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Isolation and Characterization of Mandibular Organ – Inhibiting Hormone from the Eyestalks of Freshwater Crab, *Oziotelphusa Senex Senex*

G. Purna Chandra Nagaraju*, G. Lakshmi Vara Prasad, and P. Sreenivasula Reddy

Department of Biotechnology, Sri Venkateswara University, Tirupati – 517 502, India

Abstract: Eyestalk neuropeptides play an important role in regulating wide variety of physiological processes in crustaceans. The neuropeptides belonging to the crustacean hyperglycemic hormone (CHH) family, influence the secretion of ecdysteroids and methyl farnesoate (MF) and in turn regulate molting and reproduction. Using MF secretion by Mandibular organ (MO) as bioassay two peptides with distinctive Mandibular organ-inhibiting hormone (MO-IH) activity was identified. Among these one peptide possesses hyperglycemic activity.

Keywords: eyestalk neuropeptides; mandibular organ; Mandibular organ-inhibiting hormone.

1. Introduction

During the last half-century, extensive studies on the crustacean hyperglycemic hormone (CHH) family peptides have validated their roles in the control of several physiological functions and further disclosed the details of biochemical and molecular mechanisms of their action [1]. The CHH family peptides are secreted by the eyestalk of sinus gland (SG) [2], which in turn controls the reproduction, molting, and metabolism. Sinus gland the neurohemal organ of crustaceans is located between the medulla externa and the medulla interna on the dorsolateral surface of the eyestalk ganglia in the crab *Oziotelphusa senex senex* [2].

Methyl farnesoate (MF), the unepoxidated form of Juvenile hormone III of insects, is synthesized and released by the mandibular organ (MO) in decapod crustaceans and appears to regulate many aspects of physiology, including the stimulation of reproduction, molting and morphogenesis [2]. Recent studies have focused on the biosynthesis of MF and its regulation, because the role of MF in crustaceans remains unclear [3].

Mandibular organ-inhibiting hormone (MO-IH) is synthesized and secreted by the X-organ-sinus gland complex of the eyestalk. Unfortunately, it has been difficult to develop a sensitive assay, such as a radioimmuno assay, for measuring circulating levels of MO-IH. It has been demonstrated that SG extracts and purified MO-IH directly inhibit the secretion of methyl farnesoate from mandibular organ *in vitro* [4], suggesting that MO-IH is physiologically important in the regulation of the crustacean molt and reproduction. This hypothesis is additionally supported by data showing that purified MO-IH delays molting and reproduction [5-7]. The purpose of these studies was to isolate and characterize a peptide with MO-IH activity

* Corresponding author: e-mail: raju@cc.ncue.edu.tw
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from the crab *Oziotelphusa senex senex*. The purified crab MO-IH could then be used for further studies concerning the physiology and mechanism of action of MO-IH

2. Materials and methods

The freshwater field crab, *Oziotelphusa senex senex* (Fabricius) was used as an experimental model for the present investigation. The crabs were collected from rice field and irrigation canals (free from pesticides and pollutants) in and around Tirupati (Andhra pradesh, South India) from the same location as far as possible. The crabs were brought to the laboratory, and maintained in the laboratory at 28 ± 1°C in tubs partially filled with aged fresh water. They were acclimatized to laboratory conditions (12:12 L: D) for at least 7 days before being used in experiments. The water in the tubs was changed daily. During their sojourn, the crabs were fed on sheep meat ad libitum. Feeding was stopped one day before the commencement of experiment to avoid changes due to prandial activity.

2.1. Preparation of modified medium 199 (M199)

To 500ml of tissue culture grade water, 9.6 g of M199 powder (with L-glutamine and Earle's salts) 0.0025 g of streptomycin, 2.2 g of $NaHCO_3$, were added. The contents were mixed gently until dissolved completely. The pH of medium was adjusted to 7.4 with 1 N NaOH. The volume is made up 1 liter with crab physiological saline, modified to compensate for the salts already present in the culture medium and stored at 4°C until use.

