Indian J. Fish., 60(2) : 103-107, 2013

Preliminary characterisation of gonad inhibiting hormone (GIH) gene and its expression pattern during vitellogenesis in giant tiger shrimp, *Penaeus monodon* Fabricius, 1798

K. K. VIJAYAN, P. REYNOLDS, D. L. MOHANLAL* AND C. P. BALASUBRAMANIAN*

Central Marine Fisheries Research Institute, Post Box No. 1603, Ernakulam North P. O., Kochi - 682 018., Kerala, India
**Central Institute of Brackishwater Aquaculture, 75 Santhome High Road, R. A. Puram, Chennai - 600 028, Tamil Nadu, India*
e-mail: cpbalasubramanian@yahoo.com

ABSTRACT

Farming of giant tiger shrimp is one of the most important and relatively recent coastal food production sectors in India. Although great strides have been made in shrimp husbandry practices in growout systems, management of reproduction in captivity has not kept pace. Although several hormones are involved in crustacean reproduction, the inhibitory effect of gonad inhibiting hormone (GIH) is thought to be more intense than any other hormones. As an essential component of understanding the reproduction of *Penaeus monodon*, we characterised the expression pattern of GIH gene in the sinus gland in relation to the female gametogenic cycle. A 633 bp sequence was cloned from the eyestalk of *P. monodon* by RT PCR. The sequence comprises an open reading frame (ORF) of 291 bp that encodes a protein of 96 amino acids. The deduced protein was used to search the GenBank database using BlastP. Protein alignment with all known crustacean GIH revealed that homology of *P. monodon* GIH shares 61, 48, 46, 46 and 35% identity with *Metapenaeus ensis*, *Nephrops norvegicus*, *Homarus americanus, Rimicaris kairei* and *Armadillidium vulgarae* respectively. The RT PCR analysis shows that GIH mRNA is expressed mainly in the eyestalk of female. In order to find the expression profile of GIH during different reproductive cycle, *P. monodon* female was categorised into four major stages of maturation, using light microscopical observations as well as morphological characteristics. Eyestalk of all animals from different stages of maturation indicated that GIH is expressed in all stages of maturation, although the expression of GIH mRNA changes during different stages of vitellogenesis. In animals with immature ovary, the relative GIH mRNA level was found to be less, which peaked in previtellogenic phase and diminished in subsequent phases. Low GIH mRNA level during the immature (oogonial) phase is particularly intriguing and has application in the commercial aquaculture to select the brooders

Keywords: Characterisation, Gene expression, Gonad inhibiting hormone, *Penaeus monodon,* Vitellogenesis

Introduction

Farming of giant tiger shrimp is one of the most important and relatively recent coastal food production sectors in India and in many tropical and subtropical developing countries. Nearly 40% of the Indian seafood export revenue comes from the aquaculture of *Penaeus monodon* (Bhat and Vinod, 2008). However, severe viral epidemics, especially white spot disease (WSD) caused by white spot syndrome virus (WSSV), have been threatening the industry since it was first reported in 1992 (Zhan *et al*., 1998). A new blue print for shrimp farming industry is required to overcome the disease threat and resume the production (Lotz, 1997). Development of specific pathogen free (SPF) shrimp stock and adoption of strict biosecurity protocols are central to the preventive strategies (Lightner, 2005). The major pre-requisite for production of SPF stock is to have continuous control over the reproduction. Although great strides have been made in husbandry practices for shrimp aquaculture in grow out systems, not much advancement made so far with respect to management of reproduction in captivity. The past fifty years of scientific research on crustacean reproduction has not so far made sufficient headway to resolve the major issues of captive reproduction of penaeid shrimps (Parnes *et al.,* 2004).

