Molecular studies on Gonad Inhibiting Hormone (GIH) gene in Indian white shrimp (*Fenneropenaeus indicus*, H. Milne Edwards, 1837) and Tiger shrimp (*Penaeus monodon*, Fabricius, 1798) and expression of recombinant GIH *(rPmGIH)*

Thesis submitted to

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

In partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Marine Biotechnology

Under the Faculty of Marine Sciences
Department of Marine Biology, Microbiology and Biochemistry

by

REYNOLD PETER
(Reg. No. 3482)

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE

(Indian council of Agricultural Research)
Post Box No. 1603, Kochi - 682018
Kerala, INDIA

October 2015

केंद्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
(भारतीय कृषि अनुसंधान परिषद)
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
P.B No. 1603, Ernakulam North P.O.
Kochi 682 018, Kerala, India

Certificate

This is to certify that this thesis entitled **"Molecular studies on Gonad Inhibiting Hormone (GIH) gene in Indian white shrimp (*Fenneropenaeus indicus*, H. Milne Edwards, 1837) and Tiger shrimp (*Penaeus monodon*, Fabricius, 1798) and expression of recombinant GIH (*rPmGIH*)"** is an authentic record of research work carried out by Mr. Reynold Peter, M. Sc, under my guidance and supervision in Central Marine Fisheries Research Institute, Kochi, in partial fulfilment of the requirement for the award of Ph. D degree under the Faculty of Marine Sciences in Cochin University of Science and Technology. The thesis or part thereof has not previously been presented for the award of any Degree in any University.

Place: Kochi
Date: 19.10.2015

Dr. P. C. Thomas
(Supervising Guide)

Phone: 0484-2394867 (O), +91 9447291113 (Per), +91-484-2394909 (Fax), Telegram: CADALMIN, EKM
e-mail: palahanict@yahoo.com
www.cmfri.org.in

DECLARATION

I do hereby declare that the thesis entitled *"*Molecular studies on Gonad Inhibiting Hormone (GIH) gene in Indian white shrimp (*Fenneropenaeus indicus*, H. Milne Edwards, 1837) and Tiger shrimp (*Penaeus monodon*, Fabricius, 1798) and expression of recombinant GIH (*rPmGIH*)*."* is an authentic record of research work carried out by me under the guidance and supervision of Dr. P. C. Thomas, Principal Scientist, Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Kochi in partial fulfilment for the award of Ph. D degree under the Faculty of Marine Sciences of Cochin University of Science and Technology and no part thereof has been previously formed the basis for the award of any diploma or degree, in any University.

Place: Kochi
Date: 19.10.2015

Reynold Peter

Dedicated to my Family…

Acknowledgements

I profusely thank my supervising guide Dr. P. C. Thomas, Principal Scientist (Rtd.), Marine Biotechnology Division, CMFRI, Kochi, for supporting me as a student, taking time to listen and answer questions, helping to design experiments and meticulously correcting the thesis amidst his busy schedule.

I am much indebted to Dr. K. K. Vijayan, Former Head, Marine Biotechnology Division and present Director, Central Institute of Brakishwater Aquaculture (CIBA) for the support and guidance offered during this study. He is my mentor and thereby allowed me the freedom to explore and grow. I am honest when I say I would not be at this point without him.

I sincerely express my gratitude to Dr. A. Gopalakrishnan, Director, CMFRI, Kochi for the support extended during the study. I extend my sincere thanks to Dr. P. Vijayagopal, Head MBTD for the help rendered during this study.

I express my thanks to Dr. Pradeep M.A, Scientist, MBTD, CMFRI for the support during the recombinant protein expression study. I express my deep sense of gratitude to Dr. M. P. Paulton, Senior Technical Officer, MBTD CMFRI, Kochi for valuable suggestions and support. I extend my sincere thanks to the scientists of MBTD, Mr. N. K. Sanil, Dr. Kajal Chakraborty and Dr. Sandhya Sukumaran for their support during the study. I also express my

thanks to Mrs. P. Vineetha, Mr. Nandakumar Rao, and Mr. Girish P. M of Marine Biotechnology Division, CMFRI for the help rendered during this study.

I extend my gratitude to Dr. V.S Basheer, Principal Scientist & OIC, PMFGR, NBFGR Kochi Unit and other friends there for the support during the study.

I am extremely happy to recollect the support given by Dr. Lijo John, Assistant Director, EIC, Dr. P. A. Vikas, Senior Technical officer, KVK, CMFRI, Mr. Ranjth Kumar R, Sub-Inspector Fisheries and Mr. Sajeshkumar N K, Lecturer, Mar Augusthinose College and former research fellow during this study. I express thanks to my laboratory colleagues, Mr. Subin, Mr. Wilson Sebastian, Dr. Jeswin, Dr. Anju Jeswin, Mrs. Preetha Shenoy, Mr. Sayooj, Mrs. Anu Sree, and Mrs. Esha Arshad for their support during the study.

I am thankful to Department of Biotechnology, Govt. of India and Indian Council of Agricultural Research, New Delhi for funding support and permitting to pursue Ph.D. Programme.

Finally, I remember my family with gratitude for too many things to list here. The support extended by my family through their prayers were precious.

⌘⌘⌘⌘⌘

Abbreviations

3`	:	three prime
5`	:	five prime
°C	:	degrees Celsius
β	:	Beta
µg	:	Microgram(s)
µl	:	Microlitre(s)
µM	:	Micromolar
AA	:	Amino acid(s)
APS	:	Ammonium persulfate
BLAST	:	Basic local alignment search tool
bp	:	Base pairs
cDNA	:	Complementary DNA
CDS		Complete coding sequence
CHH	:	Crustacean Hyper Glycemic Hormone
cm	:	Centimetre(s)
DEPC	:	Diethyl pyro carbonate
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxy ribonucleotide
E. coli	:	*Escherichia coli*
EDTA	:	Ethylene diamine tetra acetic acid
et al.	:	And others
g	:	Gram(s)
GIH	:	Gonad Inhibiting Hormone
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
kDa	:	KiloDalton(s)
L	:	Litre(s)
MCS	:	Multiple cloning site
MIH	:	Molt Inhibiting Hormone
min	:	Minute(s)

mg	:	Milligram(s)
ml	:	Millilitre(s)
mm	:	Millimetre(s)
mM	:	Millimolar
mRNA	:	Messenger RNA
NCBI	:	National Center for Biotechnology Information
ng	:	Nanogram(s)
ORF	:	Open reading frame
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PCR	:	Polymerase chain reaction
RACE	:	Random Amplification of cDNA Ends
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
RT-PCR	:	Reverse transcription Polymerase chain reaction
SDS	:	Sodium dodecyl sulfate
sec	:	Second(s)
TBE	:	Tris-borate-EDTA
TE	:	Tris-EDTA
TEMED	:	Tetramethylethylenediamine
UPM	:	Universal Primer Mix
UV	:	Ultraviolet
V	:	Volt(s)
UTR	:	Untranslated region
XOSG	:	X-organ Sinus Gland

Contents

List of Figures and Tables

Tables

⌘⌘⌘⌘⌘

Chapter –I

Introduction

Introduction

Shrimp aquaculture is an important global industry helping the humankind to meet the food security challenges. Shrimps are rich in protein, and contribute as a delicious food for human consumption all over the world. The global production of shrimps amounts to over 4 million tons annually, involving a trade of over 10 billion US dollars. Shrimp prevails to be the most significant solitary commodity in worth, accounting for about 15% of the overall value of the globally traded fishery products (FAO, 2014). In India also shrimp farming has emerged as one of the most significant coastal food production industrial sectors. During the past decade the industry shot up substantially, and this in turn has led to the development of complications associated with it. These include the environmental related issues resulting from the intensive culture, and outbreak of diseases leading to the decline in production. Even though many of these concerns are addressed to a large extent through scientific and eco-friendly measures, establishment of sustainable seed production technology is an area that needs further attention and focused efforts. Domestication of the shrimp species needs to be invariably achieved for the sustainable existence of the industry.

The shrimp aquaculture industry is predominantly dependent upon wild spawners for seed production at present. Though, the life cycle and different larval stages of commercially important shrimps had already been worked out in detail, through hatchery production of larvae from the spawners caught from wild, the much needed manipulation techniques to make the animal mature and spawn in the captive environment is yet to be perfected or improved for a large number of commercially valuable species. A thorough understanding of

reproductive mechanisms in target species is the essential pre-requisite for developing appropriate techniques for the reproductive manipulations within the hatchery reared populations of brooders. At present, the level of understanding on the subject is scanty and insufficient to work out a successful strategy that could be readily adapted in culture practices among crustaceans. Such vital information on crustaceans, as worked out in many fish species, is essential to face the challenges in crustacean aquaculture (Donaldson, 1996). This could be achieved only through modern biological tools to understand the reproductive mechanisms and searching for new avenues to manipulate the animals under captivity to meet the round the year demand for good quality seeds.

Tiger shrimp, *Penaeus monodon* and Indian white shrimp, *Fenneropenaeus indicus*, are the two commercially important shrimp species of India. Both species equally confronts the problem of overexploitation as well as post larval virus infection. Most of the decline in the production could be attributed to disease outbreaks caused by viruses. The only solution is to develop specific pathogen free (SPF) stocks and follow strict biosecurity protocols (Lightner, 2005).The major pre-requisite for production of SPF stock is to have control over the reproduction in terms of quantity and quality of seeds produced. Even with the commitment of substantial resources to achieve this goal by both the industry and research establishments, insufficient commercial availability and utilization of domesticated brood stock remains without much change (Coman, 2006).

Hatchery trials with domesticated stocks suggest that they are significantly less sensitive to induced spawning, and egg-hatching rates are

lower when compared with their wild counterpart. This implies that the captive environment is deterring natural reproduction to certain extent. The only technique undertaken globally for induced maturation in both the domesticated and wild-caught females maintained in captivity is the unilateral eyestalk ablation. This leads to alterations in the level of the neuropeptide hormones produced by the X-organ of the eyestalk, which have vital role in the control of the gonadal maturation and spawning in crustaceans. However, this happens to be a physiologically destructive as well as irreversible and crude technique for hormonal manipulation.

Though, ablation empowers certain control over larval production, this is not always rewarding and can bring about reduction in egg and larvae quality because of repeated spawning of ablated females (Marsden *et al.,* 2007). Moreover, with the appearance of a multitude of prospective pathogens in marine shrimps, this procedure also began failing. Therefore, when it comes to the sustained supply of healthy post larva with improved disease endurance and high production potential there is an urgent need for developing efficient and animal friendly techniques.

This in turn calls for concerted efforts for in depth studies for understanding the biological and molecular factors controlling the shrimp ovarian maturation and larval development, using the modern molecular biology techniques for achieving induced maturation and spawning, and thereby gain fool proof control over the system.

As with other decapods, reproduction in shrimps is under the influence of multiple endocrine systems. In various scientific studies, it has already been recorded that neuropeptide hormones produced within the eyestalk of

crustaceans by neurons of the medulla terminalis X-organ have vital functions in the control of the gonadal maturation and spawning in crustaceans (Keller *et al.,* 1985; Keller, 1992; de Kleijn *et al.,* 1992; Klein *et al.,* 1993; Rosas *et al.,* 1993; Yang *et al.,* 1996). Neuropeptide hormones from the medulla terminals X-organ are transmitted along axons to the sinus gland, the place where these are stashed before released into the circulation. Further, the CMG family hormones, Crustacean Hyperglycemic Hormone (CHH), Molt Inhibiting Hormone (MIH) and Gonad Inhibiting Hormone (GIH) are involved in the control of normal reproductive physiology of the shrimps. They share similar features including amino acid sequence homology, conservation of the 6-cysteine residues and structural resemblances. Among these, the Gonad Inhibiting Hormone (GIH) is negatively regulating the gonadal maturity and this feature is the basis of the unilateral eyestalk ablation procedure employed extensively for the induced maturation of shrimps under captivity. Eyestalk ablation results in lowering the concentration of GIH and this subsequently induces the gonadal maturation. Therefore, the search for an alternative mechanism necessitate a thorough understanding of the properties of the GIH and its genetic characterization. Once the GIH gene is sequenced and characterized, it could be cloned in expression vectors to produce recombinant GIH protein in the laboratories. System could be evolved to produce and harvest recombinant GIH, and its application for induced maturation replacing the conventional eyestalk ablation.

In view of the above facts, studies on the molecular aspects of the gene coding for gonad inhibiting hormone (GIH) in two commercially important shrimp species *viz. P. monodon* and *F. indicus* were undertaken for the doctoral programme. The study is divided into the following systematic steps to achieve

the target envisaged. (i) Elucidation of the complete mRNA sequence encoding the Gonad Inhibiting Hormone (GIH) gene (ii) Construction of recombinant expression vectors with GIH gene inserts and (iii) Expression of the recombinant GIH in a suitable expression system. The outputs from this study could be further used for developing an antagonist molecule to GIH hormone as an alternative to eyestalk ablation and to elucidate the binding partner as well as pathway studies.

⌘⌘⌘⌘⌘

Chapter –II

Review of Literature

Review of Literature

Conventionally, capture fisheries remain a significant source of nutrition for the common man globally. Over the past decade, however, the heightened fishing pressure have radically exhausted this pricey useful resource (FAO, 2014). On account of this, farming of aquatic organisms or aquaculture is becoming more important for the food security of the mankind. Aquaculture is one of the world's most rewarding and fast growing food production industry, serving up a reasonable, healthy and protein rich food. Apart from this, the aquaculture possesses the potential to get rid of the obligation on the ecosystem by bringing down the dependency on wild fish stocks. Aquaculture, most certainly, is an industry that has the potentiality to provide the ever increasing and long lasting demand for aquatic food products.

2.1 Importance of aquaculture

Presently, aquaculture is exceedingly diversified when it comes to the type of organisms cultured and the technologies put to use. Farming usually involves the interference in the rearing strategy for improvement in production. During the past decades, aquaculture production has increased by 7–11% on an annual basis. The production of fish and shellfish through aquaculture is an increasingly important source of high-quality animal protein, with the worldwide production reaching 66.6 million tons in 2012 (FAO, 2014). Seafood that is generated by the aquaculture currently makes up about one half of just about all the seafood directly used up by human beings. There are over 200 aqacultured varieties around the globe (Tacon, 2003) indicating the rich biodiversity. In recent years, crustaceans which include marine as well as freshwater prawns, lobsters, crabs and crayfish are becoming valuable

aquaculture merchandise estimated to be valued at about US$ 9.37 thousand million (Tacon, 2003). This is primarily due to the fact that most of the crustaceans, predominantly marine species, remain high-value species as they are reckoned to be a luxurious food item. Worldwide aquaculture output of crustaceans was determined to be 5.7mt, 9.6% of the global aquaculture production (FAO, 2012).

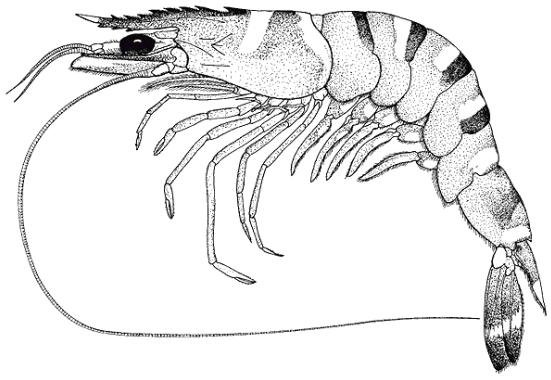
A species needs to satisfy a number of biological and economical factors to become commercially feasible for aquaculture. Though, the life cycle of several commercially important aquaculture species have already been worked out, the culture of several of them, especially the marine fish and shrimp, remains reliant on the collection of larvae, post larvae, or even gravid females from the wild environment. In the event, if the broodstock are domesticated there can be the added benefit of predictable and assured egg/larval supplies in addition to an opportunity to perform genetic selection for improving productivity. Domestication, otherwise known as 'closing the life cycle' appears to be substantial for the financial success of aquaculture.

2.2 Shrimp aquaculture

Shrimps constitute the most valuable group of crustaceans for aquaculture with regard to overall production as well as value. Shrimp aquaculture is a leading contributer in the world's aquaculture productivity since the early 70's. Because it gives a tremendous quick financial return, shrimps aquaculture showed a rapid expansion along with various associated business and turned into a multimillion dollar enterprise. Rosenberry (2004) stated that the output of cultured prawns had accomplished levels on par with the capture fisheries, with the industry producing around 2 million tons per

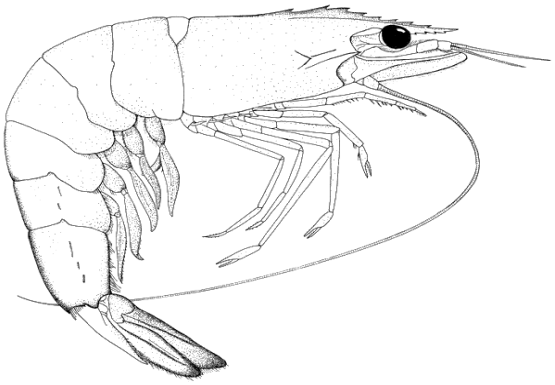
annum. Aquaculture, the shrimp culture in particular, have got persistent growth. Especially, the marine shrimp culture has grown to become one of the important crustacean aquaculture crop across the globe, the perceived importance of which is mirrored in the 250% increase in the production from 2000 to 2006 (FAO, 2009).

Tiger shrimp, *Penaeus monodon* and Indian white shrimp, *Fenneropenaeus indicus*, are two of the foremost industrial shrimp species of the India. They are found within the Indo-West Pacific from eastern and south-eastern Africa, through India, Malaysia and Indonesia to southern China and northern Australia. In taxonomic classification, they belongs to the Phylum Arthropoda, Super-class Crustacea and class Malacostraca, Order Decapoda and Family Penaeidae.



Courtesy: FAO (2015)

Figure 2.1: *Penaeus monodon*



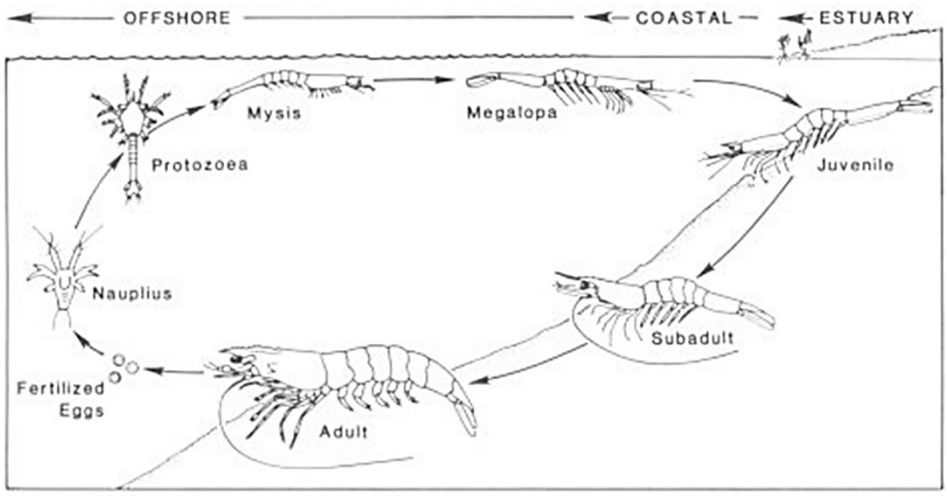
Courtesy: FAO (2015)

Figure 2.2 : *Fenneropenaeus indicus*

Both the species experience the predicament of over exploitation as well as virus infections in their postlarvae. In order to produce specific pathogen free healthy postlarvae with diseases resistance,and to enhance the yield from shrimp aquaculture, research in the area of shrimp maturation and larval development are to be taken up. From the many scientific studies, it has already been revealed that hormones plays vital part in the management of ovarian maturation as well as larval progression.

2.3 Reproduction and larval development

Numerous aquaculture shrimp species have already been domesticated and many species proved challenging to domesticate owing to the inferior reproductive efficiency in captivity (Primavera, 1984; Coman *et al.,* 2006). For the effective domestication of any species, it is necessary to have the capacity to bring up succeeding generations in captivity. To accomplish this it is crucial to have knowledge of the life cycle of the candidate species in addition to the biological necessities of every developmental stages.



Courtesy: Bailey-Brock & Moss (1992)

Figure 2.3: *Life cycle of Penaeid shrimps*

Like the majority of the penaeids, the life cycle of *P. monodon* and *F. indicus* comprises an estuarine phase for the post larvae and juvenile stages followed by a marine phase, which involve offshore migration of sub adults. Full ovary maturation and spawning transpires in the marine phase offshore where water quality parameters are stable for the developing eggs as well as early larval stages.

Most crustaceans begin to reproduce before somatic growth is completed (Hartoll, 1985). Reproduction in crustaceans is a physiological process that is entwined with the molting process. In feamle crustaceans reproduction comprise of oogenesis in conjunction with oviposition (Anikulmar and Adiyodi, 1985). Oogenesis is usually divided into 2 distinct phases: previtellogenesis and vitellogenesis. Vitellogenesis is a consistent routine of rapid deposition of yolk in oocytes, and this occurs whenever the animals are in reproductive stage (Charmantier and Charmantier-Daures, 1998).

Penaeids are equipped with a thelycum with lateral plates that covers the spermatophore. Mating of these species occurs within hours of a mature female moulting (Primavera, 1984) during the time the female is 'soft' after shedding off her shell, that the male could inject the spermatophore. Spawning takes place when the environmental conditions are optimal for embryonic development (Anikulmar and Adiyodi, 1985). Like the molting cycle, vitellogenesis is regulated by various hormones, with the inclusion neuropeptides secreted form the eyestalk (Huberman, 2000).

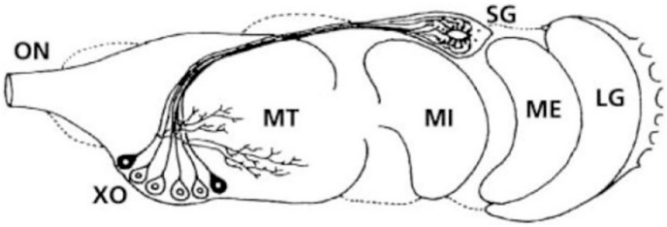
2.4 Endocrine regulation of reproduction

The endocrine system in crustaceans, which is of extreme significance in the maintaining of homeostasis, growth, development and reproduction, comprised predominantly of neuroendocrine hormones. In recent years, considerable initiatives were made to understand fully the endocrine systems of crustaceans, especially the ones with commercial importance (Huberman, 2000).

A number of crustacean hormones have been identified and consequently sequenced. Many genes responsible for various reproductive processes have also been identified (Dircksen *et al.,* 2001). With progression in molecular scientific knowledge, the intricacies of the crustacean endocrine system model has elevated to include species-specific hormones along with the multi functionality. Various hormones, analogous to vertebrate hormones have been looked into in connection with ovarian development in crustaceans. Most of the research on endocrine control over crustacean reproduction has focused on the inhibitory hormones; neuropeptides that negatively control physiological processes.

2.5 X-organ Sinus gland Complex

The crustacean eyestalk (Figure 2.4) is a neurosecretory center. Hanstrom (1939) first observed and called the structure of unidentified functionality situated on the proximal surface of the medulla terminals to be X organ (XO). The sinus gland (SG) is not a gland but a complicated neurohemal organ put together by the swollen axon terminals of neurosecretory cells, axons from cells other than neurosecretory cells and glial cells that warp around the swollen terminals of the neurosecretory cells (Skinner, 1985). This neurosecretory unit is known as X-Organ Sinus Gland Complex (XOSG).

