Development of CHH family recombinant hormones and RNAi for induced maturation of *Penaeus monodon*

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Under the Faculty of Marine Sciences

Department of Marine Biology, Microbiology and Biochemistry

by

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Certificate

This is to certify that the research work presented in the thesis entitled "**Development of CHH family recombinant hormones and RNAi for induced maturation of *Penaeus monodon***" is based on the original work done by Ms. Vrinda S. under our guidance, at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin –16, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy and that no part of the work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in the thesis entitled "**Development of CHH family recombinant hormones and RNAi for induced maturation of *Penaeus monodon***" is based on the original work done by me under the joint guidance of Dr. Rosamma Philip, Assistant Professor, Department of Marine Biology. Microbiology and Biochemistry and Prof. I.S. Bright Singh, Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin - 682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin- 16
December 2012

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"It is good to have an end to journey toward; but it is the journey that matters, in the end." Ernest Hemingway.

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Vrinda S.

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Chapter

1

GENERAL INTRODUCTION

1.1 Crustacean Endocrinology

Shrimp aquaculture globally has developed remarkably over the past two decades. In this backdrop, new technological innovations in hormonal manipulation of shrimp reproduction have turned out to be so critical for effective and sustainable stock management. Consequently, a recent flurry of research has been oriented towards isolation and characterization of members of the crustacean hyperglycemic hormone (CHH) family of neuropeptides which are known to occur in Arthropoda, and in particular, in crustaceans. The functions attributed to members of CHH family are diverse, and include regulation of haemolymph glucose concentration (from which the name CHH is derived), moulting (moult-inhibiting hormone, MIH) and reproduction (vitellogenesis-inhibiting hormone / Gonad- inhibiting hormone, VIH/GIH). Other hormones such as mandibular organ-inhibiting hormone and ion transport peptides are equally important. Research on the endocrine control of crustacean metabolism, growth and reproduction has both biological and economical implications. Nevertheless, most of the research in crustacean endocrinology has been oriented towards solving a few cardinal biological issues circumventing growth and reproduction as there is an ever increasing worldwide demand for aquatic food. This demand has lead to worldwide expansion of culturing economically important crustaceans such as lobsters, crab and shrimp. For the sustenance of aquaculture production it is inevitable to generate more information on the hormonal control of growth and reproduction.

Considering the hormonal manipulation for induced ovarian development, eyestalk ablation has been the only tool so far by the aquaculture practitioners all over the world. The principle lies in the removal of X-organ sinus gland complex to eliminate the function and action of inhibitory hormones of reproduction and maturation. However, commercial shrimp hatcheries have been facing serious difficulties in achieving induced maturation through eyestalk ablation, in spite of using the best breeders sourced from the seas. This unethical and destructive intervention can be replaced with efficient and user friendly molecular tools to bring forth gonadal maturation under captivity. The recent developments in the field of hormonal manipulation of shrimp reproduction involve the development of recombinant hormones and their antagonists. Besides hormone antagonists, RNAi has been used for the experimental manipulation of gene expression and to prove their function at genomic level. In the present work we have generated CHH family hormone recombinants and their antagonists for post translational transgene silencing and RNAi for post transcriptional transgene silencing to accomplish maturation and spawning under captivity.

The general introduction outlines the neuroendocrine system of crustaceans involved in various endocrinological and physiological events such as metabolism, growth and reproduction in crustaceans.

1.1 Crustacean Endocrinology

Crustaceans have populated the marine, freshwater, and terrestrial environments. Physiology of these organisms has evolved to suit their respective habitats, and they respond to cues in their environment that enhance their chances of survival and reproduction. The basic plan of the crustacean endocrine system consists of classical epithelial-type endocrine glands, and endocrine structures of neural origin, the neurosecretory cells and neurohemal organs. The neuroendocrine component is of major significance with respect to the number and roles of hormones (neuro hormones) produced. In fact, a large majority of crustacean hormones appear to have

a neural origin. The first concrete evidence for hormonal activities in crustaceans was obtained in the 1920s when Koller (1925, 1927) and Perkins (1928) showed that crustaceans produce hormones that effect color changes in these animals. Crustaceans utilize a variety of neuro-endocrine signaling cascades to regulate their physiology. A general review of crustacean endocrinology is warranted in an effort to identify manifestations of this system that are largely conserved among animals as well as to recognize those peptides that are unique to this taxon. Most endocrine studies of the Crustacea have been performed in the malacostracans. While general observations and conclusions drawn from studies of crabs, shrimps, crayfishes, and lobsters can likely be applied to other taxa of crustaceans, a few manifestations such as endocrine signaling are clearly unique to some groups.

1.1.1 The Endocrine Structures

Most of what is known about the crustacean endocrine system has been derived from studies with decapods. Nevertheless, there is a considerable body of knowledge about the endocrinology of two additional groups of malacostracans, the amphipods and isopods. The major endocrine structures controlling the endocrine functions of decapod crustaceans include the eyestalk (X-organ Sinus gland complex), Y-Organ, androgenic gland, ovary and mandibular organ.

1.1.1.1 *Eyestalk- X-organ Sinus gland Complex as neural center in crustaceans*

The major neuroendocrine control center of crustaceans is the sinus gland system. The sinus gland, first described by Hanstrom (1933), lies in the eyestalk of most stalk-eyed species, having migrated out of the body proper, at least in higher crustaceans, along with the lamina ganglionaris, medulla externa, medulla interna, and medulla terminalis. Hanstrom (1939) named the structure of unknown function located on the proximal surface of the medulla terminalis as X-organ (XO).

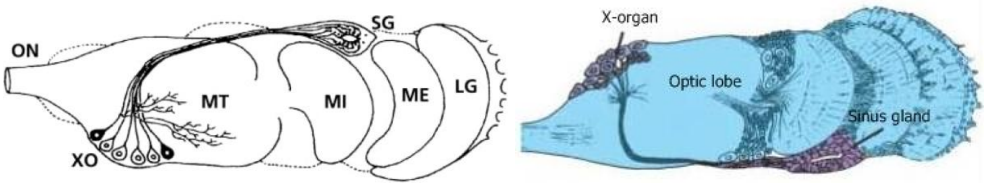

Fig.1. Diagrammatic representation of the ganglia and the X-organ/sinus gland complex in eyestalks of decapod crustaceans (dorsal view). LG- lamina ganglionaris, ME- medulla externa, MI medulla interna, MT- medulla terminalis, ON- optic nerve, SG-sinus gland, XO- X-organ perikarya. Courtesy: Böcking *et al*., 2002 and Hartenstein, 2006.

The X-organ is composed of a group of monopolar neurosecretory cells. The fact that the sinus gland does not itself produce the hormones that it contains but rather is a neurohemal organ, storing and releasing neurohormones, was first revealed by the study of Bliss (1951) and Passano (1951). The axonal endings from the cells terminate into the neurohemal organs called sinus gland (Skinner K, 1985). This neurosecretory system is called X-organ sinus gland complex (XOSG) (Fig.1). The ultra structure of this gland has been reviewed by Cooke and Sullivan (1982). In the eyestalks of decapods, this X-organ lies at the proximo ventral edge of the medulla terminalis. There is, however, some controversy over whether neurosecretory perikarya located outside this X-organ also contribute axons and their terminals to the sinus gland. For example, Andrew *et al.* (1978), after using the cobalt backfilling technique on axons entering the sinus gland of the crayfish *Orconectes virilis*, stated "to have found no evidence that neurosecretory axons enter the sinus gland from any area other than the medulla terminalis X-organ". In summing up several reports, Cooke and Sullivan (1982) concluded that at least 90% of the sinus gland terminals belong to cells whose perikarya are in the medulla terminalis X-organ, with the remainder of the terminals belonging to neurosecretory cells that are not associated with this X-organ.

Neuropeptides/neurohormones are synthesized in X organs and secreted from the cells which are collected/stored in the sinus gland, and are released into the haemolymph upon appropriate stimulation. The hormones include CHH (Jaros and

Keller, 1979; Jaros, 1979; Van Herp and Buggenum, 1979), MIH (Dircksen *et al*., 1988; Klein *et al*., 1993a), GIH (de Kleijn *et al*., 1992), RPCH (Bellon-Humbert *et al*., 1986) and PDH (Mancillas *et al*., 1981). This unique peptide family in crustaceans is involved in blood sugar regulation, inhibition of ecdysteroid synthesis, regulation of reproduction and growth (Cooke and Sullivan, 1982; Beltz, 1988; Keller, 1992; Fingerman, 1992; Chang, 1993). The CHH/MIH/GIH family, collectively known as CHH family hormones, has different functions despite their similarities in structure (Keller, 1992; Sun, 1994; Chang, 1997; Lacombe *et al*., 1999; Böcking *et al*., 2002). A schematic diagram to summarise the action of eyestalk factors is given in Fig. 2.

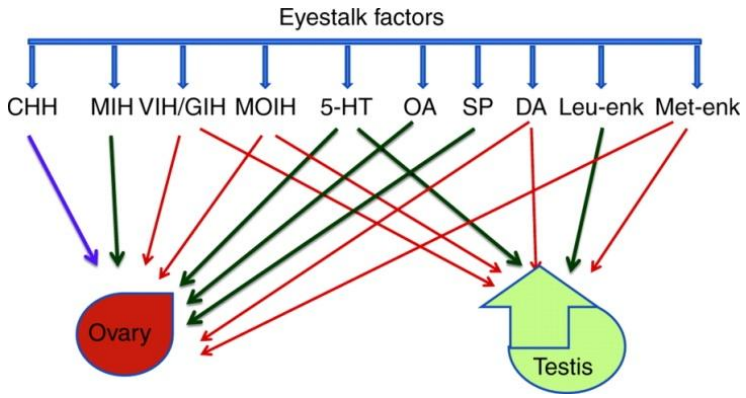

Fig.2. The effect of eyestalk factors on reproduction in crustaceans. CHH: crustacean hyperglycaemic hormone; MIH: moulting inhibiting hormone; VIH: vitellogenesis inhibiting hormone; GIH: gonad inhibiting hormone; MOIH: mandibular organ inhibiting hormone; 5-HT: 5-hydroxytryptamine; OA: octopamine; 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. Courtesy: Nagaraju, 2011.

1.1.1.2 *Y-Organ*

Epithelial, i.e., nonneural, endocrine glands are also extremely important to crustaceans. One such is the Y-organ, first described by Gabe (1953) from 58 species of malacostracans. Y-organs are the source of moulting hormone. This paired gland is located in the maxillary segment (Gabe, 1971). There have been several ultra structural

studies of Y-organs (Aoto *et al*., 1974; Hinsch *et al*., 1980; Buchholz and Adelung, 1980; Taketomi and Hyodo, 1986).

The glands consist of only one type of cells whose structure is suggestive of vertebrate steroid-secreting cells. For example, smooth endoplasmic reticulum is more commonly encountered than the rough form. However, crustaceans cannot synthesize cholesterol, but instead convert dietary cholesterol into the steroid moulting hormone ecdysone. Thus, it is not surprising that Hinsch *et al*. (1980) concluded that although Y-organ cells of *Cancer antennarius* resemble steroid-secreting cells of vertebrates, the resemblance is to the cells that have only minimal synthetic capacity. The Y-organ cells presumably absorb cholesterol from the haemolymph and convert it to moulting hormone as needed. A second epithelial endocrine gland is the androgenic gland, which is present only in males.

1.1.1.3 *Ovary*

The female reproductive system consists of paired ovaries (Fig. 3), oviducts, gonophores and an external sperm reception area. The ovarian lobes are connected by a central bridge of ovarian tissue. The lobes are symmetrically arranged and lie in the cephalothorax, dorsal to the stomach and hepatopancreas. An oviduct arises laterally from each ovary at a point just beside the position of the heart. It extends ventrally and opens through gonophores in the abdomen. In the fully mature state, the shapes of ovary cannot be distinguished as the ovary fills the thoracic region of the body cavity completely.

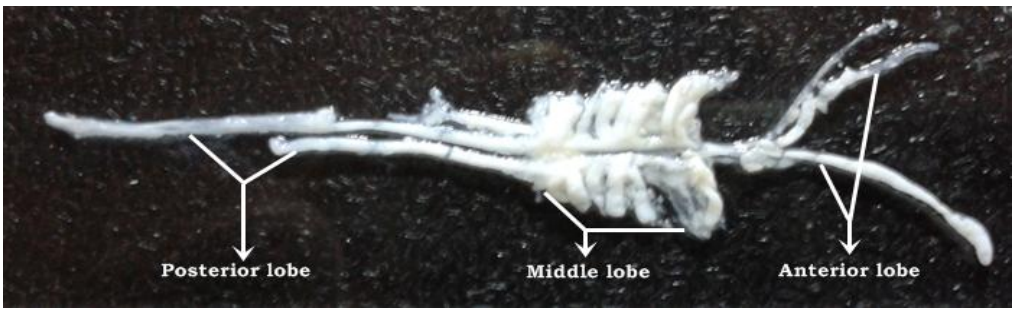

Fig. 3. Previtellogenic Ovary of *P.monodon*

In majority of female crustacean's ovarian differentiation take place when the ovary is translucent to opaque white (previtellogenic ovary). During vitellogenesis the color of the ovary changes from pale yellow (vitellogenic stage I) to orange (vitellogenic stage II) then to brown (vitellogenic stage III) and finally to dark brown, prior to spawning. The maturation of ovary is associated with an increase in the size of the ovary, as oocytes proliferate and accumulate yolk. The ovaries are the source of a hormone, called the ovarian hormone, which induces the development of female secondary sexual characteristics such as the ovigerous setae, to which eggs attach after ovulation, and the brood pouch (Charniaux- Cotton, 1952; Nagamine and Knight, 1987).

1.1.1.4 *Mandibular Organ*

An enigmatic, possible endocrine gland is the paired mandibular organ, first described by Le Roux (1968). These structures, which lie close to the Y-organs, have in fact been misidentified as Y-organs by some investigators. In contrast to the Y-organs that consist of a single type of cell, mandibular organs have been reported to consist of two cell types (Aoto *et al*., 1974).

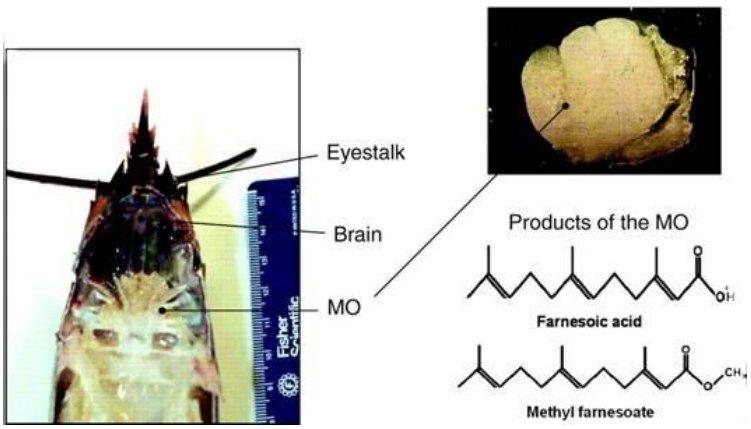

Fig.4. Brain and mandibular organ location in the lobster *Homarus americanus* and MO products such as farnesoic acid and methyl farnesoate. Courtesy: Nagaraju, 2011.

The mandibular organs may have a role in moulting and/or reproduction, because the cells display changes in ultra structure during vitellogenesis and in some but not reported in all the species during moulting cycle (pre-moult versus post-moult) (Aoto *et al*., 1974; Hinsch, 1981; Taketomi and Kawano, 1985). Also, these organs get hypertrophied following eyestalk ablation (Hinsch, 1977; Le Roux, 1983). Their ultra structure is suggested to have a lipid-secreting structure (Hinsch and Hajj, 1975). Recently, the mandibular organs of five decapods, including the spider crab *Libinia emarginata*, were found to produce methyl farnesoate, which can be converted to juvenile hormone (Borst *et al*., 1985). This compound was also present in the haemolymph of this crab (Laufer *et al*., 1985). Methyl farnesoate is highly lipophilic, which is inconsistent with the ultra structure of these organs, and may indeed in crustaceans be converted to juvenile hormone, a well-established hormone in insects.

1.1.2 Role of hormone in growth and moulting

In crustaceans, the development cycle is of utmost importance during the life course of the organism. This development cycle which is best known as the moulting cycle, consists of multiple moults throughout the organisms lifetime. Moulting which involves in the shedding of the exoskeleton is called ecdysis (Block *et al*., 2003). This is required because the endoskeleton consistently grows while the current exoskeleton is maintained at a static size. The reason for this is because the exoskeleton is composed mainly of minerals, and therefore must be shed and replaced with a new larger exoskeleton**.** Through the moulting cycle, the animals show dramatic changes in many aspects, including body structure, biology and behavior.

Each moulting cycle of crustaceans is composed of different stages including pre-ecdysis (pre-moult, D stage), ecdysis (E stage), postecdysis (post-moult, A-B stages), and inter-moulting (C stage) stage (Drach, 1939; Skinner, 1962). Usually, the duration of A, B and E stages are short in the whole cycle and duration of the intermoult is longer than the pre-moult. Various criteria are in use for staging the crustacean moult cycle. These include the degree of hardness of the exoskeleton,

changes in matrices of the setae (Drach, 1939), the growth of re-generating limb buds (Skinner, 1962; Stevenson *et al.,* 1968; Stevenson and Henry,1971; Hopkins, 1982), the progressive development of gastroliths in the digestive organs (McWhinnie, 1962; Hopkins, 1977; Rao *et al*., 1977) histological changes of organs and tissues (Travis, 1957; Skinner, 1962 ; Stevenson, 1968 ; Stevenson *et al*., 1968 ; Benhalima *et al*., 1998), and the development of setae (Lyle and MacDonald, 1983; Moriyasu and Mallet, 1986). Additionally, environmental factors such as temperature (Chittleborough, 1975), photoperiod (Quackenbush and Herrnkind, 1983), food supply (Chittleborough, 1975), space (Cheng and Chang, 1994) and various stresses (Weis *et al*., 1992) influence either the moult interval or the moult increment, or both.

1.1.2.1 *Ecdysteroid- The moulting hormone synthesized by Y-organs*

Ecdysteroids are synthesized and secreted by paired endocrine glands, the Y-organs (Gabe, 1953; Buchholz and Adelung, 1980; Birkenbeil and Eckert, 1983). They were first demonstrated as moulting glands associated with crustacean moult cycle by Echalier (1954). Ecdysteroids were identified and classified as steroid hormones associated with moulting hormone. Y-organs secrete ecdysteroids synthesized from cholesterol. The steroid arthropod moulting hormone was first isolated from insects and it was called ecdysone (Butenandt and Karlson, 1954). The structure of the more active and most predominant form of the hormone was subsequently determined to be 20-hydroxyecdysone (20E). The first isolation of 20E from a crustacean was from the spiny lobster, *Jasus lalandii* (Hampshire and Horen, 1966; Horn *et al.,* 1966). It is now apparent that ecdysone and 20E are the two most predominant members of a family of steroids that possess moulting hormone activity (Lafont, 1997). Members of this hormone family are collectively called the ecdysteroids. However, experiments have shown that in some species besides ecdysone, the Y-organ also secrete different ecdysteroids, identified as 25-deoxyecdysone (25DE) and 3-dehydroecdysone (3DE). The crustacean ecdysteroids are very polar (20-OH-ecdysone has six hydoxyl and one keto group) and there is no evidence for

carrier proteins in the haemolymph. Thus, they circulate freely and can enter cells by simple diffusion, but in addition, there is also an energy-dependent and carrier-mediated process of hormone entry (as in cray fish and crab) as demonstrated by Spindler *et al*. (1984). Receptor binding sites have been found in the DNA of *Metapenaeus ensis* (Chan, 1988), lending further evidence to the hypothesis that steroid hormones mediate their action by differential transcription of specific genes.

The ecdysteroid titer in haemolymph fluctuates during the moulting cycle (Fig. 5). At C stages, the hormonal concentration remains low. The titer rises during premoult and reaches a peak level in the stage D2 followed by an abrupt decline before ecdysis. After ecdysis the titer drops to intermoult level in the post-moult stages. Thus, the ecdysteroids initiates the cascade of physiological events that precede the moult. The control of the moulting is, ultimately, to modulate the ecdysteroids synthesis.

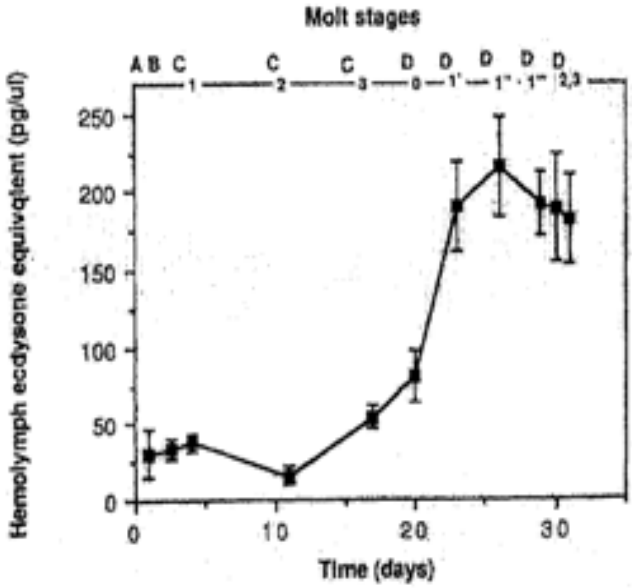

Fig.5. Ecdysteroid titers in the haemolymph of juvenile *Penaues vannamei* during the moulting cycle. Courtesy: Chan *et al*., 1988.

In crustaceans numerous studies have proved that the rate of ecdysteroid synthesis is negatively modulated by the neurohormone moult - inhibiting hormone

(MIH) produced in the eyestalk (Aiken and Waddy, 1980; Skinner DM, 1985; Skinner *et al*., 1985, Lachaise *et al*., 1993; Chang, 1989, 1993; Vernet and Charmantier-daures, 1994 and Charmantier *et al*., 1997).

1.1.2.2 *Methyl Farnesoate - The juvenile hormone*

Another compound presumably involved in regulating steroidogenesis is methyl farnesoate (MF) produced by the mandibular organ (Borst and Laufer, 1990). Elucidation of the role of MF in development and reproduction is currently an active field of investigation. There is some evidence that MF may have a role in larval development by acting as a hormone that retards development (a juvenilizing factor). In adults, MF may function in a reproductive capacity (Chang *et al*., 2001). There is also evidence that methyl farnesoate can stimulate moulting by increasing ecdysteroid production. This was demonstrated *in vitro* for the Y-organs of *Cancer magister* Dana (Tamone and Chang, 1993).

1.1.3 Reproduction in crustaceans

1.1.3.1 *Regulation of reproduction in Crustaceans*

The reproductive regulation in crustaceans is diversely mediated by both external and internal factors. External factors influencing the reproductive activity are seasonal and include the environmental conditions like temperature, photoperiod, food availability and salinity (Adiyodi and Adiyodi, 1970). In the case of farm grown crustaceans, factors like ammonia concentration, pH of water, exchange of water and dissolved oxygen are major factors affecting the growth and reproduction. All the major external factors mentioned influence the reproductive activity in crustaceans. In the broad sense, photoperiod regulates the timing of ovarian growth. It was found that in crayfish *Orconectes virilis*, the ovarian cycle of maturation was accelerated with photoperiod. In freshwater crab *Oziotelphusa sensex sensex*, the ovarian growth was stimulated with decreased light. Temperature is another factor affecting the ovarian growth and as well as

egg hatching. Warmer water temperature induces egg laying in *Orconectes* (Aiken, 1969) and increases the frequency of egg hatching in *Artemia salina* (Von Hentig, 1971). Food availability or abundance is a vital factor for normal oocyte development and provides materials for the synthesis of yolk proteins and lipids.

The endogenous factors affecting the gonad maturation in female crustaceans is regulated by both neuroendocrine and non-endocrine secretions. Gonadal maturation in crustaceans is principally controlled by two antagonistic neuropeptides: GIH (gonad inhibiting hormone/ vitellogenesis inhibiting hormone, VIH in females) secreted by the X-organ sinus gland complex (XO-SG) of the eyestalk, and gonad stimulating hormone (GSH) said to be produced from the brain and thoracic ganglion. The juvenile hormone, methyl farnesoate and ecdysteroid secreted by mandibular organs and Y-organs also control female reproduction.

1.1.3.2 *Reproduction and vitellogenesis in female crustaceans*

In the adult female crustaceans, reproduction is a physiological process that entwines with moulting. Hartonll (1985) considered the fact that most crustaceans begin to reproduce before somatic growth is completed. Gonadal and somatic growth in female crustaceans differs with species. Nelson (1991) reviewed that in the genus *Penaeus* (species such as *P. aztecus*, *P. indicus*, *P. japonicus*, *P. merguensis*, *P. monodon*, *P. stylirostris* and *P. vannamei*); there is more than one oocyte maturation and spawning phase within one moult cycle. Contrary to this, the reproduction in crabs and lobsters take place during the long intermoult period.

In the most important commercial penaeid shrimp species, ovarian development is mainly characterized by the accumulation of the major yolk protein (vitellin) and the formation of cortical rods in the oocytes (Meusy and Payen, 1988; Clarke *et al*., 1990). Vitellogenin (VTG), the precursor of vitellin is synthesized in

the ovary and hepatopancreas. Vitellogenin is transported to oocytes and accumulated in the ooplasm as vitellin. Vitellin forms the nutritional source during embryogenesis. Completion of yolk accumulation is followed by the formation of cortical rods, which are formed radially around the periphery of the oocyte plasma membrane, and mature oocytes are spawned. As the spawning takes place the content of the cortical rods are released around the egg which forms a layer of jelly enveloping the eggs. The post spawning (45 min) stage involves dissipation of the jelly layer and the formation of hatching envelope by exocytosis of the electron-dense material from the egg surface. The layer of jelly functions as a barrier preventing polyspermy. Vitellogenesis occurs in hard shelled shrimp at the intermoult stage C4, shortly after moulting and continues until immediately before final maturation in penaeid shrimps. Vitellin has been purified and characterized in various species of penaeid shrimps (Wilder *et al*., 2002). Kawazoe *et al*. (2000) characterized vitellin in kuruma prawn (*Metapenaeus japonicus*).

The ovarian development in crustaceans is known to be under the inhibitory control of the neuroendocrine system, the X-organ sinus gland complex present in eyestalks (Keller, 1992; De Kleijn and Van Herp, 1998). Eyestalk ablation evidenced the inhibitory role of neuroendocrine control and stimulation in ovarian development. The eyestalk hormone was purified and characterized as vitellogenesis-inhibiting hormone (VIH) in the American lobster *Homarus americanus* (Soyez *et al*., 1987). VIH peptides have been reported in the spiny lobster, *Jasus lalandii* (Marco *et al*., 2002) and the prawn, *Macrobrachium rosenbergii* (Yang and Rao, 2001). Purified *Homarus* VIH protein inhibited shrimp ovarian development *in vivo* (Soyez *et al*., 1987) while recombinant *Homarus* VIH (Ohira *et al*., 2005) and purified *Jasus* VIH (Marco *et al*., 2002) inhibited shrimp VTG synthesis *in vitro*. In *H. americanus*, VIH peptide levels in haemolymph and VIH mRNA levels in the X-organ sinus gland complex decreased during yolk accumulation (De Kleijn *et al*., 1998). These results suggest that the decrease of

VIH is a critical factor for inducing yolk accumulation. While VIH has not yet been identified in penaeid shrimps, several CHH family peptides have been determined (Nagasawa *et al*., 1999). Some of these have inhibitory effects on protein synthesis (including VTG and SOP) and VTG mRNA synthesis in *vitro*-incubated ovarian fragments (Khayat *et al*., 1998; Avarre *et al*., 2001; Tsutsui *et al*., 2005). Thus, the synthesis of VTG and SOP (shrimp ovarian peritrophin) seems to be mainly under control by CHH family peptides. However, evidence to support this hypothesis is limited, and further studies are necessary.

1.1.3.3 *Ecdysteroids and reproduction*

Ecdysteroids, which are produced by the Y-organ, commonly act as the moulting hormones, but there is evidence that they may also have a role in reproduction. Both the ovaries and testes have been shown to be alternate sources of ecdysteroids in certain crustacean species (Lachaise, 1990). These steroids have been found in the follicle cells, oocytes and embryos of a number of species that include the brine shrimp *Artemia salina* (Spindler *et al*., 1984), the amphipod *Orchestia gammerellus* (Blanchet *et al*., 1979), the crabs *Cancer maenas* (Lachaise and Hoffman, 1982), *C. sapidus* (McCarthy and Skinner, 1979), *C. anthonyi* and *C. magister* (Chang, 1989), *Libinia emarginata* (Laufer and Deak, 1990), *Acanthonyx lunulatus* (Chaix and DeReggi, 1982), and the macruran *Astacus leptodactylus* (Spindler *et al*., 1984). The role of ecdysteroids in reproduction is not yet clear. There are reports that vitellogenesis appears to be closely related to increased ecdysteroid titers in the haemolymph (Chaix and DeReggi, 1982; Lachaise *et al*., 198l), yet other studies show no correlation (Chang, 1984; Laufer *et al*., 1988). Ecdysteroids enhanced cellular proliferation in primary cultures of lobster testes (Brody and Chang, 1989). These findings suggest that ecdysteroids may function by stimulating growth of the gonad and spermatogenesis. Perhaps there is an antagonistic relationship between MF and ecdysteroids in reproduction, or perhaps they function at different phases of the spermatogenic cycle, thus requiring their presence at different times.