The most acknowledged consensus of crustacean reproductive endocrinology is that reproduction is controlled by two antagonistic hormones, one inhibits (vitellogenin inhibiting hormone or gonad inhibiting hormone, V/GIH) and the other stimulates (vitellogenin stimulating hormone, gonad stimulating hormone, V/GSH) (Adiyodi and Subramoniam, 1983). This simple endocrine axis has been questioned by many recent researchers who postulated a multihormonal system involving several neuroenodcrine and endocrine pathways (Van Herp *et al.,* 1997, de Kleijn *et al.,* 1998). Although several hormones are involved in the crustacean reproduction, the inhibitory effect of GIH is thought to be

more intense than any other hormones (Vaca and Alfaro, 2000). The presence of gonad inhibiting hormone was postulated by Panouse (1943) by observing eyestalk removal induced vitellogenesis in *Palaemon serratus*. This endocrinological procedure was adopted world wide by shrimp researchers and aquaculturists to obtain predictive spawning under captivity. This is almost similar to finfish aquaculture, where classical endocrinological work on finfishes *i.e.,* hypophysation or administration of pituitary gland extract was adopted to achieve induced breeding under captive conditions. However, eyestalk ablation procedure has not so far been replaced by any potent and physiologically nondestructive induced maturation procedure (Marsden *et al*., 2008) in comparison with the advance made in finfish aquaculture, where the classical endocrinological procedures were replaced with sophisticated techniques (Donaldson, 1996). Administration of GIH antagonist to neutralise or block the inhibitory effect of GIH is thought to be a better alternative to replace eyestalk ablation procedure (Okumura, 2004). However, the knowledge on GIH hormone is surprisingly less, possibly due to the extreme difficulty in obtaining chromatographically purified peptide fraction, as at least 1000 eyestalks are required for complete biochemical isolation (Meusy and Payen, 1988). Screening of genetic information, therefore, is perhaps the better assay for comprehensive elucidation, and further, it has been proved that recombinant proteins of crustacean neuropeptides can be utilised for raising antibodies (Edomi *et al.,* 2002). In this context, as a first step in developing antagonist to GIH, the cDNA of *P. monodon* GIH gene was partially characterised and its temporal expression pattern was studied through oogenesis.

Materials and methods

Animals

Female *P. monodon* (average body weight: 98.6 ± 5 g) at different stages of gonad maturation were obtained from the broodstock/spawner fishery and grow out production systems along the Tamil Nadu and Kerala coast..

RNA extraction

Total RNA was extracted from the X-organ sinus gland complex following modified guanidium thiocynate method (Chomczynski *et al.,* 1987). Briefly, X-organ-sinus gland complex was homogenised in 0.5 ml solution D (guanidium thiocynate 4 M, sodium citrate 25 mM, sarcosyl 0.5%), and equal volume of phenol as well as 1/5 volume of chloroform were added to the homogenate. The homogenate was incubated on ice for 15 min. and centrifuged at 12,000 rpm (10 min., 4° C). The aqueous phase was transferred to fresh tube and total RNA was precipitated by adding equal volume of isopropanol. The RNA pellet was rinsed with 75% ethanol and briefly dried. The quality of RNA was assessed by electrophoresis on 1.2% agarose gel.

The cDNA was produced by reverse transcription (3.5 µM anchored oligo $(dt)_{24}$, 1 U μl^{-1} RNasin, 10 mM DTT, 0.5 mM dNTP mix and 1 U μl^{-1} MMLV reverse transcriptase in 1X PCR buffer). The RT reaction was incubated at 42 °C for 1 h and terminated at 70 °C for 15 min. Primers were designed based on CMG (crustacean hyperglycemic hormone, molt inhibiting hormone, gonad inhibiting hormone) family gene sequence information of *P. monodon* and closely related species. Using these primers, PCR was performed under the following conditions: 95 °C/5 min; 35 cycles of 94 °C/30 s; 55 °C /30 s and 72 °C/45 s.