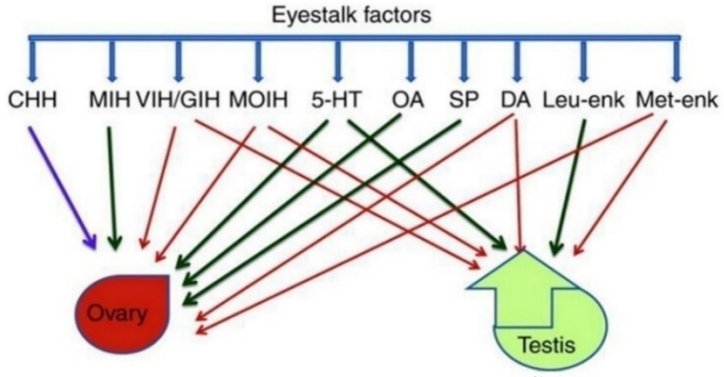


Courtesy: Bocking et al. (2001)

Figure 2.4: *Diagrammatic representation of eyestalks of decapod crustaceans (dorsal view). LG- lamina ganglionaris, ME- medulla externa, MI medulla interna, MT- medulla terminalis, ON- optic nerve, SG-sinus gland, XO- X-organ*

X organ-Sinus Gland Complex in the optic ganglia of the eyestalk is considered the most important neuroendocrine control center in crustaceans (Charmantier *et al.,* 1997). The endocrine control over reproduction starts with the environmental stimuli that induce the XOSG complex to secrete neurohormones. The reactions can vary greatly with species, age as well as season (Adiyodi and Subramouian, 1983).

It is widely known that the reproductive process is under the control of a variety of hormones produced in the crustacean eyestalks (Keller, 1992). They consist of crustacean hyperglycemic hormone (CHH) (Jaros, 1979; Van Herp and Buggenum, 1979; Keller *et al.,* 1985), molt-inhibiting hormone (MIH) (Dircksen, *et al.,* 1988; Klein *et al.,* 1993), and gonad inhibiting hormone (GIH) (de Kleijn *et al.,* 1992). Although, numerous achievements have been made in sequencing neuropeptides and determining their specified functions during the past decade, the mode of action and target tissues of the sinus gland neuropeptides remains mostly unclear even at this time. Hormones released by the sinus gland possess unique influence on reproductive process (Caillouet, 1972), absorption effectiveness and oxygen utilization (Rosas *et al.,* 1993), blood glucose levels (Keller *et al.,* 1985) and molt frequency (Yang *et al.,* 1996).



Courtesy: Nagaraju (2011)

Figure 2.5: *The effect of eyestalk factors on reproduction in crustaceans. CHH:crustacean hyperglycaemic hormone; MIH: moulting inhibiting hormone; VIH: vitellogenesis inhibiting hormone; GIH: gonad inhibiting hormone; MOIH: mandibular organ inhibiting hormone; 5-HT: 5-hydroxytryptamine; OA: octopamine; SP: spiperone; DA: dopamine; Leu-enk: leucine-enkephalin; Met-enk: methionine enkephalin. Green arrows indicate positive influence; red arrows indicate negative regulation; purple arrow indicates either positive or negative regulation.*

2.6 CMG family hormones

CMG family of neuropeptide hormones is constituted by three physiologically important neuropeptide hormones, the crustacean hyperglycemic hormone (CHH) which controls glucose concentration in the hemolymph (Kegel *et al.,* 1989; Keller, 1992; Martin *et al.,* 1993), molt-inhibiting hormone (MIH) which negatively regulates ecdysteroid synthesis (Webster, 1998) and gonad inhibiting hormone (GIH) which inhibits vitellogenesis in females (Soyez *et al.,* 1991; Grève *et al.,* 1999). GIH has also been detected in male (de Kleijn *et al.,* 1998; Martin *et al.,* 1999), and is involved in androgenic gland growth (Martin *et al.,* 1999).

All these peptides share six conserved cysteine residues, same characteristics comprising structural, sequence similarity, amino acid conservation, and collectively referred to as the CMG family hormones (Keller, 1992; Yang *et al.,* 1995; Lacombe *et al.,* 1999). In addition to several similarities in their structure, some hormones in the CMG family are multifunctional i.e. exhibit more than one biological activity (Lee *et al.,* 1995). However, the certainty concerning the primary physiological functions of different peptides still needs to be clarified. The nominal dissimilarities in hormone structure may influence functional activity (Davey *et al.,* 2000). Just like their name suggest, a number of these neuropeptides possess negative influence over various interlinked functions (Wainright *et al.,* 1996; Huberman 2000). The equilibrium between stimulatory and inhibitory hormone titres is likely to determine which functions are triggered .

CMG peptides are divided into two subtypes based on the absence (type I) or presence (type II) of a glycine (Gly) residue at position 12 in the mature

peptide (Yang, 1996; 1997). The precursors of CMG-family peptides also differ between type I and type II. The precursors of type I peptides consist of a signal peptide, a CHH precursor related peptide (CPRP), and a hormonal part, while those of type II peptides consist of only a signal peptide and a hormonal part (Webster, 2012). The CPRPs are typically found in precursors of type I peptides, but their biological function is not yet known. CMG-family mature peptides have 72 to 78 amino acid residues with six conserved cysteine residues that form three intramolecular disulfide bonds. Additionally, type I peptides are characterized by an amidated C-terminus, although some type II peptides also have an amidated C-terminus (Katayama *et al.,* 2013). According to this grouping, CHHs can be classified as type I peptide, and most MIHs, GIHs as type II peptides.

2.7 Gonad inhibiting hormone

In crustaceans, ovarian maturation is negatively controlled by the neuropeptide, gonad inhibiting hormone (GIH) produced by the X-organ sinus gland complex of eyestalk by inhibiting production of vitellogenin synthesis (Hopkins, 2012). GIH peptide actively participate in ovarian development and is a key hormone pertaining to reproduction in crustaceans as the inhibitory effect of GIH is more intense than any other hormones controlling reproductive maturation (Vaca and Alfaro, 2000). The classic experiment of Panouse (1943) first proved GIH exist from the observation that eyestalk ablation brought vitellogenesis in *Palaemon serratus* (Fingerman*,* 1987).

Gene coding for the GIH peptide from terrestrial isopod the woodlouse, *Armadillidium vulgare* (Grève *et al.,* 1999)*,* from astacidaean lobsters, *H. Americanus* (de Kleijn *et al.,* 1994)*, Homarus gammarus* (Ollivaux *et al.,* 2006),

Nephrops norvegicus (Edomi *et al.,* 2002) and from shrimps *P. monodon, Metapenaeus ensis* (Gu *et al.,* 2002), *P. monodon* (Treerattrakool *et al.,* 2008), *Rimicaris kairei* (Qian *et al.,* 2009) *Litopenaeus vanammei* (Chen *et al.,* 2014) and *Macrobrachium nipponense* (Qiao *et al.,* 2015) have been characterised and cloned so far.

GIH was reported to be expressed in the eyestalks (de Kleijn *et al.,* 1994; Edomi *et al.,* 2002; Ollivaux *et al.,* 2006) in the supraesophageal ganglion (brain), superficial axon terminals of the sinus gland (Grève *et al.,* 1999; Edomi *et al.,* 2002). GIH was not only expressed in female lobsters, but also in male lobsters (de Kleijn *et al.,* 1992) and in larvae (Rotllant *et al.,* 1993). So far only two recombinant GIH have been reported, from *Nephrops norvegicus* (Edomi *et al.,* 2002) *Litopenaeus vannamei* (Tsutsui *et al.,* 2013).

The expression of GIH in relation to the reproductive cycle has been studied in lobsters (de Kleijn *et al.,* 1998) and shrimp (*P. monodon)* (Vijayan *et al.,* 2013). The expression of GIH mRNA in immature stage was significantly lower when compared to that in previtellogenesis and vitellogenesis stages. Females in previtellogenic stage of ovary were showing significantly higher levels of GIH mRNA expression. The relatively higher GIH mRNA level in the previtellogenic and vitellogenic stages are in agreement with the proposed inhibitory function of this peptide (Vijayan *et al.,* 2013).

2.8 Role of GIH in shrimp aquaculture

Traditionally, shrimp brood stock captured offshore were spawned in captivity and employed as seed stocks for stocking in the commercial production systems (Moss *et al.,* 2012). The wild shrimps usually are periodic

spawners with distinct ecological signals stimulating ovary development and spawning directly via neurosecretory centers (Khoo, 1988). Unavailability of sufficient numbers of high quality seed at the proper time is regarded as the primary factors that restrict the productivity in aquaculture industry. For this purpose, absolute control over reproductive maturation of the species under captivity is crucial to ensure that a dependable year-round availability of seed could be guaranteed. Whilst regulating environmental conditions is a significant hatchery protocol, it offers constrained results in triggering sufficient spawning to match commercial production schedules. Systematically regulated maturation and spawning of viable eggs for timely production of high-quality shrimp nauplii has become an evasive goal for shrimp culture industry over many years.

Research on reproduction in crustaceans lags behind that of other aquacultured species ending with inadequate comprehension and less alternative technologies to control reproduction (Wilder *et al.,* 2010). Hormonal manipulation of shrimp reproduction is minimal, and only eyestalk ablation is employed to induce ovarian development and spawning in shrimp culture worldwide. While eyestalk ablation is extensively practiced, effectiveness of the procedure is debatable (Huberman, 2000).

Eyestalk ablation is used in hatcheries as a crude technique of hormonal manipulation to induce spawning in many crustaceans, including *P. monodon* (Primavera, 1984). The process involves the excision of one or both eyestalk to decrease the quantity of GIH being produced or secreted by the X-organ and sinus gland complex (Longyant *et al.,* 2003). However, this has an effect on

practically all areas of crustacean physiology, which are controlled by the X Organ Sinus Gland Complex (Quackenbush, 1986).

Though with eyestalk ablation, *P. monodon* is able to spawn 4-6 times per female per molt cycle (Marsden *et al.,* 1997), there exists proof that ablation leads to eventual decline in larval survival (Palacios *et al.,* 1999) and fecundity (Emmerson, 1983). Eyestalk ablation is a non-reversible, physiologically destructive procedure, which results in an inevitable reduction in egg quality and death of the animal (Benzie, 1998). With ablation disturbing glucose metabolism (CHH) and ecdysis (molting, MIH), it is likely to affect mobilisation of nutrients as well (Harrison, 1990). An eventual plunge in spawn frequency was observed for *P. vannamei* (Palacios *et al.,* 2000) implicating additional physiological processes apart from nutrient depletion. Besides, the recent reports of cDNA microarray analysis to examine effects of eyestalk ablation at the transcriptomic level and pathway mapping analysis to identify potentially affected biological pathways in the black tiger shrimp (Uawisetwathana *et al.,* 2011) revealed that the eyestalk ablation significantly altered the transcripts whose functions were involved in immune responses, electron transport mechanism, and calcium signal transduction, that could lead to adverse physiological effects. Therefore, modern and animal friendly approaches, based on molecular biology techniques, needs to be developed for achieving induced maturation and spawning and thereby to gain more desirable control over the system.

2.9 Alternatives to eyestalk ablation

For the past three decades, scientists and hatchery operators have focused on developing endocrinological manipulation to induce reproduction

without eyestalk ablation in crustaceans. The recent developments in the field of hormonal manipulation of shrimp reproduction like the development of recombinant hormones and their antagonists can be applied effectively to replace the destructive eyestalk ablation technique currently followed to induce gonadal maturation under captivity.

Alternative techniques like manipulating environmental factors such as temperature, salinity and photoperiod, administration of neurotransmitters, recombinant proteins and double-stranded RNA (RNA*i*) to reduce gonad inhibitory peptide transcript have been attempted to stimulate ovarian development. The results from these experiments revealed the potential (Chang *et al.,* 2001; Fanjul- Moles, 2006; Nagaraju, 2007; Tiu *et al.,* 2007; Mazurová *et al.,* 2008; Nagaraju and Borst, 2008; Treerattrakool *et al.,* 2008) to stimulate gonad maturity. Okumura (2007) suggested a convincing alternative for the physiologically destructive eyestalk ablation technique by neutralizing or blocking the endogenous inhibitory effect of GIH by an antagonist.

Numerous scattered research findings on the CMG family hormones, particularly GIH are compiled in this review to provide a varied representation of the regulatory functions in crustaceans. In spite of numerous scientific studies on CMG family hormones, our comprehension regarding their capabilities appears to be unfinished. It appears that, a multi-disciplinary strategy to redefine the functions with references to their expression and mode of actions, for instance receptor characterization are meaningful as well as vital in comprehending regulatory mechanisms underlying the distinctive process.

In view of the above facts, studies on the molecular aspects of the gene coding for gonad inhibiting hormone (GIH) in the commercially important

shrimp species *viz*. *P. monodon* and *F. indicus* needs to be undertaken in the endeavour for developing better hormonal manipulation techniques in the vast field of crustacean endocrinology and then its practical application for assured shrimp reproduction in aquaculture. The study needs the systematic steps to achieve (i) Elucidation of the complete mRNA sequence encoding the Gonad Inhibiting Hormone (GIH) gene (ii) Construction of recombinant expression vectors with GIH gene inserts (iii) Recombinant expression and purification of the GIH in expression system and (iv) Application of the gene constructs and the recombinant GIH for induced maturation and spawning. The outputs from this study could be further used for developing an antagonist molecule to GIH hormone as an alternative to eyestalk ablation and to elucidate the binding partner as well pathway studies.

⌘⌘⌘⌘⌘

Chapter –III

Materials and Methods

Materials and Methods

Detailed account of the materials and various methodologies used in conducting the study are presented in this chapter. It describes the procedure followed for collection samples of the species under study *viz., Penaeus monodon* and *Fenneropenaeus indicus*, tissue storage, isolation of RNA and its preservation. Amplification of the cDNA of GIH gene using Reverse transcription Polymerase Chain Reaction (RT-PCR) is described along with the thermal cycling conditions. Procedures involved in the gel elution, purification and cloning of the PCR products, and DNA sequencing are also described. Details of the gene specific primers designed and custom synthesized for carrying out RACE-PCR to generate full-length GIH sequence information are given along with the thermal cycling conditions. The bioinformatics software used to perform *in-silico* analysis for primer designing, sequence homology searches, signal peptide analysis; protein structure prediction and phylogenetic analysis are also described.

A detailed account of the construction of recombinant plasmid for expressing GIH gene of *P. monodon* such as preparation of gene constructs with and without signal peptide region, their cloning in expression vector, and purification of expression plasmids are given in this chapter. Methodologies standardized for the expression of recombinant GIH protein, and its affinity purification are also outlined.

3.1 Collection and storage of tissue samples

Samples of both the candidate species *Penaeus monodon* (150 to 165 mm total length) and *Fenneropenaeus indicus* (110 to 125 mm total length) were collected from the wild with the help of local fisherman venturing in seas in fishing boats. Freshly caught live specimens belonging to different reproductive maturity stages were brought to the laboratory in areated polyethyne bags. The specimens with previtellogenic ovaries were used to dissect out eyestalks surgically using sterile instruments treated with DEPC water, and the tissues were stored in cryovials containing 1.5 ml RNA later solution (Ambion Biosciences) at -80°C deep freezer for total RNA extraction.

3.2 Total RNA extraction

The eyestalks of individual shrimp with previtellogenic ovaries were dissected and pigment containing compound eye along with most of the cuticle and non-neural tissues was removed and discarded. The remaining nueral portion of the eye stalk was rinsed briefly in 75% ethanol and were used for extraction of the total RNA by using the modified guanidine thiocyanate method (Chomczynski and Sacchi, 1987).

Preparation of glassware and reagents

Glasswares and plasticwares

All glass wares used for RNA isolation were treated with 0.1% Diethyl pyrocarbonate (DEPC) solution prepared in Milli-Q water for 4 h at 37°C and then kept at 150°C in hot air oven for 3 h. Sterile disposable RNase free plastic wares were used for the preparation and storage of RNA. Utmost care was taken to prevent the action of RNase during the entire isolation process by ensuring

the biochemical and microbiological sterility of the workbench by alcoholic sterilisation. Disposable gloves were used during the preparation of reagents, isolation and analysis of RNA.

Reagents:

All reagents were prepared using molecular grade chemicals (SIGMA Inc, USA) in 0.1% DEPC treated sterilized Milli-Q water. The autoclavable reagents were made sterile at 121°C for 15 min at 15 lbs PSI.

Phosphate Bufered saline (PBS)

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4

1.47 mM KH_2PO_4

Adjust to a final pH of 7.4

Dissolved the chemicals in minimum volume of DEPC treated water and made upto 1 liter and sterilized by autoclaving.

Solution D

4 M Guanidium thiocyanate

25 mM Sodium citrate

0.5% w/v N-lauryl sarcosine

0.1 M β-mercapto ethanol*

Filter sterilized through 0.2µm filter and store at room temperature

*Add β-mercaptoethanol just before use.

Phenol

Saturated with 0.1M citrate buffer, pH 4.3

Chloroform

Isopropanol

75% Ethanol

Protocol

- **Tissue homogenization:**
  - A single pair of eyestalk neural tissue was homogenized in 500 µl PBS in a 1.5 ml micro centrifuge tube using a sterile pestle.
  - Homogenized samples were incubated on ice for 5 min to ensure the dissociation of cell wall and pigments.
  - The homogenate was then centrifuged at 10,000 rpm for 5 min at 4°C and the upper aqueous phase was saved and the pellet discarded.
- **Phase separation:**
  - The aqueous phase (0.4 ml) was transferred to a fresh 1.5 ml micro centrifuge tube.
  - Solution D (0.4 ml) was added to the preparation and the tube was vortexed vigorously for 15 sec followed by incubation on ice for 3 min.
  - Saturated phenol (0.4 ml) and chloroform (0.1 ml) was added and vortexed vigorously for 30 sec followed by incubation on ice for 15 min.
  - The preparation was centrifuged at 12,000 rpm for 15 min at 4°C
- **RNA precipitation:**
  - The upper aqueous phase was transferred to a fresh 1.5 ml micro centrifuge tube and equal volume of isopropyl alcohol was added to precipitate the RNA and kept at -80°C for 10 min .
  - Precipitated RNA was recovered by centrifugation at 12,000 rpm for 10 min at 4°C.
- **RNA wash:**
  - Precipitated RNA pellet was washed once with 1ml of 75% ethanol and centrifuged at 12,000 rpm for 5 min at 4°C.
  - The resulting pellet was saved carefully and the ethanol was decanted .

- **Dissolving RNA:**
  - The RNA pellet was air dried and dissolved in the 20 µl sterile RNA storage solution (Ambion Biosciences)

3.3 Quantification of RNA

The total RNA isolated was spectrometrically quantified using the BioPhotometer® plus with Hellma TrayCell (Eppendorf , Hamburg, Germany) by measuring the optical density ratio of A260 nm to A280 nm. Quality of total RNA isolated was also checked by running in 1% Agarose gel electrophoresis.

3.4 Oligonucleotide Primers for cDNA synthesis

The oligonucleotide primers were custom synthesised from Sigma Genosys, India. All primers were specifically designed using Primer Premier 6.0 (PREMIER Biosoft, USA).

3.5 Bacterial Culture Media

Luria-Bertani (LB) broth

10 g/liter tryptone
5 g/liter yeast extract
10 g/liter NaCl

Dissolved the components in Milli-Q water, adjusted the pH to 7.5 with 1 N NaOH, and sterilized by autoclaving at 121°C for 15 min at 15 lbs PSI.

Luria-Bertani (LB) agar

For preparing LB agar medium, agar was added to a final concentration of 1.5% to LB broth. Heated the mixture to boiling to dissolve agar and sterilized by autoclaving at at 121°C for 15 min at 15 lbs PSI.

M9 Minimal media

50 ml 20X M9 salts

20 ml 20% glucose

1 ml 1 M $MgSO_4$

0.5 g/liter NaCl

Dissolved the components in 930 ml of autoclaved Milli-Q water

20X M9 salts

20 g NH_4Cl

60 g KH_2PO_4

120 g $Na_2HPO_4 \cdot 7H_20$

Autoclaved at 121°C for 15 min at 15 lbs PSI.

Super Optimal broth with Catabolite repression (SOC) medium

2% tryptone

0.5% yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM Glucose

Autoclaved at 121°C for 15 min at 15 lbs PSI.

Ampicillin

100 mg/ml in Milli-Q water, filter sterlised and stored in aliquots at -20°C

Kanamycin

50 mg/ml in Milli-Q water, filter sterlised and stored in aliquots at -20°C

Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 M)

Dissolved 238 mg/ml in Milli-Qwater, filter sterlised and stroed in aliquots at –20°C

3.6 Preparation of Competent Cells

Chemically competent cells of cloning hosts TOP10 (Invitrogen, USA), and expression hosts (BL-21(DE3) (Novagen, USA) were prepared by calcium chloride method (Sambrook *et al.,* 1989) and stored at -80°C refrigerator in aliquots.

Briefly, bacterial cells were streaked on LB agar plate for obtaining single colonies. A single colony was inoculated in 10 ml LB media and grown overnight at 37°C with shaking at 150 rpm. An aliquot of 5 ml of overnight culture was inoculated into 50 ml LB and incubated at 37°C for 2 h at 150 rpm to get *E. coli* cells at log phase. The culture (50 ml) was centrifuged at 6000 rpm for 10 min at 4°C. The supernatant decanted and cells were re-suspended by gentle mixing in 100mM $CaCl_2$ ($1/4^{th}$ original culture volume). The cell suspension was incubated in ice for 30 min with intermittent swirling and mixing. The cell suspension was centrifuged at 6000 rpm for10 min at 4°C. The supernatant was decanted and the cell pellet was suspended in 1 ml of 0.1 M $CaCl_2$. The competent cells were aliquoted to sterile micro centrifuge tubes to store at -80°C with addition of 10% glycerol.

3.7 Preparation of Clones of Expression Vector

The expression vector plasmids pET28b+ (Novagen, USA) and pET28b+GFP (Kindly provided by Dr. M. A. Pradeep, CMFRI) were used to transform competent cloning host TOP10 (Invitrogen, USA) by heat shock

method (Sambrook *et al.,* 1989) and glycerol stocks of clones of all the vectors were prepared and stored in -80°C refrigerator.

3.8 Reverse transcriptase PCR (RT-PCR) for First strand cDNA synthesis

RT-PCR involves use of reverse transcriptase enzyme to synthesize cDNA from mRNA. The RT-PCR reaction mainly involves two major steps, 1) Synthesis of the first strand cDNA and 2) Synthesis of second strand by PCR amplification.

Chemicals and enzymes

Anchored Oligo-dT [$d(T)_{24}VN$]

First Strand cDNA synthesis Kit (Fermentas, Germany)

PCR Thermocycler: S1000 (Bio Rad, Hercules, CA, USA)

Synthesis of the first strand cDNA

After ensuring the qualitative integrity by gel electrophoresis and quantification by spectrophotometer, one micrograms of total RNA isolated from eyestalk was reverse transcribed with First Strand cDNA Kit (Fermentas, Germany) using anchored Oligo$(dT)_{24}$ primer following the instructions given by the manufacturer. The reaction mixture comprised of 1X RT buffer, 3 mM $MgCl_2$, 0.5 mM dNTP, 1 μM anchored Oligo$(dT)_{24}$ primer and 1 U Reverse Transcriptase. The total RNA was initially incubated with anchored oligo dT and at 65°C for 5 min to remove secondary structures and to anneal poly A tail with anchored Oligo $(dT)_{24}$. The reaction mix was subjected to the following temperature profile; 70°C for 5 min, then 25°C for 5 min, 42°C for 60 min and 70°C for 10 min in a S1000 PCR machine (Bio Rad, Hercules, CA, USA). The cDNA synthesized was immediately used or stored at -20°C until use.

3.9 Preliminary characterization of the GIH genes

3.10 Amplification of partial GIH gene of *P. monodon* by RT-PCR

Primer selection and design

The general strategy followed for the PCR amplification was to select the gene specific primers from closely related species and amplify the conserved part of gene and design new species-specific primers from the sequence information.

The primers were designed for the *P. monodon* GIH gene based on the available GIH gene sequence deposited in NCBI, GenBank database from *Metapenaeus ensis*, *Homarus americanus, Nephrops norvegicus* (Table.3.1).

