies revealed that the action of AKH is comparable with vertebrate hormone at functional level. AKHs resemble glucagon [9], a peptide hormone from the α-pancreatic islets cells in vertebrates and the vertebrate catecholamine adrenalin [10]. Another vertebrate candidate whose function can be compared with AKHs is vertebrate adiponectin, a hormone discovered from vertebrate adipose tissue [11] which increases the oxidation of fat and thereby reduces the intracellular triglyceride content of the liver and muscle and increases the cellular sensitivity to insulin. Reports also showed that, the receptors of AKH are structurally and evolutionarily related to gonadotropin releasing hormone receptors from vertebrates

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[12]. The present investigation was carried out to study the effect of brain-retrocerebral extract of *Orthaga exvinacea* on lipid, carbohydrate and protein metabolism using various *in vivo* (heterologous bioassay in *Iphita limbata*) and *in vitro* experiments and to separate the peptide hormone by employing HPLC analysis. Furthermore, the role of this hormone in lipid, carbohydrate and protein metabolism was also confirmed by fraction testing studies.

MATERIALS AND METHODS

Experimental insects

Larvae of *O. exvinacea* were collected locally from their natural habitat, mango trees, and were transferred to plastic basins and reared in the insectary by feeding mango leaves. Sixth instar larvae were separated from the colony and used for experiments. The insects of both sexes were used for hormone extraction and bioassay 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 and various tropical fruits. Mature adults were used for the experiments.

Preparation of brain-retrocerebral extract and Synthetic Locmi-AKH

The brain retrocerebral complexes were removed with the help of fine forceps under a stereozoom binocular microscope. The tissues were immediately put in to ice cold 80% methanol and stored at -4°C until extraction. 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. The synthetic peptide Locmi-AKH (GenScript Corp., USA) 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 (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 haemolymph (2 µl) was collected directly from the cut end of antenna in to the precalibrated capillary tube. The sample was transferred in to the bottom of 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. Similar experiments were carried out using 5 µl of insect saline instead of extract. Haemolymph samples collected in various experiments were used for the quantitation of lipids, carbohydrates and proteins. The total concentration of lipids in the haemolymph samples were measured by phosphovanillin method [13], total sugar concentration by anthrone method [14] and protein concentration by Lowry's method [15].

In vitro analysis using fat body of *O. exvinacea* to investigate the metabolic response of AKH in lipid and sugar and protein metabolism

For *in vitro* incubations, 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 changes in lipid, sugar and protein titers were measured colorimetrically with appropriate standards.

High Pressure Liquid Chromatography analysis

The dried extract made from the retrocerebral complexes from *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 brain-retrocerebral 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 (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 pore size Millipore filter. The eluants were monitored at 210 nm using a UV-VIS detector. One minute fractions starting from 1 min up to 20 min were collected manually, dried by vacuum concentrator, and were used for testing their biological activities. Similarity of 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 Locmi- AKH in a similar HPLC run.

In vitro bioassay for testing the effects of different HPLC fractions on lipid, carbohydrate and protein metabolism

One minute fraction of the extract of retrocerebral complexes of *O. exvinacea* fractionated on HPLC, were collected manually. The effects of these fractions on lipid, carbohydrate and protein metabolism were determined by *in vitro* bioassays using phosphovanillin, anthrone and Lowry reagents respectively. The experimental and control samples were analysed by spectrophotometric methods. Values obtained from various bioassay experiments were expressed as mean $\pm$ standard deviation values as well as percentage difference of the experimental over controls (E/C%) and were subjected to statistical analysis for significance. The data were obtained from the research work conducted in the Department of Zoology, University of Kerala, India.

RESULTS AND DISCUSSION

The hyperlipaemic effects of the extracts were measured by estimating change in the lipid content after the injection of extract. The result of the analysis is summarized in table 1. The brain retrocerebral extract induced significant hyperlipaemic effects 115.41±0.108 ($P < 0.001$). The change in lipid concentration by the synthetic peptide was analysed in the same way. It was observed that the synthetic peptide also produced significant hyperlipaemia 116.11±0.181 ($P < 0.05$). In the control, the injection of 5 µl of insect saline did not evoke any hyperlipaemic activity (-8%). The increase in haemolymph sugar concentration was studied before (control) and 60 min after injection (experiment), as mentioned above. Table 2 represents the result of hyperglycaemic bioassay. The data showed that both the native and synthetic peptide induced similar

hyperglycaemic effects 118.15±0.182 (P <0.05) and 118.74±0.176 (P <0.05) respectively, whereas in the control, no significant effects are observed.