cDNA cloning and semi quantitative RT PCR

The amplified products were extracted from 1.5% agarose gel, cloned into pGEM T easy vector and sequenced using T7 and SP6 promotor primers. RT-PCR was performed for *P. monodon* GIH (Primers: Pem GIH F ATGAAAACATGGCTGCTATTAGCG and Pem GIH R GCACTGAGACCAGGGAAAAC, 633 bp**)** and β actin (Primers: 5'CCCAGAAGAAGAGAGGTA3' and 5'GCGTATCCTTCGTAGATGGG3' 300 bp) using RNA (2 µg) samples from eyestalk at immature, previtellogenic, vitellogenic and ripe ovarian stages. The primers used for semi quantitative RT PCR were: PmGIHGeneF : ATGAAAACATGGCTGCTATTAGCG; Pm1.1R : TGGGATGCTTTCAGAGAAGG (316 bp). Each set of primer was tested in a range from 20 to 40 cycles using 1:1 to 1:10 dilutions of cDNA mix derived from samples to know the number of cycles where the product accumulation was in linear phase. RT-PCR conditions used were 95 °C

Table 1. Percentage identity of nucleotide sequence encoding crustacean gonad inhibiting hormones (GIH)

Name	*P. monodon*	*M. ensis*	*R. Kairei*	*N. norvegicus*	*H. gammarus*	*H. americanus*	*A. vulgarae*
P. monodon	100						
M. ensis	61	100					
R. kairei	46	42	100				
N. norvegicus	46	46	60	100			
H. gammarus	44	45	59	96	100		
H. americanus	45	53	66	95	99	100	
A. vulgarae	35	38	41	42	42	42	100

(5 min), -95 °C (1 min), 58 °C (40s), 72 °C (1 min) and final extension at 72 °C for 10 min. PCR products were resolved using 1.5% agarose gel and stained with ethidium bromide. Gels were quantitatively analysed (image J), and data expressed as GIH/ β actin ratio. The sequence of GIH was obtained by translating GIH cDNA using the translate tool (www.expasy.ch). Protein alignment was done by Clustal W (www.ebs.ac.uk), and phylogenetic analysis was performed by Clustal X as well as MEGA 4 software with all reported GIH sequences. The phylogenetic tree was constructed using neighbor joining method and tree topology was evaluated by 500 replication boot straps.

Histology of ovary

The ovarian tissues were preserved in Davidson's fixative for 24-48 h, and stored in 70% ethanol until further processing. The tissues were then dehydrated for 1 h each in a series of butanol/ethanol mixtures and infiltrated in three changes of Paraplast Plus. Serial sections (6 µm), were cut and stained in Harry's Hematoxylin and Eosin-Y. The oocyte developmental stage was determined according to previously reported criteria (Yano, 1987): oogonial stage (large nucleus with thin rim of cytoplasm), pre-vitellogenic oocyte (oocyte with homogenously hematoxylin stained cytoplasm), vitellogenic oocyte (oocyte with eosin positive yolk globules in the cytoplasm) and mature oocyte (oocytes with cortical rods in the periphery of oocyte cytoplasm).

Results

A 633 bp sequence was cloned from the eyestalk of *P. monodon* by RT PCR. The sequence comprises an open reading frame (ORF) of 291 bp that encodes a protein of 96 amino acids. The deduced protein was used to search the GenBank database using BlastP. The sequence has been submitted to GenBank (Accession No: GQ915281). Protein alignment with all known crustacean GIH revealed that homology of *P. monodon* GIH shares 61, 46, 46, 44, 45 and 35% identity with *Metapenaeus ensis, Rimicaris kairei, Nephrops norvegicus, Homarus gammarus, H. americanus,* and *Armadillidium vulgarae* respectively (Table 1; Fig. 1). In order to elucidate the expression profile of GIH gene during different reproductive phases, *P. monodon* females were grouped into four major stages of maturation using morphological and light microscopical characteristics (Fig. 2). Eyestalk of animals at different stages of maturation showed that GIH was expressed in all stages of maturation, although the expression of GIH mRNA changes during different stages of vitellogenesis. In animals with immature ovary (ovaries with 100% oogonial cells and difficult to discern from the adjacent somatic tissue in sub adult shrimps of 25-30 g), the GIH mRNA level was found to be significantly less, which peaked in previtellogenic phase and diminished in subsequent phases (Fig. 3). The expected RT PCR product of 317 bp was also observed in eye stalk tissue of males.