1.1.4 CHH family neuropeptides -Inhibitory and stimulatory roles in reproduction

An investigation of a possible hormonal control of carbohydrate metabolism in crustaceans, (Abramowitz *et al*., 1944) showed that injection of eyestalk extracts (ES) into the blue crab, *Callinectes sapidus* dramatically elicited hyperglycaemia. This dynamic factor was proved to be very potent (0.001ES equivalents giving a significant response), heat stable, highly concentrated in the SG, and was named a "diabetogenic factor". Further research and findings eventually replaced the name to crustacean hyperglycemic hormone (CHH). CHH isolated from shore crab, *Carcinus maenas*, was the first to be fully characterized (Kegel *et al*., 1989). Following this the 72 amino acid sequence of a peptide from *Homarus americanus* proved to have similar sequence identity (61% identical residues) as that of *Carcnius*-CHH. The *Homarus*-peptide interestingly exhibited both CHH and MIH (moult inhibiting hormone) activity. With these key findings two important aspects of structure and function was predicted for the CHH and the homologous peptides: (1) the hormones are multifunctional and (2) they have overlapping biological activities. A second peptide from *C. maenas* was identified which had certain structural features that set it apart from *Carcnius*-CHH (Webster, 1993). Prior to this, new sequences were available and thus emerged a new peptide family. The CHHs from *Callinectes*, *Homarus* and *Carcinus*, MIH/MIH related peptide from *Homarus* and VIH (Webster, 1993; Webster *et al*., 2000) could be considered as prototypes of two subgroups of this family (Keller, 1992). Thus, the entire grouping is now introduced as CHH superfamily with two subfamilies, type I peptides (CHH sensu stricto and ion transport peptides-ITPs) and type II peptides (Moult inhibiting hormone-MIH, Gonad/vitellogenesis- inhibiting hormones -GIH/VIHs) and mandibular organ-inhibiting hormone (MOIH) (Lacombe *et al*., 1999; Böcking *et al*., 2002; Chan *et al*., 2003; Chen *et al*., 2005; Fanjul-Moles, 2006; Giulianini and Edomi, 2006). CHH/MIH genes from penaeid shrimps (Gu and Chan, 1998a, b; Gu *et al*., 2000; Udomkit *et al*., 2000) as well as other decapods (Chan *et al*., 1998;

Tang *et al*., 1999, Dircksen *et al*., 2000) revealed striking homologies in the genome organization.

The prepropeptides of type I CHHs contain the CHH precursor related peptide (CPRP) sequence, which is located between the signal peptide and mature peptide sequences (Böcking *et al*., 2002; Wilcockson *et al*., 2002). Type II CHHs lack the CPRP sequence and have a glycine residue at position #12 of the mature peptide sequence (Böcking *et al*., 2002; Lee *et al*., 1995; Nakatsuji *et al*., 2009). The Gly12 is located in a short α1 helix in type II CHHs that is absent in type I CHHs (Katayama *et al*., 2003). The invariant peptide backbone always consists of six cysteines, two arginines, one aspartic acid, and a phenylalanine residue in identical position. The six cysteines form three disulfide bridges, and the bonds between individual Cys residues are identical in all the peptides (Böcking *et al*., 2002). Post translational modification results in α-amidation of a valine residue at the C-terminus of type I eyestalk; (Fanjul-Moles, 2006). Type I CHH peptides that have an N-terminal glutamine can undergo a slow cyclization to form pyroglutamine (Hsu *et al*., 2008). By contrast, the termini of type II CHH peptides are usually not modified (Böcking *et al*., 2002; Nakatsuji *et al*., 2009). Multiple isoforms of type I CHHs are generated as alternatively spliced variants or as different gene products (Chen *et al*., 2004; Hsu *et al*., 2008). Multiple copies of CHH genes occur in decapod genomes, probably resulting from gene duplication; the duplicated genes evolved into the type I and type II lineages (Chan *et al*., 2003; Chen *et al*., 2005; Montagné *et al*., 2010). Species in the suborder Dendrobranchiata, represented by penaeid shrimp, usually have more CHH genes than in those in the suborder Pleocyemata, represented by caridean shrimps, lobsters, crabs, and crayfish (Chan *et al*., 2003). The differences in the gene and precursor structures firmly support this division.

Remarkable upsurge of interest in the past two decades has resulted in the identification of approximately 80 peptides of the CHH family from about 40

crustacean species (Webster *et al*., 2012). The identification has been more from conceptual translation from cDNAs or *in silico* mining of transcriptomes and genomes rather than from peptide sequencing. The identified peptides can unequivocally be placed in one of the two sub families (Webster, 2012). There are 109 nucleotide sequences encoding CHH family members in the GenBank database. Fig .6 shows the alignment of CHH family neuropeptide family of various decapod prawns. With regard to biological activities, several novel hormones have more recently been added to the early recognized roles in the regulation of carbohydrate metabolism (CHH), secretagogue action on the hepatopancreas, inhibition of moulting (MIH) and gonad maturation (GIH/VIH). These comprise the inhibition of methyl farnesoate secretion (MOIH activity) (Liu *et al*., 1997; Wainwright *et al*., 1999), water uptake during ecdysis (Chung *et al*., 1999), and ionic and osmotic regulation (Soyez *et al*., 1994). These results once again underscore the pleiotropic activities of CHH-superfamily peptides. Much further work is, however, needed to elucidate the details and mechanisms of action, at the organ and cellular level, which underlie the variety of biological effects. It is safe to predict that more biological activities will eventually be discovered.

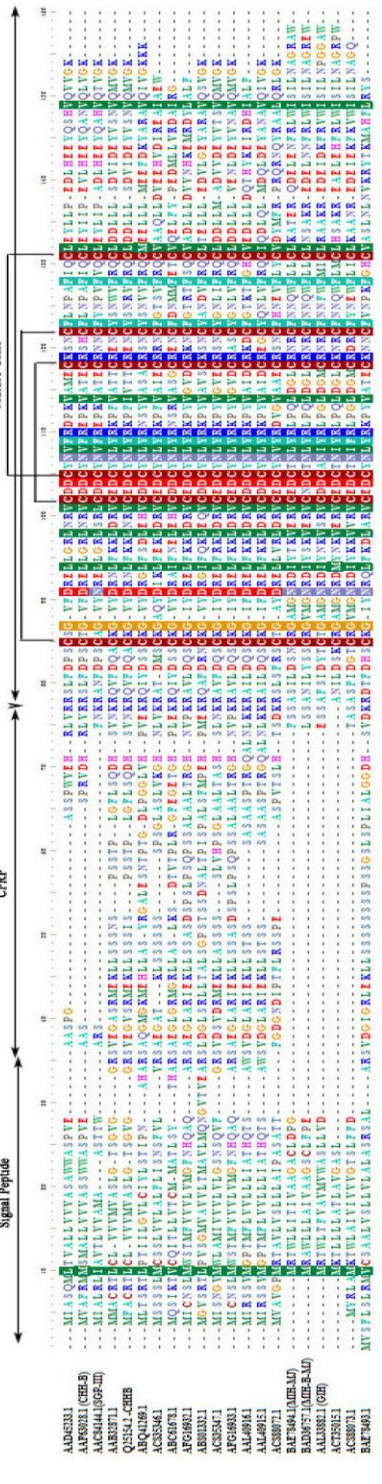

Fig. 6. Multiple amino acid alignment of CHH family members from various decapods retrieved from GenBank. The three regions are the signal peptide, CPRP, and mature CHH peptide. Highly conserved residues are shaded in Red (cysteine residues) and similar residues are shaded various colours. The dibasic cleavage site is identified by the inverted arrow. The locations of the six cysteines that form three disulfide bridges are indicated with brackets. Type II CHH family members (MIH, GIH, and VIH) lack the CPRP and have a glycine at position #12. Abbreviations and GenBank accession numbers: Cp, *Cancer productus* (CHH1,ABQ41269; CHH1b, ABQ41271); Cs, *Callinectes sapidus* (CHH, ABC61678); Gs, *Galathea strigosa* (CHH, ABS01332); Ha, *Homarus americanus* (CHHA, AAB32871; CHHB, Q25154); Mr, *Macrobrachium rosenbergii* (CHHg, AAL40916; CHHe, AAL40915); Me, *Metapenaeus ensis* (CHHA, AAD45233; CHHB, AAF63028; GIH, AF294648); Mj,*Marsupenaeus japonicus* (CHH, BAE78493; MIHC, BAE78494; MIHB, BAD36757); Pj, *Pandalopsis japonica* (CHH1PO, JQ317932; CHH1ES, JQ317933; CHH2, JQ317934); Pm, *Penaeus monodon* (CHH1 ACS88072; CHH3, AAC84144; MIH1 ACS88073 GIH, ACT35015); Rk, *Rimicaris kairei* (CHH1, ACS35346; CHH2, ACS35347; VIH, ACS35348).

1.1.4.1 *Crustacean hyperglycemic hormone - An overview*

The most abundant neuropeptide in the eyestalk neurohemal tissue or the sinus gland (SG), of decapod crustaceans is the crustacean hyperglycemic hormone (CHH). As their name implies, the CHHs are the primary regulators of haemolymph glucose levels in crustaceans. Their central role is regulation of carbohydrate metabolism reviewed by Keller and Sedlmeier (1998). Additionally, they have been implicated in the regulation of other processes, including lipid metabolism, hydro-mineralization, reproduction and moulting (Keller 1992; Liu *et al*., 1997; Webster, 1998). The relatively simple bioassay procedure made CHH a well studied hormone in this family. The primary structure of CHH isolated by HPLC was elucidated by Edman sequencing (Kegel *et al*., 1989; Chang *et al*., 1990; Kegel *et al*., 1991; Huberman *et al*., 1993; Aguilar *et al*., 1995; Yang *et al*., 1997). The prehormone structure of CHH was obtained by cDNA cloning (Weiderman *et al*., 1989; Tensen *et al*., 1991a; 1991b; Martin *et al*., 1993; Yasuda, 1994; Sun, 1994; De Kleijn 1994; Ohira 1997a; Gu and Chan, 1998a). Studies at the DNA level demonstrated multiple copies of peptide encoding genes, which explains the existence of isoforms. These isoforms have been isolated as mature peptides from SGs of single animals, e.g., up to seven slightly different CHHs in penaeid shrimp species (Davey *et al*., 2000; Khayat *et al*., 1998; Yang *et al*., 1997). Since they do not differ significantly in hyperglycaemic activity (Khayat *et al*., 1998; Yang *et al*., 1997), any distinctive physiological relevance, and their differential expression remains obscure. Another important contribution was the finding that different CHH-peptides can be generated by alternative splicing from a common transcript (Dircksen *et al*., 2001). Finally, post translational isoform generation by stereo isomerisation of particular residues in CHHs has been demonstrated in *Homarus* and crayfish species (Bulau *et al*., 2002; Soyez *et al*., 1994; Yasuda *et al*., 1994)

CHH is a neuropeptide with a very similar structure to MIH, thus it plays a role in moult regulation (Webster, 1998). Webster and Keller (1986) found that it inhibited ecdysteroid synthesis in *Carcinus maenas* Y-organs, but that the effect was

much lesser than MIH. This moult inhibiting effect of CHH has now been shown to be widespread in decapods (Van Herp, 1998; Webster, 1998). The effects of MIH and CHH appear to be synergistic (Webster, 1998). Although MIH and CHH are structurally distinct in brachyurans, the distinction is not necessarily clear in other decapods (Van Herp, 1998 and Webster, 1998). Increased levels of CHH in response to stress has been observed following emersion stress in *Cancer pagurus* Linnaeus (Webster, 1996) and starvation in the crayfish *Orconectes limosus* (Rafinesque) (Keller and Orth, 1990). Raised CHH levels have been suggested as a possible factor in extending the intermoult period beyond its normal length with, reduction in food supply (Oh and Hartnoll, 2000).

1.1.4.2 *CHH neurosecretory cells – Localization and expression*

In decapod crustaceans, the X-organ is the major neurosecretory site formed of about 150-200 somata, this constitute a heterogenous population of neurosecretory cells producing different types of major neuropeptides (90%). Additionally a limited number of neurosecretory somata from the eyestalk, brain and thoracic ganglion reach the sinus gland *via* the sinus gland nerve (Cooke and Sullivan, 1982). Various immunocytochemical studies have identified CHH/ MIH peptides in the XO-SG complex (Jaros, 1979; Van Herp and Van Buggenum, 1979; Gorgels-Kallen and Van Herp, 1981; Gorgels-Kallen and Meij, 1985; Dircksen *et al*., 1988; Webster, 1998; Marco and Gäde, 1999). The CHH/MIH peptides expressed in the conspicuous subgroup of perikarya were undistinguishable by morphological criteria, but they were clearly distinguishable by specific antibodies or hybridization probes. Various studies revealed that the CHH/MIH/GIH family peptides were expressed in single cells (De Kleijn *et al*., 1992; Chan *et al*., 2003), whereas some revealed their expression in different neurons too (Dircksen *et al*., 1988; Klein *et al*., 1993a). Electrophysiological methods, sensitive hormone determination, dispersed cell culture studies and quantitative determination have quantified and identified different CHH-producing cells (Stuenkel *et al*., 1991;

Keller *et al*., 1994, 1995; Richmond *et al*., 1996; Stuenkel and Cooke, 1988; Grau and Cooke, 1992; Meyers *et al*., 1992; Escamilla-Chimal *et al*., 2002). Interesting studies revealed that neural and non-neural cells such as glial cells and paraneurons seem responsible for the release of the hormone, which changes according to the developmental and growing stage of the animal (Chung *et al*., 1999; Escamilla-Chimal *et al*., 2001, 2002; Serrano *et al*., 2004; Chung and Webster, 2003).

XO-SG is considered the main locus of neuropeptide production; other sites also have been detected. CHH have been detected using RIA (Radio Immuno Assay) in pericardial organs (PO) of *Carcinus maenas* (Keller *et al*., 1985). Two cDNAs encoding two different CHH pre prohormones from the sinus gland and ventral cord express the same CHH mRNA (De Kleijn *et al*., 1995). Using immunocytochemistry CHH has been revealed in the second roots of thoracic ganglia, the suboesophagic ganglion of *Homarus* (Chang *et al*., 1999) and in the retina of the crayfish *Procambarus clarkii* (Chimal *et al*., 2001). Using the peptide chemistry, immunocytochemistry, cloning and sequencing methods, it has been reported that 24 neurosecretory cells of PO produced a novel CHH like peptide (PO-CHH) and two CHH - precursor-related peptides (Chung *et al*., 1999; Webster *et al*., 2000; Chung and Webster, 2004; Dircksen and Heyn, 1998; Dircksen *et al*., 2000 and Dircksen *et al*., 2001). However, PO-CHH did not show hyperglycaemic activity *in vivo* suggesting other function than that of SG-CHH. Similarly, extra-occular CHH in the hyperglycaemic activity has been questioned. A few authors have proposed that non-SG-CHH could mediate in localized regulation of cellular glucose metabolism or contribute to the regulation of secreting organ localized glucose metabolism (Chung and Webster, 2005; Chang *et al*., 1999; Chang *et al*., 2001).

1.1.4.3 *Structure of CHH gene derived precursors and peptides*

The advancement of biochemical and molecular techniques has resulted in the characterization of various precursors of CHH superfamily, showing that all the hormones have signal peptide, but only the preprohormone of CHHs contains a

precursor- related peptide that is located between the signal peptide and the hormone (Weidemann *et al*., 1989; Klein *et al*., 1993b; De Kleijn and Van Herp, 1995). Despite the advancements in the gene related studies, the gene organization, the expression pattern and evolutionary relationship of the peptides have not been completely resolved and the number of CHH genes in any species has not been determined yet. With the molecular tools about 40 genes have been reported from different species of decapods such as shrimp, crab, crayfish and lobster. Various molecular and biochemical techniques applied to study the CHH family neuropeptides in different decapod crustaceans coincide with the fact that peptides belonging to this family are polymorphic due the changes in the amino acid residues from the L- to the D- configuration. Different neuropeptides belonging to the CHH subfamily have been identified. Two groups comprising more than one isoform in Penaeidae, CHH-A and CHH-B, and at least two forms in Astacidaea and Palinura, CHH-I and CHH-II (Chan *et al*., 2003).

The peptide backbone always consists of six cysteines, two arginines, one aspartic acid and a phenylalanine residue in identical position. The six cysteines form three disulfide bridges, and the bonds between individual cysteine residues are identical in all the peptides (Böcking *et al*., 2002). In the three groups of decapods *i.e*., crayfishes, lobsters and shore crabs, the peptides are characterized by a sequence of 72 to 73 amino acid residues, C-terminal amidation and pyroGlu as N-terminal blocking group. These peptides have a molecular weight of about 8400 Da and share between 61% and 81% homology (Kegel *et al*., 1991; Fanjul-Moles, 2006). Katayama and Nagasawa (2004) reported that in the prawn *Marsupenaeus japonicus* mutation of CHH with glycine residue insertion showed weaker hyperglycaemic effect than the recombinant control. The authors suggested that the insertion of glycine residue is one of the indices for structural and functional divergence of the CHH family peptides.

CHH isoform (CHH-I) from *Procambarus buveri*, a 72 residue peptide (8388Da) shared 98% identity with the CHHs from *Orconectes limosus* and 83.3% identity with *Homarus americanus* isoform, but only 61.1% identity with *C. maenas* (Huberman *et al*., 1993; Aguilar *et al*., 1995). The sequence differences are most significant as they result in variations in interspecific hyperglycaemic activity (Fanjul-Moles, 2006). Considering the kinetics of the hyperglycaemic effect, the biological effects of two isoforms differ. With microsequencing and mass spectrometry two isoforms of CHH and CHH related peptide were identified and reported in *Homarus americanus*, *Cancer pagarus* and *Macrobrachium rosenbergii* (45%-68% identity with other crustaceans) (Tensen *et al*., 1991a; Chung *et al*., 1998; Sithigorngul *et al*., 1999; Chen *et al*., 2004). Direct sequence evidence suggested that these two isoforms came from a CHH gene transcribed in an alternative splicing manner. They demonstrated that the expression pattern of cDNA cloned from the eyestalk of this prawn (*Macrobrachium rosenbergii*) is positive in tissues of heart, gills, antennal gland and thoracic ganglion. Chen *et al.* (2003) reviewed various reports of CHH genes in decapod crustaceans and proposed that there are more than six CHH-cDNAs reported for shrimp, which can be divided into CHH-A and CHH-B, each group consisting more than one isoform. The data obtained suggests that there exist two major forms of CHH-like cDNAs in all the decapods. Mettulio *et al*. (2004 a, b) cloned cDNAs encoding CHH-A and CHH-B of Norway lobster *Nephrops norvegicus*, both CHHs differed in the preprohormone but not in the mature peptide sequence. The bioassay with the CHH expressed in bacteria showed similar hyperglycaemic activity similar to native CHH present in eyestalk extract. Bioassay with CHH modified by point deletion mutation led to the conclusion that progressive deletion of five highly conserved motifs in C-terminal reduced/lacked the hyperglycaemic activities, retaining all other motifs were important for the functional specificity. All studies in decapod crustaceans confirm the polymorphism of CHH. This polymorphism results in striking variation in the hyperglycaemic activity.

1.1.4.4 *Physiological aspects of CHH*

The physiology of the crustaceans is complex due to overlapping of multiple processes secreted from the XO-SG; these secretions influence the functions of each other (inhibitory / stimulatory). The processes change during different life stages so as to reach the adult stage. CHH is one of the most important hormones involved in the development of the organism (larval stage to adult). Mentioned above in detail is the multifunctional role of the isoforms of CHH in the structure. These isoforms play quite an intricate role in the hyperglycemic activity of CHH hormone. CHH plays the central role in carbohydrate metabolism, the stimulatory role, while it plays inhibitory role in moulting, reproduction and osmoregulation. With regard to the carbohydrate metabolism, CHH induces hyperglycaemia and hyperlipidemia in the haemolymph, playing a pivotal role in providing glucose and lipids to meet the energy requirements of the crayfish (Kummer and Keller, 1993). The CHH activity along the life cycle of decapods varies; they participate in the adaptive mechanisms to stress conditions playing a dual role in feedback control system (Santos and Keller, 1993a). The hormone synthesis and secretion is homeostatically controlled both *in vivo* (Santos and Keller, 1993b; Santos *et al*., 2001) and *in vitro*. *In vitro* experiments (Glowik *et al*., 1997) proved that CHH-producing neurons are under the negative feedback control of the glucose haemolymph and respond to D-glucose with hyperpolarization that inhibits the release of CHH, the consequence is glycolysis from glycogen and storage of D-glucose which in turn activates a K+ current. This K+ current induces hyperpolarization, enabling the glucose to directly regulate the release of CHH into the haemolymph, *i.e*., the hypoglycemic effect. CHH secreting cells depolarize, inducing the release of CHH and mobilization of glucose from glycogen. Experiments from *in vivo* studies suggest a second negative feedback loop; lactate resulting from an increasing glycolytic flux may stimulate a release of CHH to stimulate glycogenolysis (Fig. 7). During the transport from the neurohemal organ to the target organs, the bioactive CHH is bound to subunits of haemocyanin (Kallen *et al*., 1990), and at the target tissues, and induces mobilization of glucose from glycogen stores of different

tissues of the body, particularly midgut and muscles and the regulation of the haemolymph fatty acids and phospholipids by catabolism of the midgut lipid stores (Kumer and Keller, 1993; Santos and Keller, 1993a; Santos *et al*., 2001). The primary action of this peptide at membrane level seems to be an activation of GMP synthesis by activating guanylate cyclase (Goy, 1990). At tissue level, CHH stimulates glycogenolysis in muscle and midgut gland, inhibiting glycogen synthase and activating glycogen phosphorylase (Fanjul-Moles, 2006; Sedlmeier, 1982, 1988; Keller and Orth, 1990).

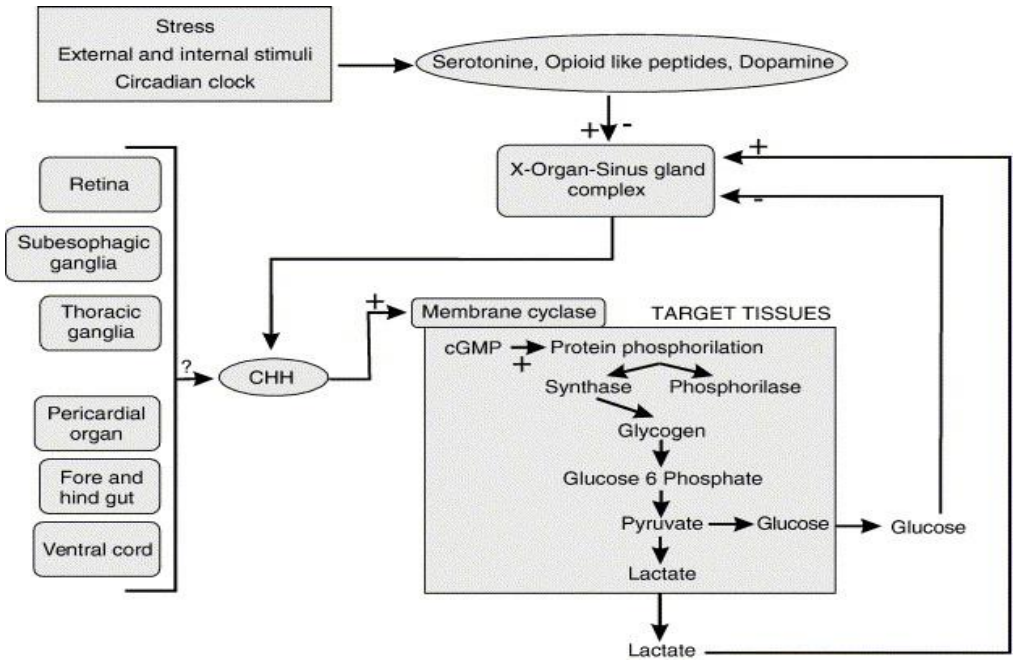

Fig.7. Drawing illustrating both the regulation of CHH level by internal and external stimuli (+stimulation, - inhibition, ? possible but not demonstrated effect) as well as different loci of this CHH production. Metabolic pathways have been simplified according to Santos and Keller (1993a).

Primarily the only recognized role of CHH was the hyperglycemic effect, but currently it has been shown and proved that CHH has multifunctional role in crustacean physiology. In addition CHH regulates ion and water balance by

increasing the transepithelial potential difference and sodium flux of isolated gills in crabs and crayfish (Morris, 2001; Spannings-Pierrot *et al*., 2000; Serrano *et al*., 2003). Reports also indicate a CHH isoform, specifically D-Phe (3) CHH effects the osmoregulatory function in freshwater crustacean. Interestingly, the CHH osmoregulatory functions relates with some of the crustacean moult cycles. In *C. maenas* and *H. americanus* before ecdysis (stressful process), rise in circulating CHH occurs (Chung *et al*., 1999; Chang *et al*., 2001) ostensibly mediating the completion of ecdysis through water uptake. The isoforms of CHH (A and B) has stimulatory effect on growth and endocytotic activity of ovocytes of crayfish, lobsters and Penaeids (*Penaeus vannamei*) (Aguilar *et al*., 1996; De Kleijn *et al*., 1998), affecting the vitellogenesis and maturation. Studies propose modulation of MF (juvenile hormone) by CHH isoforms (Liu *et al*., 1997; Silva Gunawardene *et al*., 2002, 2003).

1.1.4.5 *CHH neuromodulation*

The synthesis and secretion of CHH is under the control of the endogenous biological clock of the animal. Both CHH and glucose levels show circadian rhythmicity (Gorgels-Kallen and Voorter, 1985; Kallen *et al*., 1998, 1990; Escamilla-Chimal *et al*., 2001). Under normal conditions, CHH levels were low during the day time while increase was seen during the first hours of night time as in *Orconectes* and *Astacus* (Kallen *et al*., 1990). The circadian changes in CHH secretion positively correlated with circadian glucose rhythm (Kallen, 1988). Dark and light cycles may affect the release of CHH directly or indirectly by neural or endocrine modulation. Glycemic stress response research in context with circadian biology of decapods will be valuable to explicate complex mechanism of sugar regulation.

1.1.4.6 *Diagnosing CHH- Bioassay*

The main function of CHH is to elevate the glucose level in haemolymph. This effect is estimated by measuring the concentration of D-glucose in

haemolymph by glucose oxidase method, a colorimetric enzymatic method. The CHH levels in haemolymph can also be measured by using CHH specific antibody. Indirect or direct ELISA may be used for measuring the levels of CHH in haemolymph.

1.1.5 Moult Inhibiting Hormone in ecdysis- An overview

The basics of the hormonal control of moult initiation by two antagonistic hormones were understood well before 1980. The surgical extirpation of the eyestalk results in a shortened moult cycle interval, while the implantation of eyestalk contents restores this interval. A factor has been implied that normally inhibits the moulting process and it has been named the MIH. A Moult Inhibiting Hormone (MIH) is produced by neurosecretory cells in the medulla terminalis in the eyestalk, and stored in the sinus gland. This understanding was based on selective ablation (Pasteur- Humbert, 1962).Whilst this was accepted as a general phenomenon in Crustacea (Vernet- Cornubert, 1961; Sochasky, 1973). MIH had not then been reliably isolated nor categorised (Freeman & Bartell, 1976; Soyez & Kleinholz, 1977). The second hormone was a moulting hormone produced mainly by the Y-organs or moult glands (Gabe, 1953). The removal and re-implantation of the moult glands had been shown to, respectively, prevent and permit resumption of moulting in a range of crustaceans (Vernet, 1976). The first characterization of MIH as a neuropeptide was reported by Rao (1965). The moulting hormone was isolated and characterised by Hampshire and Horn (1966) who named it crustecdysone. In the 1970s, moulting hormone levels were being directly measured in individual animals and tissues using radioimmunoassay and chromatography (Bebbington and Morgan, 1977; Chang *et al*., 1976; Stevenson *et al*., 1979). Injection of crustecdysone had been shown to trigger precocious moulting (Dall and Barclay, 1977). Lachaise *et al.* (1993) and Skinner (1985) provided a very comprehensive review of the structure and activity of the moult glands producing C-27 steroid hormone termed ecdysteroids. A major advance since 1980 has been the isolation and characterisation

of MIH, which is now known to be a neuropeptide. This proved difficult because the eyestalk produces an entire family of structurally similar neuropeptides with different functions.

The initial advance was the development of a bioassay which measured the effect of MIH in reducing ecdysteroid synthesis by Y-organs *in vitro* (Soumoff and O'Connor, 1982). This led to the characterisation of MIH from the shore crab *Carcinus maenas* (Linnaeus) (Webster, 1986;Webster and Keller, 1986), followed by elucidation of its complete amino acid sequence (Webster, 1991).The peptidic nature of MIH has been established for many brachyuran and macruran species. Aguilar *et al*. (1996) isolated the MIH from the sinus gland of the Mexican crayfish, *Procambarus bouvieri*, and compared its sequence with the other four known peptides at the time from *Homarus americanus, Carcinus maenas, Callinectes sapidus* and *Penaeus vannamei*. Their lengths varied between 72-78 amino acids residues and their molecular masses between 8 and 9 kDa. All had six cysteines that form three disulphide bonds. Although so much is known of the chemistry of MIH, there is still uncertainty regarding the exact mechanism by which it inhibits ecdysteroid production (Lachaise *et al*., 1993). MIH activity and the secretory structures involved have now been identified in larval *Carcinus maenas* by the use of immunocytochemical methods (Webster and Dircksen, 1991). This indicates that the mechanism of moult control is similar in both the larval and post larval stages. There is clearly more to moult control than the simplistic MIH/ecdysteroid interaction.