Injection of brain-retrocerebral complex extract and synthetic AKH caused reduction in the amount of haemolymph proteins in the plant bug, *I. limbata*. The injection of crude extract resulted in a significant reduction of proteins to 53.31±2.92 per cent (P<0.05) over the controls. Synthetic AKH also produced a similar significant reduction in protein synthesis 49.12±3.61 per cent (P<0.05). The control insects injected with insect saline instead of hormone extract showed a similar reduction. Here the inhibition rate was to 58.26±2.11 per cent of controls. The results are provided in table 3. From the experiments it becomes evident that the synthesis of haemolymph proteins in the insect fat body is inhibited by the injection of the hormone extract as well as the saline in to the insect. However, the hormone extract seemed to be exerting a better inhibitory effect than the saline injection.

The brain-retrocerebral complex extract and the synthetic AKH showed significant effect on fat body lipid mobilization, up to 17% (117.63±0.35, P <0.05) when comparing with that of insect saline. While considering the role of AKH in fat body sugar release, the crude extract produced 18% increase (118.44±1.09) in carbohydrate mobilization. The synthetic peptide elicited 19% (P <0.05) increase when comparing with that observed in insect saline on fat body sugar mobilization. Both the native hormone extract and synthetic peptide showed inhibitory effects (more than 50 per cent inhibition) on proteins released in to the incubation medium. The brain-retrocerebral complex extract produced an inhibition of about 56 per cent over the controls. The quantity of proteins was reduced to 43.36±6.12 per cent of controls whereas the synthetic hormone produced an inhibition of nearly 57%. i.e., to 43.44±5.58 per cent of controls. Both the inhibitions are highly significant (P<0.001).

Thus it appears that the synthetic as well as the native adipokinetic peptide (extracted from the brain-retrocerebral complex of *O. exvinacea*) have similar effects on the metabolism of lipids, carbohydrates and proteins. The hormones stimulate the release of lipids and carbohydrates in to the haemolymph (*in vivo*) or the incubation medium (*in vitro*). The hormone, however, inhibits the synthesis and/or release of proteins in to the haemolymph or the incubation medium. Figure 1 shows the HPLC profile of the brain retrocerebral complex extract of *O. exvinacea* and synthetic Locmi AKH. As seen in the chromatogram, the synthetic peptide was eluted at the same retention time as that of the material having the retention time 10 min in the retrocerebral extract of *O. exvinacea.*

One minute fractions from HPLC were collected using a fraction collector and were individually tested for their effects on lipid, sugar and protein metabolism. The samples were prepared by reconstituting each of the freeze dried HPLC fractions of brain-CC extract in 200 µl of distilled water. Results showed that fractions 8, 10, 13, 17 and 19 have significant hyperlipaemic activity. Highest hyperlipaemic 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 hypolipaemic, but the effects were not significant. None of the other fractions showed any significant adipokinetic activity. The results are summarized in table 4 and figure 2.

The HPLC fractions when tested for their hyperglycaemic activity on fat body revealed that the fractions 6, 10, 12 and 18 induced hyperglycaemia. There were 30%, 38%, 18% and 14% increase in sugar release respectively, all of which were statistically significant. The hyperglycaemia induced by materials in other fractions was 23%, (7 min), 21% (9 min) and 25% (19 min), and none of these effects were statistically significant. Table 5 and figure 3 shows the hyperglycaemic effects of the materials in different fractions separated on HPLC. When tested for their effects on the protein turn

over in the incubation medium, it was found that the protein levels were very much affected by almost all fractions compared to the control. A significant reduction in the amount of proteins was observed. Highest inhibition was shown by fraction 10 (up to 41%, P<0.05), which also caused the maximum lipid and sugar mobilization *in vitro*. Results are provided in table 6 and figure 4. From the data presented, it can be concluded that the materials in fraction 10 showed significant adipokinetic and hyperglycaemic activities with increase of lipid and sugar, whereas suppression in the amount of protein synthesized or released was also observed, over controls.

Table 1. Lipid released from the haemolymph of *I. limbata* in response to crude and synthetic extract of *O. exvinacea in vivo*.