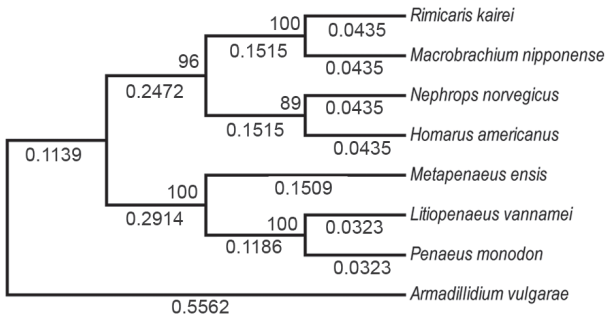

Fig. 1. Phylogeny of GIH gene in decapod crustaceans based on maximum likelihood analysis of amino acid dataset. Numbers at nodes are bootstrap values based on 1000 replicates

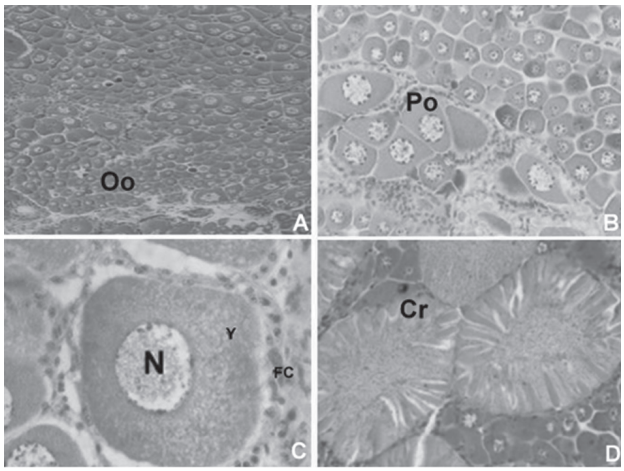

Fig. 2. Histological sections of *Penaeus monodon* ovary at different stages of development
(A): immature ovary showing oogonia clusters; (B): pre-vitellogenic stage; (C): vitellogenic stage and (D): ripe stage. Cr: cortical rod; FC: follicle cells; N: nucleus; Po : pre-vitellogenic oocytes; Y: yolk granules

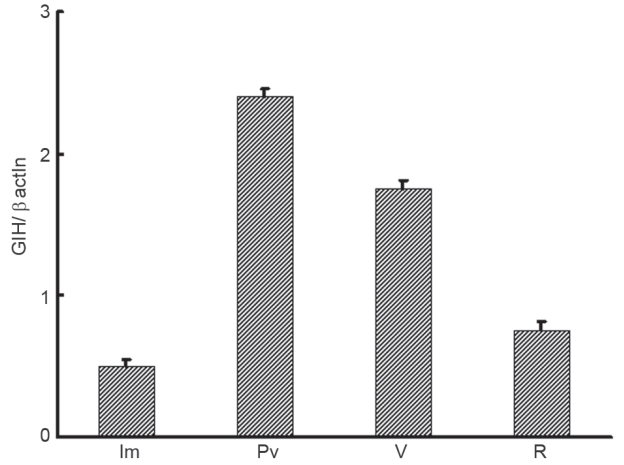

Fig. 3. Expression levels of GIH mRNA at various maturation phases of female *Penaeus monodon*
Im: immature ovary, Pv: Pre-vitellogenic ovary, V: Vitellogenic ovary; R: Ripe ovary