1.1.5.1 *Structure of MIH gene and peptide*

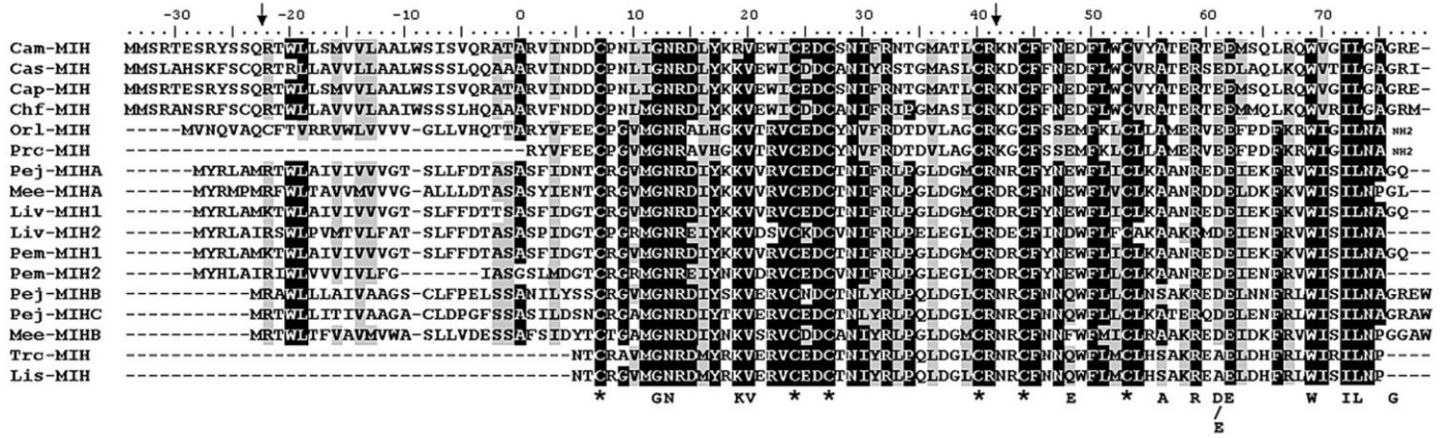

Fig.8. Multiple sequence alignment of representative MIH/MIH-like peptide precursors retrieved from GenBank. Six cysteine residues are indicated by asterisks (*). Sites of intron insertion, revealed by analyses of Chf-MIH, Mee-MIHA, Mee-MIHB, Pem-MIH1, Pem-MIH2, Pem-SGP-C1, Pem-SGP-C2, Live-MIH1, and Liv-MIH2, are marked by arrows (↓). Conserved residues proposed by Katayama *et al.* (2003) to be functionally critical for MIH activity are shown below the sequences. Note the substitutions often occurring in the peptides of group B (Mee-MIHB, Pej- MIHB, Pej-MIHC, Lis-MIH, Trc-MIH) at positions 48, 56, and 61. Amino acid residues are numbered relative to the first residue (+1) of the mature peptides. Sequences analyzed are from the following sources: Cam-*Carcinus maenas* (Klein *et al*., 1993); Cas-*Callinectes sapidus* (Lee *et al*., 1995); Cap-*Cancer pagurus* (Lu et al., 2001); Chf *Charybdis feriatus* (Chan *et al*., 1998); Orl-*Orconectes limosus* (Bulau *et al*., 2005); Prc-*Procambarus clarkia* (Nagasawa *et al*., 1996); Pej-*Penaeus japonicus* (Ohira *et al*., 1997; 2005; Yamano and Unuma, 2006); Mee-*Metapenaeus ensis* (Gu and Chan, 1998; Gu *et al*., 2002); Liv-*Litopenaeus vannamei* (Chen *et al*., 2007); Pem-*Penaeus monodon* (Yodmuang *et al*., 2004); Trc-*Trachypenaeus curvirostris* (accession number: AF312978); Lis-*Litopenaeus stylirostris* (accession number: AF312976). Note that data regarding the signal peptide of Orl-MIH, and signal peptides and N-terminal ends of mature peptides of Trc-MIH and Lis-MIH are lacking.

The MIH gene/ MIH-like peptide precursors have been mainly determined by conceptual translation of the cDNA sequences. The precursor sequences contain the signal peptide (22-35 residues) and a mature MIH/MIH like peptide. Signal peptide of the brachyuran precursors is longer than those of astacoidean or penaeid precursors (35 vs 22-29 residues) (Fig. 8). Genes of MIH/MIH-like peptide of various animals have been elucidated, including Chf-MIH, Mee-MIHA, Mee-MIHB,Pem-SGP-C1,Pem-SGP-C2, Pem-MIH1, Pem-MIH2, Liv-MIH1 and Live-MIH2; the 3 exon/2 intron organization of these genes appears to be highly conserved (Chan *et al*., 1998; Gu and Chan, 1998a; Gu *et al*., 2002; Krungkasem *et al*., 2002; Yodmuang *et al*., 2004; Chen *et al*., 2007). The intron I is inserted between the codons for the 6^{th} and 7^{th} amino acid of the signal peptide for penaeid genes except in Mee-MIHB, the insertion occurs between the 1^{st} and 2^{nd} residue due to shorter signal peptide. For brachyuran genes, intron II is invariably inserted after the second base of the codon for the 41^{st} residue of mature peptide for all genes (Fig. 8). Thus the exon I contains the sequence for the 5'-untranslated region (UTR) and a part of the signal peptide, exon II encodes the remaining part of the signal peptide and the N-terminal half (upto the 40^{th} residue) of the mature peptide, and exon III contains the sequences for the remaining C-terminal of the mature peptide and 3'-UTR. The spatial patterns of expression of MIH/MIH-like peptide genes vary considerably. The transcripts of subgroup A peptides (Pej-MIHA, Mee-MIHA, Pem-MIH1, Pem-MIH2, Pem-SGP-C1, Pem-SGP-C2, Liv-MIH1, and Liv-MIH2) are mainly or exclusively expressed in the eyestalk ganglia, whereas those of subgroup B peptides (Mee-MIHB, Pej-MIHB) predominantly in extra-eyestalk tissues (e.g., the thoracic ganglia, abdominal ganglia and brain). The pattern of differential tissue expression is consistent with the hypothesis that peptides of different groups might play different physiological roles (Nakatsuji *et al*., 2009). Nakatsuji *et al.* (2009) cited the identification of 26 MIH/MIH-like peptides identified in decapod crustaceans of various taxa, the primary structure ranging from 74-79 amino acids. Primary sequence characteristics include the presence of 6

cysteine residues at strictly conserved positions (Fig. 1.8.). Three disulphide bonds are formed between these cysteine residues (Cys^{7}-Cys^{44}, Cys^{24}- Cys^{27} and Cys^{27}-Cys^{53}) and are critical for stabilizing molecular structure (Katayama *et al*., 2001, 2003). Other conserved residues present in the majority of MIH/MIH-like sequences are those at positions 12, 13, 19, 20, 56, 59, 61, 69, 72, 73 and 76 (Fig. 8).

The C-terminal end of MIH appears more variable than the N-terminal end. Where relevant data exist, the C-terminus is either free (Cam-MIH, Webster, 1991; Cap-MIH, Chung and Webster, 1996; Pej-MIHA, Yang *et al*., 1996) or amidated (Prc-MIH, Nagasawa *et al*., 1996; Orl- MIH, Bulau *et al*., 2005; Jal-MIH, Marco *et al*., 2000), and the N-terminus is always unblocked. In many cases, the primary structure of MIH/MIH-like molecules was deduced from cloned cDNA sequences and possible terminal modifications are thus unknown. The C-terminus of Orl-MIH, as directly determined by peptide sequencing, is amidated (Ala75–NH_2) instead of the Ala75–Gly76–Arg77 predicted by the cDNA sequence (Bulau *et al*., 2005). On the other hand, the C- termini of Cam-MIH, Cap-MIH, and Pej-MIHA are free (Webster, 1991; Chung and Webster, 1996; Yang *et al*., 1996) and the same as predicted by the cDNA sequences (Klein *et al*., 1993b; Ohira *et al*., 1997b; Lu *et al*., 2001). The functional significance of an amidated C-terminus in MIH has not been determined. For CHH, the C-teminal amide affects secondary structure and is involved in conferring hyperglycaemic activity (Katayama *et al*., 2002; Mosco *et al*., 2008). Recent studies in penaeids have revealed that the phenomenon of molecular polymorphism is also present in type II peptides. Thus, there are 4 MIH/MIH-like molecules identified so far in *Penaeus monodon* (Krungkasem *et al*., 2002; Yodmuang *et al*., 2004), 3 for *Penaeus japonicus* and *Fenneropenaeus chinensis* (Yang *et al*., 1996; Ohira *et al*., 1997b; Wang and Xiang, 2003; Ohira *et al*., 2005; Yamano and Unuma, 2006), and 2 for *Litopenaeus vannamei* and *Metapenaeus ensis* (Gu and Chan, 1998b; Gu *et al*., 2002; Chen *et al*., 2007). The penaeid sequences vary from the brachyuran MIH (astaccoidean MIH and Jal-MIH).

Penaeid peptides can be further separated into 2 subgroups, A and B. The moult inhibiting activity of peptides in subgroup A is more effective in activity than that of con-specific peptides in subgroup B (i.e., Mee-MIHA vs Mee-MIHB, Gu *et al*., 2002; Pej-MIHA vs Pej-MIHB, Ohira *et al*., 2005). Thus the peptides of different subgroups might diverge functionally with peptides of subgroup A acting physiologically as MIH and subgroup B functions as regulatory or inhibitory to moulting (Gu *et al*., 2002; Ohira *et al*., 2005). It has been proposed that the subgroup has sequence similarity to vitellogenesis-inhibiting hormone and thus may be involved in the regulation of gonad maturation (Gu *et al*., 2002; Ohira *et al*., 2005). Studies of Chen *et al.* (2007) revealed the varying gene expression of Liv-MIH1 and Live-MIH2 during the moult cycle, suggesting that both the peptides act physiologically as MIH. These data are consistent with the proposition that peptides of subgroup A, to which Liv-MIH1 and Liv- MIH2 belong, function as MIHs, and supports the emerging consensus that multiple MIHs exist in a given species, as exemplified by the presence of multiple subgroup A peptides in each of the 3 species, *L. vannamei*, *P. monodon*, and *F. chinensis* (Krungkasem *et al*., 2002; Wang and Xiang, 2003; Yodmuang *et al*., 2004; Chen *et al*., 2007).

The studies above show multiple MIH/MIH-like molecules in penaeid species, but additional studies are needed to conclude and determine whether there is functional divergence among the peptides.

1.1.5.2 *MIH signalling pathway*

MIH and CHH inhibit YO ecdysteroidogenesis through separate signalling pathways that converge at cGMP (Chung and Webster, 2003; Fanjul-Moles, 2006). The membranes of YO have distinct receptors for binding to CHH and MIH (Asazuma *et al*., 2005; Chung and Webster, 2003; Chung *et al*., 2010; Webster, 1993). The identity of MIH receptor is not firmly confirmed but that of CHH is recognized as guanylyl cyclise (GC). Thus, the CHH binding directly leads to an increase in cGMP (Chung and Webster, 2006). In contrast to CHH binding, MIH

signalling involves increase in cAMP, followed by a larger and sustained increase in cGMP (Baghdassarian *et al*., 1996; Bőcking *et al*., 2002; Covi *et al*., 2008; Nakatsuji *et al*., 2006, 2009; Saïdi *et al*., 1994; Sedlmeier and Fenrich, 1993; Von Gliscynski and Sedlmeier, 1993). The increase in cGMP suggests that MIH activates soluble GC, the activation of a membrane GC would result in immediate increase of cGMP. cAMP and cGMP both inhibit YO ecdysteroidogenesis *in vitro* (Covi *et al*., 2009). Chang and Mykle (2011) indicated that nitric oxide (NO)-sensitive GC (GC-I) is involved. GC-I agonists (NO donors and YC-I) can inhibit YO *in vitro* (Covi *et al*., 2008; Mykles *et al*., 2010). Additionally YOs express a Ca^{2+}/CaM-dependant NO synthase (NOS) and the catalytic (β) subunit of GC-I (Kim *et al*., 2004; Lee and Mykles, 2006; McDonald *et al*., 2011).

Much of the data support the organization of the MIH signalling pathway into a cAMP/Ca2+-dependent ‘‘triggering’’ phase and a NO/cGMP-dependent ‘‘summation’’ phase linked by calmodulin (CaM) (Covi *et al*., 2012; Lee *et al*., 2007a,b; Mykles *et al*., 2010). A transient increase in cAMP triggers the influx of Ca^{2+}, which activates CaM (Fig. 9). A sustained activation of NOS results from the combined effects of direct binding of Ca^{2+}/CaM to NOS and the dephosphorylation of NOS by calcineurin, which is aCa^{2+}/CaM-dependent protein phosphatase. Phosphorylation of NOS reduces its activity; dephosphorylation by calcineurin enhances NOS activity (Kone, 2001). The NOS is phosphorylated in the activated YO, which is consistent with inactivation of NOS by protein kinases in the absence of MIH (Lee and Mykles, 2006). MIH is released in pulses, with each pulse having a half- life of 5-10min; this results in low MIH titers (<5fmol ml^{-1}) in the haemolymph of most intermoult animals (Chang and Mykles, 2011; Chung and Webster, 2005). The model provided below (Fig. 9) shows the mechanism for inhibition of the YO between pulses of MIH release.

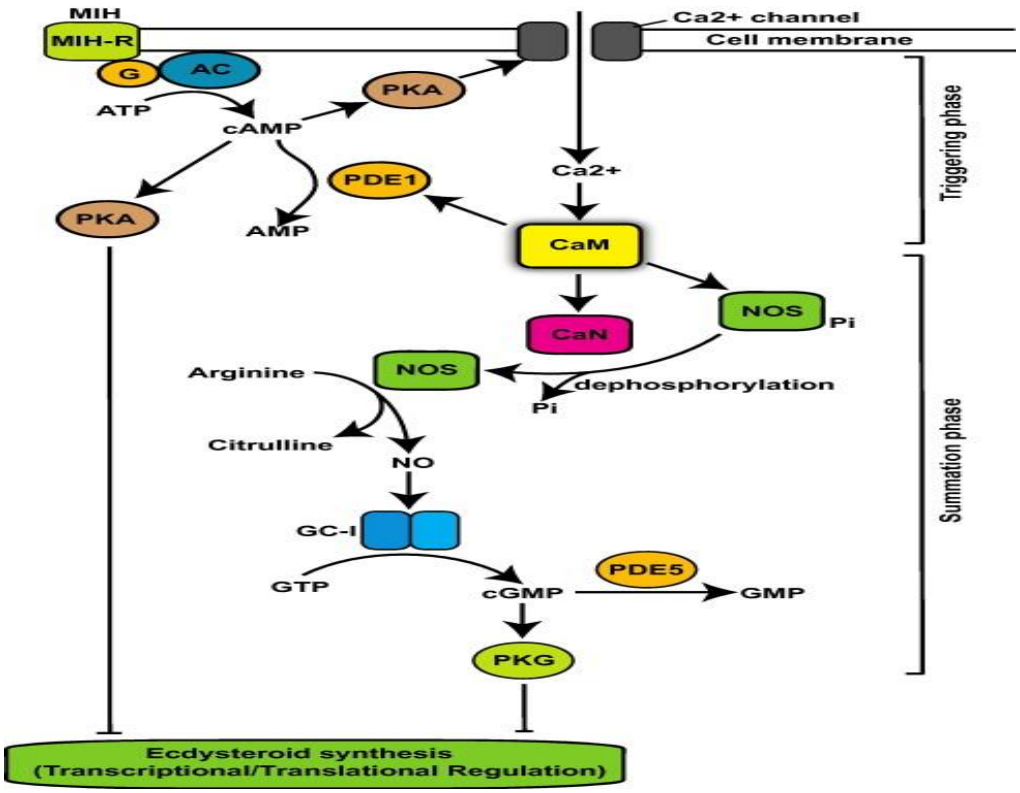

Fig.9. MIH signaling - Proposed MIH signaling pathway regulating ecdysteroidogenesis in decapod crustacean moulting gland. The "triggering" phase is initiated by binding of MIH to a G protein-coupled receptor (MIH-R) and activation of adenylyl cyclase (AC); cAMP increases intracellular Ca^{2+} via cAMP-dependent protein kinase (PKA) phosphorylation of Ca^{2+}channels. Sensitivity to MIH is determined by phosphodiesterase 1 (PDE1) activity, which varies during the moulting cycle. The "summation" phase is mediated by NO and cGMP. Calmodulin (CaM) links the two phases by activating NO synthase (NOS) directly and indirectly via calcineurin (CaN). Dephosphorylation of NOS by CaN can potentially prolong the response to MIH. CaM can also activate PDE1 to inhibit the triggering phase (PDE1 can also hydrolyze cGMP, thus inhibiting the summation phase). CGMP-dependent protein kinase (PKG) inhibits ecdysteroidogenesis. Chronic activation of PKA may directly inhibit ecdysteroidogenesis. Chronic elevated intracellular cAMP can inhibit ecdysteroidogenesis directly, perhaps by inhibiting protein synthesis. *Other abbreviations:* G, G protein; GC-I, NO-sensitive guanylyl cyclase; PDE5, cGMP PDE. Coutesy: Chang and Mykles, 2011.

1.1.5.3 ***Function of MIH***

It is increasingly clear that rates of ecdysteroid secretion are influenced not only by MIH, but also by stage-specific changes in the responsiveness of the Y-organs to MIH. Sefiani *et al.* (1996) observed a marked decline in the sensitivity of shrimp (*Penaeus vannamei*) Y-organs to sinus gland extract during premoult stages D2 and D3. A decline in the responsiveness of Y-organs to MIH during middle and late premoult was subsequently reported for crabs (Chung and Webster, 2003) and crayfish (Nakatsuji and Sonobe, 2004). The cellular mechanism(s) underlying the changes in responsiveness of Y organs to MIH are not well understood. Radioligand binding assays revealed no change in the number of MIH receptors in Y-organs during a moult cycle in *C. maenas* (Chung and Webster, 2003), indicating that changes in sensitivity to MIH are likely due to events downstream of receptor binding and may involve changes in intracellular signalling pathways. The observation that 3-isobutyl-1- methylxanthine (IBMX), a non-selective inhibitor of cyclic nucleotide phosphodiesterase (PDE), suppressed ecdysteroid secretion by Y-organs (Mattson and Spaziani, 1985) suggests that intracellular PDE activity may be involved in regulation of Y-organ responsiveness. To assess the possible involvement of PDE in determining the responsiveness of Y-organs to MIH, Y-organs were removed from crayfish (*P. clarkii*) at various stages of the moult cycle and incubated *in vitro* with MIH, IBMX, or both (Nakatsuji *et al*., 2006). The responsiveness of Y organs to MIH alone was high during the intermoult stage, declined in early premoult, was low during middle and late premoult, and then increased during post-moult, confirming previous reports of stage specific changes in responsiveness.

Stage-specific changes in the responsiveness of Y-organs to MIH appear to play an underappreciated role in the regulation of ecdysteroid production. Recent data indicate that the changes in glandular responsiveness to MIH are due, at least in part, to changes in glandular PDE activity. Because glandular PDE activity is Ca+/calmodulin dependent, it seems clear that an understanding of the mechanisms

that underlie the regulation of free calcium levels in Y-organs is critical to a comprehensive understanding of the regulation of ecdysteroidogenesis.

1.1.6 Gonad-inhibiting hormone/ Vitellogenin-inhibiting hormone- An overview

Eyestalk is the source of another reproductive inhibitory hormone, the gonad inhibiting hormone (GIH) (Quackenbush and Herrnkind, 1983), which may be the same as the vitellogenin-inhibiting hormone (VIH) (Soyez *et al*., 1987). Panouse (1943, 1944) described that the removal of one ES accelerated ovarian maturation and spawning, since then this has been used for artificial/faster maturation. In female crustaceans, the late phase of gonadal maturation to form mature ova is named vitellogenesis (Adiyodi, 1985). The targets of GIH/ VIH are possibly the ovaries and hepatopancreas, which are the sites of yolk protein synthesis (Paulus, 1984; Wolin *et al*., 1973; Paulus and Laufer, 1987; Quackenbush, 1989), partially purified eyestalk extracts, inhibit ovarian synthesis of vitellogenin *in vitro* (Quackenbush and Keeley, 1988; Eastman-Reks and Fingerman, 1984). GIH represses synthesis of vitellogenin in adipose tissue or its uptake by oocytes (Meusey and Payen, 1988). Charniaux-Cotton and Kleinholz (1964) suggested that GIH inhibits only the secondary phase of the maturation of oocytes and probably has no effect on the primary growth. MIH is suggested to be controlling the primary phase.

Huberman (2000) in his review suggested the shrimp *S. ingentis* to be a very useful organism for the assay of GIH as it undergoes several cycles of reproduction without intervening moult cycles in summer months. Chang *et al.* (1992) injected females of shrimp *S. ingentis* following a spawn with SG extracts from non reproductive female shrimps and observed a significant inhibition in ovarian development and spawning. Soyez *et al*., (1987) isolated a 7500Da peptide from the SG of *Homarus americanus* and assayed the GIH activity *in vivo* in the shrimp *Palaemon varians*, by means of ooccyte diameter measurement.

GIH is not species specific; this was proved by Aguilar *et al.* (1992) by isolating 8333Da peptide from SG of *Procambarus bouvieri*. This peptide had depressing activity on the vitellogenin synthesis of cultured *Litopenaeus vannamei* ovarian culture. The partial sequence and amino acid composition indicated it to be a member of CHH family hormones. Two isoforms of lobster *Homarus americanus* HoaGIH were isolated (Soyez *et al*., 1991) and their precursors were characterized (De Kleijn *et al*., 1994). The two isoforms isolated had the same sequence and consisted of 78 amino acids. The difference may be due to stereo inversion as seen in CHH. The mature peptide had three additional amino acids at the C-terminal end probably representing the amide - blocking signal. GIH contained no CPRP- like peptide, but only a 31-amino acid signal peptide in the preprohormone. Though the alignment of CHH and MIH with GIH showed only 19% similarity, but the six cysteines were common in the three mentioned hormones. GIH had an additional glycine at the eleventh position, thus more similar to MIH.

In situ and immunocytochemistry analysis showed that HoaGIH is expressed in both male and female (De Kleijn *et al*., 1992) and larvae (Rotllant *et al*., 1993). Analysis by Northern blot and ELISA demonstrated the expression level of HoaGIH is related to the reproductive cycle. The level of peptide and mRNA in X-organs Sinus gland was found to be low in the immature stage, whereas the GIH haemolymph level was high during the immature and previtellogenic stages. The high levels of GIH in haemolymph will inhibit the onset of vitellogenesis (concluded by De Kleijn *et al*., 1998). Little is known about the cellular mechanism and detailed structure involved by which GIH inhibits vitellogenesis. Below is a schematic description of the hormone control in vitellogenesis in crustaceans (Hasegawa *et al*., 1993)

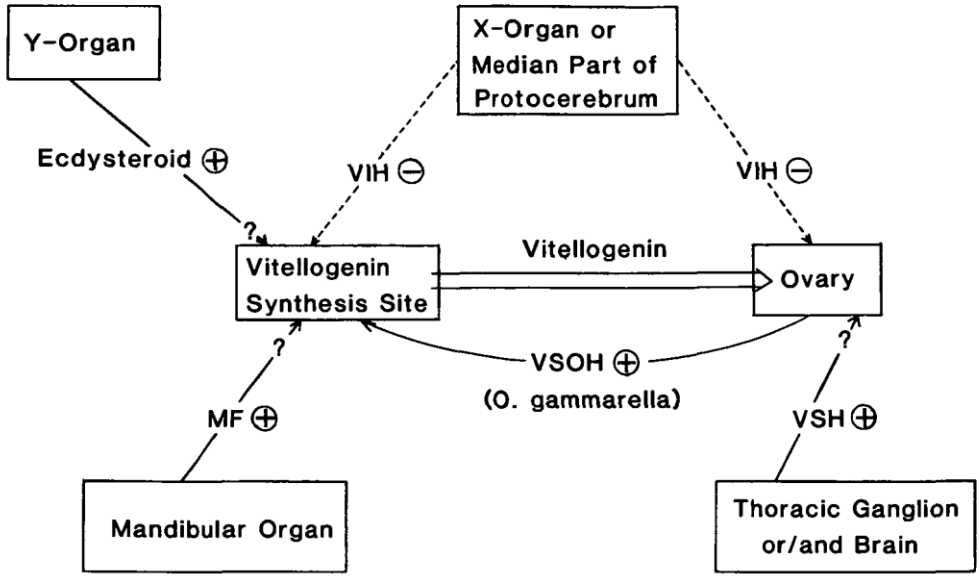

Fig.10. Schematic representation of the hormonal control of vitellogenesis in Crustacean. VIH: vitellogenesis inhibiting hormone; VSH- vitellogenin stimulating hormone; VSOH- vitellogenin-stimulating ovarian hormone; MF- methyl farnesoate. Courtesy: Hasegawa *et al*., 1993.

1.1.7 RNAi in crustaceans- Post transcriptional transgene silencing

The discovery of RNA interference (RNAi) in 1998 revolutionized the field of genomics. The initial RNAi studies in nematode worm *Caenorabditis elegans* was the key point exhibiting post transcriptional transgene silencing. The studies demonstrated that double- stranded RNA (dsRNA) can cause sequence specific degradation of endogenous mRNA (Fire *et al*., 1998). Since the discovery of RNAi in *C.elegans*, this gene silencing method was examined in other species and continues still. The post transcriptional gene silencing has been shown to work in diverse group of organisms and it is believed that RNAi was an ancient process that initially occurred before the divergence of plants and animals. RNAi has been reported in various arthropods, mainly in insects (reviewed by Fjose *et al*., 2001).

The application of RNAi in Penaied shrimp was initiated for viral control studies and suggested that this gene-silencing technique might be an applicable and powerful tool for studying gene functions in crustaceans (Tirasophon *et al*., 2005; Westenberg *et al*., 2005; Yodmuang *et al*., 2006). Recently it has been proven that RNA interference mediated gene silencing is operative in shrimp cells in culture (Tirasophon *et al*., 2005) and in the whole shrimp organism (Lugo *et al*., 2006). Consequently, the technique has revolutionized research in 'reverse genetics' by introducing dsRNA to organisms or cells as it could knockdown a gene and produce its phenotypic loss (Amdam *et al*., 2003, Cogoni and Macino, 2000, Blandin *et al*., 2002, and Fire *et al*., 1998). Tiu *et al*., 2007 used the approach of recombinant protein and RNA interference to study the reproductive functions of gonad stimulating hormone in *Metapenaeus ensis.* During the same period Treerattrakool *et al.* (2008) first reported the use of dsRNA to elucidate the function of GIH in *Penaeus monodon.* They demonstrated the influence of Pem-GIH on Vg gene expression and thus implied its role in gonad inhibition and maturation.

1.1.8 Conclusion

In this review, the numerous, scattered research findings on the neurological, physiological and endocrinological aspects of CHH family hormones are collated to provide a broad picture of their regulatory roles in crustacean physiology. The presence of various isoforms of CHH/MIH/GIH and their multifunctional activity proves the importance of the CHH family hormone in the life cycle of crustaceans. Despite the various genomic and proteomic studies on CHH family hormones, their functionality seems to be incomplete. Apparently, a multi-disciplinary approach to redefine the hormonal roles of the CHH neuropeptides family in terms of their expression, haemolymph titer and mode of actions including receptor characterization are imperative and essential in understanding regulatory mechanisms underlying the diverse physiology of crustaceans. Furthermore, using functional genomics approaches like RNA interference (RNAi) and manipulating genome are known to provide an insight into their

precise actions during a particular life stage. Having this insight in vision the following objectives were undertaken to take forward the endeavour in the field of crustacean endocrinology and its application in aquaculture:

1) Recombinant/fusion protein expression of CHH family hormone genes: Application, detection and *in vivo* bioassay

2) Antagonists (polyclonal) against the mature recombinant protein of CHH family hormone genes: *In vivo* bioassay and ELISA - development

3) *In vitro* double-stranded RNA mediated eyestalk neuropeptide (CHH1, MIH1 and GIH) gene knockdown in the shrimp *Penaeus monodon*

4) *In vivo* generated Double-stranded RNA mediated eyestalk neuropeptide (CHH, MIH and GIH) gene knockdown in the shrimp *Penaeus monodon*.

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Chapter

2

RECOMBINANT/FUSION PROTEIN EXPRESSION OF CHH FAMILY HORMONE GENES: APPLICATION, DETECTION AND *IN VIVO* BIOASSAY

2.1 Introduction

A decade of studies on decapod crustacean's neuroendocrine organs and neurosecretion has contributed considerably to the concept of neuroendocrine regulation. The most interesting aspects are the regulation of glycaemia, moulting and gonad development. Regulation of glycaemia has been studied extensively throughout in decapod crustaceans. Abramovitz *et al.* (1944) first reported the hyperglycaemic response of crude eyestalk extract injection in crustaceans followed by various other authors (Kleinholtz *et al.*, 1967; Kleinholtz, 1976; Keller and Andrew, 1973). Crustacean hyperglycaemic hormone has been isolated from several crustaceans including crabs (Kegel *et al.*, 1989; Chung *et al.*, 1998), lobster (Tensen *et al.*, 1991b, c), crayfishes (Kegel *et al.*, 1991; Huberman *et al.*, 1993) and prawn (Sithigorngul *et al.*, 1999). Udomkit *et al.* (2000) identified and characterized cDNA encoding CHH in *Penaeus monodon*. The recombinant protein expressed in yeast had the ability to elevate glucose level in the haemolymph of eyestalk ablated *P.monodon* and this was designated as Pem-CHH1 (Treerattrakool *et al.*, 2003). Udomkit *et al.* (2004) identified two other Pem-CHH cDNAs and designated them Pem-CHH 2 and Pem-CHH 3, the recombinant proteins were expressed in yeast and their biological activities were proved in *P.monodon*. Gu *et*

al. (2000) characterized MeCHH-A and MeCHH-B in *Metapenaeus ensis* and proved the biological activity of recombinant protein (rCHH-A and rCHH-B) expressed in a bacterial system.