Table 3.1: Primers used for cross species amplification of GIH gene.

No	Primer Name	Primer sequences (5`-3`)	Primer Source
1	PMeF	CAGGAAGTGTCTCCAAGC	*Metapenaeus ensis (AF294648)*
	PMeR	TGTCAGAGCATCGCAGTA	
2	PLoF	GAGCCTTCCCTGTCATCA	*Homarus americanus (X87192)* *Nephrops norvegicus (AF163771)*
	PLoR	GGCGACTAAAATTCTACCAT	
3	PMoF	GCTGGCGATAGTGATTGT	*Metapenaeus ensis (AF294648)* *Homarus americanus (X87192) &* *Nephrops norvegicus (AF163771)*
	PMoR	GCGTTCAGGATGCTGAT	

Two pair of primers mentioned in the publication by Treerattrakool *et al.* (2008) and a set of 8 forward primers and 7 reverse primers were custom designed based on the GIH sequence of *P. monodon* from Thailand Waters

(GenBank accession: DQ643389) were custom synthesized to amplify GIH gene from *P. monodon* of Indian waters (Table 3.2).

Table 3.2: Primers designed based on Pem-GIH sequence (DQ643389)

No	Primer Name	Primer sequences (5`-3`)	Primer Source
1	GIH F	GAACGTCTCGTATAAAAGGTCTGCG	Treerattrakool *et al.,* (2008)
2	GIH R	GGTCGACTTTATTTTAACGGAAAATTAAT	
3	matGIH F	AACATCCTGGACAGCAAATGCAGGG	
4	matGIH R	CCGGCATTGAGGATGCTGAT	
5	PemF	GCCCCTGCTACATACTTCA	DQ643389
6	Pm1.1F	AGGTCTGCGAGCGAGCTAC	
7	Pm1.2F	CTGGATCAGCATCCTCAATG	
8	Pm1.3F	CGAACTCGAGCATTTCAGAC	
9	Pem2F	AGGTCTGCGAGCGAGCTAC	
10	Pm3F	AACGTCTCGTATAAAAGGTCTGC	
11	Pm4F	AGGTCTGCGAGCGAGCTA	
12	Pm5F	CCACACAGCTCCACAGGCA	
13	PemR	GGACCTATCCACTCCTAAA	
14	Pm1.1R	TGGGATGCTTTCAGAGAAGG	
15	Pm1.23R	GCACTGAGACCAGGGAAAAC	
16	Pem2R	GCACTGAGACCAGGGAAAAC	
17	Pm3R	CATTTCCAATGATCACGTTTAATAG	
18	Pm45R1	AAGCATCCGAATCGGCCTAGAGAT	
19	Pm45R2	AAGGAATACAGCCAAGGCGTCGAA	

Table 3.3: Primers used for amplification of GIH CDS in *P. monodon.*

Si No	Primer Name	Primer Sequence (5`-3`)
1	PmGIH Gene F	ATGAAAACATGGCTGCTATTAGCG
2	Pm1.1R	TGGGATGCTTTCAGAGAAGG

The first strand cDNA synthesized was used as the template to amplify the *P. monodon* GIH with the primer mentioned in Table 3.3 using *Pfu* DNA polymerase. The reaction mixture composed of 1x PCR buffer with, 2 mM $MgSO_4$, 0.2 mM each dNTP, 0.5 μM of each primers and 1.25 U *Pfu* DNA polymerase and 1 μg of template cDNA. The PCR reaction conditions were 95˚C for 3 min then 35 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 60 sec followed by a final extension at 72˚C for 10 min.

3.11 Amplification of partial GIH gene of *F. indicus* by RT-PCR

Primer selection and design

The first strand cDNA synthesized was used as the template to amplify the GIH gene from *F. indicus* using the primers used to amplify GIH gene from *P. monodon* (PmGIH Gene F & Pm1.1R) following reaction conditions as above.

The nucleotide sequence of the PCR product obtained was analyzed and specific primers viz. FiGIH Gene F & FiGIH Gene R were designed based on that sequence to amplify the GIH coding sequence from *F. indicus.*

Table 3.4: Primers used for amplification of GIH CDS in *F. indicus*.

Si No	Primer Name	Primer Sequence (5`-3`)
1	FiGIH Gene F	ATGAGAACATGGCTGCTATTAACG
2	FiGIH Gene R	TCACCATGGCCGGCCGGCATT

These primers (Table 3.4) were used to amplify the partial GIH gene from *F. indicus* using the same reaction mix and cycling conditions as above.

Submarine electrophoresis, purification and quantification of RT-PCR Product.

The products from PCR reactions were analyzed on a 1.5% agarose gel in 1x TBE buffer with ethidium bromide (µg/ml). Samples mixed with sample dye were loaded and the electrophoresis carried out in a sub marine electrophoresis unit (Hoefer Inc., USA) with a constant voltage of 80 V. The gel was documented in ChemiDoc™ XRS+ System (Bio Rad, Hercules, CA, USA).

The amplified product was cleaned up using the MinElute® PCR Purification Kit (Qiagen GmbH, Hilden, Germany) system as described by the manufacturer.

Procedure

- Five volumes of buffer PB was addedto 1 volume of the PCR reaction and mixed.
- MinElute column was placedin the 2 ml collection tube.
- Sample was applied to the MinElute column and centrifuged for 1 min at 10000 rpm.
- Flow-through was discarded and MinElute column was placed back into the same collection tube.
- 750 µl Buffer PE was added to the MinElute column to wash away unvanted molecuules and centrifuged for 1 min at 10000 rpm.
- Flow-through was discarded and the MinElute column was placed back in the same collection tube.
- The column was centrifuged for 1 min at maximum speed.
- The MinElute column was placed in a sterile 1.5 ml microcentrifuge tube.

- The DNA was eluted by adding 10 µl Buffer EB (10 mM Tris-HCl, pH 8.5) to the center of the membrane, let the column stand for 1 min, and then centrifuging for 1 min.

The purified PCR product was then quantified using the BioPhotometer® plus with Hellma TrayCell (Eppendorf , Hamburg, Germany).

3.12 Cloning and sequencing of the PCR amplified products

Bacterial Strain used for cloning:

TOP10 (Invitrogen, USA) *E. coli* cloning host cells were made chemically competent and used for transformation with the recombinant plasmid.

CloneJET™ PCR Cloning Kit

pJET 1.2 Blunt-end vector *(50 ng/µl)*

In order to clone PCR amplified products, the pJET1.2/blunt vector (Fermentas, Germany), was used. PJET1.2/blunt is a linearized cloning vector, which accepts inserts from 6 bp to 10 kb.

Blunt-end PCR products generated by proofreading DNA polymerases (*eg. Pfu*) can be directly ligated with the pJET1.2/blunt cloning vector. All common laboratory *E.coli* strains were directly transformed with the ligation product. Only recombinant clones containing the insert appear on culture plates as the re-circularized or self-ligated pJET1.2/blunt vector expresses a lethal restriction enzyme in the host after transformation and hence blue/white screening was not required.

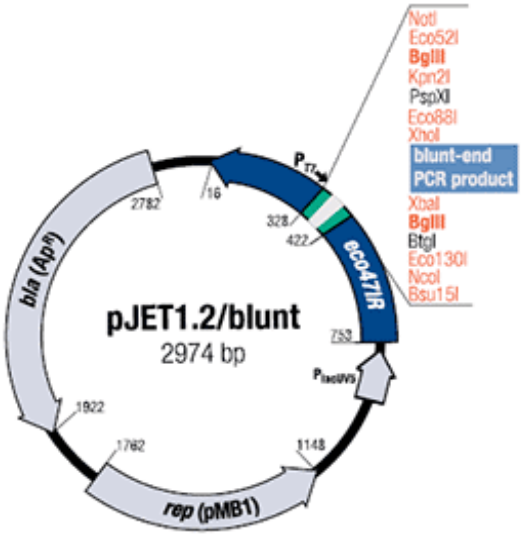


Figure 3.1: Schematic diagram of PCR cloning vector pJET1.2

Ligation of cDNA

The ligation reaction of the PCR amplified cDNA with the cloning vector was carried out as described in the manufacturer's protocol (Fermentas, Germany). The ligation protocol in brief was as follows:

2X reaction buffer	10.0 µl
PCR product (PmGIH/FiGIH cDNA)	x µl
pJET1.2/blunt end cloning vector (50ng/µl)	1.0 µl
Milli-Q water	x µl
T4 DNA ligase	1.0 µl
Total	**20.0 µl**

After giving a short spin, the ligation mixture was incubated at 22°C for 10 min and directly used for transformation.

Transformation protocol

- An aliquot of 5 µl of ligation mixture was added to 50 µl of competent cells and mixed gently by flicking. The mixture was incubated on ice for 30 min.
- The tubes were then transferred to water bath maintained at 42°C and held for exactly 90 sec. The tubes were immediately transferred back on ice and allowed to chill for 1 min.
- To the above mix, 50 µl of SOC media was added and mixed. The tubes were incubated at 37°C for 1 h in a rotary shaker to allow the bacteria to recover and develop the antibiotic resistance. After incubation, 75 µl of transformed mixture was spread evenly on LB agar plates with ampicillin (100 µg/ml) and incubated at 37°C for 16-20 h.

Confirmation of the cloned GIH genes

Colony PCR

The transformants obtained on LB agar plates with ampicillin (100 µg/ml) were screened using colony PCR with vector specific primers pJET1.2F (5`-CGA CTC ACT ATA GGG AGA GCG GC-3`) and pJET1.2R (5`-AAG AAC ATC GAT TTT CCA TGG CAG-3`) to confirm the presence of the insert DNA.

A small portion of selected colonies picked up from the transformed plate using sterile toothpicks were dispensed into the PCR reaction mix composed of 1x PCR buffer with, 2 mM $MgSO_4$, 0.2 mM each dNTP, 0.5 µM of each primers (pJET1.2F and pJET1.2R) and 0.5 U *Pfu* DNA polymerase. The PCR reaction conditions were 95°C for 5 min then 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec followed by a final extension at 72°C

for 10 min. Electrophoresis was performed on 1% agarose gel prepared in 1x TBE buffer and stained with ethidium bromide.

Colonies with expected sized products were inoculated into LB broth containing ampicillin (100 µg/ml) and incubated in shaking incubator at 37°C for 12-16 h after which the cells were pelleted and used for plasmid isolation.

Plasmid Isolation

Column based GeneJET Plasmid Miniprep Kit (Fermentas, Germany) was used to isolate recombinant plasmids from *E. coli* transformants. The protocol for plasmid DNA isolation was followed as supplied by the manufacturer.

Protocol

- Pelleted cells from 1-5 ml of overnight culture by centrifuging at high speed for 1 min and discarded the supernatant.
- Suspended cells in 200 µl resuspension solution. Pipetted up and down. 200 µl of lysis solution was added and inverted gently to mix.
- 350 µl of neutralization solution was added, inverted, and mixed 4-6 times. Pelleted the debris at high speed for 10 min.
- Transferred cleared lysate into binding column, centrifuged for 1 min. at high speed, and discarded flow-through.
- 750 µl of the wash solution was added to column and centrifuged for 1 min at high speed and discarded flow-through. Spinned for 1 min. again to dry column.
- Transferred the column to a new collection tube and eluted the plasmid DNA by adding 50 µl nuclease-free water and centrifugation at high speed for 1 min.

Preservation of recombinant Clones & Plasmids

Positive colonies were picked and grown in LB media (with 100µg/ml ampicillin) to log phase, followed by transfer of the culture to a sterile glycerol solution in cryovials to a final concentration of 10%. The vials after labeling were stored at –80°C. The eluted recombinant plasmid DNA were examined by electrophoresis for integrity and quantified using spectrophotometer and stored at -20°C.

3.13 Characterization of complete mRNA of Gonad Inhibiting Hormone (GIH) gene of Penaeid shrimps, *P. monodon* and *F. indicus* by Random Amplification of cDNA Ends (RACE)

Since the initial PCR using the cDNA followed by its sequencing revealed only partial sequence information of the GIH gene the technique of RACE was used to amplify the complete mRNA of GIH gene from *P. monodon* and *F. indicus*. The RACE products were cloned and sequenced for the complete characterization of the GIH gene.

SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) was used for carrying out the RACE. Details of the primers used for RACE-PCR, protocols for generating RACE-Ready first-strand cDNA, amplification of the 5` & 3` ends from first-strand cDNA and **c**loning and sequencing of RACE-PCR products are presented below.

Primers used for RACE

SMARTer II A Oligonucleotide

5`–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3`

3`-RACE CDS Primer A

5`–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ V N–3`

(N = A, C, G, or T; V = A, G, or C)

5`-RACE CDS Primer A

5`–$(T)_{25}$VN–3`

(N = A, C, G, or T; V = A, G, or C)

10X Universal Primer A Mix (UPM)

Long (0.4 µM):

5`-ctaatacgactcactatagggcAAGCAGTGGTATCAACGCAGAGT-3`

Short (2 µM):

5`–ctaatacgactcactatagggc –3`

Nested Universal Primer A (NUP)

5`–AAGCAGTGGTATCAACGCAGAGT–3`

Gene Specific Primers (GSPs)

PmGIH Gene F – 5`–ATGAAAACATGGCTGCTATTAGCG–3`

Pm1.1R – 5`–TGGGATGCTTTCAGAGAAGG–3`

FiGIH Gene F- 5`–ATGAGAACATGGCTGCTATTAACG–3`

FiGIH Gene R- 5`–TCACCATGGCCGGCCGGCATT–3`

Generating RACE-Ready first-strand cDNA

Total RNA isolated from the eyestalk neural tissue was used for synthesising first-strand RACE-Ready cDNA. The 5` RACE and 3`RACE reactions were carried out according to the manufacturers protocol of the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA). Two separate 10 µl reactions were set up to convert 1µg of total RNA into RACE Ready first-strand cDNA.

Protocol

1. For each 10 µl 5`- & 3`-RACE-Ready cDNA synthesis reactions, the following reagents were mixed and spin briefly in a microcentrifuge, and then set aside at room temperature until Step 7.

5X First-Strand Buffer	2.0 µl
DTT (20 mM)	1.0 µl
dNTP Mix (10 mM)	1.0 µl
Total Volume	**4.0 µl**

2. The following reagents were combined in separate microcentrifuge tubes:

For preparation of 5`-RACE-Ready cDNA	**For preparation of 3`-RACE-Ready cDNA**
1.0–2.75 µl RNA*	1.0–3.75 µl RNA*
1.0 µl **5**`-CDS Primer A	1.0 µl **3**`-CDS Primer A

3. Sterile Milli-Q water was added to the tubes from Step 2 to a final volume of 3.75 µl for 5` RACE and 4.75 µl for 3` RACE.
4. Contents were mixed and the tubes were briefly spun in a microcentrifuge.
5. The tubes were incubated at 72°C for 3 min, and then cooled at 42°C for 2 min. After cooling, the tubes were briefly spun for 10 seconds at 14,000 g to collect the contents at the bottom.
6. To just the 5` RACE cDNA synthesis reaction(s), the SMARTer II A oligo was added 1 µl per reaction.
7. Prepared enough of the following Master Mix for all 5`- & 3`-RACE-Ready cDNA synthesis reactions. The reagents were mixed at room temperature in the following order:

Buffer Mix from Step 1	4.0 µl
RNase Inhibitor (40 U/µl)	0.25 µl
SMART Scribe Reverse Transcriptase (100 U)	1.0 µl
Total Volume	**5.25 µl**

8. 5.25 µl of the Master Mix from Step 7 was added to the denatured RNA from Step 5 (3`-RACE cDNA) and Step 6 (5` RACE cDNA), for a total volume of 10 µl.
9. The contents of the tubes were mixed gently by pipetting, and the tubes were spun briefly to collect the content at the bottom.
10. The tubes were incubated at 42°C for 90 min in Veriti® Thermal Cycler (Applied biosystems).
11. After the incubation, the tubes were heated at 70°C for 10 min.
12. The first-strand reaction product were dilute with Tricine-EDTA Buffer and stored at -20°C until use.

3.14 Amplification of the 5` & 3` ends from first-strand RACE ready cDNA

The reactions were carried out according to the protocol of the Advantage 2 PCR Kit (Clontech, USA). Enough PCR Master Mix was prepared for all of the PCR reactions ensuring sufficient volume. The same master mix was used for both 5` and 3`RACE reactions. For each 50µlPCR reaction, the following reagents were mixed

PCR-Grade Water	34.5 µl
10X Advantage 2 PCR Buffer	5.0 µl
dNTP Mix (10 mM)	1.0 µl
50X Advantage 2 Polymerase Mix	1.0 µl
Total Volume	**41.5 µl**

Mixed well by vortexing and briefly spun the tube in a microcentrifuge.

For 5` RACE a 2.5 µl aliquot of 5` RACE ready cDNA of *P. monodon* and *F. indicus* were amplified with primers UPM as forward primer and Pm1.1R and Fi GIH gene R as reverse primers respectively, and for 3` RACE a 2.5 µl aliquot of 3` RACE ready cDNA of *P. monodon* and *F. indicus* were amplified with primers PmGIH gene F and Fi GIH Gene F as forward primer respectively and UPM as reverse primer (Table 3.5). PCR reaction were performed with Advantage 2 PCR Kit according to manufacturer's instructions.

Table 3.5: Composition of RACE PCR mix.

Component	**5`-RACE**	**3`-RACE**
5`-RACE-Ready cDNA	2.5 µl	-
3`-RACE-Ready cDNA	-	2.5 µl
UPM (10X)	5 µl	5 µl
GSP1 (10 µM) Pm1.1R/ PmGIH gene R	1 µl	-
GSP2 (10 µM) PmGIH gene F/ Fi GIH Gene F	-	1 µl
Milli-Q water	-	-
Master Mix	41.5 µl	41.5 µl
Final Volume	**50 µl**	**50 µl**

For 5`-RACE & 3`-RACE the amplifications were performed in Veriti® Thermal Cycler (Applied Biosystems) with 25 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 3 min.

3.15 Cloning and sequencing of RACE-PCR products

RACE PCR products were cloned in to pJET1.2/blunt cloning vector and transformed into TOP10 (Invitrogen, USA) competent cells. After colony PCR conformation the select recombinants were sub cultured, plasmid DNA was isolated, and custom sequenced using vector specific primers.

3.16 Construction of recombinant vector system for protein expression

Bacterial Strains and Vectors for Expression

BL21 (DE3) (Novagen, USA)

The BL21 (DE3) bacteria are the most widely used *E. coli* expression host for recombinant protein expression using the T7 promoter. It was with chromosomal copy of the gene for T7 RNA polymerase (T7 gene) and was deficient in the Lon and OmpT proteases. BL21 (DE3) *E.coli* strain was a lysogen of bacteriophage DE3, a λ derivative that has the immunity region of phage ***21*** and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase. The T7 RNA polymerase was under the control of the lacUV5 promoter (Studier and Moffat, 1986; Studier *et al.,* 1990) and the LacI produced by the *lacI* gene represses the expression of the T7 RNA polymerase. Consequently, the expression of the T7 RNA polymerase was inducible by isopropyl-β-D-thiogalactoside (IPTG). Addition of IPTG to the growing culture of the BL21 (DE3) strain containing the expression plasmid induced the expression of the T7 RNA polymerase, which in turn transcribed the gene of interest inserted downstream to T7 RNA promoter in the plasmid expression vector.

Plasmids:

pET-28b+ (5368 bp) Bacterial expression vector plasmid with T7 promoter and kanamycin resistance obtained from Novagen (Figure 3.2). This vector was designed for expression of the recombinant protein in *E. coli* system as fusion protein and tags for purification. The expression can be controlled by T7 promoter under the induction of IPTG.

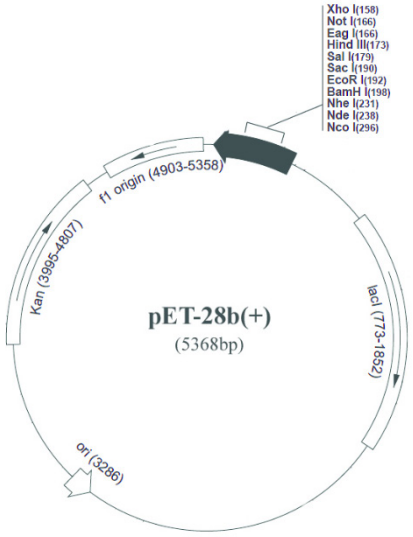


Figure 3.2: Schematic diagram of expression vector pET-28b+

pET-28b+GFP (6073 bp) plasmid was kindly provided by Dr.M.A Pradeep. This vector was designed for expression of the recombinant protein in *E. coli* system as fusion protein with Green Flurescent Protein (GFP) gene of jellyfish *Aequorea victoria* amplified using specific primers from pEGFP-N1 vector (Clontech, USA). The expression can be controlled by T7 promoter under the induction of IPTG.

Culture media for *E. coli* expression system

LB (Luria-Bertani) broth and agar medium and M9 minimal medias were utilized for the culture of recombinant *E.coli* BL21 (DE3) cells. Under selective condition, Kanamycin antibiotic final concentration of 50 µg/ml was added into the culture medium according to the characteristics of the recombinant plasmid.

Designing of primer for amplification of cDNA encoding *P.monodon* GIH (PmGIH) for cloning in expression vector.

For *E. coli* expression system, the recombinant plasmids were constructed using pET-28b+ and pET-28b+GFP as expression vectors.

For directional cloning of the gene into expression vector, the open reading frame of the gene with and without its signal peptide coding region was inserted downstream to the promoter region of the expression vector by using the respective cloning site present in the multiple cloning sites (MCS) of the vector. Restriction enzyme (RE) sites were choosen such that the gene could be inserted into the vector for recombinant expression with no or minimal extraneous vector sequences being expressed. Further the presence of internal restriction sites in the gene was analyzed for the chosen restriction enzymes. Furthermore, two restriction enzymes used should not produce compatible ends on digestion. Primers containing restriction enzyme sites were designed with appropriate number of extra nucleotides required for proper action of the restriction enzyme.

Table 3.6: Primers used for Cloning GIH genes in ExpressionVectors.

Sl. no	Primer name	Primer sequence (5`-3`)	Restriction Site	Expression Vector
1	**GIH_Nco1.nosig_F**	ATTATTCCATGGCTAACATCCT GGACAGCAAATGCAGGGG	*Nco*I	***pET28b+***
	GIH_ Not1.Nstp_ R	TATATAGCGGCCGCCCACGGCC GGCCGGCATTGAGG	*Not*I	
2	**GIH_Nco1_F**	GGTGGTCCATGGGCAAAACATG GCTGCTATTAG C	*Nco*I	***pET28b+***
	GIH_ Not1.Nstp_ R	TATATAGCGGCCGCCCACGGCC GGCCGGCATTGAGG	*Not*I	
3	**GIH_Nco1_F**	GGTGGTCCATGGGCAAAACATG GCTGCTATTAG C	*Nco*I	***pET28b+GFP***
	GIH_ HindIII.Nstp_ R	ATATATTTAAGCTTCCACGGCC GGCCGGCATTGAG	*Hind III*	

Pm GIH without signal peptide for pET-28b+

Primers GIH_Nco1.nosig_F (5`-ATT ATT CCA TGG CTA ACA TCC TGG ACA GCA AAT GCA GGG G-3`) and GIH_ Not1.Nstp_ R (5`-TAT ATA GCG GCC GCC CAC GGC CGG CCG GCA TTG AGG-3`) was designed specifically to amplify the mature peptide coding region encoding PmGIH to be inserted into expression vector pET-28b+ (Novagen, USA).

Pm GIH With signal peptide for pET-28b+

The complete coding sequence (CDS) of GIH gene including the signal peptide region was amplified using Primers GIH_Nco1F (5`-GGT GGT CCA TGG GCA AAA CAT GGC TGC TAT TAG C-3`) contained the Nco1 site and GIH_ Not1. Nstp_ R (5`-TAT ATA GCG GCC GCC CAC GGC CGG CCG GCA TTG AGG-3`) designed with the Not1 restriction sites specifically to be inserted into expression vector pET-28b+ (Novagen, USA).