Source	Lipid release (µg/µl)		E/C%	Significance*
	Control (C)	Experiment (E)		
Insect saline	3.53	3.27	92.63±2.77	N.S
Brain-retrocerebral extract	2.53	2.93	115.41±0.11	P<0.001
Synthetic AKH	2.52	2.93	116.11±0.18	P<0.05

Values are expressed as means ± standard deviation values (n=6); * indicates the significance of difference between the control and experimental.

Table 2. Sugar released from the haemolymph of *I. limbata* in response to crude and synthetic extract of *O. exvinacea in vivo*.

Source	Sugar release (µg/µl)		E/C%	Significance*
	Control (C)	Experiment (E)		
Insect saline	2.95	2.78	94.27±2.73	NS
Brain-retrocerebral extract	2.72	3.21	118.15±0.18	P<0.05
Synthetic AKH	2.39	2.84	118.74±0.17	P<0.05

Values are expressed as means ± standard deviation values (n=6); * indicates the significance of difference between the control and experimental.

Table 3. Protein released from the fat body of *I. limbata* in response to crude brain-CC extract of *O. exvinacea* and synthetic extract *in vivo*.

Source	Protein release (µg/µl)		E/C (%)	Significance* (P)
	Control (C)	Experiment (E)		
Insect saline	13.01	7.58	58.26±2.11	N.S
Brain-retrocerebral extract	11.48	6.12	53.31±2.92	<0.05
Synthetic AKH	10.40	5.15	49.12±3.61	<0.05

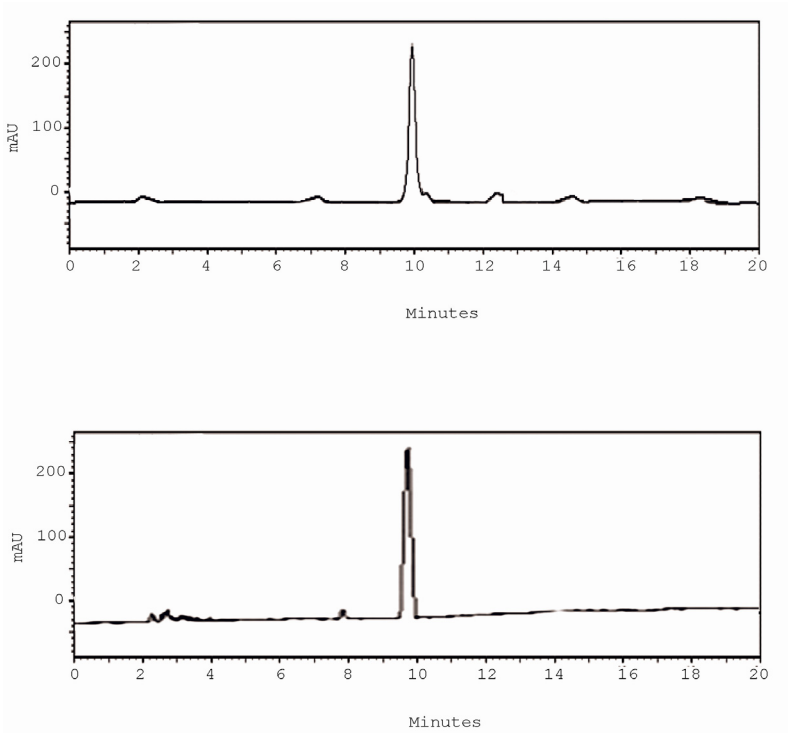
Values are expressed as means ± standard deviation (n=6); * indicates the significance of difference between the control and experimental. The haemolymph samples were collected for protein estimation before (control) and 60 min after (experiment) the injection of either insect saline, brain-CC extract (1 gpe) or synthetic AKH (500 pmole).

Table 4. *In vitro* analysis using fat body of *O. exvinacea* to investigate the metabolic response of AKH in lipid metabolism.

Source	Lipid release (µg/mg)		E/C%	Significance*
	Control (C)	Experiment (E)		
Insect saline	63.35	60.95	96.21±6.03	N.S
Brain-retrocerebral extract	83.22	97.9	117.63±0.35	P<0.05
Synthetic AKH	75.11	89.72	119.45±7.93	P<0.05

Values are expressed as means ± standard deviation values (n=8); * indicates the significance of difference between the control and experimental.

(a)



(b)

Figure 1. The HPLC profiles of crude brain-CC extract of *O. exvinacea* (a) and synthetic Lom-AKH-I (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).