Moulting in crustaceans is inhibited by moult inhibiting hormones and they are presumed to inhibit moulting by suppressing the synthesis/ secretion of the moulting hormone, ecdysteroids, secreted by the Y-organ (Lachaise *et al*., 1993). MIHs characterized from *C. maenas, P. japonicus, P. bouveri* and *P. clarkia* have been shown to suppress ecdysteroid secretion by Y-organ *in vitro* (Yang *et al*., 1996; Aguilar *et al.*, 1996; Webster and Keller, 1986; Terauchi *et al.*, 1996). The *in vivo* studies of MIH was reported initially only in *Homarus americanus* (Chang *et al.*, 1990) rendering a petite design of their biological activity. The difficulty to obtain large amount of MIH from sinus glands for experimental study led Ohira *et al.* (1999) to express MIH of *P. japonicus* in *E.coli*. The *in vivo* biological activity of the recombinant MIH produced was proved by Okumura *et al.* (2005) in *Marsupenaeus japonicus*. cDNA encoding Pem-MIH of *Penaeus monodon*, was cloned and characterized, the biological activity of the recombinant Pem-MIH expressed in yeast was demonstrated *in vivo* and the expression levels in several types of tissues were examined (Yodmuang *et al.*, 2004). The current knowledge with regard to the endocrine control of crustacean vitellogenesis is very limited, given that only two VIHs/GIHs, *viz.* Hoa-VIH (*Homarus americanus*-VIH) and Arv-VIH (*Armadillidium vulgare*-VIH), have been characterized. Ohira *et al.* (2006) produced biologically active recombinant Hoa-VIH (rHoa-VIH) with amidated C-terminus using *E.coli* system to circumvent the problem of obtaining sufficient quantity of VIH/GIH for physiological studies.

In the present study, we did the sequence analysis and homology modelling of CHH family hormone genes. Isolated the mature region of the CHH1, MIH1 and GIH genes and constructed recombinant translation expression vector (pET32a+) for recombinant / fusion protein expression. The recombinant / fusion proteins of CHH1, MIH1 and GIH were purified, detected and applied *in vivo* in *P.monodon*.

2.2 Materials and Methods

2.2.1 Experimental animals

P.monodon (10-15g) obtained from local hatchery (Nandana aquaculture Pvt.Ltd) were stocked and maintained in a laboratory recirculating aquaculture system (RAS) for shrimp integrated with nitrifying bioreactor (Kumar *et al.*, 2009, 2011) in sea water having 15g l^{-1} salinity. Water quality was maintained within a narrow range (pH 6.8-7.8; total ammonia < 0.1 mg l^{-1}; nitrite < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness >5000-6000 mg l^{-1}). The animals were fed with commercially available pelleted feed (Higashimaru, India) containing 53 % protein, 3 % fat, 12 % fiber, 20 % ash and 12 % moisture.

2.2.2 Total RNA extraction, cDNA synthesis

Total RNA was extracted using TriReagent (Sigma, USA) from the eyestalk of *P. monodon*. Eyestalk was extricated from the animal, the exoskeleton was slowly removed with forceps and the melanised area of the eyestalk was gently separated exposing the white tissue. Eyestalk from 3-4 animals were macerated in 500 µl TRI reagent (Sigma, USA) using RNAse free plastic pestle followed by the addition of additional 500 µl TRI reagent to make a final volume 1000 µl. The sample was kept for 5 min at room temperature (RT) to ensure complete dissociation of nucleoprotein complexes. An aliquot of 0.2 ml chloroform was added to TriReagent mixture and shaken vigorously for 15 sec.The reaction mix was allowed to stand for 15 min at RT followed by centrifugation at 12000x g for 15 min. From the three layers formed after centrifugation, colourless aqueous phase (upper) containing total RNA was transferred carefully to a fresh tube. An aliquot of 0.5 ml isopropanol was added and stored for 10 min at RT and centrifuged at 12000 x g for 10 min at 4 °C. RNA was found precipitated on the sides and bottom of the tube after centrifugation. The supernatant was discarded and the pellet was washed twice with 75 % ethanol. The pelleted RNA was air dried and dissolved in 20 µl DEPC (Diethyl pyrocarbonate) treated water by repeated pipetting at 55 °C. The extracted RNA was subjected to DNase treatment with RNase free DNase 1 (New England Biolabs, UK) by

adding 0.2 U of enzyme to 1 µg of RNA and incubated at 37 °C for 10 min followed by 75 °C for 10 min for inactivation. RNA concentration and quality were determined by optical density (OD 260/280 nm) measurement using a UV-Visible Spectrophotometer (Hitachi, Japan).

An aliquot of 5 µg RNA was subjected to cDNA synthesis. The 20 µl reaction mix contained M-MuLV reverse transcriptase (200 U), RNase inhibitor (8 U), Oligo $(dT)_{12}$ primer (40 pmoles), dNTP mix (1 mM), RTase buffer (1x) and $MgCl_2$ (2 mM) and the cDNA was synthesized by incubating the reaction mix at 42 °C for 1 hr followed by inactivation at 65 °C for 20 min. All reagents were purchased from New England Biolabs, UK.

2.2.3 PCR amplification of CHH family hormone genes

Initially the primers were designed to amplify the complete coding sequence (CDS) of the three genes. The primers were designed from the already submitted sequences of CHH1 (AF233295), MIH1 (AY496454) and GIH (DQ643389) of *P. monodon* in the NCBI database. The primers designed are as mentioned in Table 1.

Table.1. Primers designed from CDS of CHH1 (AF233295), MIH1 (AY496454) and GIH (DQ643389) of *P. monodon*

Primer	Primer sequence (5'-3')	Annealing Tm (° C)	Product size (bp)
CHH1	CCTGGAAGTTGCTGACCGTCGCTC	60	432
	CTTGCCGAGCCTCTGTAGGGCGG		
MIH1	CGCGTCTCCTTGGGTTCATTCCGTCC	64.1	357
	CTGACCGGCGTTCAGGATGCTGATCC		
GIH	TCCACAGGCAGCGGCCCCTGC	58	336
	CCACGGCCGGCCGGCATTG		

PCR was conducted in a 25 µl reaction volume containing 1µl of 10X buffer, 2.5 mM dNTP (2.5 µl), 1 µl of 10 pmol μl^{-1} of forward and reverse primer, 1.0 µl of 0.5U μl^{-1} of *Taq* DNA polymerase and 1 µl cDNA template. The PCR amplification

programme consisted of heating the mixture to 95 °C for 5 min before the addition of *Taq* DNA polymerase. The following PCR cycle included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 60 °C (CHH1), 64.1°C (MIH1) and 58 °C (GIH) for 45 sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. An aliquot of 10 µl PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

2.2.4 Plasmid vector used for the cloning and vector construction

The pGEM®-T and pGEM®-T Easy vectors (Fig.1) are linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (eg. *Taq* DNA polymerase).

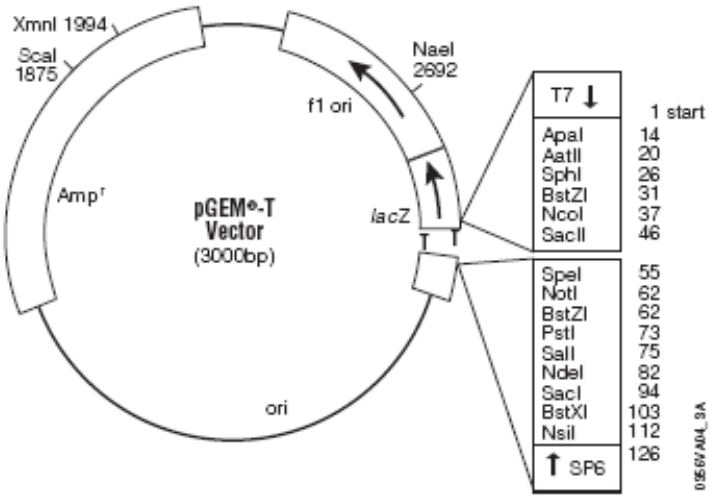


Fig.1. The pGEM®-T and pGEM®-T Easy Vector map with MCS

These vectors are high-copy-number vectors, containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding

region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates.

2.2.4.1 *TA vector construction of shrimp CHH1, MIH1 and GIH gene in pGEM-T Easy vector*

The A tailed PCR product of CHH1, MIH1 and GIH were ligated with pGEM-T easy vector (Promega, USA) by following the manufacturer's instructions. Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T vector (50 ng μl^{-1}), 3.5 µl PCR product, 1 µl ligation buffer (10X), 1 µl ligase (3Weiss units μl^{-1}) and 4 µl MilliQ were incubated at 4 °C overnight. This allowed the ligation of PCR products with pGEM-T easy vector.

2.2.4.2 *Transformation into E. coli DH5α*

The *E. coli* DH5α competent cells were thawed by placing on ice for 5-10 min. Ligation reaction (10 µl) was added to a sterile 15 ml culture tube already on ice, 50-100 µl of competent cells were transferred into the 15 ml tubes (containing ligation mix) on ice. The tubes were gently flicked to mix and placed on ice for 20 min .The cells were given heat shock at 42 °C for 90 sec in a water bath and immediately returned to ice for 2 min. Super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose) was added (600 µl). $MgCl_2$ and glucose were added just before transformation to the tubes containing cells transformed with ligation mixture, incubated for 1-1.5 hrs at 37 °C with shaking at 220-230 rpm, plated 100 µl of each transformation culture onto duplicate/triplicate LB/ampicillin (100 µg ml^{-1}) /X-gal (80 µg ml^{-1}) /IPTG (100mM) and incubated the plates overnight (12-16 hrs) at 37 °C.

2.2.4.3 *PCR confirmation of gene insert in the selected clones*

Clones were selected and patched on LB/ampicillin (100 µg ml^{-1}) /X-gal (80 µg ml^{-1}) /IPTG (100mM) plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed, primer

could amplify it from either side. The 25 µl reaction PCR reaction mixture contained 2.5 µl 10X buffer, 2.5 µl dNTP (2.5 mM), 1µl Taq polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of T7 forward and SP6 reverse primers each (10 pmol μl^{-1}) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C for Taq polymerase addition, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 45 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

2.2.4.4 *Propagation of E. coli containing the pGEM-T vectors constructs*

E. coli DH5α with pGEM-T vectors were propagated in LB ampicillin media (100 µg μl^{-1}) and incubated at 37 °C with shaking at 220 rpm (12 hrs).

2.2.4.5 *Plasmid extraction*

Plasmid was extracted using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) following manufacturer's instructions. Briefly, an aliquot of 2 ml culture after overnight incubation was pelletised at 12,000 xg for 1 min. The pellet was resuspended in 200 µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and centrifuged at 12,000 xg for 10 min to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centifuged at 12,000 x g for 1 min. Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins, salt and other contaminants. The column was transferred to a fresh collection tube to elute the plasmid DNA, 100 µl 10 mM Tris-Cl was added and centrifuged at 12,000 xg for 1 min.The plasmid DNA was stored at -20 ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

2.2.4.6 *Sequencing and sequence analysis of CHH1, MIH1 and GIH genes of P.monodon*

The cloned plasmid was sequenced at Microsynth GmBH (Switzerland). The sequence was subjected for BLAST search (Nucleotide) in NCBI database for confirmation of the genes sequenced.

2.2.4.7 *Sequence analysis and homology modelling*

DNA sequences obtained from the positive clones of CHH1, MIH1 and GIH were assembled using the Gene Tool software. The homology search of DNA sequences and deduced amino acid sequences were completed using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997). The ORF translation and calculation of the theoretical MW of the deduced protein were formulated using ExPASy Proteomics server (http://us.expasy.org/tools/pi_tool.html). The cluster analyses of deduced amino acids were carried out using cluster 3 (http://bonsai.hgc.jp/~mdehoon/ software/cluster/). Comparative modelling was performed by searching the Protein Data Base (PDB) of known protein structures using the target sequence of each of the structures in the database. Homology modelling was constructed using the Swiss model (http://swissmodel.expasy.org/).

2.2.5 Identification of mature region of CHH1, MIH1 and GIH gene

The amino acid sequences of CHH1 (Accession GQ221085), MIH1 (Accession GQ221086) and GIH (Accession GQ228835) were analyzed using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The FASTA format sequence of CHH1, MIH1 and GIH were translated using the translate tool of ExPASy (SIB Bioinformatics Resources Portal). The amino acid sequences were pasted in FASTA format. The output file was analyzed using the Gene Tool software for separating the signal peptide sequence and the mature sequence. The mature region was identified and the specific primers were designed for the PCR.

2.2.5.1 *Designing primers with restriction sites*

Specific restriction primers for the mature region of CHH1, MIH1 and GIH genes were designed. Primers were designed specifically to obtain the mature region

in-frame for further recombinant protein expression. *EcoR* I having the sequence GAATTC was added to the 5' end and *Xho* I (CTCGAG) to the 3'end of CHH1 and MIH1. The restriction analysis of the GIH sequence showed the presence of *Xho* I restriction site, thus *EcoR* I was added to both the 5'and 3'end of the sequence. The primers designed have the sequence as given in Table 2.

Table. 2. Primers of mature region designed from CDS of CHH1 (Accession GQ221085), MIH1 (Accession GQ221086) and GIH (Accession GQ228835) of *P. monodon*

Primer	Primer sequence (5'-3)	Tm (°C)	Product Size (bp)
CHH1	F- GAATTCAGCCTATCCTTCAGGTCTTGCACGGGC	60	222
	R- CTCGAGCTTGCCGAGCCTCTGTAGGGCGG		
MIH1	F- GAATTCAGTTTCATAGACGGCACTTGTCGAGGCGT	60	231
	R- CTCGAGCTGACCGGCGTTCAGGATGCTGATCC		
GIH	F- GAATTCAACATCCTGGACAGCAAATGCAGGGGTGC	60	237
	R- GAATTCCCACGGCCGGCCGGCATTG		

2.2.5.2 *PCR amplification of mature CHH family hormone genes*

The mature regions of CHH1, MIH1 and GIH were amplified using the primers with restriction sites (5'-*EcoR* I- 3'- *Xho* I in CHH1 and MIH1, 5' and 3' *EcoR* I in GIH) as specified in Table 2. Briefly, PCR was conducted in a 25 µl reaction volume containing 2.5 µl of 10X *Pfu* Buffer with 20 mM $MgSO_4$, 2.5 mM dNTP (2.5 µl), 1 µl of 10 pmol $\mu l^{-1}$of forward and reverse primer and 0.3 µl of 2.5 U μl^{-1} of *Pfu* DNA polymerase and 1 µl cDNA template. The PCR amplification programme consisted of heating the mixture to 95 °C for 5 min before addition of *Pfu* DNA polymerase enzyme. The following PCR cycle included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 60 °C for 45 sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. Aliquots (10 µl) of PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

2.2.6 Plasmid vectors used for the cloning and expression in the work.

2.2.6.1 *pJET 1.2 Blunt-end vector*

pJET1.2/blunt is a linearized cloning vector (Fig.2), which accepts inserts from 6 bp to 10 kb. The 5'-ends of the vector cloning site contain phosphoryl groups, therefore, phosphorylation of the PCR primers is not required.

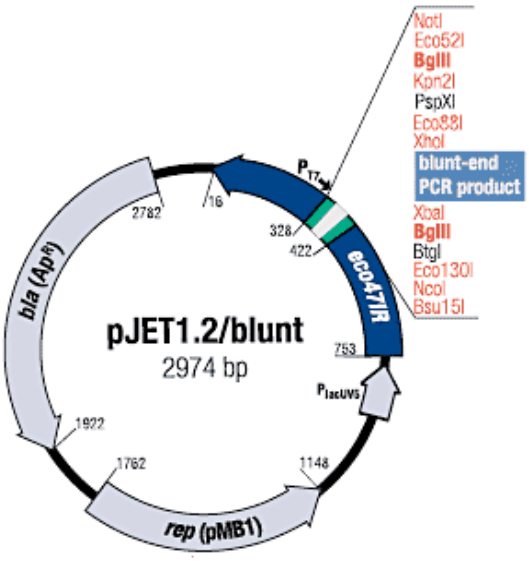


Fig. 2. pJET1.2/blunt vector map with MCS region

Blunt-end PCR products generated by proofreading DNA polymerases (*Pfu*) can be directly ligated in just 5 min with the pJET1.2/blunt cloning vector. All common laboratory *E.coli* strains can be directly transformed with the ligation product. Recircularized pJET1.2/blunt vector expresses a lethal restriction enzyme after transformation and is not propagated. As a result, only recombinant clones containing the insert appear on culture plates. Therefore, blue/white screening is not required.

2.2.6.2 *pET32a+ Translation vector*

pET32a+ translation vector (Fig.3) and pET series as a whole is a powerful system developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7

transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction.

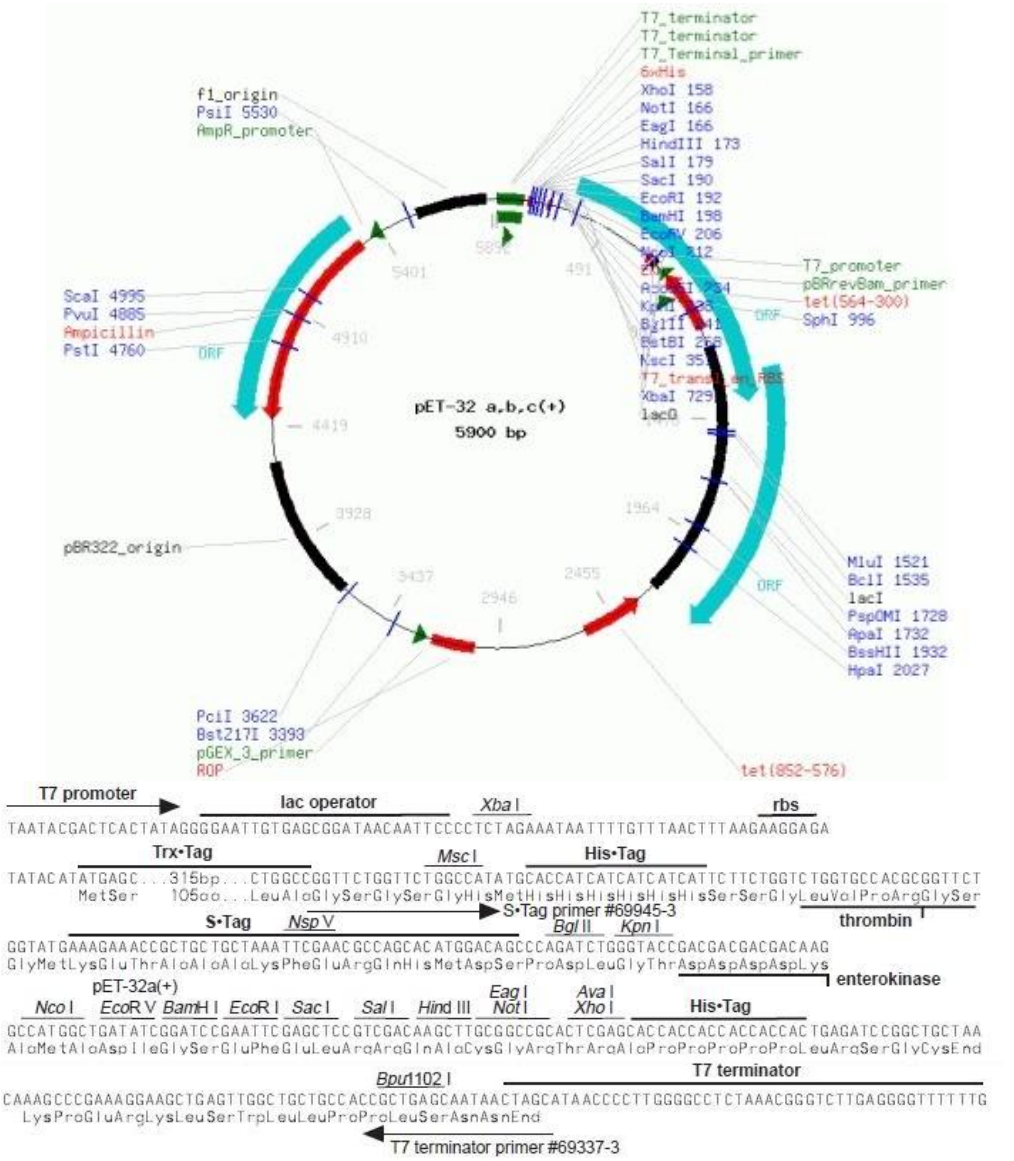


Fig. 3. pET32a+ Translation vector map - Details of cloning and expression region

Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, target protein expression may be initiated either by infecting the host with λCE6, or by transferring the plasmid into an expression host (*e.g.*, *E. coli* BL21 (DE3) pLysS) containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control. In the second case, expression is induced by the addition of IPTG to the bacterial culture.

The pET-32 series is designed for cloning and high-level expression of peptide sequences fused with the 109aa Trx•Tag™ thioredoxin protein. Cloning sites are available for producing fusion proteins also containing cleavable His•Tag® and S•Tag™ sequences for detection and purification of fusion protein. The translation vector names are distinguished from the transcription vector names by the addition of a letter suffix following the name, *e.g.*, pET-32a(+), which denotes the reading frame relative to the *BamH* I cloning site recognition sequence, GGATCC. All vectors with the suffix "a" express from the GGA triplet, all vectors with the suffix "b" express from the GAT triplet, and all vectors with the suffix "c" express from the ATC triplet of the *BamH* I recognition sequence. Vectors with a "d" suffix also express from the "c" frame, but contain an upstream *Nco* I cloning site in place of the *Nde* I site in that series for insertion of target genes directly into the ATG start codon.

2.2.6.3 *Propagation of E. coli containing the plasmid vectors and plasmid extraction*

pJET 1.2 vector and pET32a+ vector were propagated in *E. coli.* DH5α in LB ampicillin (100 µg μl^{-1}) medium at 37 °C with shaking at 220 rpm. Plasmid extraction was carried out as detailed earlier in this chapter.

2.2.7 Blunt-end vector construction of shrimp CHH1, MIH1 and GIH genes in pJET1.2

Blunt ended PCR products of CHH1, MIH1 and GIH were ligated with pJET 1.2 blunt vector (Fermentas, GmBH, Germany) by following the manufacture's

instructions. Briefly, 10 µl ligation mixture containing 0.5 µl pJET vector (50 ng µl $^{-1}$), 3.5 µl PCR Product, 1 µl ligation buffer (10X), 1 µl ligase enzyme (1U µl $^{-1}$), 1 µl of 50% PEG and MilliQ (to make 10 µl) was incubated at 22 °C overnight. This allowed for the ligation of PCR products with pJET 1.2 blunt ended vector.

2.2.7.1 *Transformation into E. coli DH5α*

The transformation was carried out as discussed earlier in section 2.2.4.2.

2.2.7.2 *PCR confirmation of gene insert in the selected clones*

The clones were selected and patched on LB/ampicillin plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed, primer could amplify it from either side. PCR reaction mixture contains 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1µl *Taq* DNA polymerase (0.5 U µl^{-1}), pinch of colony, 1µl of pJET1.2 Forward (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 Reverse (5'-AAGAACATCGATTTTCCATG GCAG-3') primers each. The mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 20 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

2.2.7.3 *Insert orientation confirmation by PCR*

The orientation of insert in the positive clones were confirmed by PCR using pJET1.2 Forward (5'-CGACTCACTATAGGGAGAGCGGC-3') and gene specific reverse primers of CHH1, MIH1 and GIH. PCR mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes

was 95 °C for 5 min followed by holding at 80 °C for *Taq* polymerase addition, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 45 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products were analyzed by 1 % agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

2.2.7.4 *Propagation of confirmed colony and plasmid extraction*

After confirmation, the transformed *E. coli* DH5α containing cloned vectors were propagated in 10 ml LB ampicillin (100 µg μl^{-1}) medium at 37 °C with shaking at 220 rpm. Plasmid extraction was carried out as detailed earlier in section 2.2.4.5 this chapter.

2.2.7.5 *Sequencing and sequence analyses of mature CHH1, MIH1 and GIH genes of P.monodon*

The cloned plasmids were sequenced (at SciGenome Labs Pvt .Ltd, Cochin, India) and the sequences were subjected for BLAST search (Nucleotide) in NCBI database for confirmation of any kind mutation errors and in-frame of the genes.

2.2.7.6 *Restriction digestion of cloned pJET 1.2 vector to release the mature regions of CHH1, MIH1 and GIH and their purification*

The purified plasmid vectors containing CHH1 (pJET-CHH1) and MIH1 (pJET-MIH1) were restriction digested with *EcoR* I and *Xho* I enzyme, while GIH (pJET-GIH) was restricted with only *EcoR* I (New England Biolabs, UK) to release the corresponding mature sequences (CHH1, MIH1 and GIH). An aliquot of 20 µl reaction mixture containing 5 µl plasmid (pJET-CHH1/pJET-MIH1), 0.5 µl enzyme (*EcoR* I and *Xho* I-10,000 U ml^{-1}), 2 µl reaction buffer, 0.4 µl of 100X BSA and 12.1 µl MilliQ water was incubated for 1 h at 37 °C and inactivated at 65 °C for 20 min. In case of pJET-GIH, 20 µl PCR reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (*EcoR* I -10,000 U ml^{-1}), 2 µl reaction buffer and 12.5 µl MilliQ water was incubated for 1 hr at 37 °C and inactivated at 65 °C for 20 min. The restriction digestion was confirmed by 1% agarose gel electrophoresis.

The restricted and released mature CHH1, MIH1 and GIH were gel purified using GenElute™ Gel extraction kit (Sigma, USA) following manufacturer's instructions. Briefly, the agarose gel that contained DNA fragment of appropriate size was excised using X-tracta gel extraction tool (Sigma, USA). The excised gel slice was taken in a 2.0 ml tube, weighed and 3 gel volumes (~450 µl) of gel solubilization solution was added and incubated at 60 ºC for 10 min with repeated vortexing in every 2 min. After incubation, 1 gel volume (~150 µl) of 100 % isopropanol was added and mixed gently until it became homogenous. This solubilized gel solution was loaded into the binding column that was pre-treated with column preparation solution and centrifuged at 12,000 xg for 1 min. An aliquot of 700 µl wash solution was added and centrifuged for 1 min at 12,000 xg , repeated the centrifugation and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2 ml MCT) and added 50 µl of preheated (at 65 ºC) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 xg for 1 min and stored at -20 ºC. The concentration of DNA was measured spectrometrically at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® flourometer (invitrogen™, USA).

2.2.8 Construction of recombinant translation vector system for recombinant protein expression

The pET32a+ translation vector was restriction digested with *EcoR* I and *Xho* I enzyme and only with *EcoR* I enzyme as in the methodology explained in the previous section (section no. 2.2.7.6.). Restriction digested plasmid was treated with CIP (Calf Intestinal Phosphatase) to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20 µl plasmid, 0.1 µl CIP enzyme and 5 µl buffer was incubated at 37 ºC for 1 hr followed by heat inactivation at 65 ºC for 20 min. Restriction digested, CIP treated vector was gel purified using GenElute™ Gel Extraction kit (Sigma, USA) as explained elsewhere in this chapter.

Restricted and purified mature CHH1, MIH1 and GIH were ligated with pET32a+ translation vector (Novagen, UK) by following the manufacture's instructions. Briefly,

10 µl ligation mixture containing 1.0 µl pET32a+ vector (50 ng μl^{-1}), 4.0 µl PCR product, 1 µl ligation buffer (10X), 1 µl T4 DNA ligase enzyme (1U μl^{-1}) and 3.0 µl MilliQ were incubated at 22 °C overnight. This allowed for the ligation of PCR products with pET32a+ translation vector.

2.2.8.1 *Transformation into E. coli DH5α*

The transformation was carried out as discussed earlier in section 2.2.4.2

2.2.8.2 *PCR confirmation of gene insert in the selected clones*

The clones were selected and patched on LB/ampicillin (100 µg ul^{-1}) plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers from either side of the multiple cloning site of the vector so that whatever be the product formed primer could amplify it from either side. The PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTPs (2.5 mM), 1µl *Taq* polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of T7-Forward (5'-TAATACGACTCACTATAGGG-3') and T7-Reverse (5'- CTAGTTATTGCTCAGCGGTG-3') primers each, was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 52 °C for 20 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

2.2.8.3 *Insert orientation confirmation by PCR*

The orientation of insert in the positive clones were confirmed by PCR using T7-Forward (5'-TAATACGACTCACTATAGGG-3') and gene specific reverse primers of CHH1, MIH1 and GIH. PCR mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C

for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 45 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products were analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

2.2.8.4 *Propagation of confirmed colony and recombinant plasmid extraction*

After confirmation, the transformed *E. coli* DH5α containing recombinant plasmids were propagated in 10 ml LB ampicillin (100 µg μl^{-1}) medium at 37 °C, with shaking at 220 rpm. Plasmid extraction was carried out as detailed earlier in this chapter. Plasmids (5-6) of each construct was extracted and transformed into *E. coli* BL21-PLys-DE3 for further screening of recombinant protein expression.

2.2.8.5 *Sequencing and sequence analyses of mature CHH1, MIH1 and GIH gene of P.monodon*

The cloned plasmids were sequenced (at SciGenom Labs Pvt.Ltd, Cochin, India) and the sequences were subjected for BLAST search (Nucleotide) in NCBI database for confirmation of any kind of mutation errors and the in-frame of the genes with the ATG of the pET32a+ system.