Pm GIH with signal peptide for pET-28b+ GFP

Primers GIH_Nco1F (5`-GGT GGT CCA TGG GCA AAA CAT GGC TGC TAT TAG C-3`) and GIH_ HindIII.Nstp_ R (5`-ATA TAT TTA AGC TTC CAC GGC CGG CCG GCA TTG AG-3`) was designed specifically to amplify the complete coding sequence (CDS) encoding PmGIH gene including the signal peptide region to be inserted into expression vector pET-28b+GFP.

The sequence shown in red colour was restriction enzyme recognition sequences. The sequence on left is 5` overhang required for proper activity of restriction enzyme. The translation will start from ATG codon present in the NcoI recognition site. Reverse primers does not contain internal stop codon giving a recombinant fusion protein with vector encoded hexahistidine residues at C-terminal.

PCR amplification of the inserts of *P.monodon* GIH (*PmGIH*) (with and with out signal peptide) for *E. coli* expression vectors pET28+ and pET28+GFP.

PCR amplification was carried out using plasmid (pJET-PmGIH & pJET-FiGIH) containing CDS of GIH as a template, and specific primers for incorporating the designated restriction sites in the PCR product. This facilitated the in-frame cloning in to the expression vectors.

A 50 µl PCR reaction was set up to amplify the gene with the following components added sequentially to a nuclease free PCR tube. The respective set of primers for inserting designated restriction sites in the PCR product for cloning in expression vector were used as described in Tables 3.6.

Components	Volume / concentration
Nuclease-free water	X µl
10X Taq buffer (with MgSO4)	5.0 µl
dNTPs (10mM)	1.0 µl
Template DNA	1.0 µl
Forward primer	0.5 µM
Reverse primer	0.5 µM
Pfu DNA polymerase (1.25U)	0.5 µl
Total mix	50.0 µl

PCR thermal cycling conditions

The PCR reaction was performed using automated S1000 Thermal cycler (BioRad, Hercules, CA, USA). The PCR reaction conditions were 95˚C for 3 min then 35 cycles of 95˚C for 30sec, 58˚C for 30 sec and 72˚C for 60 sec followed by a final extension at 72˚C for 10 min.

Agarose Gel Electrophoresis of PCR Product, Purification and Quantification

Agarose gel electrophoresis of the PCR Product was carried out in 1.5% agarose gel at 8 V/cm for an hour. After completion of electrophoresis, the gel was examined and documented under ChemiDoc™ XRS+ System (BioRad, Hercules, CA, USA).

The amplified product was cleaned up using the MinElute®PCR Purification Kit (Qiagen GmbH, Hilden, Germany) as described by the manufacturer. The purified PCR product was then quantified using the BioPhotometer® plus with Hellma TrayCell (Eppendorf, Hamburg, Germany).

Restriction Digestion of Vectors and PCR Products for Subcloning

Using the recombinant cloning vector (pJET-PmGIH) as template, the Pm GIH gene was amplified using the three set of primers designed with restriction sites in their 5` ends (Table 3.6).The amplified PCR products were digested with respective restriction enzymes.

For cloning of PmGIH genes into pET28b+ & pET28b+GFP vectors, the purified vector plasmids as well as the PCR products were double digested using the restriction enzymesunder conditions given in Tables 3.7 & 3.8.

Table 3.7 : Components of restriction digestion mix for pET28b+ vector & PCR Products

Components	***Volume***
pET28b+ vector	X µl
10X RE buffer (3.1)	2 µl
NcoI	0.5 µl
NotI	0.5 µl
Milli-Q water	Xµl
Total volume	**20 µl**

Components	***Volume***
PCR product	X µl
10X RE buffer (3.1)	4 µl
NcoI	0.5 µl
NotI	0.5 µl
Milli-Q water	X µl
Total volume	**20 µl**

Table 3.8 : Components of restriction digestion mix for pET28b+GFP vector & PCR Product

Components	*Volume*
pET28b+GFP vector	X µl
10X RE buffer (2.1)	2 µl
NcoI	0.5 µl
Hind III	0.5 µl
Milli-Q water	Xµl
Total volume	20 µl

Components	*Volume*
PCR product	X µl
10X RE buffer (3.1)	4 µl
NcoI	0.5 µl
Hind III	0.5 µl
Milli-Q water	X µl
Total volume	20 µl

Reaction mixture for the double digestion of both PCR product and vectors were incubated at 37°C for 16 h. After digestion, the enzymes were inactivated at 80°C for 20 min (for vector digestion) and at 65°C for 20 min (for PCR product digestion).

Extraction of the digested products from the Agarose Gel

After restriction digestion, the digested vectors and the PCR products were separated using 1% agarose gel electrophoresis. The separated products were observed under UV transilluminator and excised immediately. Digested products were purified from the gel as per manufacturer's protocol using the MinElute® Gel Purification Kit (Qiagen GmbH, Hilden, Germany) and quantified by using BioPhotometer® plus with Hellma TrayCell (Eppendorf , Hamburg, Germany).

Protocol for DNA extraction from gel:

1. After electrophoresis, excised DNA band from gel with a clean, sharp scalpeland placed gel slice in a 1.5 ml pre weighed microcentrifuge tube.
2. Weighed the gel slice in the tube and 3 volumes of Buffer QG to 1 volume of gel was added.

3. Incubated at 50°C for 10 min until the gel slice has completely dissolved with intermittent mixing by vortexing the tube every 2–3 min.
4. After the gel slice dissolved completely 1 gel volume of isopropanol was added to the sample and mixed by inverting the tube several times.
5. MinElute columnwas placein the provided 2 ml collection tube in a suitable rack.
6. To bind DNA, the sample was applied to the MinElute column, and centrifuged for 1 min at 10,000 x g.
7. The flow-through was discarded and the MinElute column was placed back in the same collection tube.
8. 500 µl of Buffer QG was added to the spin column and was centrifuged for 1 min at 10,000 x g.
9. The flow-through was discarded and was placed to the MinElute column back in the same collection tube.
10. 750 µl of Buffer PE was added to the MinElute column to washand centrifuged for 1 min at 10,000 x g.
11. The flow-through was discarded and the MinElute column was centrifugedfor an additional 1 min at ≥10,000 x g.
12. The MinElute column was placed into a 1.5 ml microcentrifuge tube.
13. 10 µl of Buffer EB or water was added to the center of the membrane and the column was let to stand for 1 min and then was centrifuged for 1 min to elute DNA
14. The column was discarded and DNA was stored at -20°C

Ligation of PCR Products with the Expression Vectors

The restriction digested PCR product thus purified was directionally ligated into the expression vector pET-28b+ and pET-28b+GFP downstream to T7 promoter before C-terminal hexa-histidine (6xHis) tag using T4 DNA ligase

(Fermentas, Germany). The digested PCR product and expression vector pET-28b+ and pET-28b+GFP were identically treated before the ligation reaction was carried out as described by the respective suppliers. The ligation mix is in given is in Table 3.9.

Table 3.9 : Ligation mix for expression vector construction

Linear vector DNA	5-10 µl (50-400 ng)
Insert DNA (PCR product)	Used a 3:1 molar ratio of insert DNA termini to vector
10X ligation buffer	2 µl
Nuclease-free water	Upto a volume of 20 µl
T4 DNA Ligase (5 U/ µl)	0.2-0.4 µl (1-2 u)

The reaction tube containing the ligation mix was spinned for 5-10 seconds. The ligation reaction was carried out for 6 to 8 hour at 22°C. Then the ligated mixture was used for transformation into TOP10 Chemically Competent *E. coli* cells (Invitrogen, USA) following the protocol described by Sambrook *et al.* (1989).

Confirmation of Positive Clones

The transformants were selected on LB agar plates containing kanamycin (50 µg/ml). The transformants were screened for the presence of insert by colony PCR using GIH specific primers matGIHF (5`-AAC ATC CTG GAC AGC AAA TGC AGG G-3`) & matGIHR (5`-CCG GCA TTG AGG ATG CTG AT-3`). Positive clones were selected for plasmid purification using QIAprep kit (Qiagen GmbH, Hilden, Germany) to reconfirm the presence of insert by automated DNA sequencing (SciGenom Labs, India). The remaining plasmids were labelled and stored at -20°C.

Preservation of recombinant Clones

Positive colonies were picked and grown in LB media (with 50 µg/ml kanamycin) to log phase, followed by transfer of the culture to a sterile glycerol solution in cryovials to a final concentration of 10%. The vials after labeling were stored at –80°C.

3.17 Expression of Recombinant Protein in *E. coli*

Transformation of recombinant plasmids into expression hosts

The recombinant plasmids named pET28b-*PmGIH_nosig*, pET28b-*PmGIH_withsig* and pET28b-*PmGIH_GFP* were transformed into chemically competent BL21 (DE3) (Novagen) expression host by heat shock method and plated on LB agar plates containing kanamycin (50 µg/ml).

Induction of expression using IPTG

A single colony from a freshly streaked plate was picked and inoculated into 5 ml LB agar plates containing kanamycin 50 µg/ml. The culture was incubated in a shaking incubator at 37°C for overnight. 1% of the overnight culture was sub-cultured in two test tubes of 2 ml LB broth medium containing kanamycin each marked induced and uninduced. The tubes with culture was further incubated at 37°C with shaking at 225 rpm until the optical density (OD) at 600 nm reached approximately 0.5- 0.6 (3-4 h approximately). Subsequently, the expression of the recombinant protein was induced by IPTG under the control of T7 promoter at a final concentration of 0.1-1 mM. The tubes were further incubated at 37°C with shaking for 4 -5 h. An aliquot of the induced culture were collected at different time points and then centrifuged at 12,000 rpm for 1 min. The cells were resuspended in a final concentration of 1x sample

buffer, and the pattern of protein expression was analyzed by Tricine SDS-PAGE.

Optimization of expression level and soluble expression

To increase the expression of recombinant protein, different concentrations of IPTG were tried. Incubation time, temperature and culture media were also optimized to facilitate the soluble expression and easy downstream processing.

Analysis of expression of recombinant PmGIH

The expression of target genes was assessed quickly by analysis of the total cell protein on a Tricine SDS-PAGE followed by Coomassie Brilliant blue staining as below.

1. 1 ml of the induced and the uninduced culture were taken and centrifuged at 10,000x*g* for 1 min.
2. The supernatant was collected in a fresh tube, and total protein was precipitated by adding equal volume of 100% ethanol.
3. The cell pellet and total protein pellet precipitated from media fraction were taken in seperate tubes and resuspended by mixing in 100 µl sterile Milli-Q water
4. 100 µl of 4X SDS sample buffer was added and mixed thoroughly.
5. Immediately the sample was heated in boiling water for 3-5 min to denature the proteins and used for Tricine SDS-PAGE analysis.

Analysis of Solubility of the Expressed Protein

1. The cells were harvested by centrifugation of 5 ml culture for 10 min at 10,000 rpm. The supernatant was decanted and the pellet was resuspended in 0.5 ml cold 20mM Tris-HCl, pH 7.5
2. The cells were lysed by freeze thaw method.

3. The entire lysate was centrifuged for 10 min at 12,000 rpm to separate soluble and insoluble fractions.
4. For Tricine SDS-PAGE analysis, 100 µl of soluble supernatant was transferred and 100 µl of 4X SDS sample buffer was added and immediately heated for 3 min. at 85°C to denature proteins and then was stored at -20°C until Tricine SDS-PAGE analysis.

Insoluble Cytoplasmic Fraction of the Expressed Protein

1. The insoluble pellet obtained above was washed by resuspending in 500 µl , 20 mM Tris-HCl, pH 7.5 and centrifuged at 10,000 rpm for 5 min. Then the supernatant was removed and wash was repeated.
2. The final pellet was resuspended in 1.5 ml 1% SDS with heating and vigorous mixing.
3. 100 µl sample was removed and was combined with 100 µl 4X SDS sample buffer and was immediately heated for 3 min. to denature the proteins for Tricine SDS-PAGE analysis.

3.18 Tricine–SDS Poly Acrylamide Gel Electrophoresis (Tricine–SDS-PAGE)

Tricine–SDS-PAGE was used to separate proteins in the mass range 1–100 kDa and was the preferred electrophoresis system for the resolution of proteins smaller than 30 kDa.

Composition of stock solutions for use in Tricine SDS-PAGE:

Acrylamide / bis acrylamide (37.5% T, 1% C) 30%

$$\text{Total monomer concentration (\% T)} = \frac{[\text{Acrylamide (g)} + \text{bis} - \text{acrylamide (g)}]}{(\text{Total volume})} \text{x } 100$$

$$\text{Cross linking monomer (\% C)} = \frac{\text{bis} - \text{acrylamide (g)}}{[\text{Acrylamide (g)} + \text{bis} - \text{acrylamide (g)}]} \text{x } 100$$

Dissolved 30 g Acrylamide and 0.8 g bis–acrylamide in 50 ml Milli-Q water and final volume was made to 100 ml with Milli-Qwater, and stored at 4°C in an amber coloured bottle.

3x Gel Buffer (3 M Tris-Cl, 0.3% SDS, pH 8.45)

Dissolved 36.4 g Tris base in 50 ml Milli-Q water. The pH was adjusted to 8.45 with 6 N HCl and final volume was made to 100 ml with Milli-Q water and finally 0.3 g SDS was addded and was stored at 4°C**.**

10x Cathode Running Buffer (0.1 M Tris, 0.1 M Tricine, and 0.1% SDS)

Dissolved 12.11 g Tris base, 17.92 g Tricine and 1g SDS in 50 ml Milli-Q water, and without adjusting the pH the final volume was made to 100ml with Milli-Q water and stored at 4°C

10x Anode Running Buffer (0.2 M Tris-HCl, pH 8.9)

Dissolved 24.22 g Tris base in 50ml Milli-Q water. pH was adjust to 8.9 with 6 N HCl and final volume was made to 100 ml with Milli-Q waterand store at 4°C.

1 M Tris-HCl, pH 6.8

Dissolve 12 g Tris base in 50 ml Milli-Q water. The pH was adjusted with 6 N HCl and final volume was made to 100 ml with Milli-Q water and stored at 4°C.

2x Tricine sample buffer

Mixed 1 ml 1 M Tris-Cl pH 6.8, 2.4 ml glycerol, 0.8 g SDS, 2 mg Coomassie blue G-250, 1 ml β-mercapto ethanol and final volume was made to 10 ml with Milli-Q water and stored at 4°C.

Ammonium persulfate(APS) 10%

Tetramethylethylenediamine (TEMED)

15% Separating gel Preparation

Milli-Q water	0.516 ml
Glycerol	1.00ml
3X gel buffer	3.33ml
Acrylamide / Bis (30% stock)	5ml
10% APS	150µl
TEMED	4 µl
Total Volume	**10ml**

5% Stalking Gel Preparation

Milli-Q water	2.4ml
3X gel buffer	1.67ml
Acrylamide / Bis (30% stock)	840µl
10% APS	75µl
TEMED	5 µl
Total Volume	**5ml**

Tricine SDS-PAGE Electrophoresis

The gel was cast in a Mini-PROTEAN®Tetra Cell (10x8cm) (Bio-Rad Laboratories, Inc, USA). After the polymerization, the gel assembly was transferred in to a tank containing the 1x running buffers. The cathode and anode tanks were filled and electrophoresis was carried at constant current of 10 mA per gel till the dye reached separating gel, then the current was increased to 20 mA/gel. After the electrophoresis the gel was removed and proteins were fixed by staining in the Coomassie stain solution.

Coomassie Blue staining and Destaining

Fixing and staining was carried out simultaneously in a solution containing 0.1% Coomassie blue R-250 in fixative (40% Methanol and 10% glacial Acetic acid). Afterwards the destaining of the gel was carried out by several changes of 40% methanol + 10% glacial acetic acid mixture to remove

the background stain. The gels were documented under ChemiDoc™ XRS+ System (BioRad, Hercules, CA, USA)

Large scale expression and purification

A single colony of the *E. coli* BL21(DE3) harboring the recombinant plasmids (pET28b-PmGIH_*Nosig*, pET28b-PmGIH_*Withsig and* pET28b-PmGIH_*GFP*) was inoculated into 10 ml of LB broth containing 50 µg/ml kanamycin and incubated overnight at 37°C with 225 rpm shaking. The overnight cultures containing pET28b-PmGIH_*Nosig &* pET28b-PmGIH_*GFP* were used to incoculate fresh LB broth containing 50 µg/ml kanamycin to yield the final dilution of 1:100. The culture containing pET28b-PmGIH_*Withsig* was used to inoculate fresh M9 minimal media containing 50 µg/ml kanamycin to yield the final dilution of 1:100. The induced cultures were incubated at 37°C with shaking at 225 rpm until OD600 of the culture reached 0.5-0.6. Once the O.D. was reached IPTG was added into the culture at a final concentration of 0.4 mM to express the recombinant protein under the control of T7 promoter. The induced culture in LB media was incubated at 37°C with shaking at 225 rpm for 4-5 hours whereas, the culture in M9 minimal media was incubated at 16°C with shaking at 225 rpm overnight to increase the percentage of target protein found in soluble form and easy down stream processing.

The bacterial cells and media fractions were harvested after induction by centrifugation. The pellet and supernatant were stored at -80°C until analysis. After cell lysis, soluble, insoluble and media fractions were subjected to 15% Tricine SDS Polyacrylamide gel electrophoresis (Tricine – SDS PAGE) (Schagger, 2006) under native and denaturing conditions.

3.19 Purification of recombinant protein expressed in *E.coli*

Buffers for purification under denaturing conditions

Lysis buffer (1 liter):

100 mM NaH_2PO_4

10 mM Tris-HCl

8 M Urea

Adjust pH to 8.0 using NaOH.

Wash buffer (1 liter):

100 mM NaH_2PO_4

10 mM Tris-HCl

8 M Urea

Adjust pH to 6.3 using HCl.

Elution buffer(1 liter):

100 mM NaH_2PO_4

10 mM Tris-HCl

8 M Urea

Adjust pH to 4.5 using HCl.

Buffers for purification under native conditions

Lysis buffer (1 liter):

50 mM NaH_2PO_4

300 mM NaCl

10 mM Imidazole

Adjust pH to 8.0 using NaOH.

Wash buffer (1 liter):

50 mM NaH_2PO_4

300 mM NaCl

20 mM Imidazole

Adjust pH to 8.0 using NaOH.

Elution buffer (1 liter):

50 mM NaH_2PO_4

300 mM NaCl

250 mM Imidazole

Adjust pH to 8.0 using NaOH.

3.20 Affinity purification of *rPmGIH* without Signal peptide

Host cells expressing 6xHis-tagged recombinant protein (*rPmGIH_nosig*) were harvested by centrifugation, and were subjected to lysis and affinity purification using the Ni-NTA Fast Start Kit (Qiagen GmbH, Hilden, Germany) under denaturing conditions. Briefly, the cells from 100 ml of induced culture were pelleted by centrifugation at 3000xg for 10 min. The cell pellet was suspended in 10 ml denaturing lysis buffer and incubated at room temperature for 60 min with intermittent mixing. The lysate was centrifuged at 14000xg for 30 min at room temperature to pellet the cellular debris, and the supernatant was loaded into a fast start column containing Ni-NTA resin. The protein-bound resin was washed serially several times with denaturing washing buffer, and the bound 6xHis-tagged recombinant protein was eluted serially three times in 1 ml elution buffer. Fractions were analysed by Tricine-SDS polyacrylamide gel electrophoresis under reducing conditions to detect the presence of recombinant protein.

3.21 Affinity purification of *rPmGIH* with Signal peptide

Media fraction from the culture of host cells expressing 6xHis-tagged recombinant protein (*rPmGIH_withsig*) harvested by centrifugation was purified by a Ni-NTA matrix affinity column under native conditions according to the protocol of the Ni-NTA Fast Start Kit (Qiagen GmbH, Hilden, Germany). Briefly, the supernatant from 100 ml of induced cell culture cleared by centrifugation at 3000x g for 10 min mixed with equal volume native wash buffer was loaded to a column (10 ml) containing 1 ml of Ni-NTA resin pre-equilibrated with the lysis buffer. The protein bound resin was washed six times with native washing buffer. The protein was serially eluted three times in 1ml elution buffer, and fractions were analysed by Tricine-SDS polyacrylamide gel electrophoresis under reducing conditions to detect the presence of recombinant protein.

3.22 Affinity purification of *rPmGIH* with GFP

Host cells expressing 6xHis-tagged recombinant protein (*rPmGIH_GFP*) harvested by centrifugation were subjected to lysis and affinity purification using the Ni-NTA Fast Start Kit (Qiagen GmbH, Hilden, Germany) under native conditions. Briefly, pelleted cells from 100 ml of induced culture by centrifugation at 3000xg for 10 min. The cell pellet was suspended in 10 ml native lysis buffer and incubated at room temperature for 60 min with intermittent mixing. The lysate was centrifuged at 14000x g for 30 min at room temperature to pellet the cellular debris, and the supernatant was loaded into a fast start column containing Ni-NTA resin. The protein-bound resin was washed serially several times with native washing buffer, and the bound 6xHis-tagged recombinant protein was eluted serially three times in 1 ml elution buffer.

Fractions were analysed by Tricine-SDS polyacrylamide gel electrophoresis under reducing conditions to detect the presence of recombinant protein.

3.23 Determination of Protein Concentration.

The affinity purified recombinant protein was then quantified using the BioPhotometer® plus with Hellma TrayCell (Eppendorf , Hamburg, Germany) by measuring the absorbance at 280 nm by using the elution buffer used as blank.

3.24 Software used for Sequence Annotation and In-Silico Analysis

Nucleotide sequence editing and alignments were carried out using BioEdit software package version 7.01. Primers were designed for cloning and recombinant expresssion using the Primer Premier 6.0 software (PREMIER Biosoft, USA). The sequence was analyzed for identity and similarity to known sequences by BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al.,* 1990) at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih. gov/BLAST).

Contig assembly for generating the complete mRNA sequence were carried out using SeqMan in DNASar Lasergene software package version 7. EMBOSS Transeq at (http://www.ebi.ac.uk/Tools/st/ emboss_transeq/) was used for finding the open reading frames, 3` & 5` UTRs and deducing the amino acid sequences. Conserved domains, motifs and protein family signatures were identified using Motif Scan and ScanProsite tool of ExPASy programme (http://prosite.expasy.org). SignalP program version 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide sequence. Molecular weight and isoelectric point of the target were

predicted out using ProtParam tool at ExPASy (http://web.expasy.org/prot param/).

Multiple sequence alignment was performed by using CLUSTAL X program (Thompson *et al.,* 1997). Phylogenetic and molecular evolutionary analyses were conducted using the Maximum Likelihood method with MEGA version 6 (Tamura *et al.,* 2013). The nucleotide sequence and the deduced amino acid sequences were annotated and submitted to GenBank using Sequin application version 13.70.

⌘⌘⌘⌘⌘

Chapter –IV

Results and Discussion

Results and Discussion

The findings of the various experiments conducted as part of this thesis are described and discussed in comparison with the scientific literature in this chapter.

The preliminary reverse transcription - polymerase chain reaction (RT-PCR) for the synthesis of the cDNA of the Gonad Inhibiting Hormone (GIH) gene resulted in the amplification of a partial cDNA segment of 316 bp and 291 bp from *P. monodon* and *F. indicus* respectively.

Characterization of the complete cDNA of the GIH gene using Random Amplification of cDNA Ends (RACE) followed by sequencing of the RACE products has shown the complete cDNA length to be 858 bp and 855 bp in *P. monodon* and *F. indicus* respectively.