Discussion

Recent progress in molecular biological techniques has led to in depth understanding on molecular structure and functions of several crustacean eyestalk neuropeptides (crustacean hyperglycemic hormone family CHH family (de Kleijn *et al.,* 1998). Compared with other CHH family peptides (*e.g.*, CHH and MIH), only limited number of GIH has been characterised to date (Treerattrakool *et al.,* 2008). Amino acid sequence of *P. monodon* GIH obtained in the present study shows high sequence identity (96-97%) with GIH of *P. monodon* deposited in the GenBank, while it shows relatively lower sequence identity to the GIH from other crustaceans (lobsters and caridean shrimps). This observation is consistent with the hypothesis of greater evolutionary divergence between dendrobranchiata and pleocyemata (Ma *et al*., 2009). Interestingly, amino acid identity of *P. monodon* with MIH of other penaeid shrimps is relatively high (56-74%). On the basis of amino acid conservation, Lacombe *et al.* (1999) proposed to subdivide the V/GIH group into sequence involved in reproduction and sequence for moulting. Later, Edami *et al.* (2002) also acknowledged this hypothesis. Present *P. monodon* GIH sequence further expands this hypothesis. The existence of GIH in male shrimp confirms previous observation made in lobsters (de Kleijn *et al.,* 1992; Edomi *et al.,* 2002).

The current study provides a tool to investigate the pattern of GIH expression during various phases of oocyte maturation. The present data confirms an increased mRNA expression of eyestalk GIH during pre-vitellogenic phase and its down regulation during advanced phase of maturation. The above pattern of changes in GIH mRNA level is generally consistent with the model that GIH negatively regulates vitellogenesis or maturation. However, data from the immature stage did not appear to fit the model. One possible explanation is that low GIH mRNA titre may not alone is responsible for the inhibition of ovarian maturation during the immature phase of reproductive cycle. Okumra (2004), reported the incapability of unilataeral eyestalk ablation to induce vitellogenin gene expression in immature *Marsupenaeus japonicus*, and this finding further confirms the hypothesis that low VIH level may not be the sole regulator of vitellogenesis or maturation. It has been widely reported that unilateral eyestalk ablation often failed to induce maturation in commercial experimental facilities (de Kleijin *et al.,* 1992), possibly due to the stage of female maturation cycle. Further studies on haemolymph GIH level are required to prove this hypothesis

Acknowledgements

We are grateful to Dr. G. Syda Rao, Director, CMFRI, Cochin and Dr A. G. Ponniah, Director, CIBA, Chennai, for providing the facilities to carry out the work. The research was supported by grants from the Department of Biotechnology, Govt. of India (4030600013 BT/PR5721/ AAQ/03/238/2005). We thank Dr. Vinay Keuteney for assistance in using bioinformatics software. Thanks are always due to Ms Sanjukta Ramachandran for her valuable assistance while conducting this experiment.

References

Adiyodi, R. G. and Subramoniam, T. 1983. Arthropoda-Crustacea. In: Adiyodi, K. G. and Adiyodi, R. G. (Eds.), *Reproductive biology of invertebrates*: *oogenesis, oviposition, and oosorption*. Wiley. Chichester, p. 443-495.

Bhat, B. V. and Vinod, P. N. 2008. Development of seafarming in India – an export perspective. In: Lovatelli, A. Phillips, M. J., Arthur, J. R. and Yamamoto, K. (Eds.). FAO/NACA Regional workshop on the future of Mariculture- a regional approach for responsible development in the Asia-Pacific Region. Guangzhou, China, 7–11 March 2006. *FAO Fisheries Proceedings.* No. 11. Rome, FAO., p. 301–306.