2.2.8.6 *E. coli BL21 (DE3) pLysS competent cell preparation*

E.coli BL21 (DE3) pLysS 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 hrs at 150 rpm. The re-inoculation helps to get *E.coli* cells in their log phase. The cells (50 ml) were centrifuged at 6000 rpm for 20 min at 4 °C. All the steps were carried out at 4 °C. The supernatant decanted and cells were resuspended by gentle vortexing with 0.1M $CaCl_2$ ($1/4^{th}$ original culture volume). The resuspended cells were placed in ice for 45 min with intermittent swirling and mixing. The cells were centrifuged at 6000 rpm for

20 min at 4 °C. The supernatant was decanted and cell pellet was resuspended in 1 ml of 0.1M $CaCl_2$. The competent cells formed were stored at -80°C with addition of 10-12 % glycerol.

2.2.8.7 *Transformation into E. coli BL21 (DE3) pLysS*

The competent cells (*E. coli* BL21 (DE3) pLysS) were thawed by placing on ice for 5-10 min. Positive plasmid constructs (pET32a+ - CHH1, pET32a+ -MIH1 and pET32a+ -GIH) were added (5 µl) to a sterile 15 ml culture tube already on ice. Aliquots of 50-100 µl of competent cells were transferred into the 15 ml tubes (containing +ve plasmid constructs) on ice. The tubes were gently flicked to mix and placed on ice for 20 min .The cells were given heat shock at 42 °C for 90 sec in a water bath and immediately returned to ice for 2 min. An aliquot of 600 µl super optimal broth with catabolite repression (SOC - 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose) was added to the tubes containing cells transformed with +ve plasmid constructs. The tubes were incubated for 1.5 hrs at 37 °C with shaking at 220-230 rpm and 100 µl of each transformed culture were plated in duplicate/triplicate on to LB/ampicillin plates. The plates were incubated overnight (12-16 hrs) at 37 °C.

2.2.8.8 *Induction (IPTG) of the recombinant protein expression of pET32a+ -CHH1, pET32a+ -MIH1 and pET32a+ -GIH constructs*

Single colonies of recombinant pET32a+-CHH1, pET32a+-MIH1 and pET32a+-GIH constructs in *E.coli* BL21 (DE3) pLysS were inoculated into 3 ml LB/ampicillin (100 µg μl^{-1}) medium and incubated at 37 °C at 250 rpm until the OD_{600} of 0.5 was obtained. Overnight incubation was avoided. As controls *E.coli* BL21 (DE3) pLysS without vector and with pET32a+ vector were also processed. The entire 3 ml culture was added to 100 ml LB/ampicillin medium and further incubated at 37 °C for 2-3 hrs until OD_{600} of 0.6 (Optimum OD for protein expression). The OD_{600} was monitored frequently during the growth phase of the culture by removing aliquots asceptically. Just prior to induction 5 ml culture was aliquoted, that

served as un-induced control. IPTG was added to a concentration of 1mM to the induced culture. The cultures (induced and uninduced) were incubated further for 3.5-4 hrs at 37 °C with shaking at 250 rpm. The cells were pelletized at 12000 xg for 2-5 min at RT. The pellet was stored at -80 °C until further processing.

The pellet was further processed with two lysis buffers for the release of the expressed recombinant protein. Lysis buffer 1(50mM KH_2PO_4 - pH 7.8, 400mM NaCl, 100mM KCl, 10 % Glycerol, 0.5 % Triton X-100, 10mM Imidazole) was added for a volume calculation of 200 µl for 4ml pelletized culture. A sonication cycle of 20 pulses with 1min gap was repeated 5 times. The cells were pelletized at 6000 rpm for 10 min at 4 °C. The supernatant was collected and stored at -20 °C. To the lysed pellet was added lysis buffer 2 (31.25 mM Tris buffer pH 6.8, 25 % glycerol, 10 % SDS). The sonication cycle was repeated as mentioned for lysis 1. The cells pelletized at 6000 rpm for 10 min at 4 °C. The supernatant was separated and stored at -20 °C.

The supernatants from the first and second lysis including the pellet were analyzed for recombinant protein expression by Coomasie stained 12 % SDS- PAGE. An aliquot of 10 µl of lysis 1 and lysis 2 supernatants of the induced and uninduced samples were boiled in 10 µl sample buffer (62.5mM Tris-Cl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % mercaptoethanol, 0.2 % bromophenol blue) for 5 min. The samples were given a short spin and supernatant was subjected to 12.5 % reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis following the method of Laemmli (1970). The protein was separated and analyzed using 5 % stacking gel and 12 % resolving gel prepared into 10 x 10.5 cm vertical gel plate of mini VE^{TM} mini vertical electrophoresis unit (Hoefer-Amersham, USA). Electrophoresis was performed in 1x Tris- glycine SDS (pH8.3) buffer at a voltage of 12mA (EPS 301, Amersham, USA). After electrophoretic separation, gel was stained in coomasie brilliant blue stain R-250 (0.025 % coomasie brilliant blue R-250, 40 % methanol and 7 % acetic acid in distilled water), de-stained in de- staining solution 1(40 % methanol and

7 % acetic acid in distilled water) and de-staining solution II (5 % methanol and 7 % acetic acid in distilled water), and photographed using Gel-DOC^{TM} XR+ imaging system (BioRad, USA). Recombinant protein expression was determined by comparing the presence and absence of protein profile with that of uninduced cells. Molecular weight of protein was determined by comparing with that of standards (PMWM- Genei, India).

2.2.8.9 *Ni-NTA (IMAC) column purification of mature recombinant proteins of CHH family hormones*

Qiagen Ni-NTA superflow cartridges pre-filled with 1ml Ni-NTA superflow and ready to use for purification of 6x His-tagged proteins using a syringe was used for the purification of CHH family recombinant proteins.

The decision whether to purify 6xHis-tagged proteins under native or denaturing conditions depends on protein location, solubility and the accessibility of the 6x His-tag. The CHH family recombinant protein purification was carried out under native conditions (variation in imidazole concentration) as well as under denaturing conditions (presence of 8M Urea with varying pH of buffers).

2.2.8.10 *Manual purification of 6xHis-tagged recombinant protein of CHH family hormones under denaturing conditions*

Ten ml syringe was filled with buffer B (8 M Urea, 100 mM NaH_2PO_4, pH 8.0). A suitable adapter was attached to the syringe. The syringe was attached to the cartridge inlet. The cartridge was equilibrated with 10 ml column volumes of buffer B. The flow rate was maintained at 1ml min^{-1}. Prior to this the lysate was diluted 1:10 times in buffer B. The syringe was removed and filled with cleared lysate. The cleared lysate was applied to the cartridge using the flow rate (1ml min^{-1}). The cartridge was washed with 10 column volumes of buffer C (wash buffer-8 M Urea, 100 mM NaH_2PO_4, pH 6.3) at the same flow rate as earlier. The recombinant protein was eluted with 5-10 column volumes of buffer E (elution buffer- 8 M urea, 100 mM NaH_2PO_4, pH 4.5).

2.2.8.11 *SDS-PAGE analysis of 6xHis-tag purified recombinant proteins*

The lysate elute, wash elute and final elutes were analysed on 12 % SDS-PAGE to confirm the complete purification steps.

2.2.8.12 *Polyacrylamide gel extraction of the recombinant protein*

In order to exclude the non-specific protein bands and the denaturants like urea in the Ni-NTA purified recombinant samples, they were subjected to polyacrylamide gel extraction. The CHH family recombinant hormones were run on 12 % SDS-PAGE. The recombinant protein bands of CHH1, MIH1 and GIH were cut using surgical blade from the unstained portion of the gel. An aliquot of 1ml extraction buffer (50 mM Tris-Cl pH8, 0.1 mM EDTA and 0.15 M NaCl), were added to the gel pieces and homogenized using a glass homogenizer. The extraction buffer was added further to immerse the gel pieces completely. The homogenized recombinant protein was incubated at 30 °C overnight with shaking at 200-250 rpm. The samples were centrifuged at 5000-10000 xg for 10 min. The supernatant was collected using gel loading tips and concentrated with the Millipore's Amicon Ultra-4 centrifugal filter device.

2.2.8.13 *Concentrating the gel extracted elutes using Millipore's Amicon Ultra-4*

Aliquots of 10-15ml each of CHH family recombinant proteins purified using gel extraction was concentrated using the Millipore's Amicon Ultra-4 (Centrifugal filter device) concentrating and desalting column. Aliquots of 4 ml samples were loaded onto the columns and centrifuged in a swinging bucket rotor (Kemi) at 3000-4000 xg maximum for approximately 10 min. The step was repeated to concentrate the 10-15 ml of the purified recombinant proteins to 0.5 ml-1 ml. The concentrated solute each was recovered by inserting a gel loading pipette tip into the bottom of the filter device. The concentrated samples were analysed on 12 % SDS-PAGE to check the purity of the purified protein.

2.2.8.14 ***Concentrating and refolding of the gel extracted elutes using Millipore's Amicon Ultra-4***

The concentrated samples were reconstituted to the original volume using the refolding buffer (50 mM Tris-Cl pH 8, 0.1 mM EDTA and 0.15M NaCl) and centrifuged in a swinging bucket rotor (Kemi) at 3000-4000 x g maximum for approximately 10 min. The concentrated sample was given 5-6 washes with the refolding buffer to remove the denaturing salts and thus refold the protein to its native form.

2.2.8.15 ***Qubit analysis of the gel extracted recombinant protein***

The recombinant proteins of CHH family hormone extracted was measured using the Quant-iT^{TM} protein assay kit using Qubit fluorometer (Invitrogen, UK). The Quant-iT^{TM} protein assay kit for use with the Qubit fluorometer make protein quantitation easy and accurate. The assay is accurate for initial sample concentrations from 12.5 μg ml^{-1} to 5 mg ml^{-1} and exhibits low protein-to-protein variation. The assays were performed at room temperature, and the signal was stable for 3 hrs.

The Quant-iT working solution was made by diluting the Quant-iT protein reagent 1:200 in Quant-iT protein buffer. The Quant-iT working solution was mixed well without any air bubbles and 199 μl was aliquoted to 0.5 ml tubes. An aliquot of 1μl each of the gel extracted recombinant protein (CHH1, MIH1 and GIH) was added to the respective tubes and mixed by mild vortexing with the care not to create air bubbles (air bubbles cause error in the readings). The tubes were incubated for 15 min at room temperature. The samples were measured using the Quant-iT protein programme. The readings were recorded. The Qubit fluorometer gave values for Quant-iT protein assay kit in μg ml^{-1}. This value corresponded to the concentration after the sample was diluted into the assay tube. The sample concentration was calculated using the following equation: Concentration of sample = QF value x (200/X) where QF value = the value given by the Qubit Fluorometer, X = the number of microliters of sample added to the assay tube.

2.2.9 *In vivo* bioassay of recombinant CHH1 protein in *P.monodon*

P.monodon (10-15 g) obtained from local hatchery were stocked and maintained in a laboratory recirculating aquaculture system (RAS) for shrimp integrated with nitrifying bioreactor (Kumar *et al*., 2009, 2011) in seawater having 15g l^{-1} salinity. Water quality was maintained within a narrow range (pH 6.8-7.8; total ammonia < 0.1 mg l^{-1}; nitrite < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness >5000-6000 mg l^{-1}). The shrimps were fed with commercially available pelleted feed (Higashimaru, India) containing 40 % protein, 3 % fat, 12 % fiber, 18 % ash and 12 % moisture. The shrimps were in the D0-D1 stage during the time of injection., The animals were eyestalk ablated 24 hrs before the injection for *in vivo* bioassay of recombinant CHH1protein

The shrimps were bilaterally eyestalk ablated with sharp scissors and held under heavy aeration to recover the stress. The shrimps were returned to the respective tanks. After bilateral eyestalk ablation the shrimps were starved for 18 hrs. Prior to injection 25 µl of haemolymph was removed for baseline glucose measurement. The purified recombinant protein was dissolved in PBS at a concentration of 50 ng μl^{-1}. An aliquot of 100 µl of the recombinant protein solution was injected into the muscle. Simultaneously eyestalk ablated shrimps were injected with eyestalk extracts. To prepare the extract, the ablated eyestalks (stored at -80 °C) were used. The rigid exoskeleton and retina were removed prior to homogenization in PBS. The supernatant was injected into the eyestalk ablated shrimps as positive control. The negative control was injected with 100 µl of PBS. The haemolymph glucose levels were measured with glucose oxidase kit (Biolab Diagnostics (I) Pvt. Ltd, India). An aliquot of 2 µl of haemolymph was combined with 198 µl of glucose buffer and incubated for 15 min at 37 °C. The glucose level was determined by measuring the absorbance at 500 nm. The level of CHH hormone was analysed with antiCHH1 antibody during the initial period of the experiment.

2.2.10 *In vivo* bioassay of recombinant MIH1 protein in *P.monodon*

The haemolymph MIH are high at the intermoult stage C and decreases during the early premoult stages D0-D1 (Nakatsuji and Sonobe, 2003), thus the shrimps at the stage D2-D3 were selected and randomly divided into two groups viz control (injected PBS) and rMIH group. Prior to injection, 25 µl of haemolymph was removed. The purified recombinant protein was dissolved in PBS at a concentration of 50 ng μl^{-1}. An aliquot of 100 µl of recombinant protein solution was injected into the pericardial cavity of the prawn. The rMIH were injected on 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th} and 13^{th} days. The haemolymph was collected 24 hrs post injection for two days to measure the level of MIH. The MIH concentration in the haemolymph was measured by ELISA (antiMIH antibody). For the rest of the injections, haemolymph was not collected but the moulting stages and moulting were observed. The determination of moult stages was based on the degree of setae development according to the method described by Chan *et al*., (1988), with modifications. Uropods were observed instead of pleopod (Chan *et al*., 1988). The moult stage determination with uropods gave clear and consistent images in animals (Cesar *et al*., 2006; Promwikorn *et al*., 2004). The uropod morphological pictures were taken with Olympus camera (FE 270, UK) under a light microscope (10X) and were analyzed accordingly.

2.2.11 *In vivo* bioassay of recombinant GIH protein in *P.monodon*

The development of recombinant GIH of *P. monodon* in a bacterial system has not been reported till date. For assessing the effect rGIH in the animal there are no physiological aspects like that of rCHH1 (glucose level) and rMIH (moult stages). Thus, the experimental design was set only to know the effect of rGIH in the animals by measuring the level of GIH in haemolymph by ELISA using the antiGIH antibody raised against the rGIH protein. The injection experimental set up was similar to that followed in the rMIH, but the animals in the D2-D3 stage were injected with rGIH on 1^{st} and 3^{rd} day, The haemolymph GIH was observed 24hrs post injection.

2.3 Results

2.3.1 TA vector construction for sequence analyses and homology modelling

The CHH family hormone genes amplified were CHH1-432 bp, MIH1-357 bp and GIH-336 bp using *Taq* DNA polymerase (Fig. 4). They were cloned successfully in the TA vector (pGEM-T). The colony PCR to check for the presence of inserts gave an approximate product size of 532 bp (CHH1), 457 bp (MIH1) and 436 bp (GIH) (Fig. 5 and 6). Three TA vectors of CHH family hormone genes (pGEM-T-CHH1, pGEM-T-MIH1 and pGEM-T-GIH) were successfully constructed for sequence analyses and homology modelling (Fig. 7, 8, 9).

The results of the CHH1, MIH1 and GIH BLAST search with the PDB revealed identity to one reference protein, moult inhibiting hormone (*Marja* MIH) from Kuruma prawn (Katayama *et al*., 2003). The reference used for model creation was the solution structure of the moult inhibiting hormone from the Kuruma prawn *Marsupenaeus japonicus* [Marja MIH; Protein Data Bank Code: 1J0T]. Homology modelling is currently restricted to protein sequences (targets) that share 30 % or more sequence identity to an experimentally solved protein structure template (Baker and Sali, 2001). The three targets had considerable sequence identity and similarity of 33.78 % (CHH1), 96.15 % (MIH1) and 70.51 % (GIH) respectively. The QMEAN score is a composite score consisting of a linear combination of 4 statistical potential terms (estimated model reliability between 0-1). The QMEAN scores predicted were 0.48 (CHH1), 0.55 (MIH1) and 0.52 (GIH) respectively. Structurally conserved regions for the model and the template were determined by multiple sequence alignment from cluster 3, revealing significantly conserved region of the mature peptide. The secondary structure of the template with CHH1, MIH1 and GIH peptides consisted of the six conserved cysteine residues which form the disulphide bond(Cys8 and Cys45, Cys25 and Cys41, Cys28 and Cys54), which showed that the models created are realistic (Fig. 10). The tertiary structure of protein was built by packing of its secondary structure elements to form discrete domains or autonomous folding

units. Comparative modelling predicted the 3D structure of CHH1, MIH1 and GIH models based primarily on its alignment to 1J0T as a template (Fig. 11). Finally, CHH1, MIH1 and GIH peptides and *Marja* MIH were predicted to contain 5 α-helix regions.

2.3.2 Identification of mature region of CHH1, MIH1 and GIH genes

The FASTA format of sequences of CHH1 (Accession GQ221085), MIH1 (Accession GQ221086) and GIH (Accession GQ228835) were translated to amino acid output using the Translate tool of EXPASY (SIB Bioinformatics Resource Portal). The translated sequences were analyzed for presence of signal peptide using the SignalP 4.0 server. The presence of signal peptide was identified in CHH1 (pos.23 and 24) with the mature chain spanning from 24-128aa (Fig. 12). In MIH1 the signals were predicted at positions 28 and 29 with mature chain spanning from 29-105aa (Fig. 13), whereas GIH signal was identified at position 17 and 18, the mature region spanning from 18-96aa (Fig. 14).

2.3.3 Blunt-end vector construction of shrimp CHH1, MIH1 and GIH gene in pJET1.2

Pfu DNA polymerase amplified mature gene products of CHH family hormone genes (Fig. 15) were gel purified (Fig. 16) and was cloned into blunt end pJET1.2 vector (2.974 Kb) for the purpose of constructing blunt end vector constructs. *Pfu* DNA polymerase works as a proof reading enzyme and thus there will not be insertion or deletion of bases which will change the frame of the gene. The CHH family hormone gene amplified using *Pfu* DNA polymerase (CHH1-222 bp, MIH1-231 bp and GIH-237 bp) with added restriction sites (the sizes were CHH1-234 bp, MIH1-243 bp, GIH- 249 bp) was cloned into the blunt-end vector. The colony PCR to check the orientation was performed using pJET-Forward primer and gene specific reverse primers (Fig. 17 CHH, Fig. 18 MIH and Fig. 19 GIH). The approximate product sizes obtained were 296 bp (CHH1), 305 bp (MIH1) and 311 bp (GIH) (Fig. 20). The blunt end CHH family vectors were successfully constructed (pJET-CHH1

(3.208 Kb) (Fig. 21), pJET-MIH1 (3.217 Kb) (Fig. 22) and pJET-GIH (3.223 Kb) (Fig. 23). The sequencing of the blunt-end vector constructs (Fig 24) showed the in-frame sequence of CHH1, MIH1 and GIH, that confirmed the insert and frame of the inserts (Figs. 25, 26, 27)

2.3.4 Construction of recombinant translation vector system for recombinant protein expression

The restriction of inserts from blunt end vector constructs pJET-CHH1 (3.208 Kb), pJET-MIH1 (3.218 Kb) and pJET-GIH (3.223 Kb) were in-frame (Fig. 28). The sequence analyses confirmed no addition of any vector sequence, thus preserving the frame of the inserts. The restricted gene inserts from the blunt-end vector constructs were further cloned into pET32a+ translation expression vector for the construction of translation expression vectors. The expression vector, pET32a+ was restricted using the same enzymes as that included in the primers of the inserts (*EcoR* I/*Xho* I- CHH1, MIH1 (Fig. 29) and *EcoR* I/*EcoR* I-GIH (Fig. 30). The inserts were screened using the vector primers (T7-F and T7-R, Vector region spans 647 bp) and the products sizes obtained were CHH1:881 bp- 647+234 bp; MIH1:890 bp- 647+243 bp; and GIH: 896 bp- 647+249 bp (Fig. 31 and 32). The orientation of the inserts in the expression vector constructs were confirmed by colony PCR with T7-Forward primer and gene specific reverse primer that resulted in CHH1 (550bp- vector region; 550+234 bp-784 bp), MIH1 (550+ 243- 793 bp) and GIH (550+ 249- 799 bp) (Fig. 33, 34, 35). Further, the in-frame of the inserts in the expression vector constructs were confirmed by sequencing the plasmids isolated (Fig. 36).The vector constructs were pET32a+CHH1 (6.134 Kb), pET32a+MIH1 (6.143 Kb) and pET32a+GIH (6.149 Kb) (Fig. 37, 38 and 39).

2.3.5 Expression and purification of the recombinant protein in bacterial system

According to the reading frame of the sequences of pET32a+-CHH1, pET32a+-MIH1 and pET32a+GIH, the deduced recombinant proteins expressed are

demonstrated in Fig. 40 (CHH1) Fig. 41(MIH1) and Fig. 42 (GIH). The CHH1 recombinant protein BLAST resulted in the protein with TRX tag and 99 % similarity with crustacean neurohormone superfamily (CHH1) (Fig. 43). The MIH1 recombinant protein BLAST resulted in the protein with TRX tag and 99 % similarity with Crustacean neurohormone superfamily (MIH1) (Fig. 44) while that of GIH recombinant protein BLAST resulted in the protein with TRX tag and 99 % similarity with crustacean neurohormone superfamily (GIH) (Fig. 45). The fusion protein consisted of histidine peptide tag, in which the six histidine is the binding site for Ni^{2+}, allowing the purification of the recombinant protein with Ni-NTA columns. The BLAST results of the recombinant vector constructs confirmed the presence of CHH1, MIH1 and GIH protein in the vector constructs.

2.3.6 Recombinant CHH1, MIH1 and GIH

The recombinant expression constructs (pET32a+-CHH1, pET32a+-MIH1 and pET32a+GIH) were transformed into *E.coli* BL21 (DE3) pLysS and the expression of the recombinant proteins were induced with 1 mM IPTG. Six constructs (clones) of each gene were induced for recombinant protein expression and the construct each of CHH1, MIHI and GIH which exhibited increased expression level was further processed for large scale expression (Fig. 46, 47, 48). It was observed that during the induction of recombinant GIH, the protein expressed was in lower concentration and only one could give a detectable protein. This vector construct was processed further. SDS-PAGE analysis of cell fractions from *E. coli* BL21(DE3) pLysS transformed with pET32a+CHH1, pET32a+MIH1 and pET32a+GIH revealed peptides of the predicted size (size of fusion protein from the vector was ~20 kDa) is 29.47 kDa (CHH1), 29.85 kDa (MIH1) and 32.16 kDa (GIH) in the insoluble fraction of IPTG-induced cells. The recombinant protein expression was detected initially with western blot: using mouse anti-His antibody/goat anti-mouse IgG HRP conjugate antibody. Results from Western blot analysis confirmed the size of the recombinant protein expressed. However, no positive bands were found in the controls (BL21 cells and

empty vector transformed BL21). The expected molecular weight of each of the fusion protein was 29.47 kDa (CHH1), 29.85 kDa (MIH1) and 32.16 kDa (GIH) using MW/p*I* calculator tool of ExPASy. Western blot results confirmed expected molecular weight closely. These results conclude that the expected recombinant proteins had the correct reading frames with histidine tag at the N-terminus (Fig. 49). In the next chapter the antibodies (antagonists) developed against the recombinant proteins were used to confirm the results of initial recombinant proteins expressed.

The recombinant proteins were purified through Ni-NTA affinity column. Cell pellet lysed with two buffers *viz*., lysis 1 and lysis 2, resulted in the extraction of recombinant protein in lysis 2 confirming the nature of the expressed protein as inclusion body. Only in the presence of denaturing reagents like urea, the inclusion body was soluble (Fig. 50-CHH1, Fig. 51-MIH1). Since the elution buffer consisted of 8 M urea in Tris buffer, the recombinant protein was supposed to be denatured. Finally, for *in vivo* injection experiment and for the development of antibody, the recombinant protein was SDS-PAGE extracted, purified and concentrated (Fig. 52, Fig. 53). The concentration of recombinant proteins obtained from 6 gel extracts were 4.31 mg ml^{-1} (rCHH1) and 3.72 mg ml^{-1} (rMIH1), while 10 gel extracts gave 1.32 mg ml^{-1} of rGIH. Fig 54 gives a comparative image of gel extracted and purified rCHH1, rMIH1 and rGIH with BL21 (DE3) pLysS and pET32a+ induced (Fig. 54). These purified recombinant proteins were ready for *in vivo* injection in *P.monodon* and for raising antibodies in mice (detailed in Chapter-4).

2.3.7 *In vivo* bioassay of recombinant CHH1 protein in *P.monodon*

The hyperglycaemic activity of recombinant CHH1 (rCHH1) were observed by *in vivo* bioassay in *P.monodon*. An aliquot of 0.5 µg $µl^{-1}$ of rCHH1 slightly increased the haemolymph glucose level 0.5 hr post injection of purified rCHH1. The glucose level was observed to rise from 57.20 ±12.59 mg dl^{-1} (0.5 hr) and continued to rise, reached a maximum of 68.70 ± 10.75 mg dl^{-1} (1.5 hr). It was also observed that by 2 hrs the glucose level diminished and normalcy was attained. To compare the

hyperglycaemic effect of rCHH1, eyestalk ablated prawns were injected with two equivalents of eyestalk extract in PBS. The trend in the rise of glucose was somewhat similar to that of rCHH1. The glucose level was observed to rise from 51.68 ± 3.27 mg dl^{-1}(0.5 hr) to reach a maximum of 65.2 ±13.31 mg dl^{-1}(1.5 hrs). Normalcy in eyestalk injected animals were observed to be attained by 2hrs. In comparison to the controls without injection and PBS injected controls, highest glucose concentrations were 32.32 mg dl^{-1} (1.5 hrs) and 28.82 mg dl^{-1} (1.5 hrs). The glucose level rise in rCHH1 and eyestalk injected group was highly significant (<0.05) (Fig. 55). The level of CHH hormone was analyzed in the samples with anti CHH1 antibody through ELISA to confirm the hyperglycaemic effect,. The level of rise in glucose of rCHH1 and ES applied samples compared with the uninjected control and PBS was highly significant (<0.05). A steady rise in the CHH levels was observed in comparison with the uninjected control and PBS control (Fig. 56).

2.3.8 *In vivo* bioassay of recombinant MIH1 protein in *P.monodon*

Under laboratory culture conditions, moulting of animals (10-15 g) was observed to take place at duration of 11.67 ± 1.03 days. D2-D3 stages were selected to get a better response of the injected rMIH. According to Chan *et al*. (1988), the D2 stage duration was for a period of 2-3 days (i.e.6-7 % of entire moulting duration) and D3 stage for 1-2 days (3-4 % of the entire moulting duration). Based on this data we observed that the D2 stage duration was extended from ~ 3 days to 8 days while D3 stage extended from ~2 days to 7 days, post injection of rMIH. Thus the shrimps injected with rMIH, the moult cycle was significantly delayed 16.67 ± 1.03 days. When compared with the control by Student's t- test, the moult cycle duration of rMIH group was significantly longer than that of the controls (Fig. 57). It was also observed that the injections could not be continued past 12 days from the start of injection as there was mortality, possibility due to the extension of moult stages and the stress related to it. The effect of recombinant MIH1 protein injection was significant, but relatively lesser in terms of MIH concentration in the haemolymph. The MIH concentration in the haemolymph showed a

significant but comparatively lesser increase (7.05 %) in comparison with the control (Fig. 58). The glucose level during the initial 24 hrs was observed, there was no significant increase in rMIH injected animals with that of control.

2.3.9 *In vivo* bioassay of recombinant GIH protein in *P.monodon*

The development of recombinant GIH of *P. monodon* in a bacterial system has not been reported till date. For assessing the effect of rGIH in the animal there are no physiological aspects like that of rCHH1 (glucose level) and rMIH (moult stages). The 24 hrs observation of haemolymph GIH showed significant increase (16.23 %) of GIH level in comparison to that of the control measuring by means of ELISA using anti- rGIH antiserum (Fig. 59). Glucose concentration was measured, but no significant difference was observed (Fig. 60).

2.4 Discussion

2.4.1 Homology modelling of CHH1, MIH1 and GIH of P. monodon

The homology modelling -a bioinformatic tool, enables the prediction of protein/ peptide structure in comparison with the target protein with only the amino acid sequence. The homology structures modelled based on the existing structures have gained considerable success in the field of proteomics. Homology protein modelling uses experimentally determined protein structures (template) to predict the 3-D of another protein that has similar amino acid sequence (target).This approach to modelling is possible since a small change in the protein sequence usually results in a small change in its 3D structure (Hubbard and Blundell, 1987). Homology results include precious information about how well each region of the model fits its templates. The NMR structure of MIH (1J0T) (Katayama *et al*., 2003) provides a significant insight into how the members of CHH family can be modelled. Among the eyestalk neuropeptides, the CHH family has similar protein structure and share conserved characteristics in amino acid sequences to form a unique family (Katayama *et al*., 2003). The three targets we modelled with 1J0T as template had considerable sequence

identity and similarity of 33.78 % (CHH1), 96.15 % (MIH1) and 70.51 % (GIH). The similarity of the CHH family protein confirms that they harbour similar folding with that of MIH (1J0T -template) of *Marsupenaeus japonicus*. Nagaraju *et al*. (2009) gave an insight into the structural prediction and analysis of VIH-related peptides from various crustacean species. Based on various other gene level studies it is well understood that all the members of the CHH family are derived from a common ancestral gene in the time course of gene mutation and duplication (Nagaraju *et al*., 2009). We found that all the six cysteine residues are relatively at the same positions (Cys8 and Cys45, Cys25 and Cys41 and Cys28 and Cys54) allowing the formation of the three intramolecular disulphide bonds that provide strong stabilization of the 3-D structure. Thus, the modelled 3D structures of the three genes CHH1, MIH1 and GIH showed significant homology to the only NMR structure of MIH (1J0T). The slight variations observed in the case of CHH may be due to the variation in the N-terminal sequence. These findings also agree with previously published homology and 3-D modelling of CHH family neuropeptides. (Chen *et al*., 2004; Katayama *et al*., 2003).