Conceptual translation of the complete coding sequence (CDS) sequence using the EMBOSS Transeq showed that it contains a 291 bp open reading frame encoding 96 amino acid residues. In-silico analysis using SignalP 4.1 program has shown the presence of a secretary signal peptide-coding region in the N-terminus of deduced polypeptide of both the species. ScanProsite tool detected the presence of CMG neurohormone family signature ([LIVM]-x(3)-C-[KR]-x-[DENGRH]-C-[FY]-x-[STN]-x(2)-F-x(2)-C.) and a C-type lectin domain signature (C-[LIVMFYATG]-x(5,12)-[WL]-{T}-[DNSR]-{C}-{LI}-C-x(5,6)-[FYWLI VSTA]-[LIVMSTA]-C) in the deduced amino acid sequences. The multiple sequence alignment using homology search algorithms and phylogenetic analysis revealed the close similarity among the reported GIH sequences.

The recombinant expression plasmids constructed with the GIH gene insert was used to transform BL21 (DE3), *E. coli* expression strain. Induction of the transformed colonies with IPTG resulted in the expression of the recombinant fusion protein. The expressed 6xHIS tagged recombinant protein was affinity purified using Ni - NTA matrix affinity column under native and denaturing conditions. SDS PAGE and Tricine-SDS-PAGE analysis of the purified *rPmGIH_nosig, rPmGIH_withsig* and *rPmGIH_GFP* showed single band products that migrate at 10.9 kDa, 12.5 kDa and 39.27 kDa respectively, which matched the theoretical molecular weight calculated from its sequence. The findings in detail are presented here under.

4.1 RNA isolation

The RNA isolation protocol used in this study was found to yield good quality RNA for the preparation of cDNA. For the qualitative and quantitative check, 2.0 µl of isolated totoal RNA was used to measure the OD at 260 nm using RNA storage solution (Ambion Biosciences) as a reference. The conversion factor for RNA was taken as 0.04 µg/µl per OD_{260} unit (Sambrook *et al.,* 1989). Ratio of OD at 260 nm and 280 nm was >1.8. It indicated that the purified RNA was good for preparation of cDNA.

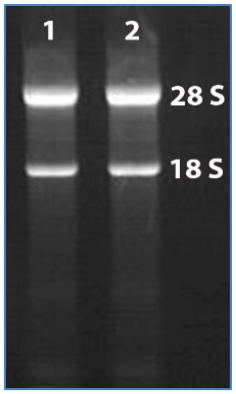


Figure 4.1 : *Total RNA isolated from eyestalk neural tissue Lane 1 P. monodon in & Lane 2 F. indicus*

The female shrimps with previtellogenic ovaries were used for isolation of total RNA as it has been reported in *P. monodon* that the GIH mRNA expression level peaked in previtellogenic phase (Vijayan *et al.,* 2013). The concentration of RNA was observed to be between 0.50 – 1 µg/µl. On agarose gel electrophoresis two distinct bands were observed indicating good quality of the total RNA preparation suitable for cDNA synthesis (Figure 4.1).

4.2 Preliminary characterization of the GIH gene from *P. monodon*

Compared with other members of the CMG family hormones, a limited number of GIH gene sequences have been characterized so far (Treerattrakool *et al.,* 2008). The isolation and amplification of GIH gene have been a difficult task because of the complex nature of the CMG family hormones, and the lack of proper information on the GIH sequence. At the time of initiation of this work, the GIH sequences available in GenBank were from the following species (Table 4.1).

Table 4.1: GIH sequences used for initial primer designing

SI No	Species name	GenBank Accession	Sequence size	CDS size	No. of Amino acids
1	*Metapenaeus ensis*	AF294648	852 bp	308 bp	102 aa
2	*Homarus americanus*	X81821	2165 bp	338 bp	112 aa
3	*Nephrops norvegicus*	AF163771	1359 bp	338 bp	112 aa

Therefore, one of the main objectives of the study was to characterize the cDNA encoding GIH in *P. monodon* and *F. indicus*, which is essential for the better understanding of the endocrine regulation of reproduction and growth in these animals. In the present study, putative GIH cDNA from *P. monodon* and *F. indicus* were successfully characterised by RT-PCR and by using RACE approach. Evidence is provided herein to show that the isolated cDNA is encoding for *P. monodon* and *F. indicus* GIH.

As neuropeptides of the CMG family are distinctive, cloning the genes of those neuropeptides mainly depends on a PCR approach using primers drawn from the sequence of neuropeptides from other closely related species. Many crustacean neuropeptides genes are cloned by this approach (de Kleijn *et al.,* 1994; 1995).

Initially primers were designed based on the GIH gene sequence of the three closely related species mentioned in Table 4.1 for cross species PCR amplification of GIH gene from cDNA. First strand cDNA synthesis was carried out using total RNA isolated from the X-organ sinus gland complex of female *P. monodon* eyestalk, using the modified Guanidium thiocyanate method (Chomczynski and Sacchi, 1987).

Out of the three pair of primers used for amplification, only two pairs generated amplified fragments. While the PMeF & PMeR pair generated a fragment of 427 bp (Figure 4.2), another pair PMoF & PMoR produced a fragment of size 516 bp (Figure 4.3).

These fragments were cloned in pJET1.2 blunt end vector and transformed into *E.coli* strain TOP10. Colony PCR was performed to select transformed colonies and plasmid isolation and sequencing using vector primers were performed for obtaining the sequence of the cloned fragments. The sequences obtained were edited using BioEdit software and searched for homology with available sequence in the publically available databases using the BLAST tool.

The sequence of the cDNA fragment amplified using the first pair of primers PMeF & PMeR and the sequence query of 427 bp product in the BLAST search did not produce any significant score with any of the reported CMG family hormones in crustaceans. However, it contained coding sequences (CDS) for unknown short peptides (Figure 4.4).

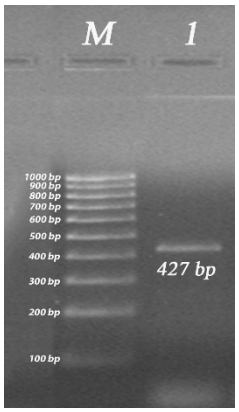


Figure 4.2: *Lane M is 100 bp DNA marker & Lane 1 is PCR product of PMeF & PMeR primers*

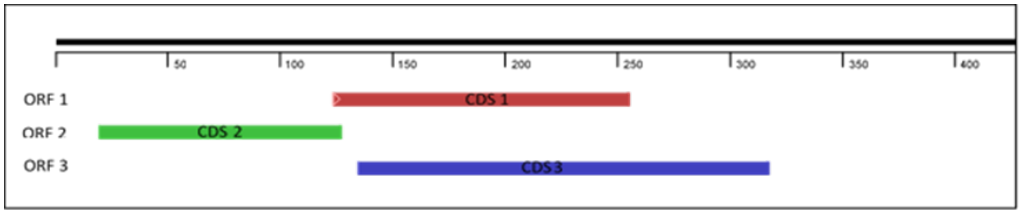


Figure 4.4 : *Graphical representation of Open Reading Frames (ORF) and Coding Sequence (CDS) detected in the nucleotide sequence of PMe primer amplified cDNA.*

The nucleotide homology of CMG family hormones among different groups of crustaceans is high and cloning neuropeptide genes of a different crustacean using cross priming technique is difficult. Even so, the approach might promote cloning different members of the CMG family of neuropeptide genes as in this case of the primers PMoF & PMoR. The sequence of the cDNA fragment amplified with the second pair of primers PMoF & PMoR contained two coding sequences (Figure 4.5). The sequence query in the blast search showed 80-98% similarity with other reported CMG family hormones of *P. monodon*, mainly to MIH.

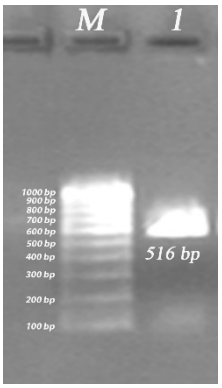


Figure 4.3: *Lane M is 100 bp DNA marker &Lanes 1 shows PCR product by primer pair PMoF & PMoR*

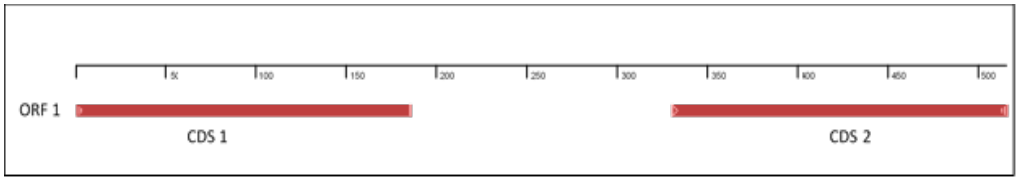


Figure 4.5 : *Graphical representation of Open Reading Frames (ORF) and Coding Sequence (CDS) detected in the nucleotide sequence of PMo primer amplified cDNA.*

The third pair of primers reported by Treerattrakool *et al.* (2008) for amplification of the complete cDNA of GIH of *Penaeus monodon* from Thailand waters was synthesized and used to amplify GIH gene from eyestalk cDNA of female *P. monodon at* previtellogenic phase from Indian waters. However, these primers (GIH F & GIH R) failed to produce any amplified product.

However, two partial cDNA fragments encoding GIH in *P. monodon* were amplified using primers designed based on a GenBank sequence of *P. monodon* GIH (DQ643389). The primers Pm1.3 F & Pm1.23 R amplified a 400 bp product (Figure 4.6), and another primer combination, Pm 1.3F and GIHR produced an amplified product of 535 bp size (Figure 4.7).

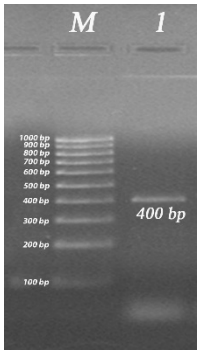


Figure 4.6 : *Lane 1is 100 bp DNA marker & lane 2 is PCR product of Pm 1.3F & Pm1.23R primer*

```
>gb|DQ643389.1|  Penaeus monodon gonad-inhibiting hormone (GIH) mRNA, complete
cds
Length=861

 Score =  717 bits (388),  Expect = 0.0
 Identities = 397/401 (99%), Gaps = 2/401 (0%)
 Strand=Plus/Plus

Query  1    CGAACTCGAGGC-TTTCAGACTCTGGATCAGCATCCTCAATGCCGGCCGGCCGTGGTGAT  59
            ||||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  CGAACTCGA-GCATTTCAGACTCTGGATCAGCATCCTCAATGCCGGCCGGCCGTGGTGAT  385

Query  60   CCTTCCTTCTCTGAAACCATCCCACGCGCCCCAGCCTCAGTCCTGATCAGGGACACCTGG  119
            | ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  386  CTTTCCTTCTCTGAAAGCATCCCACGCGCCCCAGCCTCAGTCCTGATCAGGGACACCTGG  445

Query  120  CAGGACGCTGGCACAGCTCTCGAGTCTCCCCCTGCGCTATTAAACGTGATCATTGGAAAT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  446  CAGGACGCTGGCACAGCTCTCGAGTCTCCCCCTGCGCTATTAAACGTGATCATTGGAAAT  505

Query  180  AGACATCTCTAGGCCGATTCGGATGCTTCGACGCCTTGGCTGTATTCCTTTATGAATACA  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  506  AGACATCTCTAGGCCGATTCGGATGCTTCGACGCCTTGGCTGTATTCCTTTATGAATACA  565

Query  240  TTTAGGAGTGGATAGGTCCTCTCTCAGTGTAATTTAAGGGCGTATTATTGCATTAGAGAA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  566  TTTAGGAGTGGATAGGTCCTCTCTCAGTGTAATTTAAGGGCGTATTATTGCATTAGAGAA  625

Query  300  AGTCTAAGTACTCTAGAATCAAAttttctttcgtttcactgctatctattgttttcaat  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  626  AGTCTAAGTACTCTAGAATCAAATTTTTCTTTCGTTTCACTGCTATCTATTGTTTTCAAT  685

Query  360  tttgtttcccatcttttacttgttttcCCTGGTCTCAGTGC  400
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  686  TTTGTTTCCCATCTTTTACTTGTTTTCCCTGGTCTCAGTGC  726
```

Figure 4.8 : *BLAST result of the sequence data of Pm1.3 F & Pm1.23R amplified product*

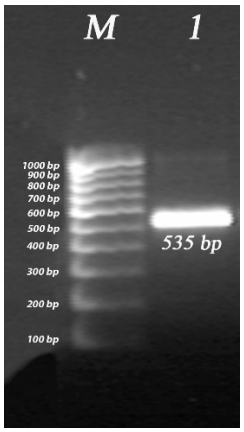


Figure 4.7: *Lane 1 100 bp DNA marker & lane 2 PCR products using Pm 1.3F & GIHR primers*

The sequence information of both of these (400 bp & 535 bp) products has shown 99% similarity with the 3` region of Pem-GIH cDNA sequence (DQ643389) (Figure 4.8).

Failure of the primers designed with the help of primer designing software to bind to 5` region of the GIH cDNA has forced us to design a primer manually. The PmGIH Gene F primer, which starts exactly at the start codon of the GIH gene, was designed manually. This PmGIH Gene F primer along with Pm 1.1 R primer amplified a cDNA fragment of 316 bp size (Figure 4.9).

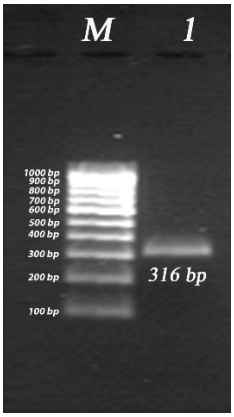


Figure 4.9 : *Lane 1 100 bp DNA marker & lane 2 PCR product using PmGIH Gene F & Pm 1.1 R primer*

The cDNA fragment of size 316 bp size amplified using the Pm GIH Gene F & Pm 1.1 R was cloned in to pJET1.2 vector and transformed into competent *E. coli* strain TOP10. Transformed colonies were selected and plasmid isolation was performed. This recombinant plasmid upon sequencing using vector primers has shown to contain the complete coding sequence (CDS) of the GIH gene from *P. monodon* (Figure 4.10). The recombinant plasmid was named as pJET-PmGIH.

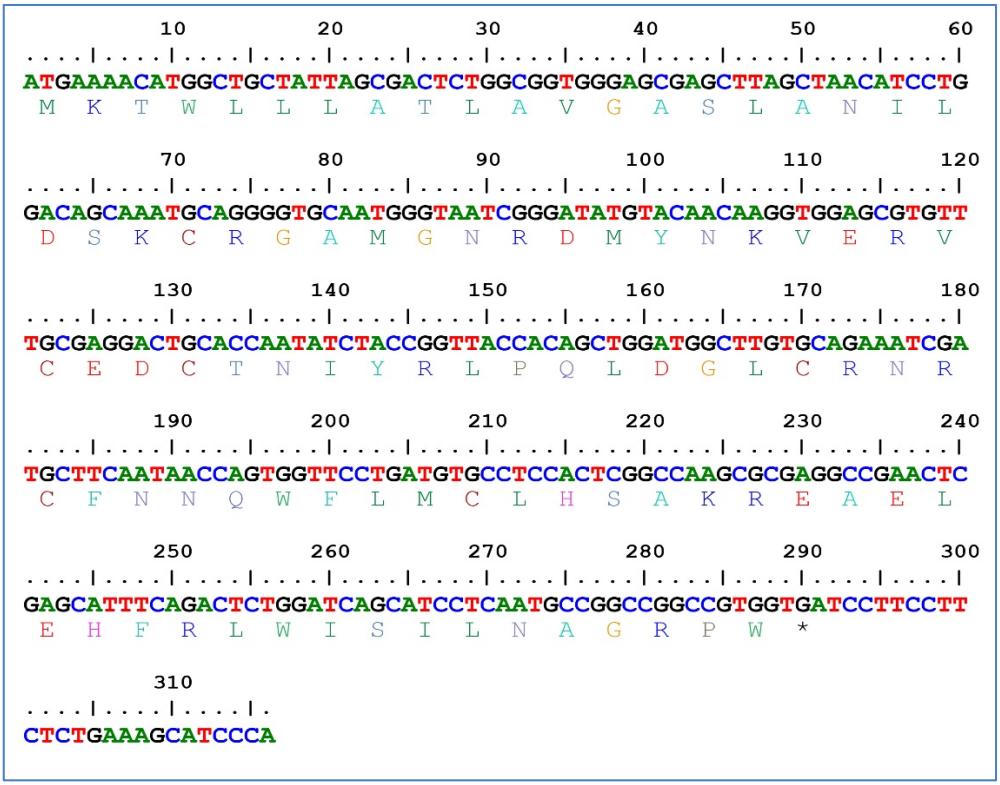


Figure 4.10: *Nucleotide sequence with translated protein sequence of pJET-PmGIH clone*

4.3 Preliminary characterization of the GIH gene from *F. indicus*

The primers PmGIH Gene F & Pm1.1R, which amplified the CDS of GIH gene in *P. monodon* was used for cross species amplification of GIH gene in *F. indicus*. First strand cDNA synthesis was carried out using total RNA isolated from the X - organ sinus gland complex of female *F. indicus* eyestalk.

The RT-PCR amplified a partial cDNA fragment of the expected size of 316 bp. This fragment was cloned in pJET1.2 vector and sequenced. The

sequence analysis revealed variations in the primer binding sites, so new primers were designed based on the sequence information generated.

The new primers FiGIH Gene F & FiGIH Gene R were designed specifically to amplify only the coding sequence (CDS) of 291 bp of the GIH gene from *F. indicus*. This cDNA fragment was cloned in to pJET1.2 vector and transformed into competent *E. coli* strain TOP10. Transformed colonies were selected and plasmid isolation was performed. The recombinant plasmid upon sequencing using vector primers has shown to contain the complete coding sequence (CDS) of the GIH gene from *F. indicus* (Figure 4.11). The recombinant plasmid was named as pJET-FiGIH.

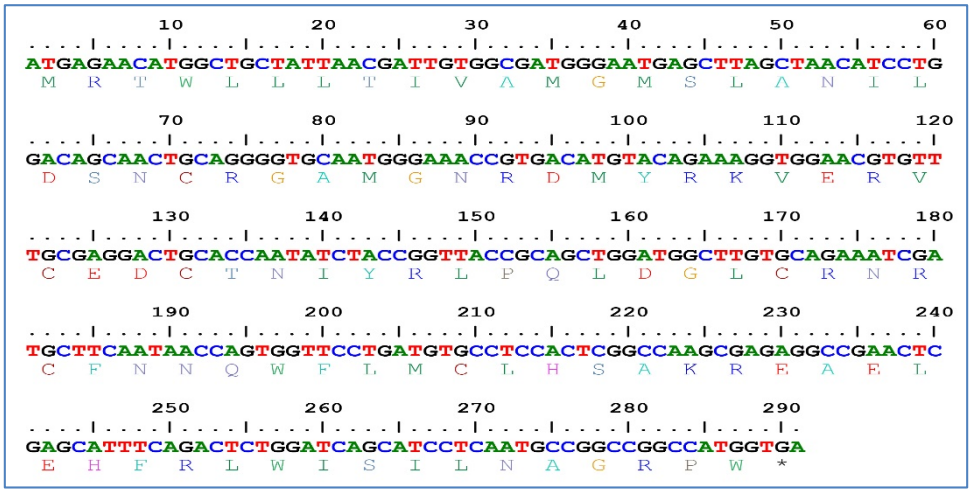


Figure 4.11: *Nucleotide sequence with translated protein sequence of pJET- FiGIH clone*

4.4 Characterization of full length mRNA of Gonad Inhibiting Hormone (GIH) gene of Penaeid shrimps, *P. monodon* & *F. indicus* using Random Amplification of cDNA Ends (RACE)

To date, the charecterisation of the GIH gene of Penaeid shrimps, *P. monodon* and *F . indicus* have not been reported from Indian waters. GIH has been identified only from only a few crustacean species such as *Metapenaeus ensis* (Gu, *et al.,* 2002), *Litopenaeus vanammei* (Chen *et al.,* 2014), *Homarus americanus* (De Kleijn *et al.,* 1995), *Homarus gammarus* (Ollivaux *et al.,* 2006), *Nephrops norvegicus* (Edomi *et al.,* 2002), *Macrobrachium nipponense* (Qiao *et al.,* 2015) and *Rimicaris kairei* (Qian *et al.,* 2009).

In this study, the molecular identification and preliminary characterization of GIH gene of *P. monodon* and *F .indicus* were carried out by reverse transcription PCR and sequencing of the product. The initial characterization was followed by RACE to develop the full-length nucleotide sequence of the complete cDNA. The amino acid sequences were deduced, and their characteristic features were worked out as detailed below.

4.5 Characterization of full length GIH cDNA of *P. monodon* by RACE

The 5` and 3` RACE PCR performed using the UPM and GSPs (PmGIH Gene F & Pm1.1 R) produced amplified products of 422 bp & 844 bp respectively including the adapter regions. Optimization of PCR parameters helped to get the specific amplicon free from nonspecific PCR product. The PCR products cloned using pJET1.2 vector and sequencing of the recombinant plasmids using vector primers generated the sequence information of the cloned products. The full-length cDNA of Pm GIH was subsequently generated by

assembling the overlapping 5` and 3` sequence reads into contigs by SeqMan software. The sequence was further edited to remove adapter and vector sequences to obtain full-length sequence of 858 bp Pm GIH cDNA.

Nucleotide and deduced amino acid sequences of Pm GIH complete cDNA

The full length of Pm GIH cDNA was 858 bp (GenBank accession no. KT906363), including the 5′ and 3′ untranslated region (UTR) containing 61 bp and 506 bp, respectively and a 291 bp open reading frame (ORF). The nucleotide sequence and the deduced amino acid sequence are shown in figure 4.12 and figure 4.13 respectively.

Conceptual translation of the coding sequence using the EMBOSS Transeq revealed the open reading frame (ORF) of 291 bp which encodes 96 amino acid prepro PmGIH (Figure 2). The molecular mass and theoretical pI estimated using the ProtParam tool were 11.18 kDa and 8.8 respectively.

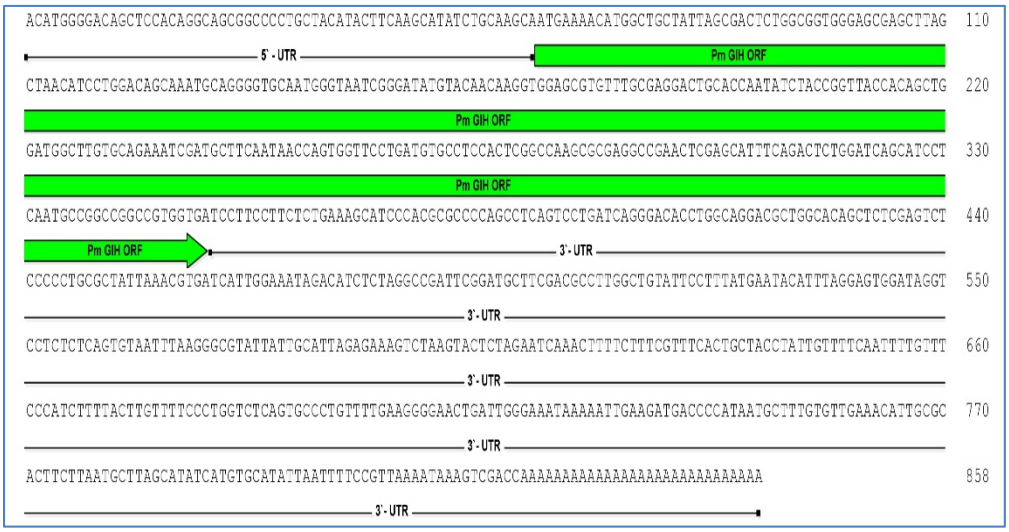


Figure 4.12: *Nucleotide sequence of full length GIH cDNA of P. monodon*

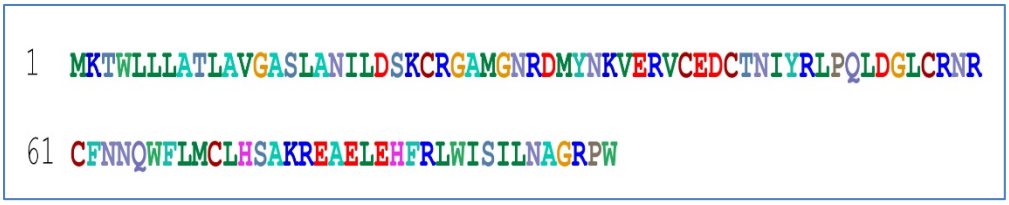


Figure 4.13: *Amino acid sequence of prepro GIH peptide from P. monodon*

Analysis of the amino acid sequence using SignalP 4.1 program identified a 17 amino acid secretary signal peptide region at the N-terminus of the deduced peptide with the cleavage site located between the amino acids Alanine and Asparagine at positions 17 and 18 respectively (Figure 4.14).