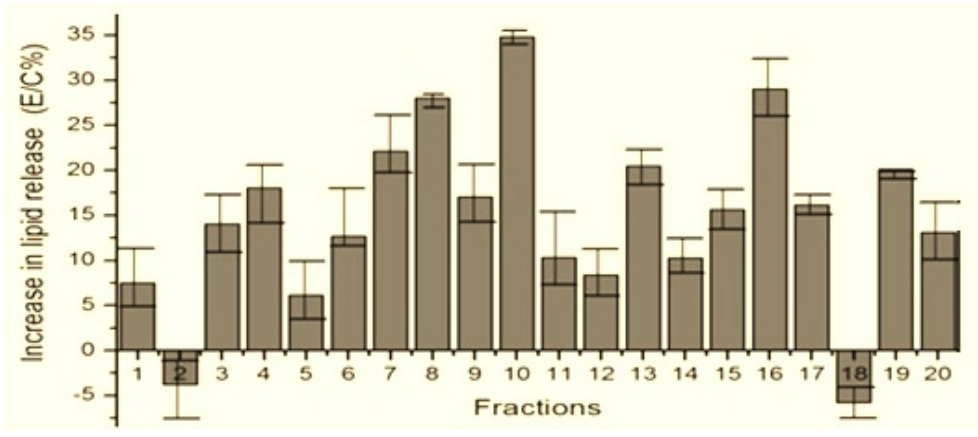


Figure 2. Hyperlipaemic activity of HPLC fractions tested *in vitro* on the fat body of *O. exvinacea*. HPLC fractions were collected and tested for hyperlipaemic activity. The increase in total haemolymph lipid is represented as E/C% (Mean±SD).

Despite lipids are important fuels for long distance flight in insects, carbohydrates are used in the initial stage of flight and also contribute substantially in the later phase [16]. Lipids are depleted largely from the glycogen reserves of fat body conferring an increased level of soluble carbohydrates in the haemolymph. Injection of locust retrocerebral extract induces haemolymph sugar (hypertrehalosemic effect) in cockroaches [17] and elevates haemolymph lipid (adipokinetic effect) in locusts [18]. When the brain-CC extract injected into locusts or in to lepidopteran insects, it produced an adipokinetic effect [19]. A remarkable hyperlipaemic response was shown by the fat body of *I. limbata* injected with CC extracts of *Spodoptera mauritia* [20] and *I. limbata* [21]. Comparable results have been reported for the moth, *Manduca sexta*, which utilizes lipids as the prime fuel for flight muscle contraction [22]. In *M. sexta*, a single AKH mediate the mobilization of carbohydrates in larvae and lipids in adults [23]. Our studies revealed that, injection of this peptide hormone in to the plant bug, *I. limbata* elicits both carbohydrate and 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 and sugar release. An *in vitro* study with fat body, to elucidate the role of AKH in carbohydrate and lipid metabolism showed that this peptide hormone have a key role in carbohydrate and lipid mobilization. The RP-HPLC purification and further fraction testing 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 *in vitro* bioassays using HPLC fractions for lipid and carbohydrate mobilization confer a prominent hyperlipaemic and hyperglycaemic effects in the separated fractions.

Table 5. *In vitro* analysis using fat body of *O. exvinacea* to investigate the metabolic response of AKH in sugar metabolism.

Source	Sugar release (µg/mg)		E/C%	Significance*
	Control (C)	Experiment (E)		
Insect saline	27.991	25.79	92.12±3.74	N.S
Brain-retrocerebral extract	18.005	21.17	117.58±1.49	<0.001
Synthetic AKH	18.774	22.24	118.44±1.09	<0.001

Values are expressed as means ± standard deviation values (n=10); * indicates the significance of difference between the control and experimental.

Table 6. *In vitro* analysis using fat body of *O. exvinacea* to investigate the metabolic response of AKH in sugar metabolism.

Source	Protein release (µg/mg)		E/C (%)	Significance* (P)
	Control (C)	Experiment (E)		
Insect saline	38.96	19.57	50.24±11.15	N.S
Brain-CC extract	36.00	15.97	44.36±6.12	<0.001
Synthetic AKH	34.63	15.04	43.44±5.58	<0.001

Values are expressed as means ± standard deviation (n=10); * indicates the significance of difference between the control and experimental. For experimental, the fat body was incubated in physiological saline containing either insect saline, brain-CC extract (4 gpe) or synthetic AKH (2000 pmole), controls contained buffer plus equal amount of distilled water instead of other solutions.