2.4.2 Expression of mature recombinant CHH family hormones

The research in the field of CHH family neuropeptides have been in the limelight from past two decades due to their importance in the field of aquaculture. The presence of only small quantity of the purified CHH family neuropeptides makes it difficult for the isolation from the eyestalk of shrimp. Another factor is the similarity in the size and structure of the three CHH family neuropeptides limiting its isolation and purification directly from the animal (Gu *et al*., 2001). Thus the recombinant DNA technology is the simplest and instant method for the production of large quantity of purified proteins. The simple technique of fusion of the target gene fragment to the 3'end of a translation vector to efficiently express the protein solves the difficulty in isolating the active neuropeptides from the animal. The recombinant protein expression of mature region was undertaken to study the physiological action of the CHH family neuropeptides. The major design of the work described in Chapter

2 was to get the complete protein including the signal peptide and mature region of the three inhibitory hormones of the CHH family, but we were unable to get the whole hormone as the bacterial system was unable to read the signal peptide region of the three genes (CHH1, MIH1 and GIH). It is concluded in various recombinant protein expression works that the presence of rare codons in the gene of interest or eukaryotic secretory signal peptide may inhibit the expression. Thus, we had to design primers mainly from the mature region of the hormone gene.

The three inhibitory hormone genes were amplified using *Pfu* DNA polymerase (proof reading property), thus the point deletion or insertion mutation possibility can be neglected. Initially the three genes were cloned in pJET1.2 Blunt end vector to avoid the insertion of bases from the vector region causing frame shift in the gene of interest. Subsequently, the three inhibitory hormone genes (CHH1, MIH1 and GIH) were cloned and sequenced in expression translation vector pET32a+. Three expression translation vector constructs could be built successfully for the production of recombinant/fusion proteins viz., rCHH1 (29.47 kDa), rMIH1 (29.85 kDa) and rGIH (32.16 kDa) in *E.coli* BL21 (DE3) pLysS system by IPTG induction. It was observed that the expression level of the three hormones varied, the expressed recombinant protein of GIH was in reduced quantity than rCHH1 and rMIH1, which indicated that the protein expressed might have different structure and function inside the bacteria and thus the level of expression has varied. The Western blot analysis of the expressed recombinant proteins demonstrated the estimated molecular weight and expression level of the recombinants. The presence of His-tag in the recombinant proteins allowed the purification with Ni-NTA columns under denaturing conditions. The recombinant/fusion protein was further subjected to SDS-PAGE extraction and refolding for *in vivo* studies in *P.monodon* and for raising polyclonal antibodies in mice.

2.4.3 Biological functions of the CHH family recombinant proteins

The biological functions of the CHH family recombinant proteins were observed by *in vivo* injection in *P.monodon*. *In vivo* injection of rCHH1 could elevate

the glucose level in the haemolymph, rMIH retarded the moulting duration and rGIH slightly enhanced the levels of GIH. These results indicated that the recombinant proteins developed posses the neurohormonal functions. The *in vivo* injection of 4 µg (140.5 pmoles) rCHH produced hyperglycaemic effect similar to that of the eyestalk extract. The hyperglycaemic effect was taking into action a little from 0.5 hr (59 %) of injection and reached maximum at 1.5hrs (~100 %) and the effect drained off slowly after 2 hrs. The concentration of applied recombinant protein can be debated.

The concentration of the injected recombinant protein in haemolymph was estimated as $1.69x10^{-6}$M (rCHH1), $1.30x\ 10^{-6}$M (rMIH1) and $0.425x\ 10^{-6}$M (rGIH). Chan *et al*. (1994) carried out *in vivo* bioassay in crayfish *Procambarus clarkii* with HPLC purified CHH1 (12.5 pmol *i.e*., 0.1 µg) and produced hyperglycaemic effect of 17 mg dl^{-1} and 14 mg dl^{-1} increase in haemolymph glucose. Injection of 64 pmole of purified Pej-CHH like peptides raised the glucose level by 10-18.5 mg dl^{-1} in 2 hrs. The hyperglycaemic activity of 5 µg of recombinant Pem-CHH2 and Pem-CHH3 within 0.5 hr of injection was 3.58 mg dl^{-1} and 3.05 mg dl^{-1} in *P.monodon* (Udomkit *et al*., 2004). Compared to the amount of HPLC purified CHH1 injected as mentioned above, the recombinant protein injected in the present study contributed a small fraction of the native form of the protein even after the refolding procedure. The correct folding of peptide is important for the function as neurohormone. Considering the fact that recombinant protein underwent denaturation and renaturation during the purification procedure (denaturing conditions-8 M Urea) which affected the correct folding of the peptides, we opted for the PAGE extraction without the addition of the denaturing agents like beta-mercaptoethanol/DTT. The presence of SDS too was tried to be eliminated by washing and concentrating in the refolding buffer repeatedly. Still the fact remains of the low percentage of correct folded peptide (six cysteines) which is difficult to be obtained *in vitro*. The positive result of the bioassay exhibiting hyperglycaemic effect (glucose concentration was 65.2 ±13.31 mg dl^{-1} (1.5 hr) for rCHH1 showed the presence of some native form of the protein to induce the function

in the animals. Studies by Chan *et al*. (1988) in *P. vannamei* revealed that the haemolymph glucose level during the D0-D1 stage was high. Thus the D0-D1 stage was taken for experimental assay of recombinant CHH1.

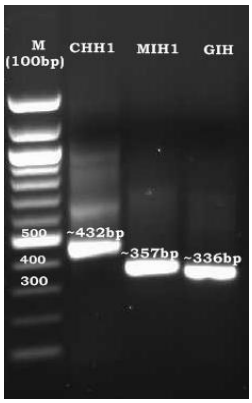
In the present study we found inhibitory effects of rMIH on the moult duration and haemolymph MIH levels *in vivo* in *P.monodon.* The development of recombinant MIH of *P. monodon* in a bacterial system has not been reported yet. The dose-response of purified rMIH of *P.monodon* was examined. In the shrimps injected with rMIH, the moult cycle was significantly delayed 16.67 ± 1.03 days in comparison to the control (11.67 ± 1.03 days). As observed the inhibition in moulting at a concentration of 1.30x 10^{-6}M (rMIH1) was 42 %. These are almost comparable to those of natural Pej-MIH, maximum inhibition (45 %, 8.6x 10^{-9}- 4.3x 10^{-8} M) (Ohira *et al*., 1999). Katayama et al. (2001) proved that recombinant molt- inhibiting hormone has similar secondary structure to the native hormone. The timing of MIH injection in the moult cycle may be important for determining the effects of MIH, as the haemolymph MIH levels and responsiveness of the Y-organ to MIH has been known to change the moult cycle. Haemolymph MIH levels are high at intermoult stage C and decreases during the premoult (D0-D2) (Okumura *et al*., 2005). Thus, for the experiments the late premoult stage (D2-D3) was opted, the rMIH injected may compensate for the reduced levels of the endogenous MIH and therefore cause moult inhibition. The rapid degradation of exogenous MIH (Chung and Webster, 2003; Nakatsuji and Sonobe, 2004) is the possible reason why rMIH had to be injected every alternate day. Technique for continuous administration of the peptides, such as osmotic pump, may be effective in enhancing the effects of exogenous (recombinant) MIH (Gu *et al*., 2001). We also observed that the extension of the moult cycle brought about mortality, which may be due to the extension of the moult duration or a particular moult stage for a prolonged time, bringing forth stress to the animal.

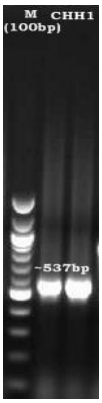
The general yield of natural MIH from one prawn amounts to only 80 ng (Ohira *et al*., 1999); which has been overcome by expression in bacterial system in the

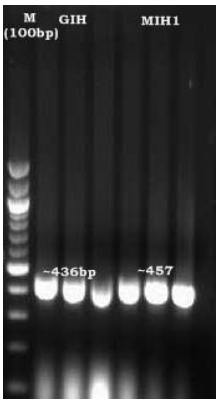
present work. We have succeeded in expressing large quantity of rMIH of *P.monodon* in *E.coli* expression system with significant biological activity. The rMIH of *P.monodon* has various applications, not only with biological activity but also in understanding the interrelated activities of MIH in reproduction, and control of ecdysteroid levels.

We report the development of recombinant GIH (0.425×10^{-6} M) of *P. monodon* for the first time in a bacterial system. The rGIH developed could significantly increase the level of GIH, even though the level of increase was small. The haemolymph GIH increased by 10 % in comparison with the control. The concentration of rGIH applied was 50 ng μl^{-1}; this might be the reason for just 10 % increase in GIH level. The dose response relation can be further investigated. Ohira *et al*. (2005) was successful in the expression recombinant VIH from *Nephrops norvegicus* in a bacterial system. They observed that rHOa-VIH-OH showed no effect on VG mRNA expression in the ovary of *M. japonicus* at a concentration of 4-400 nM, while rHOa-VIH-amide inhibited VG mRNA levels slightly at 40 nM (79.1 %) and significantly at 400 nM (50.9 %) compared with the non- treatment group. Edomi *et al*. (2002) expressed recombinant *N. norvegicus* VIH in *E.coli*, this recombinant VIH was used for antibody generation of VIH. However, the biological activity of this recombinant VIH expressed was not reported. We have been successful in developing the rGIH of *P.monodon* in bacterial system, thus large quantity of rGIH could be produced for various studies. The rGIH developed was used for raising antibody of GIH (detailed in Chapter 3). This is the first report of production of recombinant GIH of *P.monodon* in bacterial system having biological activity.

Generally the isolation of crustacean neurohormones especially the CHH family hormones that are present in small quantity from the animals for large scale biological assays and various other interrelated studies could overcome by the present work. The development of recombinant proteins of CHH family hormones will be useful tool for further physiological studies in better understanding the endocrine mechanisms regulating hyperglycaemia, moulting and vitellogenesis.





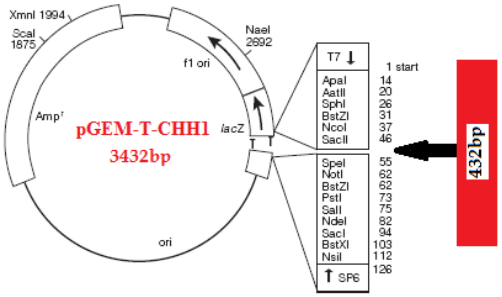


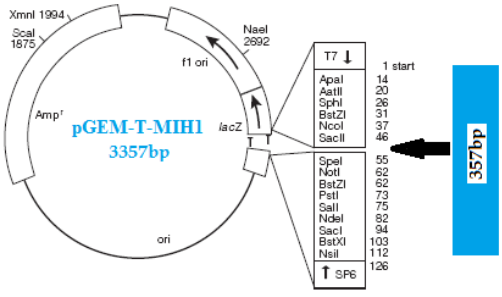
(Fig. 4) **(Fig. 5)** **(Fig. 6)**

Fig.4. PCR amplified CHH1, MIH1 and GIH genes

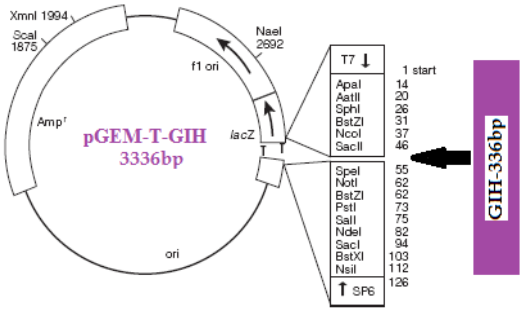
Fig.5. Colony PCR of CHH 1 in pGEM-T with T7 and Sp6 primers

Fig.6. Colony PCR of MIH 1 and GIH in pGEM-T with T7 and Sp6 primers





(Fig. 7) **(Fig. 8)**



(Fig. 9)

Fig.7. TA vector construct of pGEM-T-CHH1,

Fig.8. TA vector construct of pGEM-T-MIH1

Fig.9. TA vector construct of pGEM-T-GIH

```
Multiple Sequence Alignment with template

gi|40744211|gb|AAR89516.1|          -----MYRLAMKTWLAIVIVVVGTSLFFDTASASFIDG-----------  MIH1
1J0T_A|PDBID|CHAIN|SEQUENCE         ------------------------------ASFIDN-----------  1J0T A
gi|359720535|gb|AEV54450.1|         ----------MKTWLLLATLAVG------ASLANILDS-----------  GIH
gi|7107418|gb|AAF36407.1|AF233      MVAVGPMRTAVLVSLLLAIPASATTFGDGNDIPTFLRSSPEASPVTSLHT  CHH1
                                                                  ..:: .

gi|40744211|gb|AAR89516.1|          ---------TCRGVMGNRDIYKKVVRVCEDCTNIFRLPGLDGMCRDRCFY  MIH1
1J0T_A|PDBID|CHAIN|SEQUENCE         ---------TCRGVMGNRDIYKKVVRVCEDCTNIFRLPGLDGMCRNRCFY  1J0T A
gi|359720535|gb|AEV54450.1|         ---------KCRGAMGNRDMYNKVERVCEDCTNIYRLPQLDGLCRNRCFN  GIH
gi|7107418|gb|AAF36407.1|AF233      SDKRSLSFRSCTGAY-DRELLVRLDRVCEDCYNVYRDVGVAAECRSNCFH  CHH1
                                          .* *.  :*::  :: ****** *::*   : . **..**

gi|40744211|gb|AAR89516.1|          NEWFLICLKAANREDEIEKFKVWISILNAGQ--                    MIH1
1J0T_A|PDBID|CHAIN|SEQUENCE         NEWFLICLKAANREDEIEKFRVWISILNAGQ--                    1J0T A
gi|359720535|gb|AEV54450.1|         NQWFLMCLHSAKREAELEHFRLWISILNAGRPW                    GIH
gi|7107418|gb|AAF36407.1|AF233      NEVFLYCVDYMFRPRQRNQYRAALQRLGK----                    CHH1
                                    *: ** *:.   *  : :::: :. *.
```

Fig.10. Multiple sequence alignment of CHH1, MIH1 and GIH with the template (1J0T-A)

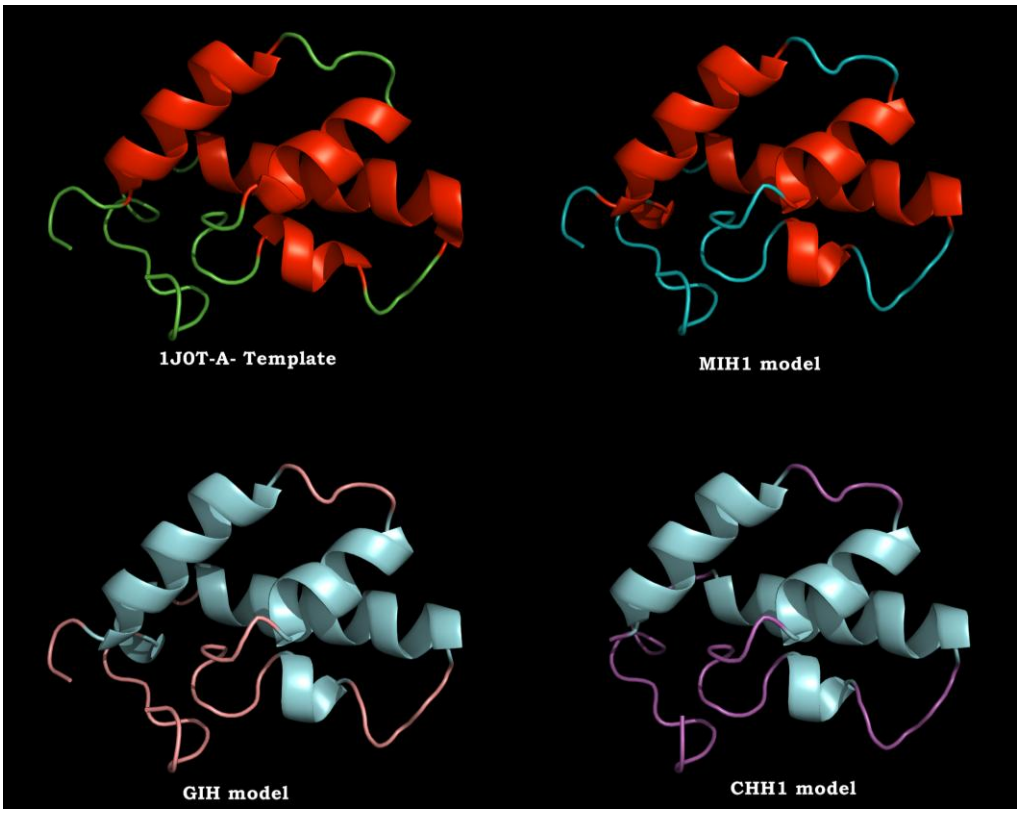
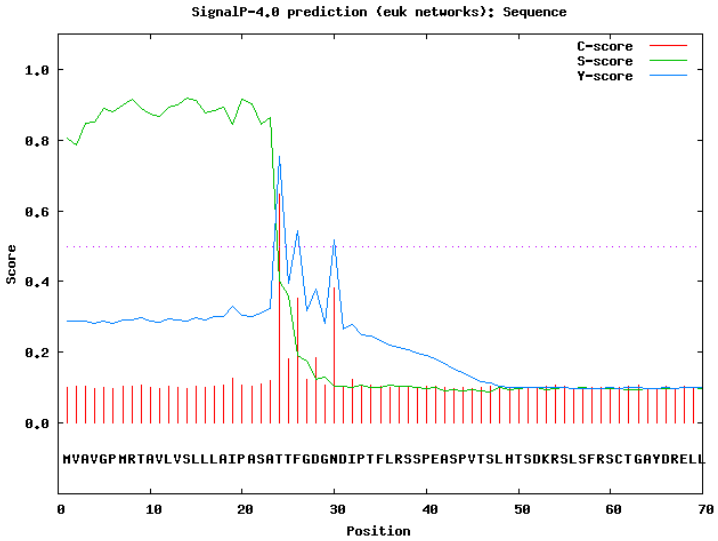
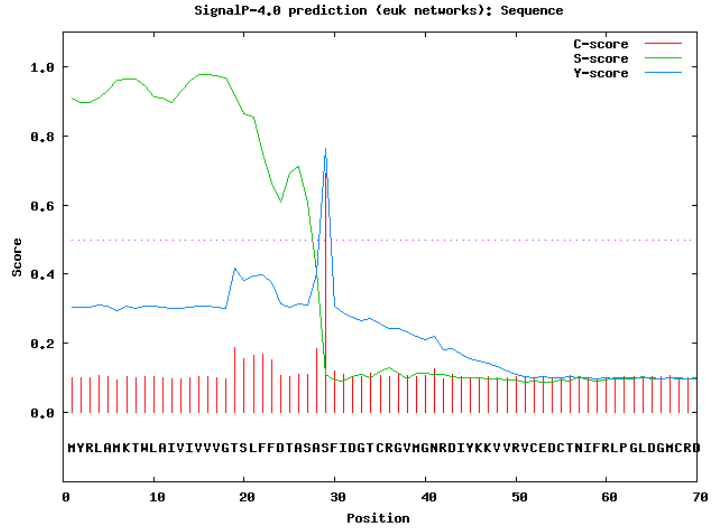


Fig.11. 3D structure of CHH1, MIH1 and GIH model based primarily on its alignment to 1J0T as a template



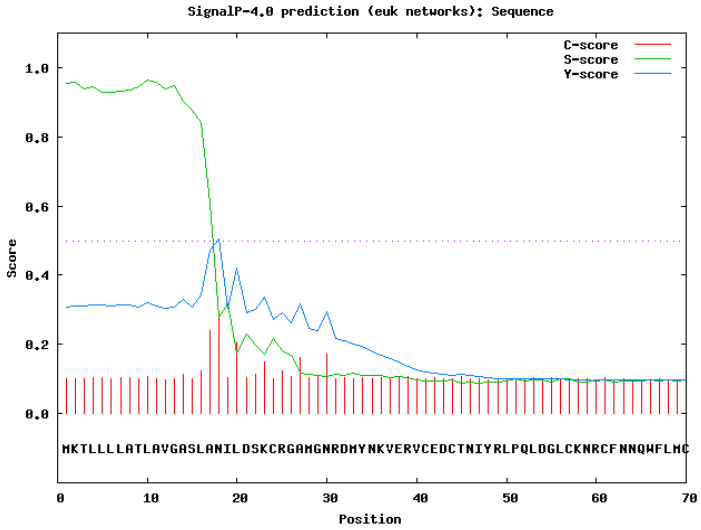
SP=`YES` Cleavage site between pos. 23 and 24
>Sequence; Mature Chain: 24-128
TTFGDGNDIPTFLRSSPEASPVTSLHTSDKRSLSFRSCTGAYDRELLVRLDRVCEDCYNV
YRDVGVAAECRSNCFHNEVFLYCVDYMFRPRQRNQYRAALQRLGK

Fig.12. CHH1 Signal Prediction



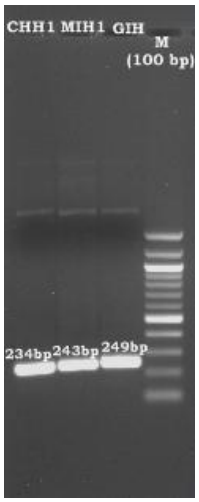
SP='YES' Cleavage site between pos. 28-29:
>Sequence; Mature Chain: 29-105
SFIDGTCRGVMGNRDIYKKVVRVCEDCTNIFRLPGLDGMCRDRCFYNEWFLICLKAANRE
DEIEKFKVWISILNAGQ

Fig.13. MIH1Signal Prediction

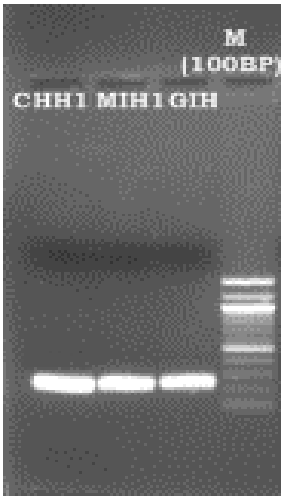


SP='YES' Cleavage site between pos. 17 and 18
>Sequence; Mature Chain: 18-96
NILDSKCRGAMGNRDMYNKVERVCEDCTNIYRLPQLDGLCKNRCFNNQWFLMCLHSAKRE
AELEHFRLWISILNAGRPW

Fig.14. GIH Signal Prediction

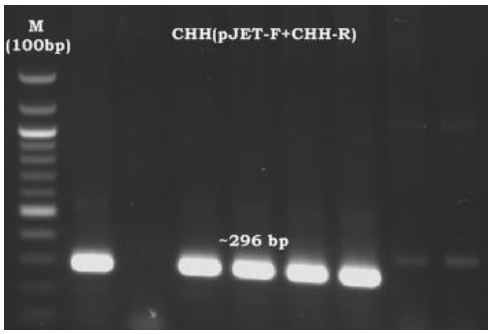


(Fig.15)

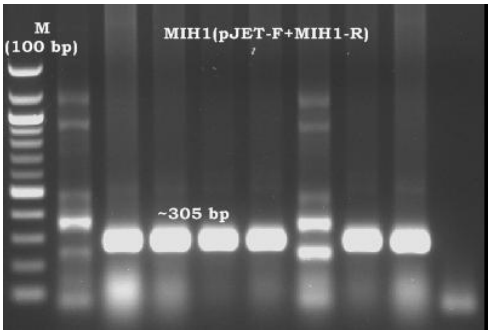


(Fig.16)

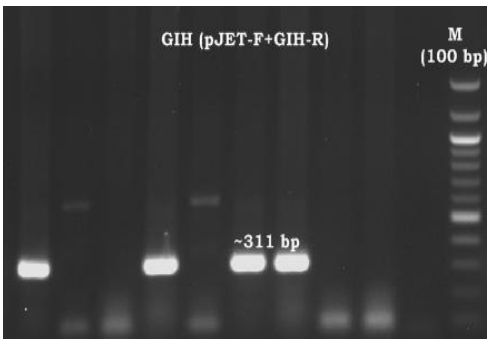
Fig.15. *Pfu* amplified mature region of CHH1, MIH1 and GIH genes
Fig.16. Gel purified mature CHH1, MIH1 and GIH genes



(Fig.17)



(Fig.18)



(Fig.19)

Fig. 17, 18 19. Colony PCR – Insert orientation confirmation by pJET-F+ CHH1-R, pJET-F +MIH1-R, pJET-F+ GIH-R

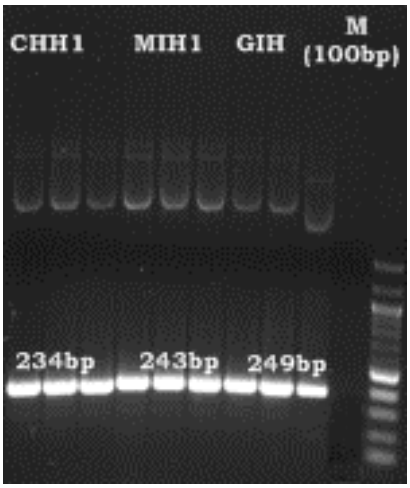


Fig.20. PCR confirmation of insert (CHH1, MIH1 and GIH) in plasmid with vector primers (pJET-F+pJET-R)

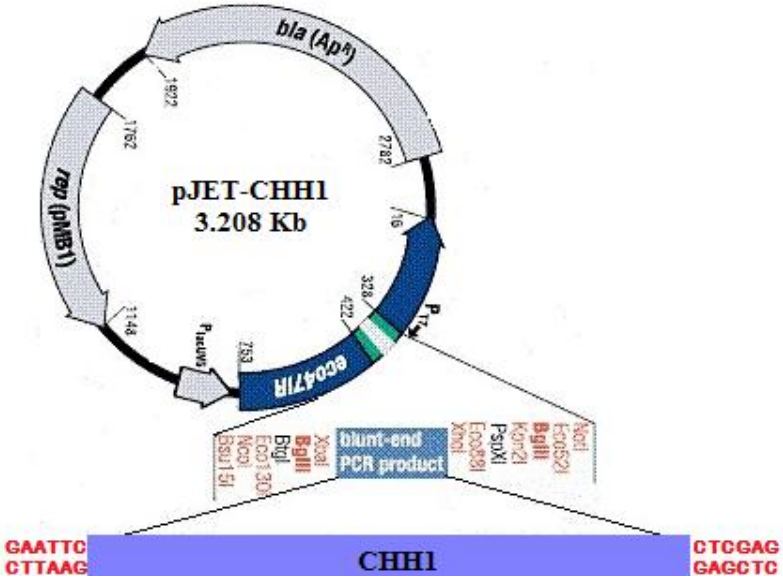


Fig.21. pJET-CHH1 vector (3.208 Kb)

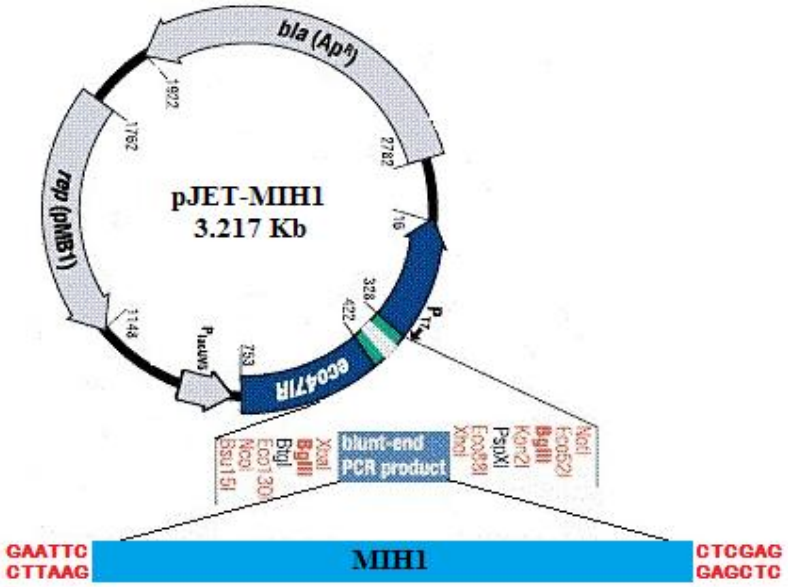


Fig.22. pJET-MIH1 vector (3.217 Kb)