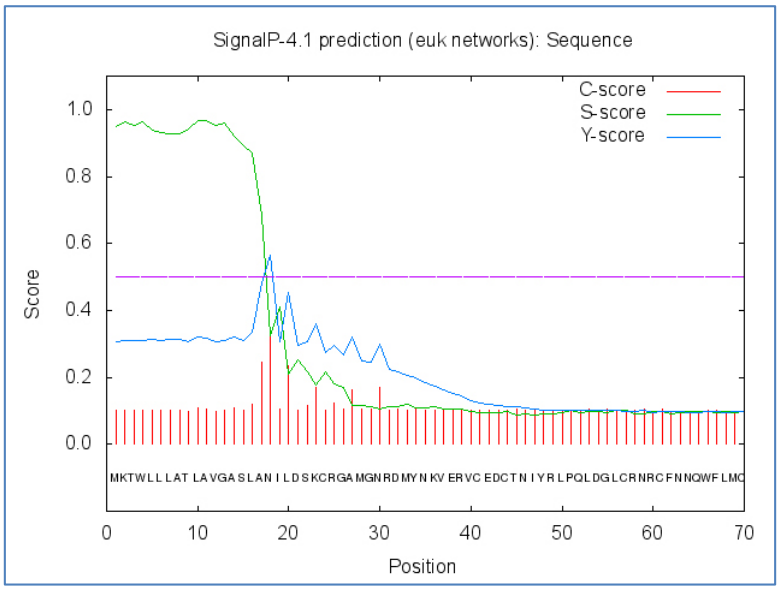


Figure 4.14: *Signal peptide analysis of P. monodon GIH as predicted by the SignalP 4.1 server.*

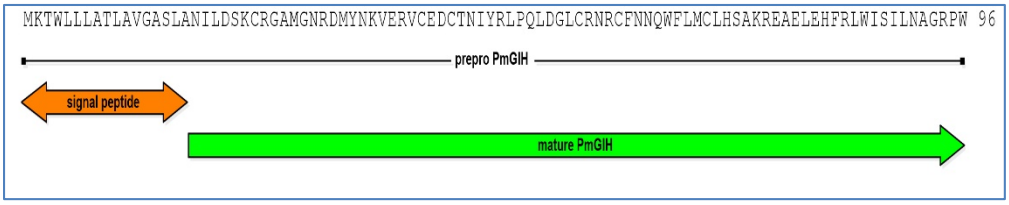


Figure 4.15: *Graphical representation of the predicted signal peptide and mature peptide regions in the P. monodon GIH amino acid sequence.*

Sequence analysis of the 79 amino acid mature peptide region predicted a molecular mass of 9.435 kDa and a theoretical pI of 8.65 (Figure 4.15).

The pair wise alignment of the complete cDNA sequence of GIH of *Penaeus monodon* generated in this study (KT906363) with that from Thailand (DQ643389) by Treerattrakool *et al.* (2008) has shown the deletion of 32 bp nucleotide sequence from the 5`- UTR region of the Pm- GIH sequence of the present study (Figure 4.16). The sequence submitted from Thailand the 5` UTR region consisted of 93 bp, whereas, the 5`-UTR of the GIH characterized in this study from the *P. monodon* from Indian waters was of 61 bp only.

In addition to this there was substitution of 6 nucleotides at the beginning of 5`-UTR (CCCCAC was substituted with ATGGG) in the Pm-GIH sequence. This difference in the sequence might be the reason why the primers designed based on the Pem-GIH sequence were unable to bind to the 5` region of the Pm-GIH during our preliminary characterization stage.

Figure 4.16: *Nucleotide sequence alignment showing the sequence divergence between GIH cDNA of P. monodon from Thailand (DQ643389) and India (KT906363)*

4.6 Characterization of full length GIH cDNA of *F. indicus* by RACE

The 5` and 3` RACE PCR performed using the UPM and GSPs (Fi GIH Gene F & Fi GIH Gene R) produced amplification products of 394 bp & 844 bp respectively including the adapter regions. Optimization of PCR parameters helped to get the specific amplicon free from nonspecific PCR product. The PCR products were purified and cloned into pJET1.2 vector and sequenced for identification and characterization of the gene.

Sequencing of the purified plasmids using vector primers gave the sequence information of both the fragments. The full-length cDNA of Pm GIH was subsequently generated by assembling the overlapping 5` and 3` sequence reads into contigs by SeqMan software using reactions. The sequence was further edited to remove adapter and vector sequences to obtain full-length sequence of 855 bp Fi GIH cDNA.

Nucleotide and deduced amino acid sequences of Fi GIH complete cDNA

The 855 bp *F. indicus* GIH full-length cDNA characterized in this study (GenBank accession no. KT373905) contained 58 bp in the 5`- UTR, 291 bp in the ORF and 506 bp in the 3`- UTR. The nucleotide sequence is shown in Figure 4.17.

Conceptual translation of the coding sequence using the EMBOSS Transeq revealed that the open reading frame (ORF) of 291 bp encodes 96 amino acid prepro Fi GIH (Figure 4.18). The molecular mass and theoretical pI estimated by using ProtParam tool were 11.35 kDa and 8.91 respectively.

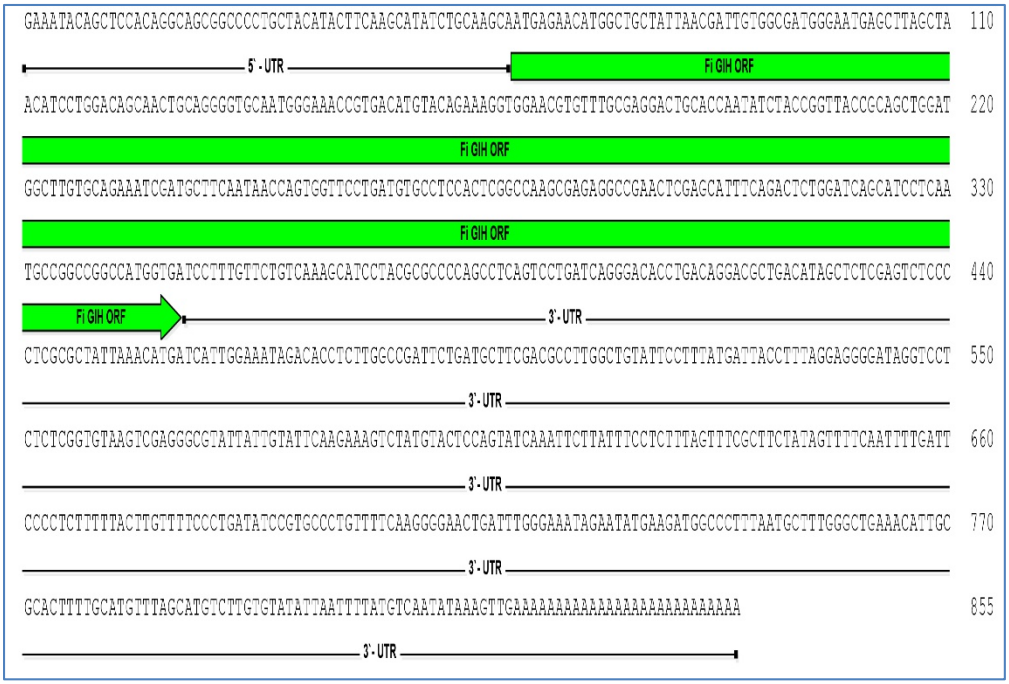


Figure 4.17: *Nucleotide sequence of full length GIH cDNA from F. indicus*

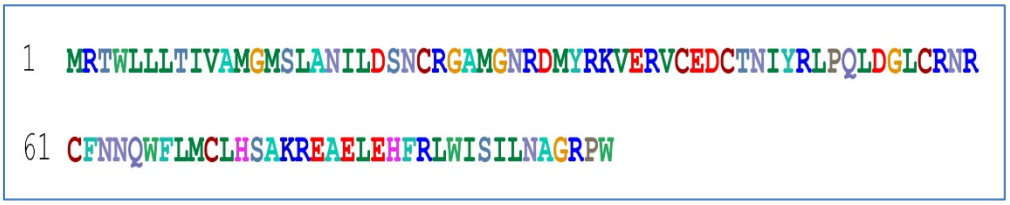


Figure 4.18: *Amino acid sequence of prepro GIH peptide of F. indicus*

Analysis of the amino acid sequence using SignalP 4.1 program revealed a 17 amino acid secretory signal peptide region at the N-terminus of the deduced peptide with the cleavage site located between the amino acids Alanine and Asparagine at positions 17 and 18 respectively (Figure 4.19).

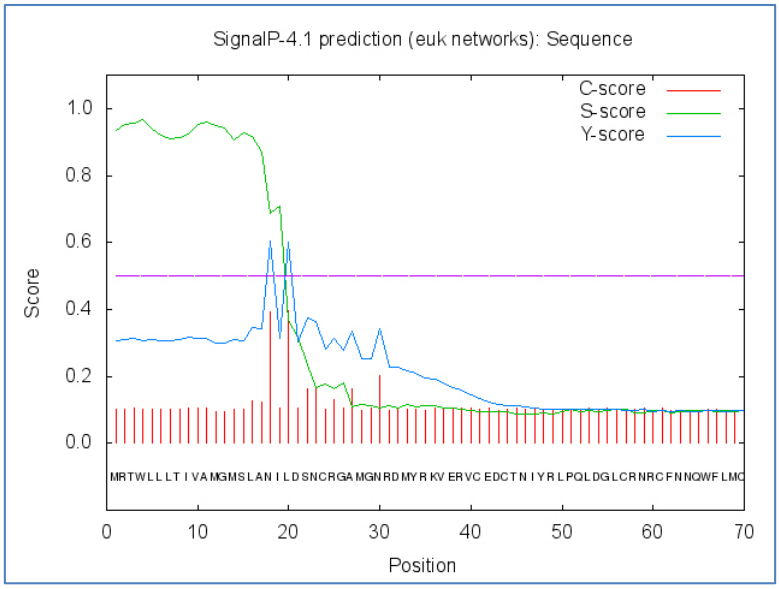


Figure 4.19: *Signal peptide analysis of F. indicus GIH as predicted by the SignalP 4.1 server.*

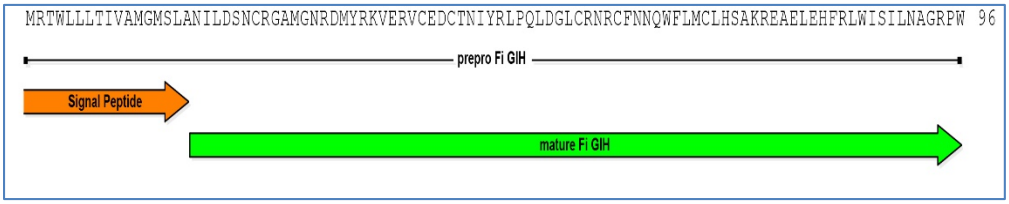


Figure 4.20: *Graphical representation of the predicted signal peptide and mature peptide regions in the F. indicus GIH amino acid*

Sequence analysis of the 79 amino acid mature peptide region predicted a molecular mass of 9.463 kDa and a theoretical pI of 8.66 (Figure 4.20).

ScanProsite tool detected the presence of the Arthropod CHH/MIH/GIH neurohormones family signature **([LIVM]-x(3)-C-[KR]-x-[DENGRH]-C-[FY]-x-[STN]-x(2)-F-x(2)-C)** in the deduced amino acid sequence of PmGIH and FiGIH at aminoacid position 53-70 (Figure 4.21).

Figure 4.21: *Arthropod CHH/MIH/GIH neurohormones family signature detected by ScanProsite in GIH of P. monodon and F. indicus*

A C-type lectin domain signature **(C-[LIVMFYATG]-x(5,12)-[WL]-{T}-[DNSR]-{C}-{LI}-C-x(5,6)-[FYWLIVSTA]-[LIVMSTA]-C)** has also been detected at position 44-70 in the deduced amino acid sequence of PmGIH and FiGIH (Figure 4.22).

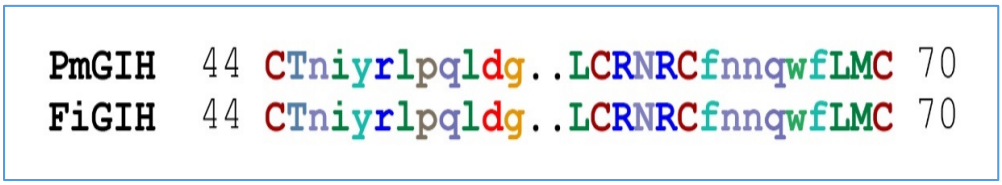


Figure 4.22: *C-type lectin domain signature detected by ScanProsite in GIH of P. monodon and F. indicus*

4.7 Phylogenetic & Evolutionary Analysis

Phylogenetic and evolutionary relationships of GIH from tiger shrimp, indian white shrimp from this study and other selected decapod crustaceans were estimated using the GIH aminoacid sequence of woodlouse *Armadillidium vulgarae* (P83627) as out-group and other GIH aminoacid sequences of selected crustacean species such as *Metapenaeus ensis* (AAL33882), *Litopenaeus vanammei* (AGX26044), *Homarus americanus* (CAA60644), *Homarus gammarus* (ABA42181), *Nephrops norvegicus* (AAK58133), *Macrobrachium nipponense* (AEJ54623) and *Rimicaris kairei* (ACS35348).

Multiple alignment analysis of amino acid sequences has shown the presence of six conserved cysteine residues (Cys^{24}, Cys^{41}, Cys^{44}, Cys^{57}, Cys^{61}, and Cys^{70}) in the mature peptide region of Pm GIH and Fi GIH sequences, all of which are conserved across the CMG family of peptides in crustaceans involved in the formation of internal disulfide bonds and a glycine (Gly^{46}) specific to CMG family II peptides. A putative alanine amidation site (Ala^{92}) was also detected in both PmGIH and FiGIH sequences. The glycine residue (Gly^{93}) provide the amid group, and the three amino acids at the C-terminus of the GIH peptide (Arg^{94}-Pro^{95}-Trp^{96}) are likely to constitute the amidation signal and also function as an active peptide precursor cleavage site (Figure 4.23).

Molecular analysis of amino acid sequences of GIH characterised from the *P. monodon* and *F. indicus* species showed that they consist of signal peptides (17 amino acid residues) and mature peptides (72 amino acid residues). They also showed a considerable degree of sequence similarity with other GIH sequences, including the preservation of six cysteine residues at the same relative locations (Wongsawang *et al.,* 2005; Nagaraju and Borst, 2008) forming three intra-molecular disulfide bonds (1-5; 2-4 & 3-6); these are major chemical forces, which help to maintain the GIH tertiary structure (Nagaraju *et al.,* 2009). The GIH sequences of *P. monodon* and *F. indicus* also had an unblocked N-terminus and an amidated C-terminus in accordance with the findings of Bocking *et al.,* (2001).

Multiple alignment revealed that *F. indicus* GIH shares 93, 97, 64, 32, 35, 39, 37, 37 and 37% identity with *P. monodon*, *L. vannamei*, *M. ensis*, *M. nipponense, R. kairei*, *N. norvegicus*, *H. americanus, H. gammarus,* and *A. vulgare* respectively. And the *P. monodon* GIH shares 93, 95, 65, 34, 34, 37,

35, 35 and 36% identity with *F. indicus*, *L. vannamei*, *M. ensis*, *M. nipponense, R. kairei*, *N. norvegicus*, *H. americanus, H. gammarus,* and *A. vulgare* respectively (Figure 4.23; Table 4.2).

Amino acid sequence of GIH from *P. monodon* and *F. indicus* obtained in the present study showed high sequence identity (64-97%) with other peaneid shrimp GIH in the GenBank, while it showed relatively lower sequence identity to the GIH from other crustaceans (lobsters and caridean shrimps). It had been as well proposed (Chan *et al.,* 2003) that GIH may very well be a recently emerged gene in crustaceans, to which teir higher sequence identity could be attributed. This observation is consistent with the hypothesis of greater evolutionary divergence between dendrobranchiata and pleocyemata (Ma *et al.,* 2009).

The ML tree places the GIH sequences in a branching order that reflects the phylogenetic relationship of the respective species. As shown in Figure 4.24, P. *monodon, F. indicus, L. vanammei and M. ensis* formed one distinct cluster, supported by high bootstrap value indicating close genetic relationship, whereas *H. americanus*, *H. gammarus, N. norvegicus*, *R. kairei* and *M. nipponense* formed another distinct cluster.

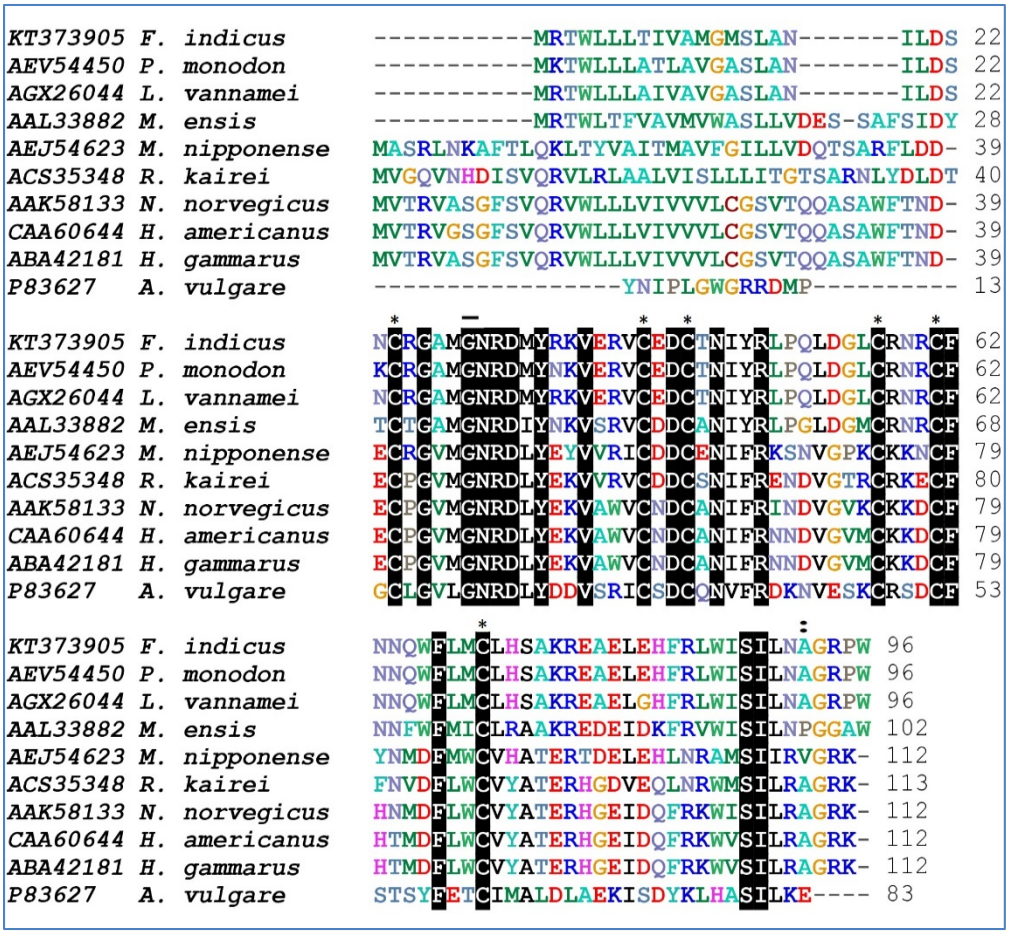


Figure 4.23: *Multiple alignment of the FiGIH and PmGIHwith other crustacean GIH aminoacid sequences. Amino acid numbers (excluding gap) are shown on the right. Invariant residues are shaded black. The position of 6 conserved cysteine residues are marked with asterisks (*),the conserved glycine residue is marked with a hyphen (-)and the putative amidation site is marked with colon (:).*

Table 4.2: Estimates of Percentage identity between aminoacid sequence of crustacean gonad inhibiting hormones (GIH)

	F.indicus	*P.monodon*	*L.vannamei*	*M.ensis*	*M.nipponense*	*R.kairei*	*N.norvegicus*	*H.americanus*	*H.gammarus*	*A.vulgare*
F.indicus	***	93	97	64	32	35	39	37	37	37
P.monodon		***	95	65	34	34	37	35	35	36
L.vannamei			***	66	32	35	40	38	38	37
M.ensis				***	33	32	40	39	39	34
M.nipponense					***	49	51	49	49	28
R.kairei						***	61	60	60	25
N.norvegicus							***	96	97	28
H.americanus								***	99	28
H.gammarus									***	28
A.vulgare										***

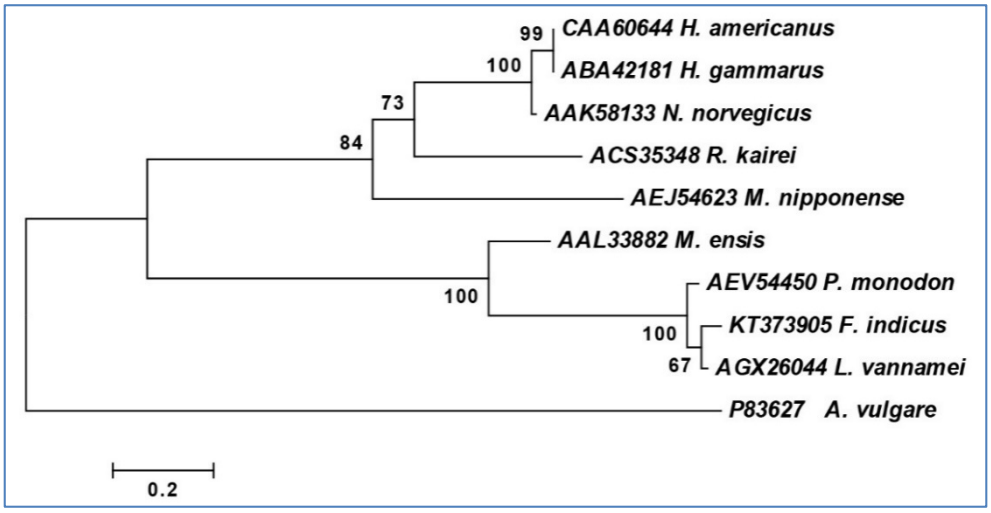


Figure 4.24:*A bootstrapped Maximum Likelihood tree obtained using MEGA6 illustrating relationships between the amino acid sequences among crustacean gonad inhibiting hormones (GIH). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.*

4.8 Construction of recombinant expression vectors with GIH gene inserts

The native neuropeptide purified by RP-HPLC has inherent advantages of having naturally folded peptide that completed all posttranslational changes for conducting physiological studies (Mosco *et al.,* 2012). However, it is extremely difficult to get chromatographically purified GIH in enough quantities; for example, single sinus gland yields 2-4 µg of neuropeptide (Chung and Webster, 2003) and therefore, many experimental animals and lengthy procedures are necessary to get GIH in enough quantities. Recombinant DNA technology is one of the simplest and fastest methods to get large quantities of purified proteins. Recombinant DNA technology can obtain greater quantities to enable further physiological investigations (Mosco *et al.,* 2012). So far, recombinant GIH have been reported only from *Nephrops norvegicus* (Edomi *et al.,* 2002) and *Litopenaeus vannamei* (Tsutsui *et al.,* 2013) and both were expressed in the insoluble form only.