2.2. Purification of eyestalk peptides

Purification of eyestalk peptide hormones was done essentially similar to the method standardized by Chang et al. [8]. In brief, sinus glands were dissected from intermolt male crabs. The sinus glands were transferred to a glass homogenizer, disrupted in 0.1N HCl and heated for 5 minutes at 80°C. The homogenate was centrifuged at 16,000 X g for 5 minutes and supernatant used for separation of peptides by using HPLC. The Peptide fractions were separated on C_{18} reverse-phase column (with a gradient of 0.1% trifluoroacetic acid in water to 80% methanol) with a flow rate of 0.1 mL/min). The eluent was monitored for absorbance at 280nm. Different peptide peaks (1.5 ml) were collected and used for bioassay.

2.3. Effect of eyestalk peptides on MF secretion *in vitro* culture

The MOs were dissected from ice-anaesthetized crabs (C_4 stage male crabs) and left and right MOs cultured individually in 2 ml of medium 199 [9] at 18°C for 12 h. To study the effect of eyestalk peptides on MF secretion, one MO from an animal was used for treatment, with the other MO being an untreated control. The use of MOs from a single animal in this manner has previously been validated [4]. Mandibular organs were incubated in 2 ml of medium 199 with isolated eyestalk peptides. After 12 h incubation, the medium was collected and 2.5 ml of acetonitrile and 0.5 ml of 0.9% sodium chloride were added to the medium. The medium was extracted three times with hexane and the hexane layers combined and lyophilized. The residue was resuspended in 200µl of methanol prior to analysis by HPLC. The amount of all-trans MF present in each extract was determined by comparing its peak area to that of the internal standard (cis-trans MF), which represented 25ng.

2.4. *In vivo* studies of eyestalk peptides

Sixty male crabs (bilaterally eyestalk ablated after 24 h) were divided into 6 groups of 10 animals each. Group 1 served as initial

control. Group 2 received 20µl of crustacean physiological saline through the arthrodial membrane of the coax of the 4th pair of walking legs and served as concurrent control crabs. Group 3, 4, 5 and 6 received 20µl per crab of each peptide peak (equivalent to two sinus glands) respectively.

All hemolymph samples were removed 2h after injection and at the same time of the day to obviate any possible variation due to circadian rhythm in the hemolymph sugar level. The hemolymph sugar level was determined using anthrone reagent [10].

2.5. Statistical analysis

Results obtained were subjected to the following statistical analyses. The Mean, Standard Error (S.E), Analysis of Variance (ANOVA) and Student – Newman – Keul's (S-N-K) tests were carried out according to Steel and Torrie [11] using basic programming techniques on IBM personal computer for different parameters.

3. Results

Figure 1 illustrates the UV absorbance of the chromatogram from the separation of 200 SGs. The chromatogram shows the presence of a number of peaks with the majority eluting after 50 min. Fractions 40-70 were separated and assayed for their ability to inhibit MO secretion.

The effect of each of the fractions on MF secretion from MO *in vitro* is shown in Table 1. The ability of each selected fractions to inhibit MF secretion from MOs *in vitro* was determined after 12 h. of incubation and compared with that of controls. It can be seen that at lest 2 peaks contained MO-IH activity, although to varying degrees of potency.

The same fractions were also tested for their ability to regulate hemolymph sugar level *in vivo* (Table 2) in eyestalk ablated (ESX) male crabs. It is clear that among fractions tested, one contained significant CHH activity.