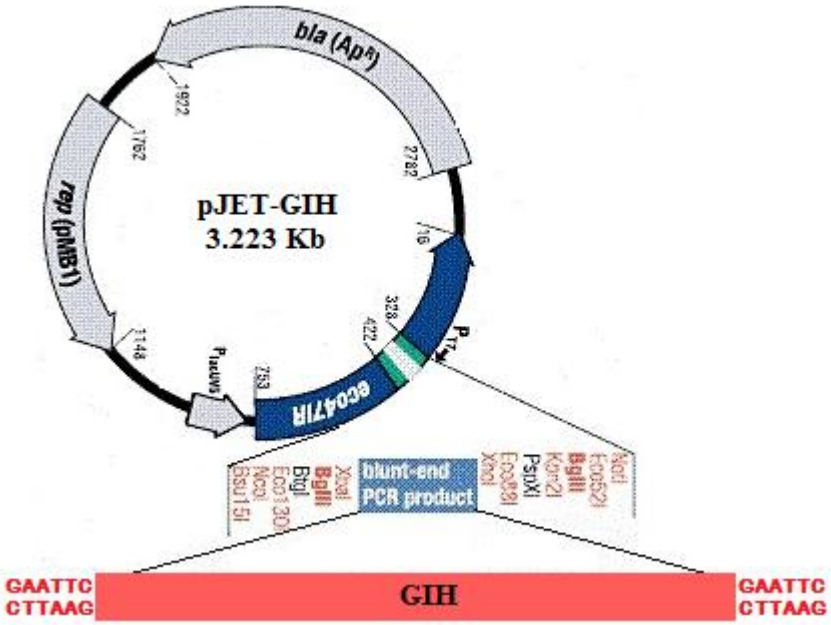


Fig. 23. pJET-GIH vector (3.223 Kb)

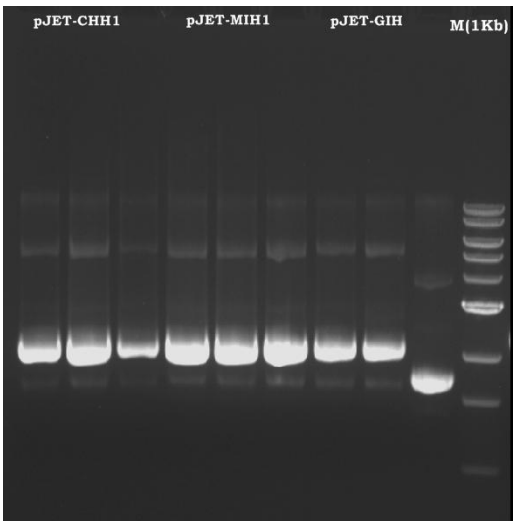
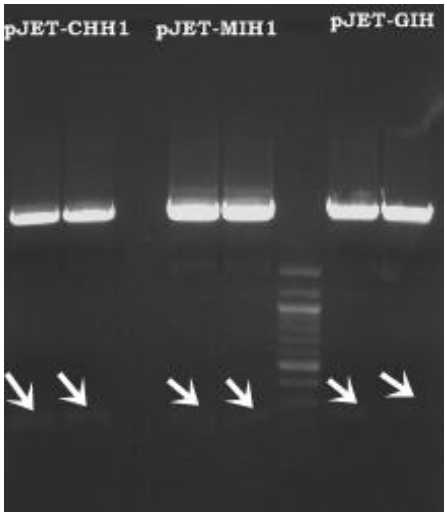


Fig.24. Plasmid extracted from the positive clones of pJET blunt end vector constructs

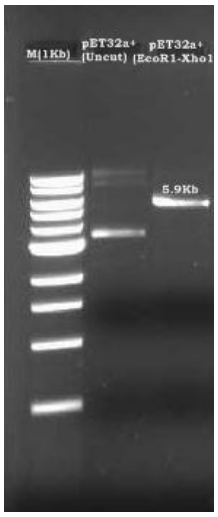
Fig.25. In-frame sequence of CHH1 in pJET-CHH1 vector construct

Fig.26. In-frame sequence of MIH1 in pJET-MIH1 vector construct

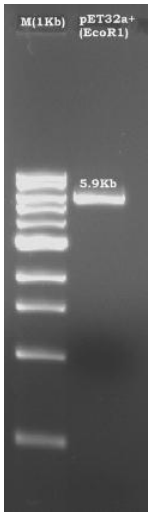
Fig.27. In-frame sequence of GIH in pJET-GIH1 vector construct



(Fig.28)



(Fig.29)

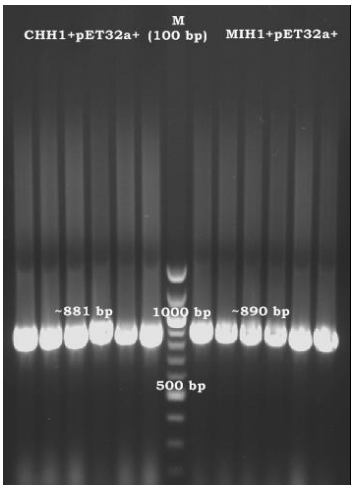


(Fig.30)

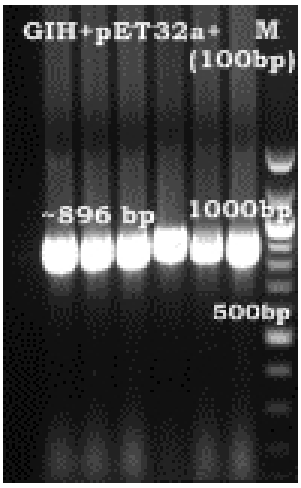
Fig.28. *EcoR* I-*Xho* I restriction of CHH1, MIH1 and EcoR1 restriction of GIH from pJET blunt end vector

Fig.29. *EcoR* I-*Xho* I restriction of pET32a+

Fig.30. *EcoR* I restriction of pET32a+

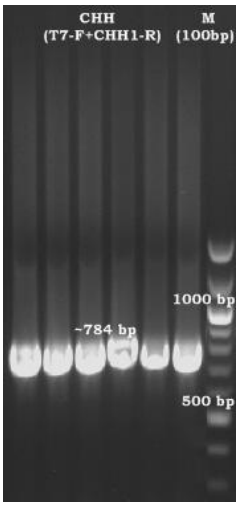


(Fig.31)

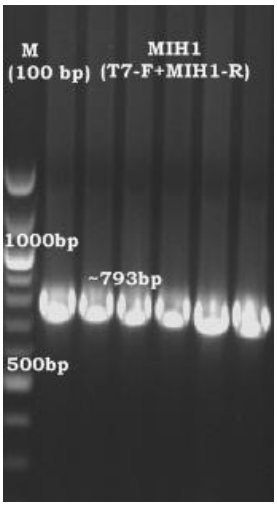


(Fig.32)

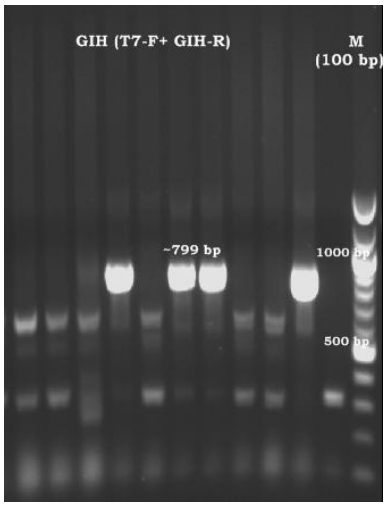
Fig.31. Colony PCR of CHH1, MIH1 in pET32a+ with T7-F and T7-R primers
Fig.32. Colony PCR of GIH in pET32a+ with T7-F and T7-R primers



(Fig.33)



(Fig.34)



(Fig.35)

Fig.33, 34, 35. Orientation of inserts CHH1, MIH1 and GIH with T7-F and gene specific reverse primer

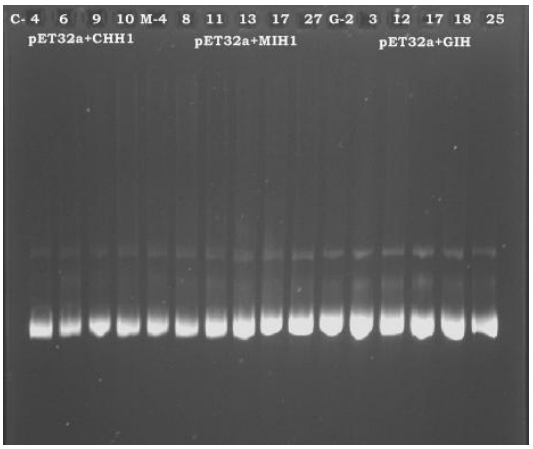
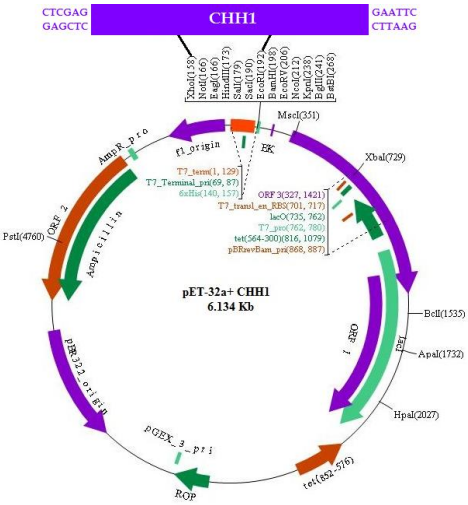
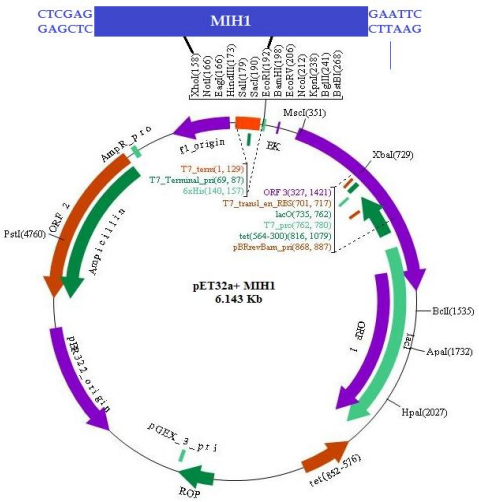


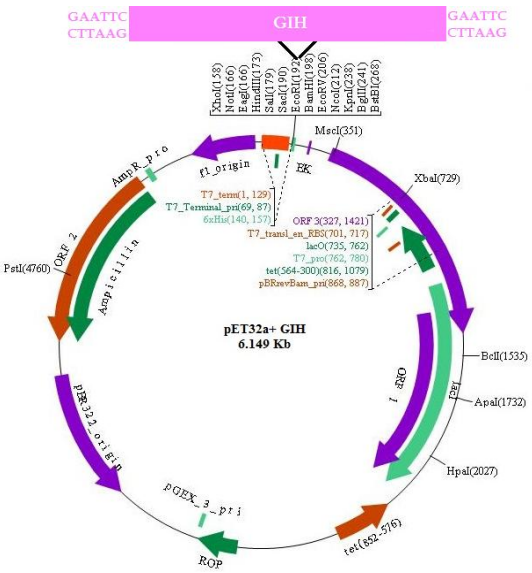
Fig.36. Plasmid from positive vector constructs in pET32a+ of CHH1, MIH1 and GIH



(Fig.37)



(Fig.38)



(Fig.39)

Fig.37. Vector construct of pET32a+CHH1 (6.134Kb)

Fig.38. Vector construct of pET32a+MIH1 (6.143 Kb)

Fig.39. Vector construct of pET32a+GIH (6.149Kb).

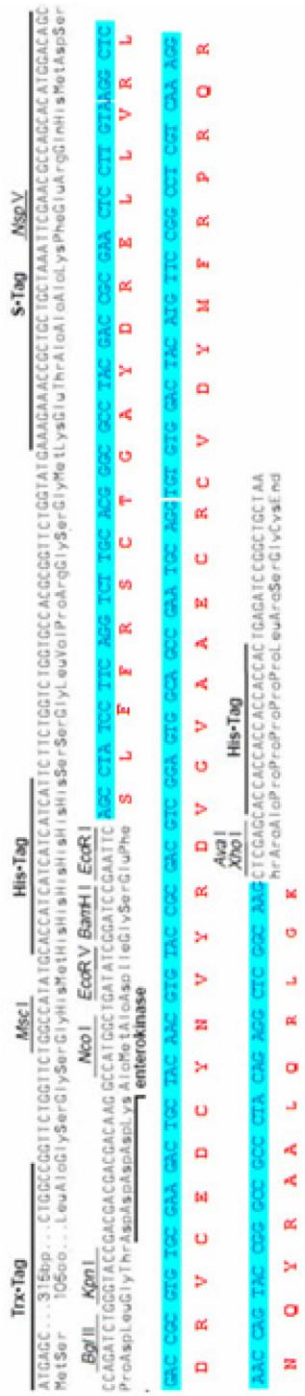


Fig.40. pET32a+-CHH1 recombinant protein- Theoretical pI/Mw: 5.45 / 29.47 kDa

Fig.41. pET32a+-MIH1 recombinant protein Theoretical pI/Mw: 5.18 / 29.85kDa

Fig.42. pET32a+-GIH recombinant protein Theoretical pI/Mw: 5.32 / 32.16 kDa

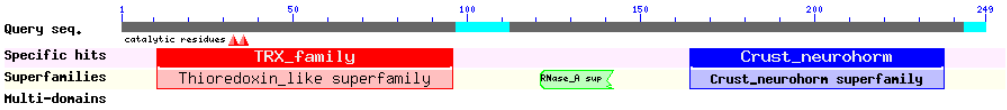


Fig.43. CHH1 recombinant protein BLAST result

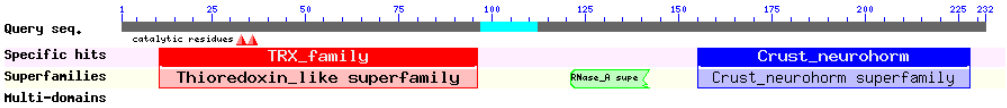


Fig.44. MIH1 recombinant protein BLAST result

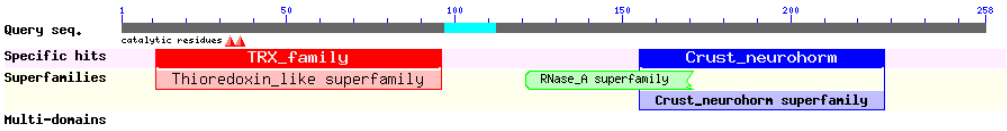
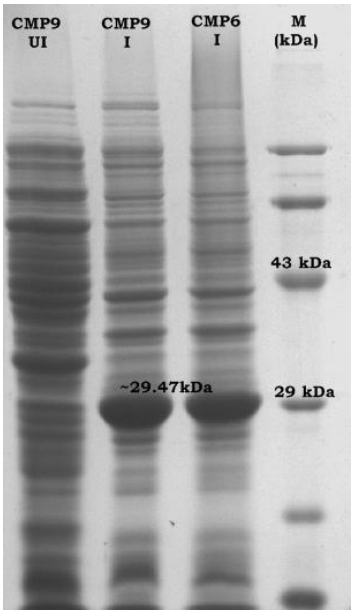
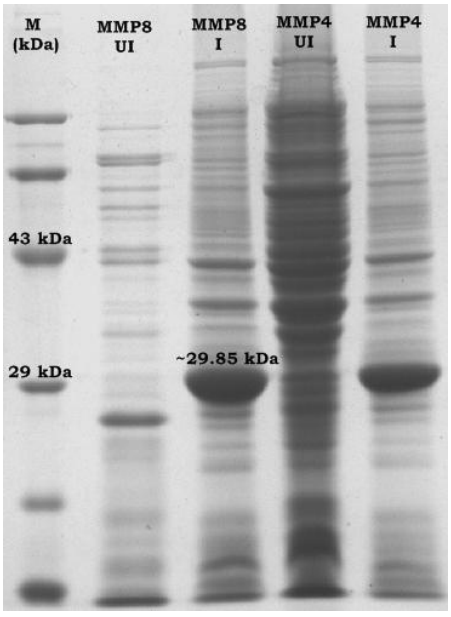


Fig.45. GIH recombinant protein BLAST result



(Fig.46)



(Fig.47)

Fig.46. Recombinant protein expression of CHH1
Fig.47. Recombinant protein expression of MIH1

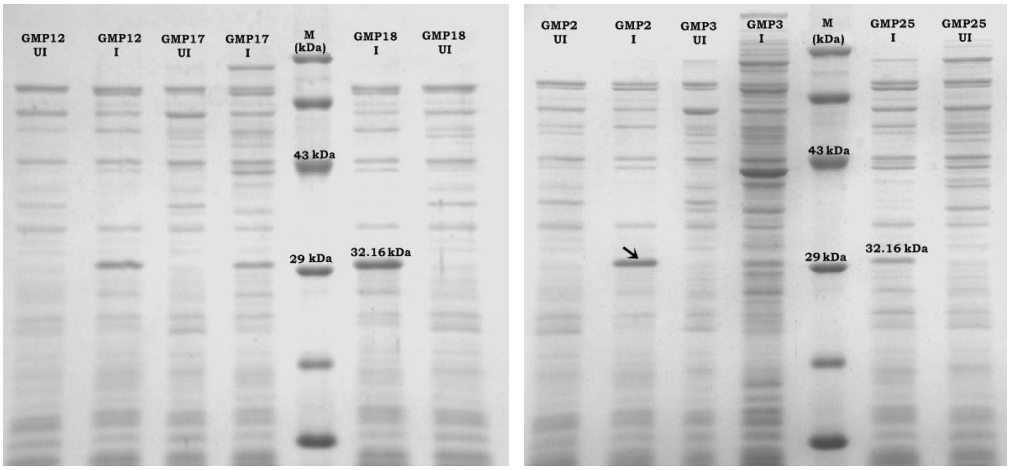


Fig.48. (Two gel images) Recombinant protein expression of GIH

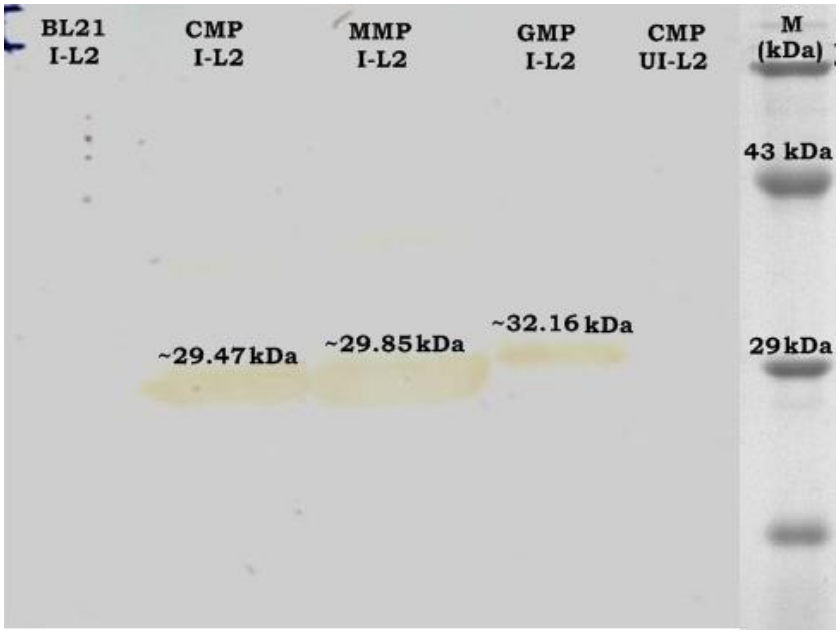
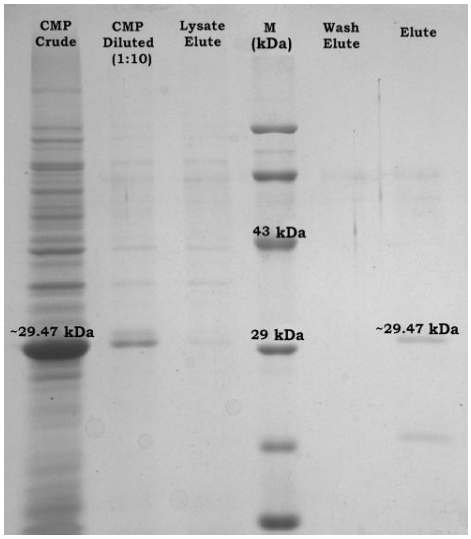
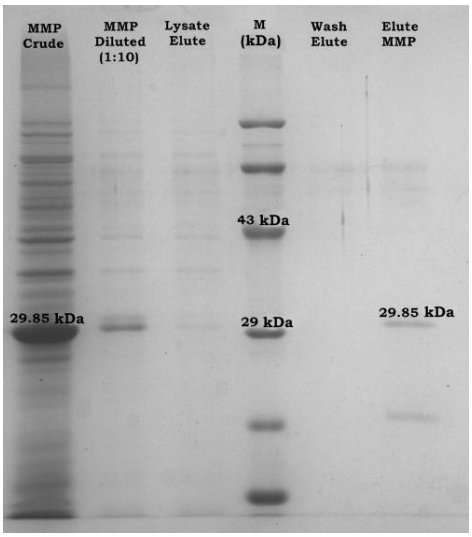


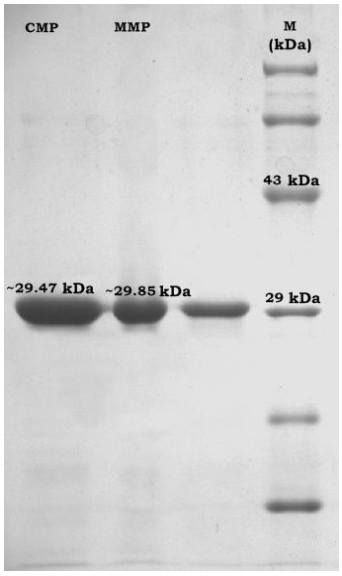
Fig.49. Western blot: using mouse anti-His antibody/Goat anti-mouse IgG HRP conjugate antibody

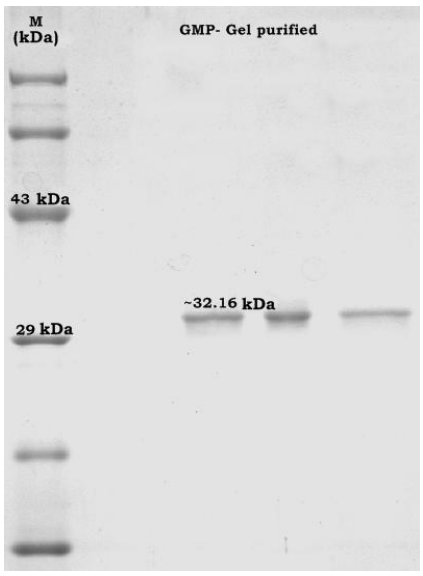




(Fig.50) **(Fig.51)**

Fig.50. The CHH1 recombinant proteins purified through Ni-NTA affinity column.
Fig.51. The MIH1 recombinant proteins purified through Ni-NTA affinity column





(Fig.52) **(Fig.53)**

Fig.52. The CHH1 and MIH1 recombinant protein was SDS-PAGE extracted, purified and concentrated
Fig.53. The GIH recombinant protein was SDS-PAGE extracted, purified and concentrated

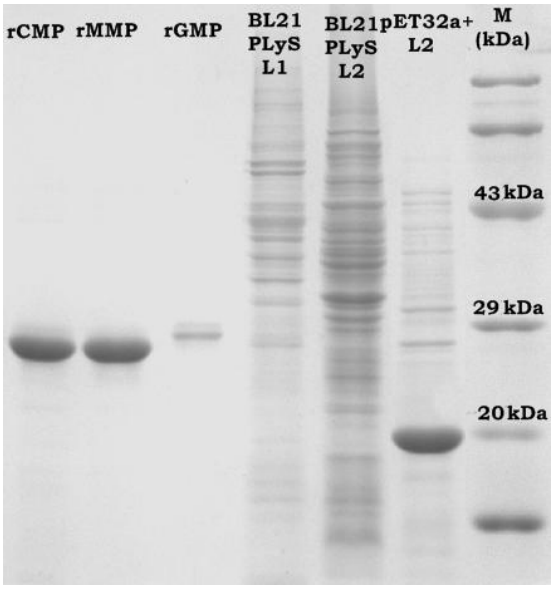


Fig.54. Comparative image of gel extracted and purified rCHH1, rMIH1 and rGIH with BL21 (DE3) pLysS and pET32a+ induced

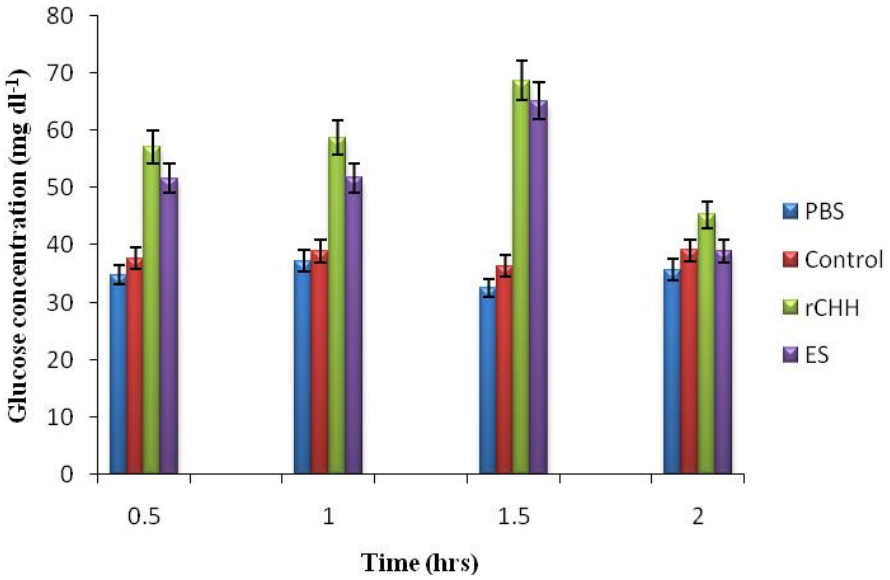


Fig.55. The level of rise in glucose of rCHH1, Control (eyestalk extract injected, ES) compared with the control (uninjected) and PBS

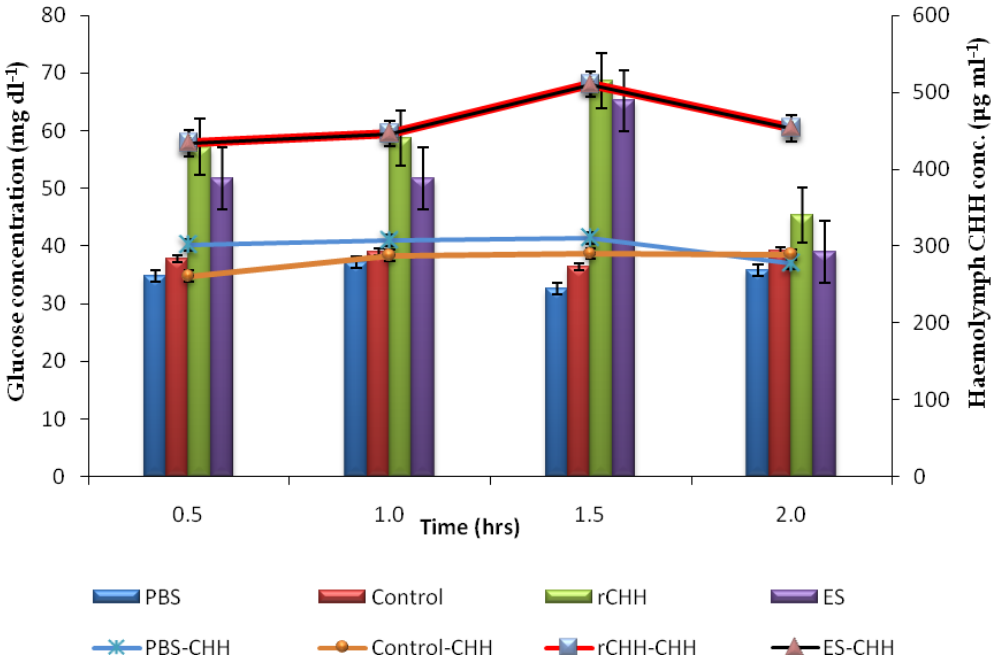


Fig.56. CHH hormone levels were analyzed with antiCHH1 antiserum. Graph showing comparison of glucose with that of haemolymph CHH level