Chomczynski, P, and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thocyanate-phenol-chloroform extraction. *Anal. Biochem*., 162: 156-199.

de Kleijn, D. P. V., Coenen, T., Laverdure, A. M., Tensen, C. P. and Van Herp, F. 1992. Localisation of messenger RNAs encoding crustacean hyperglycemic hormone and gonad inhibiting hormone in the X-organ sinus gland complex of the lobster, *Homarus americanus*. *Neurosci.,* 51: 121-128.

de Kleijn, D. P. V. and Van Herp, F. 1998. Involvement of the hyperglycemic neurohormone family in the control of reproduction in decapod crustaceans. *Invertebr. Reprod. Dev.,* 33: 263-272

Donaldson, E. 1996. Manipulation of reproduction in farmed fish. *Anim. Reprod. Sci*., 42: 381-392

Edomi, P., Azzon, E. , Mettulio, R., Pandolfelli, N., Ferrero, E. A. and Giulianini, P. G. 2002. Gonad-inhibiting hormone of the Norway lobster (*Nephrops norvegicus*): cDNA cloning, expression, recombinant protein production and immunolocalisation. *Gene,* 284: 93-102

Lacombe, C., Grève, P. and Martin, G. 1999. Overview on the sub-grouping of the crustacean hyperglycemic hormone family. *Neuropeptides,* 33 (1): 71-80.

Lightner, D. V. 2005. Biosecurity in Shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquacult. Soc*., 36: 229-248

Lotz, J. M. 1997. Viruses, biosecurity, and specific pathogen free stocks in shrimp aquaculture. *World J. Microbiol. Biotechnol*., 13: 405-413

Ma, K. Y., Chan, T.-Y. and Chu, K. H. 2009. Phylogeny of penaeoid shrimps (Decapoda: Penaeoidea) inferred from nuclear protein-coding genes. *Mol. Phylogenet. Evol*., 53: 45-55

Marsden, G., Hewitt, D., Boglio, E., Mather, P. and Richardson, N. 2008. Methyl farnesoate inhibition of late stage ovarian development and fecundity reduction in the black tiger prawn, *Penaeus monodon. Aquaculture,* 280: 242-246

Meusy, J. J. and Payen, G. G. 1988. Female and male reproduction in malacostracan crustacean. *Zool. Sci.,* 5: 217-265

Okumura, T. 2004. Perspectives on hormonal manipulation of shrimp reproduction. *J. A. R. Q.,* 38: 49-54

Panouse, J. B. 1943. Influence de I'ablation du pedoncule oculaire sur la croissance de I'ovaire chez la crevette *Leander seerratus*. *C. R. Acad. Sci*., 217: 553-555

Parnes, S., Mills, E., Segall, C., Raviv, S., Davis, C. and Sagi, A. 2004. Reproductive readiness of the shrimp, *Litopenaeus vannamei* grown in brackishwater system. *Aquaculture,* 236: 593-506

Soyez, D., Le Caer, J. P., Noyel, P. Y. and Rossier, J. 1991. Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster, *Homarus americanus*. *Neuropeptides*, 20: 25-32

Treerattrakool, S., Panyim, S., Chan, S. M., Withyachumnarnkul, B. and Udomkit, A. 2008 Molecular characterisation of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. *FEBS J.,* 275: 970-980

Vaca, A. and Alfaro, J. 2000. Ovarain maturation and spawning in the white shrimp, *Peaneus vannamei*, by serotonin injection. *Aquaculture*, 182: 373-385.

Yano, I. 1987. Effect of 17β-hydroxy-progesterone on vitellogenin secretion in kuruma prawn, *Penaeus japonicus. Aquaculture,* 61: 49-57.

Van Herp, F. and Soyez, D. 1997. Arthropoda-Crustacea. In: Adiyodi, K. G. and Adiyodi, R. G. (Eds.), *Progress in Reproductive endocrinology,* Oxford and IBH, New Delhi, p. 247-275.

Zhan, W. B., Wang, Y. H., Fryer, J. L., Yu, K. K., Fukada, H. and Meng, Q.- X. 1998. White spot syndrome virus infection of cultured shrimp in China. *J. Aquat. Anim. Health,* 10: 405-410.

Date of Receipt : 27.04.2012
Date of Acceptance : 04.02.2013