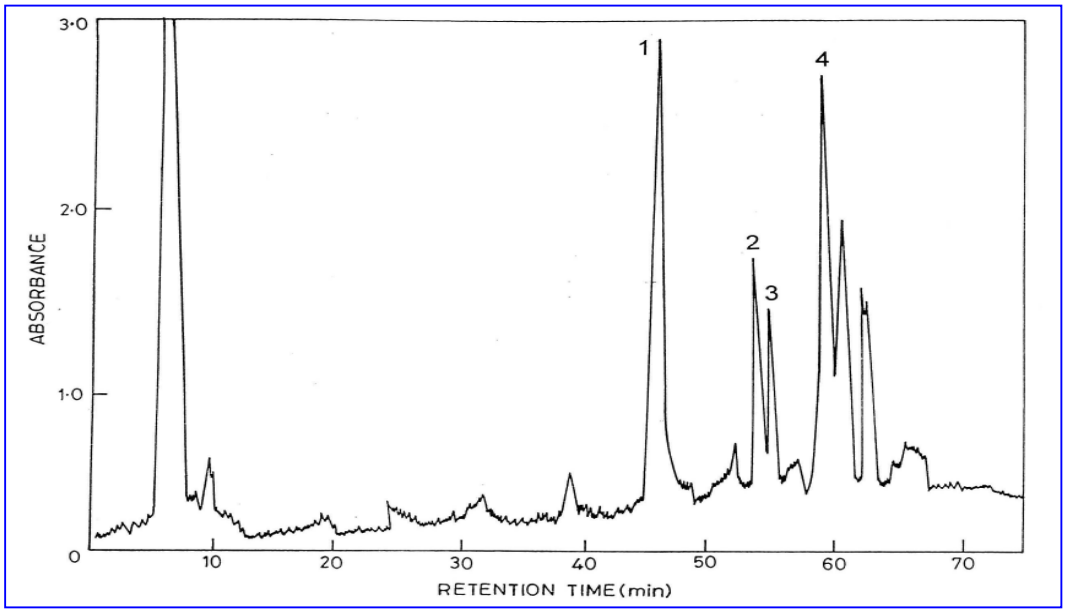

Figure 1. UV absorbance of the chromatogram from the separation of 200 SGs

Table 1. Effect of eyestalk peptide fractions on MF secretion *in vitro* after 12 h

	MF secretion ng/MO
Untreated control	22.37 ± 6.91
Eyestalk peptide fractions	
Peptide fraction 1	26.64 ± 4.31 (19.09)
Peptide fraction 2	11.31 ± 3.14* (-49.44)
Peptide fraction 3	6.94 ± 1.17* (-68.97)
Peptide fraction 4	25.31 ± 3.18 (13.14)

Values are means ± S.E.
Values in parentheses are % change from untreated control samples.
*Significant difference from untreated control, $p<0.0001$, (N = 4).

4. Discussion

Fifty-nine years ago, Abramowitz et al. [12] showed that the eyestalk of crustaceans contained a hyperglycemic factor (CHH) that termed as 'diabetogenic factor'. Since then, CHH has been isolated and sequenced from several crustaceans including, crayfishes, lobsters, prawns, shrimps and crabs [13].

The molt inhibiting hormone (MIH), gonad inhibiting hormone (GIH) and MO-IH are members of the ever-expanding CHH family of crustacean neuropeptides [14]. The CHHs have been shown to regulate carbohydrate metabolism in the shore crab, *Carcinus maenas*, the kumuran prawn *Penaeus japonicus*, the lobster, *Homarus americanus*, the freshwater field crab *Oziotelphusa senex senex* and fiddler crab *Uca triangularies* [15-21]. Receptor-binding studies in the crab *C. maenas* and the crayfish *Orctonectes limosus* indicate that CHH may have different target organs and may influence reproduction directly as well as indirectly [14, 22]. The GIH inhibits the onset of vitellogenesis in the lobster, *H. americanus* [23] and MO-IH inhibits the MF synthesis and secretion by MO in the edible crab *C. pagurus* [4, 24]. All these neuropeptides are structurally related and consequently from a peptide family referred to as the CHH-family [13].

Table 2. *In vivo* effect of eyestalk peptide fractions on hemolymph sugar levels in crab, *Oziotelphusa senex senex*

Groups of crabs tested	**Hemolymph sugar level (mg/100 ml)**
Eyestalk less crabs (ESX)	112.67 ± 10.83
ESX crabs injected with saline	110.89 ± 12.81 (-1.58)
ESX crabs injected with peptide fraction 1	117.68 ± 9.77 (4.45)
ESX crabs injected with peptide fraction 2	86.49 ± 11.31 (-23.24)
ESX crabs injected with peptide fraction 3	181.13 ± 10.94* (60.76)
ESX crabs injected with peptide fraction 4	113.73 ± 9.19 (0.941)

Values are means ± S.E.
Values in parentheses are % change from ESX crabs.
*Significant difference from untreated control, $p<0.0001$, (N = 10).