Forward and reverse primers were designed based on the sequence of Pm GIH gene sequence available with us, and the cloning sites available in the pET28b+ and pET28b+GFP expression vectors. Two set of primers were designed for cloning of PmGIH gene - with and without signal peptide region in the pET28b+ expression vector by inserting the NcoI restriction site in the forward primer and NotI site in the reverse primer. This strategy enabled in-frame fusion of PmGIH with the vector encoded T7 promoter region at the 5` end and hexa histidine tag at the N terminal. For pET28b+GFP expression vector the forward primer included NcoI and reverse primer included XhoI restriction sites. Using these primers, PCR amplification resulted in the required length of product when the recombinant plasmid pJET-PmGIH was used as a

template. In case of pET28b+GFP, the cloning strategy resulted in the construction of GIH followed by the GFP in frame with the hexa histidine tag. (Figure 4.25)

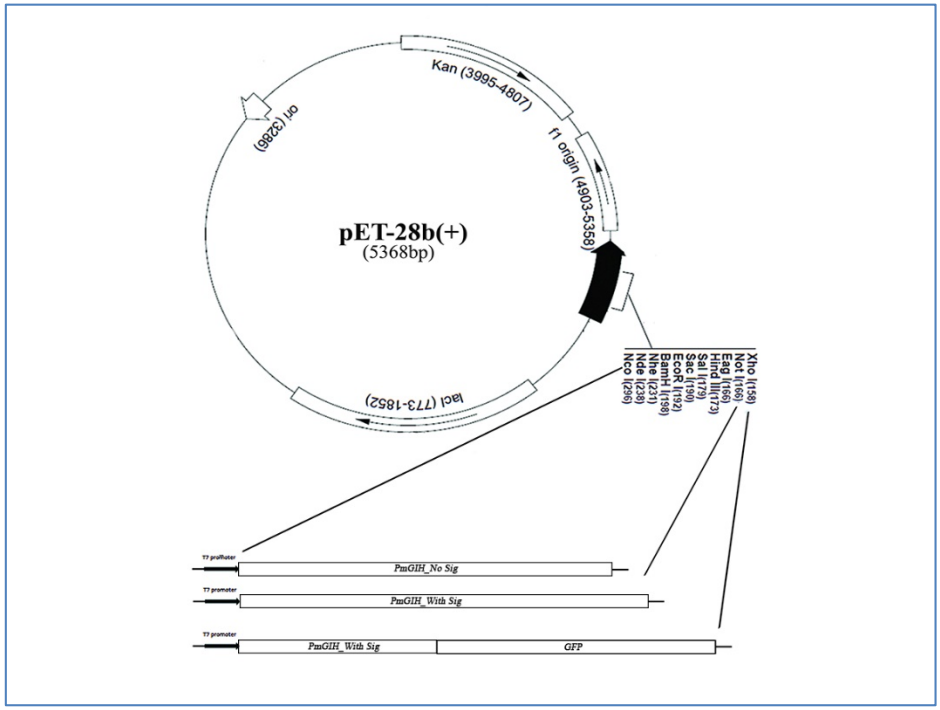


Figure 4.25: *Schematic diagram of expression vector pET-28b+ with PmGIH_Nosig, PmGIH_Withsig and PmGIH_GFP constructs.*

The expression vector constructed were cloned and recombinant expression plasmids were isolated. Sequencing of the purified expression plasmids pET28b-PmGIH_*Nosig*, pET28b-PmGIH_*Withsig* & pET28b-PmGIH_*GFP* carried out to check proper insertion of the fragment in the vector did not show any mutations, the GIH peptide coding sequence was in the correct reading frame.

The sequence analysis of the pET28b-*PmGIH_Nosig* construct predicted that the recombinant protein would contain 92 amino acids: 79 amino acids of the mature GIH peptide with 2 vector amino acids at the N-terminal and 11 vector amino acids, including six Histidine residues at the C-terminal. The expected molecular weight of the recombinant product was calculated to be 10.92 kDa (Figures 4.26 and 4.27).

```
1   ATGGCTAACA TCCTGGACAG CAAATGCAGG GGTGCAATGG GTAATCGGGA TATGTACAAC
61  AAGGTGGAGC GTGTTTGCGA GGACTGCACC AATATCTACC GGTTACCACA GCTGGATGGC
121 TTGTGCAGAA ATCGATGCTT CAATAACCAG TGGTTCCTGA TGTGCCTCCA CTCGGCCAAG
181 CGCGAGGCCG AACTCGAGCA TTTCAGACTC TGGATCAGCA TCCTCAATGC CGGCCGGCCG
241 TGGGCGGCCG CACTCGAGCA CCACCACCAC CACCACTGA
```

Figure 4.26: *Nucleotide sequences of pET28+PmGIH_Nosig recombinant DNA construct.*

```
1  MANILDSKCR GAMGNRDMYN KVERVCEDCT NIYRLPQLDG LCRNRCFNNQ WFLMCLHSAK

61 REAELEHFRL WISILNAGRP WAAALEHHHH HH
```

Figure 4.27: *Amino acid sequence of rPmGIH _nosig protein.*

The sequence analysis of the pET28b-*PmGIH_Withsig* construct predicted that the recombinant protein will contain 108 amino acids: 96 amino acids of the GIH prepro peptide with one vector amino acids at the N-terminal and 11 vector amino acids, including 6 His residues at the C-terminal with an expected molecular weight of 12.51 kDa (Figures 4.28 and 4.29).

```
1   ATGGGCAAAA CATGGCTGCT ATTAGCGACT CTGGCGGTGG GAGCGAGCTT AGCTAACATC
61  CTGGACAGCA AATGCAGGGG TGCAATGGGT AATCGGGATA TGTACAACAA GGTGGAGCGT
121 GTTTGCGAGG ACTGCACCAA TATCTACCGG TTACCACAGC TGGATGGCTT GTGCAGAAAT
181 CGATGCTTCA ATAACCAGTG GTTCCTGATG TGCCTCCACT CGGCCAAGCG CGAGGCCGAA
241 CTCGAGCATT TCAGACTCTG GATCAGCATC CTCAATGCCG GCCGGCCGTG GGCGGCCGCA
301 CTCGAGCACC ACCACCACCA CCACTGA
```

Figure 4.28: *Nucleotide sequences of pET28+PmGIH_Withsig recombinant DNA construct.*

```
1   MGKTWLLLAT LAVGASLANI LDSKCRGAMG NRDMYNKVER VCEDCTNIYR LPQLDGLCRN
61  RCFNNQWFLM CLHSAKREAE LEHFRLWISI LNAGRPWAAA LEHHHHHH
```

Figure 4.29: *Amino acid sequence of rPmGIH _withsig protein.*

And the sequence analysis of pET28b-*PmGIH_GFP* construct predicted the recombinant protein will contain 345 amino acids: 96 amino acids of the GIH prepro peptide with one vector amino acids at the N-terminal plus 238 amino acids of the GFP and 11 vector amino acids, including 6 His residues at the C-terminal with an expected molecular weight of 39.27 kDa (Figure 4.30 and 4.31).

```
1    ATGGGCAAAA CATGGCTGCT ATTAGCGACT CTGGCGGTGG GAGCGAGCTT AGCTAACAT
61   CTGGACAGCA AATGCAGGGG TGCAATGGGT AATCGGGATA TGTACAACAA GGTGGAGCG
121  GTTTGCGAGG ACTGCACCAA TATCTACCGG TTACCACAGC TGGATGGCTT GTGCAGAAA
181  CGATGCTTCA ATAACCAGTG GTTCCTGATG TGCCTCCACT CGGCCAAGCG CGAGGCCGA
241  CTCGAGCATT TCAGACTCTG GATCAGCATC CTCAATGCCG GCCGGCCGTG GAAGCTTGT
301  AGCAAGGGCG CCGAGCTGTT CACCGGCATC GTGCCCATCC TGATCGAGCT GAATGGCGA
361  GTGAATGGCC ACAAGTTCAG CGTGAGCGGC GAGGGCGAGG GCGATGCCAC CTACGGCAA
421  CTGACCCTGA AGTTCATCTG CACCACCGGC AAGCTGCCTG TGCCCTGGCC CACCCTGGT
481  ACCACCCTGA GCTACGGCGT GCAGTGCTTC TCACGCTACC CCGATCACAT GAAGCAGCA
541  GACTTCTTCA AGAGCGCCAT GCCTGAGGGC TACATCCAGG AGCGCACCAT CTTCTTCGA
601  GATGACGGCA ACTACAAGTC GCGCGCCGAG GTGAAGTTCG AGGGCGATAC CCTGGTGAA
661  CGCATCGAGC TGACCGGCAC CGATTTCAAG GAGGATGGCA ACATCCTGGG CAATAAGAT
721  GAGTACAACT ACAACGCCCA CAATGTGTAC ATCATGACCG ACAAGGCCAA GAATGGCAT
781  AAGGTGAACT TCAAGATCCG CCACAACATC GAGGATGGCA GCGTGCAGCT GGCCGACCA
841  TACCAGCAGA ATACCCCCAT CGGCGATGGC CCTGTGCTGC TGCCCGATAA CCACTACCT
901  TCCACCCAGA GCGCCCTGTC CAAGGACCCC AACGAGAAGC GCGATCACAT GATCTACTT
961  GGCTTCGTGA CCGCCGCCGC CATCACCCAC GGCATGGATG AGCTGTACAA GCTCGAGCA
1021 CACCACCACC ACCACTGA
```

Figure 4.30: *Nucleotide sequences of pET28+PmGIH_GFP recombinant DNA construct.*

```
1   MGKTWLLLAT LAVGASLANI LDSKCRGAMG NRDMYNKVER VCEDCTNIYR LPQLDGLCRN
61  RCFNNQWFLM CLHSAKREAE LEHFRLWISI LNAGRPWKLV SKGAELFTGI VPILIELNGD
121 VNGHKFSVSG EGEGDATYGK LTLKFICTTG KLPVPWPTLV TTLSYGVQCF SRYPDHMKQH
181 DFFKSAMPEG YIQERTIFFE DDGNYKSRAE VKFEGDTLVN RIELTGTDFK EDGNILGNKM
241 EYNYNAHNVY IMTDKAKNGI KVNFKIRHNI EDGSVQLADH YQQNTPIGDG PVLLPDNHYL
301 STQSALSKDP NEKRDHMIYF GFVTAAAITH GMDELYKLEH HHHHH*
```

Figure 4.31: *Amino acid sequence of rPmGIH _GFP protein.*

4.9 Expression and purification of the recombinant GIHs in *E.coli*

The BL21(DE3) strain of *E. coli* was transformed with each of the expression vectors pET28b-PmGIH_*No sig*, pET28b-PmGIH_*With sig* and pET28b-PmGIH_*GFP*. The recombinant clones were screened by PCR amplification and a single positive clone from each plate were selected for expression studies.Optimization of gene expression was carried out with respect to inducer concentration, time and temperature of induction. IPTG concentration of 0.4 mM was producing good results and was used to induce protein expression.

In the case of pET28b-PmGIH_*Nosig*, production of sufficient quantity of biologically active soluble form of recombinant GIH without signal peptide could not be accomplished by using different approaches in spite of optimization of protocols using different strategies for enhancing the solubility. Majority of the recombinant GIH without signal peptide was getting accumulated in the inclusion bodies. Therefore, it was purified from the inclusion bodies through denaturing Ni-NTA affinity column chromatography. The yield of the purified *rPmGIH_nosig* was 3-4 mg/litre of the induced culture. Tricine-SDS-PAGE analysis of the purified *rPmGIH_nosig* (Figure 4.32) showed single band products that migrate at 10.9 kDa, which matched the theoretical molecular weight calculated from its sequence. Not detected this protein band in un-induced culture.

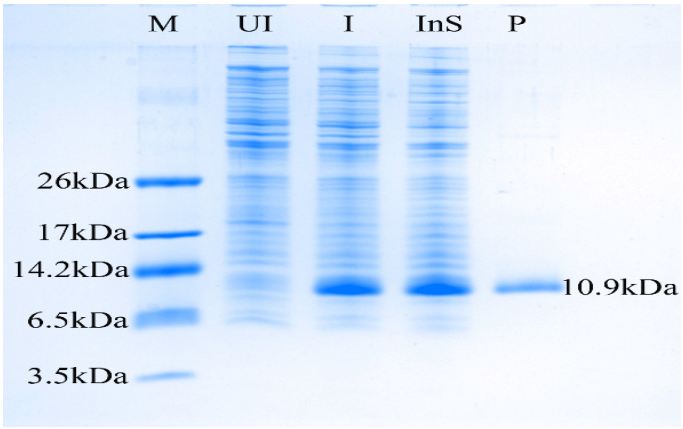


Figure 4.32: *Tricine SDS-PAGE (15%) of the rPmGIH without signal peptide M-marker, UI-Uninduced total cellular protein, I- Induced total cellular protein, InS - insoluble fraction, P– purified rGIH without signal peptide.*

Traditionally, secretory protein expression is often associated with eukaryote expression systems that need efforts that are more demanding, running costs and facilities. Moreover, associated post-translational modifications and heterologous sample preparations can sometime interfere in downstream applications. To allow for an easy, straight forward and highly cost-effective approach, it is desirable to establish *E. coli* based methods (Kotzsch *et al.,* 2011). Secretory expression of target proteins into the culture media combines the advantages of easy recovery and the possibility of tailoring the growth conditions for preservation of activity and stability of recombinant proteins.

Recombinant CMG family peptide are generally expressed in the insoluble fraction when using *E. coli* as expression system, and therefore, in order to confer the biological activity it should be subjected to refolding reaction

(Tsutsui *et al.,* 2013). It has been the crucial problem in producing recombinant protein using *E. coli* expression system. Culture condition of recombinant *E. coli* can influence the expression of gene and solubility of expressed protein. High-level expression usually causes formation of an inclusion body. Reduction of the cultivation temperature of the host after induction was tried in the present study as it had been suggested as a way to avoid or decrease the inclusion body formation. Gopal and Kumar (2013) have reported that solubility of recombinant protein was increased by prolonged induction at low temperatures with decreased amounts of IPTG. Incubation time, temperature and culture media optimized to facilitate the soluble expression of recombinant GIH were 16 °C overnight incubation with shaking at 225 rpm in M9 minimal media.

It was interesting to observe that in case of BL21(DE3) host which produce the recombinant GIH with signal peptide, the cell density got decreased as indicated by decreased optical density at 600 nm of the culture after induction with IPTG. To verify this the cell density of uninduced and induced cultures expressing recombinant GIH with signal peptide pre and post induction were compared by measuring the cell densities by taking optical density at OD600 in different time intervals, which showed (Figure 4.33) a drastic reduction in the cell density of the induced culture from third hour onwards.

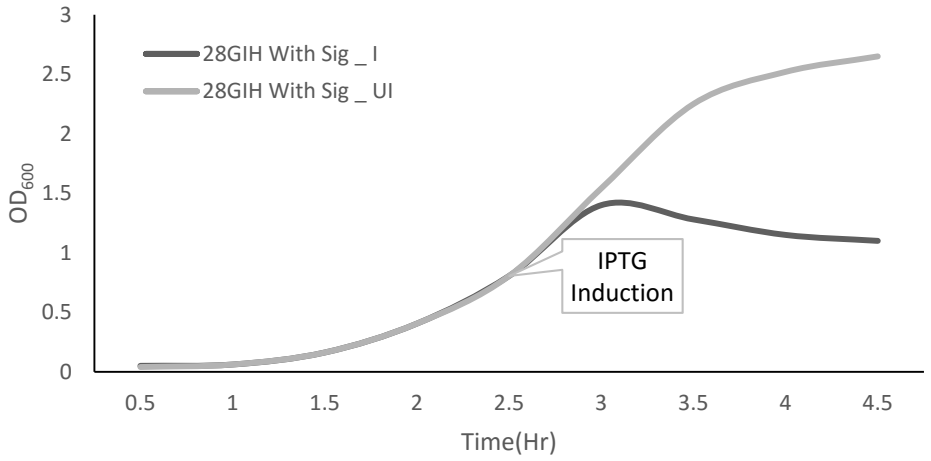


Figure 4.33: *Comparison of growth pattern of induced and uninduced BL21(DE3) host cells expressing recombinant GIH with signal peptide*

This decrease in cell density could be attributed to the lysis of host cells by the production of recombinant peptide in soluble form secreted in to the media due to the presence of the secretory signal peptide.

Prevention of cell lysis was necessary for obtaining recombinant GIH in sufficient quantities. Media and growth conditions were optimised to minimise lysis of the host cells by the expressed recombinant protein. Using M9 minimal growth media and lowering the incubation temperature to 16°C after induction for overnight with gentle shaking helped in reducing the cell lysis and production of soluble recombinant GIH protein in sufficient quantity to carry out affinity purification.

Rapid lysis of the host cells observed during the expression of recombinant PmGIH with signal peptide have been explained to be due to the

presence of C-type lectin domain conferring antibacterial property to the recombinant protein. Calcium dependent (C-type) lectins shares homology in the carbohydrate recognition domains (Weis *et al.,* 1998) and are involved in pathogen recognition and cellular interactions. Expression of Lectin genes has been reported in the hepatopancreas of the pacific white shrimp *Penaeus vannamei* and the Atlantic white shrimp *Penaeus setiferus* (Gross *et al.,* 2001). Wong *et al.* (2008) detected the higher expression of these genes in immature *Metapenaeus ensis* and put forward their importance in signalling in the hepatopancreas for synthesis and mobilization of vitellogenin and nutrients. The presence of a C-type lectin domain in the GIH has raised many questions about its role, mode of interaction, possible site of action etc. However, its precise role and possible interactions in vitellogenesis remains to be further explored.

On the other hand, we can explore the possibility that the nervous system, equipped with peptides that exhibit potent antimicrobial properties, utilize neurotransmitters and hormones to defend directly the organism from microbial assault. Many neuropeptides and peptide hormones are akin to antimicrobial peptides in their amino acid composition, size *etc*. and their antimicrobial activity suggests they have a direct role in innate defence (Brogden, 2005). Several anatomical settings like the eyestalk, in which neuropeptides, known to play a critical role in physiological functioning, could be responsible for a previously unrecognized direct anti-infective, innate immune role. This signals the possibility that the nervous system, through utilization of these peptides, has the capacity to deliver anti-infective agents directly to innervated sites, localized with great spatial specificity and delivered rapidly. The nervous and neuroendocrine systems, in principle, have the potential to serve a direct immune function.

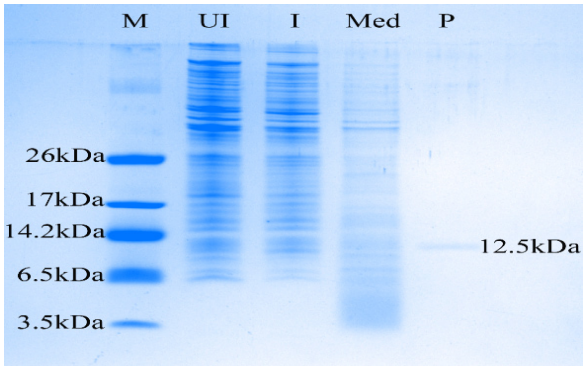


Figure 4.34: *Tricine SDS-PAGE (15%) of the rPmGIH with signal peptide M-marker, UI-Uninduced total cellular protein, I-Induced total cellular protein, Med - Media fraction, P - purified recombinant GIH with signal peptide.*

The recombinant GIH with signal peptide was purified using native Ni-NTA affinity column chromatography. The yield of the purified rPmGIH_*withsig* was 1-2 mg/litre of culture. Tricine-SDS-PAGE analysis of the purified *r*PmGIH_w*ithsig* (Figure 4.34) showed single band products that migrate at ~12 kDa, which matched the theoretical molecular weight calculated from its sequence. Not detected this protein band in un-induced culture.

In the case of pET28b-PmGIH_*GFP* expression, production of biologically active form of recombinant GIH with GFP as fusion protein with signal peptide was observed in the *E. coli* cell surface after the induction.The recombinant peptide was getting accumulated in the cell wall. Therefore, it was purified from the cell pellet through native Ni-NTA affinity column chromatography. Yield of the purified *rPmGIH_GFP* was 2-3 mg/litre. Tricine-SDS-PAGE analysis of the purified *rPmGIH_nosig* (Figure 4.35) showed single band products that migrate at 39.29 kDa, which matched the theoretical

molecular weight calculated from its sequence. Not detected this protein band in un-induced culture.

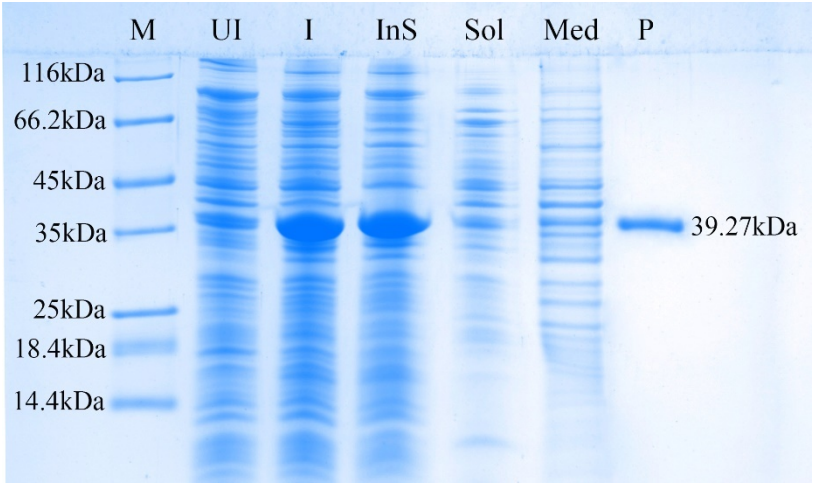


Figure 4.35: *Tricine SDS-PAGE (15%) of the rPmGIH with GFP M-marker, UI-Uninduced total cellular protein, I-Induced total cellular protein, InS - insoluble fraction, Sol- soluble fraction, Med - Media fraction, P– purified recombinant GIH-GFP.*

In the present work, the recombinant GIH (*rPmGIH*s) of *P. monodon* has been produced using *E. coli* expression system, and succeeded in producing a reasonable quantity. Moreover, the *rPmGIH_withsig* was obtained as secretory protein in the media fraction; the steps involved in the refolding activity could be avoided. The *E. coli* expression system reported here can produce sufficient quantities of recombinant PmGIH for down strean research activities like for the production of specific antibodies, which when administered could act as antagonist blocking the inhibitory action of GIH hormone *in vivo*. Specific immunoassay systems developed using the recombinant proteins can elucidate the neuroendocrine mechanisms regulated

by GIH through *in vivo* bioassays. It can also find application in the characterization of the binding partners and associated pathways etc. The recombinant molecules developed can lead to further understanding and expansion by further experimentations in the frontiers of molecular mechanisms regulating reproduction in crustaceans.

⌘⌘⌘⌘⌘

Chapter –V

Summary and Conclusion

Summary and Conclusion

The technique currently undertaken globally for induced maturation in both the domesticated and wild-caught females shrimps maintained in captivity is unilateral eyestalk ablation. Since this crude method is physiologically destructive as well as irreversible technique, modern and animal friendly approaches based on molecular biology needs to be developed for hormonal manipulation to induce maturation leading to ovary development and spawning. Even though many attempts in this direction are being carried out globally, a concrete technology is yet to be developed for ready application in shrimp aquaculture.