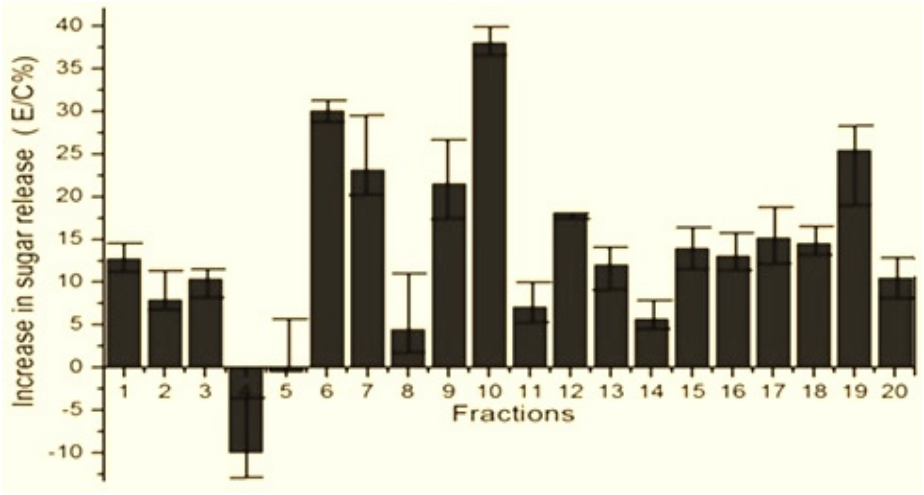


Figure 3. Hyperglycaemic activity of HPLC fractions tested *in vitro* on the fat body of *O. exvinacea*. HPLC fractions were collected and tested for hyperglycaemic activity. The increase in total haemolymph sugar is represented as E/C% (Mean±SD).

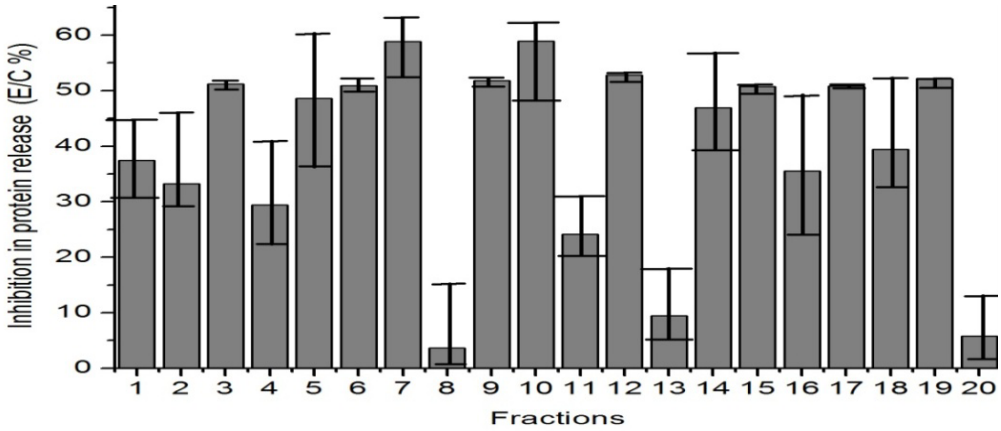


Figure 4. Activity of HPLC fractions in protein metabolism on the fat body of *O. exvinacea* tested *in vitro*. HPLC fractions were collected for testing the effects of different fractions on protein release. The inhibition in total haemolymph protein release is represented as E/C% (Mean±SD).

The relevance of body fat regulation is emphasized by dysfunctions resulting in obesity and lipodystrophy in humans. Packaging of storage fat in intracellular lipid droplets, and the biological mechanisms guiding storage-fat mobilization, is conserved between mammals and insects. The basic premise of this investigation points to those neuropeptide hormones that can serve as potent messengers in insects to control various metabolites. From the present study it can be summarized that AKH stimulates intermediary metabolism in the fat body, leading to fat mobilization resulting in hyperglycaemia and hyperlipaemia. New approaches in molecular biology should be carried out to exploit the fat mobilizing effect of this hormone in order to target this lipid mobilizing hormone effectively for therapeutic purposes for controlling obesity. By using the information available on

these lines, in future, we can consider this hormone a better candidate for fat body regulation in animals including higher vertebrates.

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