Landau et al. [25] observed that the secretory activity of the MOs can be stimulated by Red pigment concentrating hormone (RPCH) or inhibited by Pigment dispersing hormone (PDH) in the crayfish *Procambarus clarkii* indicating their possible importance for the control of reproduction. The biological activity of these inhibitory peptides was assessed principally by *in vitro* bioassays. Each peptide generally associated with two or more physiological functions [26].

Several studies have indicated and confirmed the presence of a MO-IH activity in the neurosecretory system of the crustacean eyestalk. In this context it is also interesting to note that CHH isoforms from the crayfish *P. clarkii* have an inhibitory effect on MF synthesis in the MOs [27]. Bioassay measuring the inhibition of MF synthesis by MO exposed to fractionate sinus gland extracts from the crab *Cancer pagurus* revealed the presence of two neuropeptides named MO-IH-1 and MO-IH-2 almost identical in structure, which suppressed MF synthesis. These inhibitors are members of the CHH family as they share 50-60% sequence similarity with the members of this group, particularly with Vitellogenic inhibiting hormone (VIH) and MIH [28]. In edible crab, *C. pagurus*, MO-IH inhibits activity of a mandibular organ cytosolic S-adenosyl-L-methionine; farnesolic acid methyltransferase enzyme there by reducing MF synthesis [4].

In the edible crab, *C. pagurus*, there are two isoforms of MO-IH (1 and 2) that differ from each other by a single amino acid substitution of lysine in MO-IH-1 for a glutamine in MO-IH-2. Subsequently, sequencing of the cDNAs encoding MO-IH-1 and –2 highlighted a further amino acid substitution of isoleucine in MO-IH-1 for a serine in MO-IH-2 in the putative signal peptide regulation [5]. Hinsch [29] observed hypertrophy of the MO in response to eyestalk ablation in male spider crab *Libinia emarginata* and also caused ultrastructural changes in nuclei, mitochondria and endoplasmic reticulum. Eyestalk removal causes hypertrophy and changes in the ultrastructure of the MO in the shrimp larvae *Palaemonetes varians* [30]. Le Roux [31] also observed hypertrophy of the MO in response to eyestalk ablation in the shrimp *Pisidia logicornis.* Similar results were also observed in the lobster, *H. americanus* [32] and in the crab *C. maenas* [33]. Synthesis of MF in MOs is negatively regulated by factors from sinus gland [34]. Liu and Laufer [35] have isolated and characterized three SG neuropeptides from the spider crab *L. emarginata* that inhibit the synthesis of MF in MO, but at the same time have hyperglycemic activity when injected into destalked fiddler crab, *Uca pugilator*. In the present study the same effect was seen when MO and SG-peptides were co-incubated *in vitro* and SG-peptides administration *in vivo* in the crab *Oziotelphusa senex senex.* These reports described above clearly support a role for SG peptides directly inhibiting the MF secretion and inducing hyperglycemic activity in the eyestalk-ablated crabs.

Addition of the spider crab (*L. emarginata*) SG extract to cultured MOs resulted in a significant decline of secreted MF *in vitro* [36]. Injection of SG extracts into lobsters (*H. americanus*) resulted in a decline in circulating MF *in vivo* showing a complex interplay between the eyestalk neuroendocrine systems [37]. The SG extracts on MO size and MF synthesis significantly inhibited in the crayfish *P. clarkii* [38]. In contrast, MO stimulatory activity has been observed with the RPCH. In the spider crab *L. emarginata*, RPCH caused elevated secretion of MF *in vitro* [25].

However, in *C. pagurus*, CHH does not inhibit MF secretion, because incubation of the MO with high concentrations (100 nM) of CHH, which profoundly inhibited MF synthesis in *C. maenas*, entirely failed to elicit any significant decrease in MF synthesis in *C. pagurus* MOs [28].

The CHH peptide hormone family from the eyestalks regulates MF secretion in the MO

(MO-IH), and it also regulates ecdysone production by affecting Y-organs through MIH activity [8]. MF may also influence the Y-organ and intern ecdysone production [39]. Organ culture studies showed that CHH family peptides are involved in the regulation of hemolymph suger level (CHH), regulation of ecdysteroid secretion (MIH), regulation of MF secretion (MO-IH), and also exhibits several overlapping physiological activities.

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