5.1 Summary

The main aim of this study was to decipher the genetic mechanism behind the hormones involved in the maturation of two commercially important shrimp species, *Fenneropenaeus indicus* and *Penaeus monodon.* Ovarian maturation in crustacean is mainly controlled by an eyestalk neuropeptide called gonad-inhibiting hormone (GIH) that is presumed to inhibit vitellogenin synthesis. Hence, the present study was undertaken for the molecular characterization of GIH gene followed by the construction of an expression vectors with a GIH gene insert, and expression of the recombinant GIH in a suitable expression system. The production of GIH through recombinant means as a secretary protein achieved from this work is the first report from the shrimp species. The information produced and the precise molecular tools developed can contribute in the manipulation of the regulatory mechanisms integral to the gonadal maturation leading to induced spawning, giving much needed impetus to the shrimp culture industry.

In this study, the cDNA synthesized by RT-PCR was used to amplify the GIH gene from both the candidate species. The preliminary characterization of partial cDNA of GIH gene of *P. monodon* and *F. indicus* using Reverse transcription Polymerase Chain Reaction (RT-PCR) yielded a 316 bp and 291 bp cDNA fragments from *P. monodon* and *F. indicus* respectively.

Further, the full-length cDNA sequences of the GIH were generated using Random Amplification of cDNA Ends (RACE). Characterization of the complete cDNA of Gonad Inhibiting Hormone (GIH) gene of *P. monodon* and *F. indicus* using RACE has revealed the complete cDNA length as 858 bp and 855 bp respectively.

Conceptual translation of the CDS sequence detected the presence of a 291 bp open reading frame encoding 96 amino acid residues in both the sequences. *In-silico* analysis using various software like; SignalP 4.1 program has shown the presence of a secretary signal peptide-coding region in the N-terminus of deduced polypeptide of both the species. ScanProsite tool detected the presence of CMG neurohormone family signature ([LIVM]-x(3)-C-[KR]-x-[DENGRH]-C-[FY]-x-[STN]-x(2)-F-x(2)-C) and a C-type lectin domain signature (C-[LIVMFYATG]-x(5,12)-[WL]-{T}-[DNSR]-{C}-{LI}-C-x(5,6)-[FYWLIVSTA]-[LIVMSTA]-C) in the deduced amino acid sequences of both the GIH sequences. Multiple sequence alignment using homology search algorithms and phylogenetic analysis revealed the close similarity among the reported GIH sequences.

Expression vector with GIH gene insert was developed for *Penaeus monodon* GIH using the pET28b+ expression vector using specific primers designed from the sequence information generated in this study, and having

restriction sites incorporated in it. In-frame fusion of PmGIH with the vector encoded T7 promoter region at the 5` end and hexa histidine tag at the N-terminal was carried out. Two set of primers were designed for cloning of GIH gene - with and without signal peptide region in the pET28b+ expression vector by inserting the NcoI restriction site in the forward primer and NotI site in the reverse primer. For pET28b+GFP expression vector the forward primer included NcoI and reverse primer included XhoI restriction sites. Using these primers, PCR amplification was carried out using the recombinant plasmid pJET-PmGIH as a template. In case of pET28b+GFP, the cloning strategy resulted in the construction of GIH followed by the GFP in frame with the hexa histidine tag.

Expression of the recombinant GIH protein of the most commercially demanding species viz., *Penaeus monodon* was carried out in *E. coli* using the pET28b+ expression described above. The genetic engineering of the GIH gene was carried out at the signal peptide region to produce the GIH protein both as secretary and as intracellular inclusion bodies. For this the recombinant expression plasmids constructed were transformed in to BL21 (DE3) *E. coli* expression strain and the recombinant protein expression was induced by adding IPTG at a final concentration of 0.4 mM to the growing culture of the recombinant colonies selected for expression.

The expressed 6x HIS-tagged recombinant proteins were affinity purified to homogeneity using Ni -NTA matrix affinity column under native and denaturing conditions. The yields of the purified proteins were in the range of 1-4 mg/liter of the induced culture. Tricine-SDS-PAGE analysis of the purified *rPmGIH_nosig, rPmGIH_withsig* and *rPmGIH_GFP,* showed single band products that migrate at 10.9 kDa, 12.5 kDa and 39.27 kDa respectively, which

matched the theoretical molecular weight predicted from the sequence of recombinant construct.

The GIH production through recombinant means as secretary protein is the first report among shrimp species.

5.2 Conclusion

The aim of the doctoral research was to understand the gene coding for gonad inhibiting hormone in *Penaeus monodon* and *Fenneropenaeus indicus* through transcriptomic and proteomic approach, which can find application in the development of a suitable antagonistic system to reduce the negative impact of GIH in reproductive maturation.

The present work has evolved a system to produce and harvest recombinant GIHs in sufficient quantities, both as a secretary protein as well as inclusion bodies. It can be used for the generation of anti-PmGIH antibody, which can be used as GIH antagonist to be employed for blocking the interplay of the GIH hormone by binding to it, and silence GIH gene following translation, which could be projected as an alternative to replace the eyestalk ablation technique for induced maturation of shrimps under captivity.

The recombinant PmGIH (*rPmGIH*) proteins produced can also be used for studying the structural and physiological functions of the hormone, as well as for elucidating its mode of action. The possibilities are unlimited; a fluorescently labeled antibody that will bind only to the GIH protein can provide information on the site of synthesis, mode of transport and site of action of the GIH hormone.

The purified recombinant *rPmGIH_GFP* protein can find applications in developing high-throughput sub cellular localization assays to localize the binding partner for GIH.

The importance of this work is in the fact the information produced and the precise molecular tools developed can contribute in the manipulation of the regulatory mechanisms integral to the gonadal maturation leading to induced spawning giving much needed impetus to the shrimp culture industry.

⌘⌘⌘⌘⌘

References

References

Adiyodi RG and Subramonium T (1983) Arthropoda: Crustacea. Reproductive Biology of Invertebrates. Adiyodi, K. G. and Adiyodi, R G. New York, John Wiley and Sons. 9, 443-495.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic Local Alignment Search Tool. J Mol Biol 403–410.

Anilkumar G and Adiyodi KG (1985) The role of eyestalk hormones in vitellogenesis during the breeding season in the crab, *Paratelphusa hydrodromous* (Herbst). Biol Bull 169:689–695.

Bailey-Brock JH and Moss SM (1992) Penaeid taxonomy, biology and zoogeography, p. 9-27. In: Fast A.W. and Lester L.J. (Eds). Marine shrimp culture: principles and practices. Developments in aquaculture and fisheries science, volume 23. Elsevier Science Publisher B.V., The Netherlands.

Benzie JAH (1998) Penaeid genetics and biotechnology. Aquaculture 164:23–47.

Bocking D, Dirkensen H and Keller R (2001) The crustacean neuropeptides of the CHH/MIH/GIH family: structures and biological activities. In The Crustacean Nervous System (ed. W. Korand), Berlin: Springer, New York.

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250.

Caillouet AC Jr (1972) Ovarian maturation induced by eyestalk ablation in pink shrimp, *Penaeus duorarum* Burkenroad. Proc. World Maricul. Soc. 3, 205-225.

Chan SM, Gu PL, Chu KH and Tobe SS (2003) Crustacean neuropeptide genes of the CHH/MIH/GIH family: Implications from molecular studies. Gen. Comp. Endocrinol. 134:214–219.

Chang ES, Chang SA and Mulder EP (2001) Hormones in the Lives of Crustaceans: An Overview. Am Zool 41:1090–1097.

Charmantier G and Charmantier-Daures M (1998) Endocrine and neuroendocrine regulations in embryos and larvae of crustaceans. Invertebr Reprod Dev 33:273–287.

Charmantier G, Charmantier-Daures, M and Van Herp F (1997) Hormonal reproduction of growth and reproduction in crustaceans. Endocrinology and Reproduction; Recent Advances in Marine Biotechnology. M. Fingerman, Nagabhushanam, R. and Thompson, M-F. New Hampshire, USA, Science Publishers Inc. 1, 109-161.

Chen T, Zhang L-P, Wong N-K, Zhong M, Ren C-H and Hu C-Q (2014) Pacific white shrimp (*Litopenaeus vannamei*) vitellogenesis-inhibiting hormone (VIH) is predominantly expressed in the brain and negatively regulates hepatopancreatic vitellogenin (VTG) gene expression. Biol Reprod 90:47.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Chung JS and Webster SG (2003) Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*: From target to transcript. Eur J Biochem 270:3280–3288.

Coman GJ, Arnold SJ, Peixoto S, Crocos PJ, Coman FE and Preston NP (2006) Reproductive performance of reciprocally crossed wild-caught and tank-reared *Penaeus monodon* broodstock. Aquaculture 252:372–384.

Davey ML, Hall MR, Willis RH, Oliver RW, Thurn MJ and Wilson KJ (2000) Five Crustacean Hyperglycemic Family Hormones of *Penaeus monodon*: Complementary DNA Sequence and Identification in Single Sinus Glands by Electrospray Ionization-Fourier Transform Mass Spectrometry. Mar Biotechnol (NY) 2:80–91.

de Kleijn DP, Coenen T, Laverdure AM, Tensen CP and VanHerp F (1992) Localization of messenger RNAs encoding crustacean hyperglycemic hormone and gonad inhibiting hormone in the X-organ sinus gland complex of the lobster *Homarus americanus*. Neuroscience 51:121–128.

de Kleijn DP, Janssen KP, Waddy SL, Hegeman R, Lai WY, Martens, GJ and Van HF (1998) Expression of the crustacean hyperglycaemic hormones and the gonad-inhibiting hormone during the reproductive cycle of the female American lobster *Homarus americanus*. J Endocrinol 156:291–8. doi: 10.1677/joe.0.1560291

de Kleijn DP, Sleutels FJ, Martens GJ and VanHerp F (1994) Cloning and expression of mRNA encoding prepro-gonad-inhibiting hormone (GIH) in the lobster *Homarus americanus* . FEBS Lett 353:255–258.

de Kleijn DP and VanHerp F (1995) Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comp Biochem Physiol B Biochem Mol Biol 112:573–579.

Dircksen H, Böcking D and Heyn U (2001) Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochem. 170:159–170.

Dircksen H, Webster SG and Keller R (1988) Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. Cell Tissue Res 251:3–12.

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

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

Emmerson WD (1983) Maturation and growth of ablated and unablated *Penaeus monodon* Fabricius. Aquaculture 32:235–241.

Fanjul-Moles ML (2006) Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: Review and update. Comp Biochem Physiol - C Toxicol Pharmacol 142:390–400.

FAO (2009) Aquaculture production. In: Yearbook of Fishery and Aquaculture Statistics. pp 84–188.

FAO (2012) The State of World Fisheries and Aquaculture 2012. Rome. 230 p.

FAO (2014) The State of World Fisheries and Aquaculture 2014. Rome. 218 p.

FAO (2015) FAO FishFinder - Web Site. FAO FishFinder. FI Institutional Websites. In: *FAO Fisheries and Aquaculture Department* [online]. Rome. [Cited 21 September 2015]. http:// www. fao. org/ fishery/ fishfinder/en

Fingerman M (1987) The Endocrine Mechanisms of Crustaceans. J Crustac Biol 7:1–24.

Gopal GJ and Kumar A (2013) Strategies for the production of recombinant protein in *Escherichia coli*. Protein J 32:419–25. doi: 10.1007/s10930-013-9502-5

Grève P, Sorokine O, Berges T, Lacombe C, Van Dorsselaer A and Martin G (1999) Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). Gen Comp Endocrinol 115:406–414.

Gross, P. S. Bartlett TC, Browdy CL, Chapman RW and Warr GW (2001) Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, L. setiferus. Dev Comp Immunol 25:565–577.

Gu PL, Tobe SS, Chow BKC, Chu KH, He JG and Chan SM (2002) Characterization of an additional molt inhibiting hormone-like neuropeptide from the shrimp *Metapenaeus ensis*. Peptides 23:1875–1883.

Hanström B (1939) Hormones in Invertebrates. Oxford University Press, London, Edinburgh, New York, Toronto, 198 p.

Harrison KE (1990) The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans, a review. J. Shellfish Res. 9, 1-28.

Hartoll RG (1985) Growth, sexual maturity and reproductive output. In Factors in adult growth, Crustacean Issues 3, pp101-128, editor: Wenner AM, Rotterdam:Balkerma.

Hopkins PM (2012) The eyes have it: A brief history of crustacean neuroendocrinology. Gen Comp Endocrinol 175:357–66.

Huberman A (2000) Shrimp endocrinology. A review. Aquaculture 191:191–208.

Jaros PP (1979) Immunocytochemical demonstration of the neurosecretory X-organ complex in the eyestalk of the crab *Carcinus maenas*. Histochemistry 63:303–310.

Katayama H, Ohira T and Nagasawa H (2013) Crustacean Peptide Hormones : Structure, Gene Expression and Function. Aqua-BioScience Monogr 6:49–90.

Kegel G, Reichwein B, Weese S, Gaus G, Peter-Katalinić J and Keller R (1989) Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. FEBS Lett 255:10–14.

Keller R (1992) Crustacean neuropeptides: Structures, functions and comparative aspects. Experientia 48:439–448.

Keller R, Jaros PP, and Kegel G (1985) Crustacean hyperglycemic neuropeptides. Amer. Zool, 25207-222.

Khoo HW (1988) Shrimp maturation in captivity: its control and manipulation. *J.Singapore Nat. Acad. Sci.* **17,** 1-11.

Klein JM, Mangerich S, de Kleijn, DP, Keller R and Weidemann WM (1993) Molecular cloning of crustacean putative molt-inhibiting hormone (MIH) precursor. FEBS Lett 334:139–42.

Kotzsch A, Vernet E, Hammarstrom M, Berthelsen J, Weigelt J, Graslund S and Sundstrom M (2011) A secretory system for bacterial production of high-profile protein targets. Protein Sci 20:597–609.

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

Lee KJ, Elton TS, Bej AK, Watts SA, Watson RD (1995) Molecular cloning of a cDNA encoding putative molt-inhibiting hormone from the blue crab, *Callinectes sapidus*. Biochem Biophys Res Commun 209:1126–1131.

Lightner DV (2005) Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. J. World Aquac. Soc. 36:229–248.

Longyant S, Sithigorngul P, Sithigorngul W, Chaivisuthangkura, Thammapalerd N and Menasveta P (2003) The effect of eyestalk extract on the vitellogenin levels in the haemoymph of the giant tiger prawn *Penaeus monodon*. Sci. Asia 29, 371-381.

Ma KY, Chan TY and Chu KH (2009) Phylogeny of penaeoid shrimps (Decapoda: Penaeoidea) inferred from nuclear protein-coding genes. Mol Phylogenet Evol 53:45–55.

Marsden G, Mather P and Richardson N (2007) Captivity, ablation and starvation of the prawn *Penaeus monodon* affects protein and lipid content in ovary and hepatopancreas tissues. Aquaculture 271:507–515.

Marsden G, McGuren JJ, Hansford SW and Burke MJ (1997) A moist artificial diet for prawn broodstock: Its effect on the variable reproductive performance of wild caught *Penaeus monodon*. Aquaculture 149:145–156.

Martin G, Sorokine O and Van Dorsselaer A (1993) Isolation and molecular characterization of a hyperglycemic neuropeptide from the sinus gland of the terrestrial isopod *Armadillidium vulgare* (Crustacea). Eur J Biochem 211:601–7.

Martin G and Juchault P (1999) Androgenic hormone specificity in terrestrial isopods (oniscidea): Systematic involvements. J. Crust. Biol. 19:684–689.

Mazurová E, Hilscherová K, Triebskorn R, Köhler H-R, Maršálek B and Bláha L (2008) Endocrine regulation of the reproduction in crustaceans: Identification of potential targets for toxicants and environmental contaminants. Biologia (Bratisl) 63:139–150.

Mosco A, Zlatev V, Guarnaccia C, Pongor S, Campanella A, Zahariev S and Giulianini PG (2012) Novel protocol for the chemical synthesis of crustacean hyperglycemic hormone analogues - an efficient experimental tool for studying their functions. PLoS One 7:e30052. doi: 10.1371/journal.pone.0030052

Moss SM, Moss DR, Arce SM, Lightner DV and Lotz JM (2012) The role of selective breeding and biosecurity in the prevention of disease in penaeid shrimp aquaculture. J Invertebr Pathol 110:247–250.

Nagaraju GPC (2007) Is methyl farnesoate a crustacean hormone? Aquaculture 272:39–54.

Nagaraju GPC (2011) Reproductive regulators in decapod crustaceans: an overview. J Exp Biol 214:3–16.

Nagaraju GPC and Borst DW (2008) Methyl farnesoate couples environmental changes to testicular development in the crab *Carcinus maenas*. J Exp Biol 211:2773–2778.

Nagaraju GPC, Kumari NS, Prasad GLV, Rajitha B, Meenu M, Rao MS and Naik BR (2010) Structural prediction and analysis of VIH-related peptides from selected crustacean species. Bioinformation 4:6–11.

Okumura T (2007) Effects of bilateral and unilateral eyestalk ablation on vitellogenin synthesis in immature female kuruma prawns, *Marsupenaeus japonicus*. Zoolog Sci 24:233–240.

Ollivaux C, Vinh J, Soyez D and Toullec J-Y (2006) Crustacean hyperglycemic and vitellogenesis-inhibiting hormones in the lobster *Homarus gammarus*. FEBS J 273:2151–60.

Palacios E, Ibarra AM and Racotta IS (2000) Tissue biochemical composition in relation to multiple spawning in wild and pond-reared *Penaeus vannamei* broodstock. Aquaculture 185:353–371.

Palacios E, Perez-Rostro CI, Ramirez JL, Ibarra AM and Racotta IS (1999) Reproductive exhaustion in shrimp (*Penaeus vannamei*) reflected in larval biochemical composition, survival and growth. Aquaculture 171:309–321.

Panouse JB (1943) Influence de l'ablation du pédoncle oculaire sur la croissance de l'ovaire chez la crevette Leander serratus. C. R. hebd. Séanc. Acad Sci., Paris 217, 553–555.

Primavera J (1984) A Review of maturation and reproduction in closed thelycum penaeids. Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps., Iloilo City, Philippines, SEAFDEC Aquaculture Department.

Qian Y-Q, Dai L, Yang J-S, Yang F, Chen D-F, Fujiwara Y, Tsuchida S, Nagasawa H and Yang, W-J (2009) CHH family peptides from an "eyeless" deep-sea hydrothermal vent shrimp, *Rimicaris kairei*: characterization and sequence analysis. Comp Biochem Physiol B Biochem Mol Biol 154:37–47.

Qiao H, Xiong Y, Zhang W, Fu H, Jiang S, Sun S, Bai H, Jin S and Gong Y (2015) Characterization, expression, and function analysis of gonad-inhibiting hormone in Oriental River prawn, *Macrobrachium nipponense* and its induced expression by temperature. Comp Biochem Physiol A Mol Integr Physiol 185:1–8.

Quackenbush LS (1986) Crustacean Endocrinology, A Review. *Can. J. Fish. Aquat.Sci.* 43, 2271-2282.

Rosas C, Fernandez I, Brito R and Diaz-Iglesia E, (1993) The effect of eyestalk ablation on the energy balance of the pink shrimp, *Penaeus notialis*. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 104, 183-187.

Rosenberry B (2004) World shrimp farming. Shrimp News Int. 17, 4.

Rotllant G, De Kleijn D, Charmantier-Daures M, Charmantier G and VanHerp, F (1993) Localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of *Homarus gammarus* larvae by immunocytochemistry and in situ hybridization. Cell Tissue Res 271:507–512.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory press. New York.

Schägger H (2006) Tricine-SDS-PAGE. Nat Protoc 1:16–22.

Skinner DM (1985) Molting and regeneration. In The biology of Crustacea, V9, pp 43-146. D.E. Bliss and L.H. Mantel (eds), Academic Press, New York.

Soyez D, Le Caer JP, Noel PY and Rossier J (1991) Primary structure of two isoforms of the vitellogenesis-inhibiting hormone from the lobster *Homarus americanus*. Neuropeptides 20:25–32.

Studier FW and Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol 189:113–130.

Studier FW, Rosenberg AH, Dunn JJ and Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 185:60–89.

Tacon AJ (2003) Aquaculture Production Trends Analysis. FAO Fisheries Circular No.886, Rev 2- Review of the state of world aquaculture by Inland Water Resources and Aquaculture Service, FAO, Fisheries Department.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Desmond HG (1997) The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

Tiu SHK, Hui JHL, Mak ASC, He JG and Chan SM (2006) Equal contribution of hepatopancreas and ovary to the production of vitellogenin (PmVg1) transcripts in the tiger shrimp, *Penaeus monodon*. Aquaculture 254:666–674.

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

Tsutsui N, Nagakura-Nakamura A, Nagai C, Ohira T, Wilder MN and Nagasawa H (2013) The ex vivo effects of eyestalk peptides on ovarian vitellogenin gene expression in the kuruma prawn *Marsupenaeus japonicas*. Fish. Sci.: 79:33-38.

Uawisetwathana U, Leelatanawit R, Klanchui A, Prommoon J, Klinbunga S and Karoonuthaisiri N (2011) Insights into eyestalk ablation mechanism to induce ovarian maturation in the black tiger shrimp. PLoS One 6:e24427. doi: 10.1371/journal.pone.0024427

Vaca AA and Alfaro J (2000) Ovarian maturation and spawning in the white shrimp, *Penaeus vannamei*, by serotonin injection. Aquaculture 182:373–385.

Van Herp F and Van Buggenum HJ (1979) Immunocytochemical localization of hyperglycemic hormone (HGH) in the neurosecretory system of the eyestalk of the crayfish *Astacus leptodactylus*. Experientia 35:1527–1529.

Vijayan KK, Reynold Peter, Mohanlal DL and Balasubramanian CP (2013) Preliminary characterisation of gonad inhibiting hormone (GIH) gene and its expression pattern during vitellogenesis in giant tiger shrimp, *Penaeus monodon* Fabricius, 1798. Indian Journal of Fisheries 60(2), 103-107.

Wainwright G, Webster SG, Wilkinson MC, Chung JS and Rees HH (1996) Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*. Involvement in multihormonal regulation of growth and reproduction. J Biol Chem 271:12749–12754.

Webster SG (1998) Neuropeptides inhibiting growth and reproduction in crustaceans. In Recent advances in Arthropod Endocrinology. Pp 33-52, eds: Coast GM and Webster SG. Cambridge University Press.

Webster SG, Keller R and Dircksen H (2012) The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. Gen Comp Endocrinol 175:217–233.

Weis WI, Taylor ME and Drickamer K (1998) The C-type lectin superfamily in the immune system. Immunol Rev 163:19–34.

Wilder M, Okumura T and Tsutsui N (2010) Reproductive Mechanisms in Crustacea Focusing on Selected Prawn Species: Vitellogenin Structure, Processing and Synthetic Control. Aqua-BioScience Monogr. 3:73–110.

Wong QWL, Mak WY and Chu KH (2008) Differential gene expression in hepatopancreas of the shrimp *Metapenaeus ensis* during ovarian maturation. Mar Biotechnol (NY) 10:91–8.

Wongsawang P, Phongdara A, Chanumpai A and Chotigeat W (2005) Detection of CHH/GIH activity in fractionated extracts from the eyestalk of Banana prawn. Songklanakarin J. Sci. Technol. 27 (Suppl. 3): 789-798.

Yang WJ, Aida K and Nagasawa H (1995) Amino acid sequences of a hyperglycaemic hormone and its related peptides from the Kuruma prawn, *Penaeus japonicus*. Aquaculture 135:205–212.

Yang WJ, Aida K and Nagasawa H (1997) Amino acid sequences and activities of multiple hyperglycemic hormones from the Kuruma prawn, *Penaeus japonicus*. Peptides 18:479–85.

Yang WJ, Aida K, Terauchi A, Sonobe H and Nagasawa H (1996) Amino acid sequence of a peptide with molt-inhibiting activity from the kuruma prawn *Penaeus japonicus*. Peptides 17:197–202.

⌘⌘⌘⌘⌘