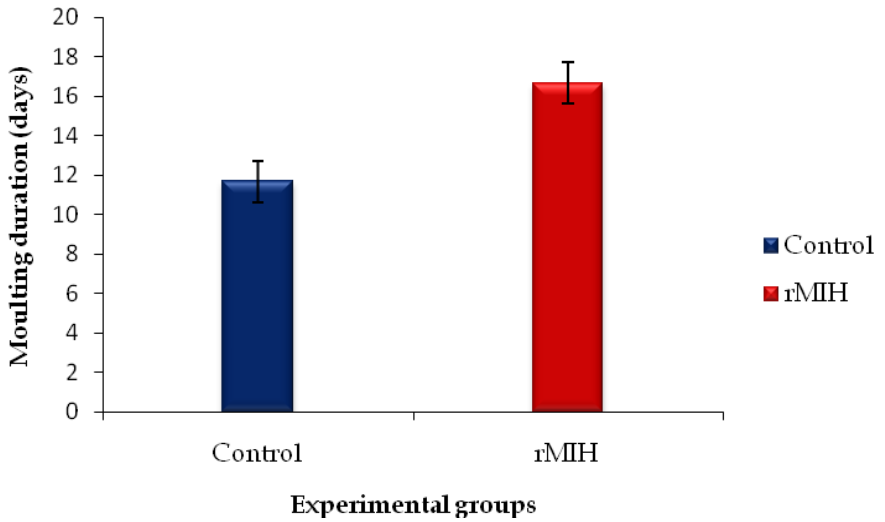


Fig.57. The moult cycle duration of rMIH comparison with control

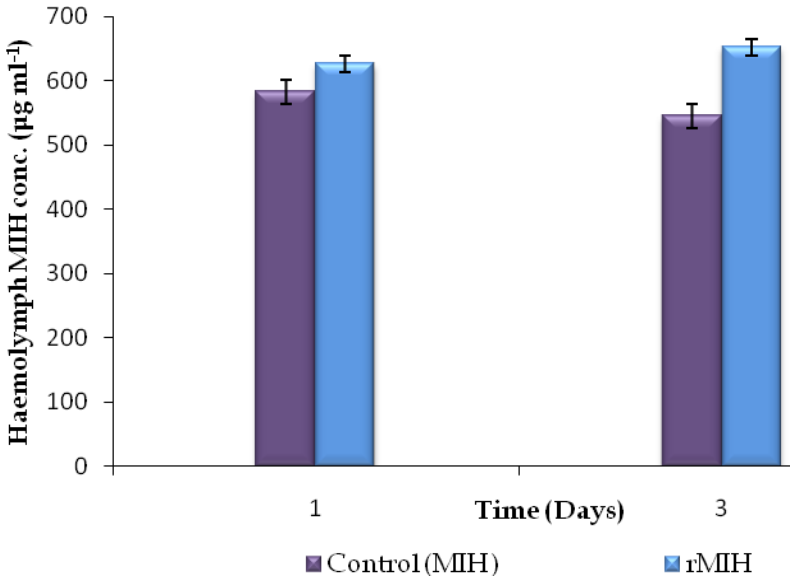


Fig. 58. Haemolymph MIH levels on application of rMIH in comparison with control

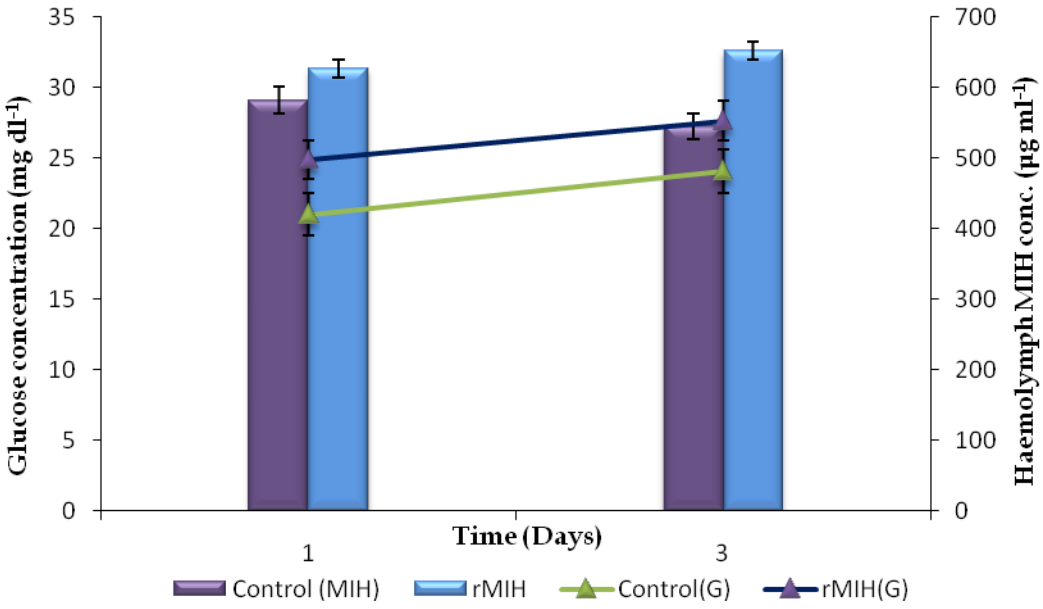


Fig.59. The glucose and haemolymph MIH level during the initial 24hrs on application of rMIH

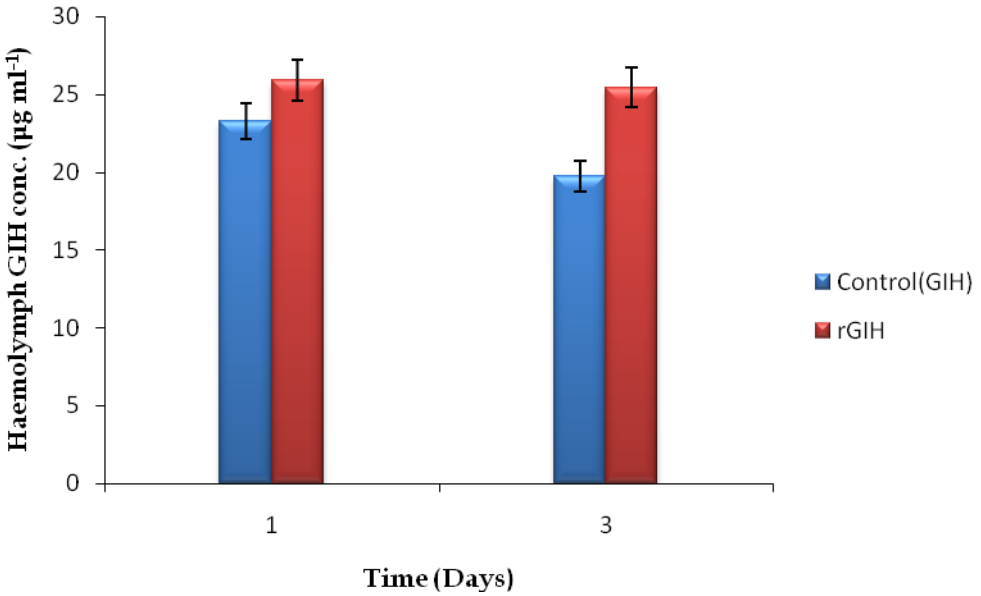


Fig.60. 24 hrs observation of haemolymph GIH concentration on application of rGIH *in vivo* in *P.monodon*

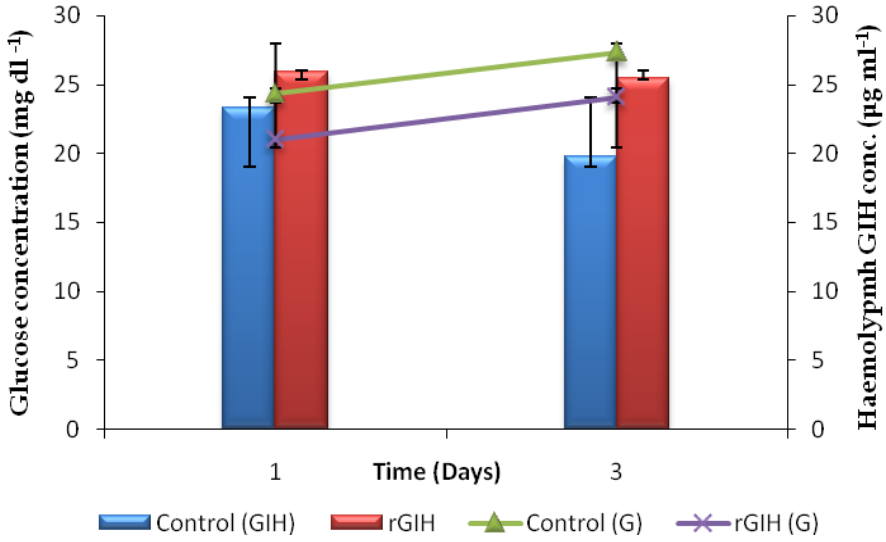


Fig.61. Haemolymph GIH and Glucose level on application of rGIH

Chapter

3

ANTAGONISTS (POLYCLONAL) AGAINST THE MATURE RECOMBINANT PROTEIN OF CHH FAMILY HORMONE GENES: *IN VIVO* BIOASSAY, IMMUNOLOCALIZATION AND ELISA DEVELOPMENT

3.1 Introduction

The most important tool for research in shrimp endocrinology is the availability of CHH family specific antibodies to study the expression of certain endocrine modulators in the reproductive/life cycle of the animals (Giulianini *et al*., 2002). In the review Huberman (2000) emphasized the basic endocrinological studies that can be applied in culturable crustacean species. All the recent studies in the field of crustacean endocrinology describe the production of recombinant hormones in both prokaryotic and eukaryotic system. The recombinant hormones produced in a prokaryotic system includes the *Penaeus japonicus* moult- inhibiting hormone (Ohira *et al*., 1999) and CHH of *Metapenaeus ensis* (Gu *et al*., 2000). Gu *et al*. (2001) had proved the functions of the CHH antibody raised against the recombinant CHH developed in bacteria.

Treerattrakool *et al*. (2006) developed antiCHH antibody of recombinant CHH and proved the functionality of the antibody by *in vivo* studies in *P.monodon*. Initially the only anti-CHH antibodies available were those against HPLC purified CHH

peptides from several decapods and also against synthetic oligopeptides (Gorgels-Kallen *et al*., 1982; Dircksen *et al*., 1988, Soyez *et al*., 1998; Sithigorngul *et al*., 1999). The unavailability and cost of commercialized CHH family specific antibodies lead us to the development of antagonists/antibody against the CHH family neurohormones (CHH1, MIH1 and GIH). CHH family recombinant proteins (rCHH1, rMIH1 and rGIH) detailed in Chapter 2 was used for the generation of polyclonal antibody in mice. The specific antibody generated can be used to study the expression levels of the three inhibitory hormones in the haemolymph during the life/reproductive stages of *P.monodon*. The antagonists developed can also be used to modulate growth and reproduction in *P. monodon*. This can be developed as a tool for post translational transgene silencing essential to replace the eyestalk ablation - a destructive way of maturation in shrimp. The antibody/antagonists developed, as described in the later part of the chapter, we hypothesize that an ELISA could be developed as a tool for assaying the levels of CHH family hormones in the haemolymph of shrimp during the various developmental stages of its life.

3.2 Materials and methods

3.2.1 Preparation of antigen (rCHH1, rMIH1 and rGIH)

The purification of recombinant proteins of CHH family hormone genes (rCHH1, rMIH1 and rGIH) through Ni-NTA affinity column, SDS-PAGE extraction, concentrating and refolding have been described in detail in Chapter 2.

3.2.2 Production of antiserum to mature recombinant protein of CHH family hormones in Female Balb/c mice

3.2.2.1 *Immunization of mice*

Eight week old female balb/c mice (5-6 no.s) were immunized with 50-100 µg of purified recombinant proteins. The process was as follows: the purified recombinant protein was diluted in 250 µl PBS to get a concentration of 100 µg. Four groups of mice (6 no.s) were injected: (a) rCHH1, (b) rMIH1 (c) rGIH and (d) PBS.

The schedule of immunization is as given below:

Day	Volume of antigen in PBS	Adjuvant	Volume of adjuvant	Route of injection
0	100 µg	Complete Freunds adjuvant	250µl	IP
15	100 µg	Incomplete Freunds adjuvant	250µl	IP
25	100 µg	-	100µl	IP

3.2.2.2 ***Mode of injection***

Intraperitoneal injection: The mouse was held by grasping the loose skin over the shoulders with thumb and forefinger, and holding the base of the tail with little finger projecting belly upwards. The site of injection was disinfected with 70% alcohol. The needle was inserted to a depth of approximately 5mm (intraperitonial), to one side of the middle or on both sides. The inoculum was injected slowly and with a brief pause needle was withdrawn, to allow the inoculum to disseminate. The area of injection was disinfected with 70% alcohol and subsequently Neosporin (antibiotic) was applied.

3.2.2.3 ***Checking the titer***

1) The mice were bled on day 27 from the tail. The tail was disinfected with 70% alcohol and tail end cut with a scalpel blade. The blood (4-5µl) was collected to an MCT. Ears were clipped to distinguish the animals in the same cage,.

2) The blood was allowed to clot at room temperature and the serum collected.

3) Antibody titre check by immunodot and ELISA.

4) The mouse with the highest titre ratio of serum titre (>1:1000) was sacrificed and blood was collected and serum separated for further experiments.

3.2.3 Cross reactivity of the antiserum of CHH1, MIH1 and GIH by ELISA

The antigen (0.1ml of 20 µg- rCHH1, rMIH1 and rGIH) was coated on to wells of ELISA plate. The plates were incubated at 4 °C overnight. The antigen was discarded; the plate was washed once with PBST (phosphate buffered saline pH 7.2 with Tween 20 (0.01 %). The free sites were blocked by coating the plate with BSA (3 % in PBS) for 1 hr. BSA was poured off and the plate was washed 3 times with PBST. An aliquot of 0.1ml mouse antiserum to recombinant CHH hormones (anti-rCHH1, anti-rMIH1 and anti-rGIH) was added and incubated for 1hr. The antiserum was discarded and the plate washed thrice with PBST. An aliquot of 0.1ml of conjugated rabbit anti mouse IgG – HRP (1:2000 dilution) in 3 % BSA prepared in PBS was added and incubated for 1 hr. The plate was washed thrice with PBST. An aliquot of 0.1ml TMB substrate (3, 3', 5, 5'-tetramethylbenzidine base, Sigma USA) was added and incubated for 10-30 min. The development of blue colour was observed and the reaction stopped by adding 50 µl of 1M H_2SO_4. The yellow colour was measured at Abs_{450} nm using ELISA reader (Tecan Infinite M200). The Abs_{450} measured was compared with the positive (control antiserum) and negative controls (PBS).

3.2.4 Immunodot assay to confirm the titre of the polyclonal antisera

Nitrocellulose membrane was cut approximately to the size of the well of a microplate. Membrane was placed into each well carefully without any damage. Duplicates were kept for each dilution. The plate was overturned to check whether the membranes were intact in the well. The antigen (rCHH1, rMIH1 and rGIH) was dotted on to the membrane using 2.5 µl microtip. The membrane pieces in the wells were allowed to dry at room temperature for 5-10 min and blocked with 3 % BSA in PBS (volume added-200 µl). The plate was incubated for 1.5-2 hrs at room temperature with shaking at low speed. The BSA was removed and the wells were washed with PBS (200 µl) for 2-3 min. The PBS was removed and the plate dried by tapping on blotting

paper. Different dilutions of test antiserum was prepared by double dilution in another plate (1:200,1:400,1:800,1:1600,1:3200).

PBS alone and serum from unimmunized mouse were used as controls. The dilutions were added to the respective wells and the plate was incubated overnight on a shaker with low speed (50-70 rpm). The plate was washed twice with PBST and once with PBS. The secondary antibody (rabbit antimouse IgG-HRP; 1:1000 dilution) diluted in 3 % BSA/ PBS (150 µl) was added to each well and incubated for 1.5-2 hrs. The plate was washed twice with PBST and once with PBS. An aliquot of 200 µl of the substrate, 4-chloro-naphthol with H_2O_2 was added. Purple blue colour development was observed for 10min.

3.2.5 Western blot: Specificity of the antisera (anti-rCHH1, anti-rMIH1 and anti-rGIH)

Western blot was carried out in ECL semidry blotter (Amersham Biosciences, USA). The antigens (10 µg of rCHH1, rMIH1 and rGIH) were electrophoresed in 12 % SDS-PAGE at 12V (described in chapter 2 section 2.2.8.8).The stacking gel section was incised and the resolving gel was equilibrated with transfer buffer. Prior to the blotting step, the blotting paper and nitrocellulose membrane were equilibrated in transfer buffer. Three layers of transfer buffer saturated blotting paper were stacked; the air bubbles formed were removed by rolling a glass pipette over the stack. The nitrocellulose membrane was placed over the blotting paper. The gel was placed carefully over the nitrocellulose membrane without any air bubble. The gel was covered with 3 layers of saturated blotting paper. The stack was placed in the ECL semidry blotter (Amersham Biosciences, USA) and blotted at 65mA for 1.5-2 hrs.

The nitrocellulose membrane was stained with Ponceau for 5-10 min to ensure the complete transfer of protein on the membrane. The membrane was given a series of wash with PBS to remove the stain. The nitrocellulose membrane

was blocked with 5% skimmed milk in PBST (PBS + Tween 20 (0.1%)) for 1.5hrs. The membrane was washed with PBST twice with gentle agitation. Incubated overnight at 4°C with antisera raised against the CHH family recombinant proteins (anti-rCHH1, anti-rMIH1 and anti-rGIH) diluted (1:25,000) in 5 % skimmed milk-PBST. The membrane was washed with PBST thrice (5-10 min). The membrane was incubated in the secondary antibody, antimouse IgG HRP conjugated (1:1000 dilution) in 5% skimmed milk-PBST for 1-1.5 hrs with gentle agitation at room temperature. The membrane was washed twice with PBST with a time interval of 5min. An aliquot of 4-chloronaphthol in 30 % H_2O_2 was added to the membrane and incubated for 10-30 min. The purple colour development was observed and the membrane was washed in distilled water to stop the reaction.

3.2.6 Immunolocalization of CHH family hormones in eyestalk

The eyestalks were cut from live anaesthetized shrimps (By dipping in ice flakes) and fixed in Davidson's fixative for 24 hrs. The exoskeleton of the eyestalk was removed before dehydration and tissue embedded in paraffin using the conventional methods. Consecutively 5 µm sections were mounted onto the slide and dried at room temperature overnight. The tissue sections were dewaxed in xylene (twice for 10min) and dehydrated through series of ethanol dilutions (100 %, 95 % and 70 %). The sections were blocked with BSA (3% in PBS) for 1 hr in humified chamber. After blocking the antiserum of anti-rCHH1, anti-rMIH1 and anti-rGIH (1: 2000 dilutions in 3 % BSA-PBS) was added and incubated overnight at 4 °C. The secondary antibody, conjugate of anti mouse IgG –FITC (Sigma, USA) (1:40 dilution) in 3% BSA prepared in PBS was added and incubated for 1 hr in dark in humified chamber. Finally the slides were stained with nuclear stain DAPI (10 µl, 0.02 µg ml^{-1}), incubated for 3 min. The slides were rinsed with distilled water, air dried and mounted with mounting media (Vectashield, USA) and observed in a UV fluorescence microscope (Olympus,

Germany). DAPI and FITC were viewed under different filters with excitation wavelength 360-370 nm and 470-490 nm, respectively. The images were processed and merged using the "Imagepro – express" software (Media Cybernetics Inc, MD, USA). Between each step, the slides were washed twice for 5 min with PBST (PBS + 0.01% Tween 20).

3.2.7 Development of ELISA for determining the haemolymph concentration of CHH family hormones (CHH1, MIH1 and GIH) in an unknown sample

3.2.7.1 *Titre of antisera and precision of the test*

Different concentrations of recombinant protein of CHH1, MIH1 and GIH (antigen) ranging from 0.00005 to 50 ng ml^{-1} (100 µl) were used to coat the 96 well micro titer plates. The protocol followed was the same as in section (3.2.3) of ELISA with the exception that the dilution of primary antiserum used for the experiment was1:50,000.

3.2.7.2 *Standard curve and analyses of data*

The standard curve was plotted with concentration of rCHH1, rMIH1 and rGIH on X-axis and absorbance of recombinant protein of CHH1, MIH1 and GIH on Y-axis. The linear region of the graph was re-plotted and linear regression of the data was performed. The regression equations obtained were used for estimating the concentration of CHH1, MIH1 and GIH in an unknown haemolymph serum samples.

3.2.8 Application of antagonists of recombinant CHH1, MIH1 and GIH in *P.monodon*

3.2.8.1 *In vivo bioassay of antagonist of recombinant CHH1 (anti-rCHH) protein in P.monodon*

P.monodon (10-15g) obtained from local hatchery were stocked and maintained in recirculating aquaculture system (RAS) for shrimp integrated with nitrifying bioreactor (Kumar *et al*., 2009, 2011) in sea water having 15g l^{-1} salinity. Water quality was maintained within a narrow range (pH 6.8-7.8; total ammonia < 0.1

mg l^{-1}; nitrite < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness >5000-6000 mg l^{-1}). The animals were fed with commercially available pelleted feed (Higashimaru, India) containing 40 % protein, 3 % fat, 12 % fiber, 18 % ash and 12 % moisture.

The animals were starved for 18 hrs before the injection of antagonist of rCHH1 protein. Prior to injection 25 µl of haemolymph was removed. The antiserum to rCHH1 having a dilution of 1:500 (in PBS) was prepared prior to injection. An aliquot of 100 µl of the diluted antiserum was injected into the pericardial cavity. The negative control was injected with 100 µl of PBS. The haemolymph glucose levels were measured with glucose oxidase kit (Biolab Diagnostics (I) Pvt. Ltd, India). The haemolymph was collected at 0.5 , 1.0 , 1.5 and 2 hrs post injection and stored at -80 °C until further processing. The haemolymph samples were centrifuged at 10,000 xg for 5 min at 4 °C. An aliquot of 5 µl of haemolymph was combined with 195 µl of glucose buffer and incubated for 15 min at 37 °C. The glucose level was determined by measuring the absorbance at 500 nm and deducing the concentration from a standard graph.

3.2.8.2 *In vivo bioassay of antagonist of recombinant MIH1 (anti-rMIH) protein in P.monodon*

The animals in the stage C of moulting were selected and randomly divided into two groups viz., control (control serum) and anti-rMIH group. Prior to injection 25 µl of haemolymph was removed for baseline analysis. The antiserum to rMIH1was diluted in PBS (1:500). Control serum was also diluted in the same pattern (1:500). An aliquot of 100 µl antiserum (1:500 dilution) was injected into the pericardial cavity of shrimp. For *in vivo* bioassay the animals were injected every alternate day (1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th} and 13^{th} day) with the anti-rMIH antibody and the moulting interval was observed. The haemolyph was collected 24hrs post injection for two days to measure the level of MIH. MIH concentration in the haemolymph was measured by ELISA using anti-rMIH antiserum. For rest of the injections,

haemolymph was not collected and the moulting stages were observed. The determination of moult stages was based on the degree of setae development according to the method described by Chan *et al*. (1988), with modifications. Uropods were observed instead of pleopod (Chan *et al*., 1988). The moult stage determination with uropods gave clear and consistent images in the animals (Cesar *et al*., 2006; Promwikorn *et al*., 2004). The uropod morphological pictures were taken with Olympus camera (FE 270, UK) under a light microscope (10X) and were analyzed accordingly.

3.2.8.3 *In vivo bioassay of antagonist of recombinant GIH (anti-rGIH) protein in P.monodon*

There are no physiological aspects like that of anti-rCHH1 (glucose level) and anti-rMIH (moult stages) to assess the effect of anti-rGIH in the animal. Thus the experimental design was set only to know the effect of anti-rGIH in the animals by measuring the level of GIH in haemolymph by ELISA using the anti-rGIH antibody raised against the rGIH protein. The injection experimental set up was similar to that followed in the anti-rMIH. The antiserum to rGIH was diluted in PBS (1:500).. Control serum was also diluted in the same pattern (1:500). An aliquot of 100µl of the diluted antiserum (1:500) was injected into the pericardial cavity. The animals in the D2-D3 stage were injected with anti-rGIH on 1st, 3rd, and 5th day. The GIH levels in the haemolymph were measured at intervals of 2nd and 4th day post injection.

3.3 Results

3.3.1 Specificity of the polyclonal antisera developed against the mature recombinant protein of CHH family hormones

The purified fusion/recombinant proteins- rCHH1, rMIH1 and rGIH were used to raise polyclonal antisera in mice. The titre value of the polyclonal antisera obtained for rCHH1 was 2, 04, 800, rMIH1 (1, 02, 400) and rGIH (1: 4, 09, 600). . The specificity of the antisera was tested by Western blot analysis: the anti-rCHH1, anti-rMIH1 and anti-rGIH antibodies recognized the antigen rCHH1 (Fig.

1), rMIH1 (Fig. 2) and rGIH (Fig. 3). The Western blot results confirmed the detection of purified recombinant

protein and the same in the supernatant of lysis 2 extracts of the induced *E.coli* BL21 (DE3) pLySs. There was no cross reactivity with other structurally related protein (Fig: 4). The sensitivity of anti-rCHH1, anti-rMIH1 and anti-rGIH was determined by indirect ELISA (Fig. 5, 6 and 7) and dot blot analysis (Fig. 8). A quantity of 0.00005 ng ml $^{-1}$ to 50 ng ml $^{-1}$ of rCHH1, rMIH1 and rGIH could be detected by 1:50,000 dilutions of the antisera from mice. The absorbance of hydrolyzed substrate was linearly proportional to logarithmic concentration of antiserum.

3.3.2 Circulating CHH1, MIH1 and GIH in the haemolymph

In the cross reactivity assay, the haemolymph was substituted for the recombinant antigen; the reduction in the absorbance at 450 nm reflected the circulating neuropeptide in the haemolymph. The female shrimps in the premoult stages (D0-D4) were selected. The variation in the levels of haemolymph CHH1, MIH1 and GIH were observed. During the D0-D4 stage, the level of CHH in the haemolymph was observed to increase in the D0 stage and a gradual decrease from D1-D4 (Fig. 9). In case of MIH1, a dip during early stage of premoult (D0-D2) and slow rise towards the late premoult (D3-D4) was observed (Fig. 10). It was noted that the haemolymph GIH level remained steady with fewer fluctuations as per the stages (Fig. 11).

3.3.3 Localization of CHH, MIH and GIH in the eyestalk of *P.monodon*

The eyestalk sections were immunostained with anti-rCHH, anti-rMIH and anti-rGIH antiserum. The neurosecretory cells with immunoreactive property of CHH, MIH and GIH formed a cluster, mainly situated at the sides of the medulla terminalis ganglion. These are the sites related to the sinus gland and X-organ. Three types of immunopositive neurosecretory cells were visible that produced CHH, MIH

and GIH. The immunofluorescence revealed that CHH neurosecretory cells were most abundant among the three kinds of neurosecretory cells (Fig. 12). It was observed that the MIH immunofluorescence was localized peripherally in the neurosecretory cells (Fig. 13). The anti-rGIH antibody located the presence of GIH neurosecretory cells as scattered (Fig. 14). The immunofluorescence revealed very well the localization of the CHH family neurosecretory cells in the eyestalk in comparison with the control (Fig. 15).

3.3.4 *In vivo* bioassay of anti-rCHH1 antisera – hypoglycemic effect

The hypoglycemic effect of anti-rCHH1 antibody was observed by *in vivo* bioassay. Anti serum at 1:500 dilution containing anti-rCHH1 decreased the haemolymph glucose level 0.5hr post injection. The glucose level was observed to be decreased from 21.60 ±5.52 mg dl^{-1} (0.5hr) and continued to diminish and reached a minimum of 17.65 ± 3.49 mg dl^{-1} (1.5hrs). Also observed that by 2hrs the glucose level was 18.08 ± 1.23 assuming that normalcy could slowly being attained. The hypoglycemic effect of anti-rCHH1 antisera was compared to the controls injected with the serum of control mice (PBS injected), the glucose concentration was 38.91 ± 5.83 mg dl^{-1} (1.5hrs) showing a significant ($p < 0.05$) hyperglycaemic effect of CHH1. The decreases in haemolymph CHH levels were 71.43 % which supports the hypoglycemic effect of anti-rCHH1 antibody (Fig. 16).

3.3.5 *In vivo* bioassay of anti-rMIH1 antisera - Moult attenuation

Moulting of animals (10-15 g) was observed to take place at a duration of 11.67 ± 1.03 days under laboratory culture conditions. The C-stage animals were selected to get a better response of the injected MIH antisera. According to Chan *et al*. (1988), the C stage duration in *P.vannamei* consists of C1 (1.5 days -5 %), C2 (6-8 days- 20 %) and C3 (4-7 days- 15 %). Based on the data available we observed that the C- stage duration was reduced post injection of anti-rMIH antiserum. The shrimps injected with anti-rMIH antiserum (1: 500 dilutions) exhibited a moult duration that was significantly reduced to

8.33 ± 0.82 days ($p < 0.0001$). When compared with the control by Student T- test, the reduction in moulting duration of anti-rMIH was highly significant (Fig. 17). A tendency to move from C-stage to D0- stage was observed. The effect of anti-rMIH antibody injection was significant, in terms of MIH concentration in the haemolymph. The MIH concentration in the haemolymph showed a significant decrease (21 %, $p < 0.05$) in comparison with the control (Fig. 18).

3.3.6 *In vivo* bioassay of anti-rGIH antisera - decline of GIH

Assessing the effect of anti-rGIH in the animal there was no physiological aspects like that of anti-rCHH (glucose level) and anti-rMIH (moulting duration). Thus a 24 hrs observation of haemolymph GIH was assessed 1st and 3rd day post injection of a GIH antisera. A significant decrease of 32.94 % ($p < 0.05$) was observed in the haemolymph GIH level (Fig. 19) by ELISA.

3.4 Discussion

3.4.1 Specificity, sensitivity and functionality of the polyclonal antibodies of CHH family hormone genes

Immunology and *in vivo* bioassay studies have established the interspecific variability of the eyestalk neuropeptides in crustaceans (Gorgels- Kallen *et al*., 1982; Leuven *et al*., 1982; Dircksen *et al*., 1988; Marco and Gäde 1999). Since the advancement of molecular tools, the amino acid sequence alignment has become the new choice for this kind of studies (Lacombe *et al*., 1999). With all the advanced molecular tools, the fundamental method adopted still, is the use of specific antibodies for the expression studies of neuropeptides during the various morphological and physiological stages of development. Antibodies are tools with which quantification of haemolymph CHH levels by means of ELISA were premeditated in reproductive biology (de Kleijn *et al*., 1998) and also in the environmental stress studies (Lorenzon *et al*., 1997, 2000; Chang *et al*., 1998; Bergmann *et al*., 2001). The antibodies were generally raised against the CHH neurohormones that were chromatographically purified peptide fractions (Gorgels-

Kallen and van Herp, 1981; Gorgels- Kallen *et al*., 1982; Dircksen *et al*., 1988), synthetic peptides corresponding to N-terminal sequence (Soyez *et al*., 1998; Ollivaux and Soyez, 2000). Gu *et al*. (2001) initially reported the functionality of anti-recombinant CHH of *Metapenaeus ensis* and the corresponding counts of immunopositive neurons in the eyestalk. It was demonstrated with the studies of Gu *et al*. (2000, 2001) that recombinant crustacean hormones expressed in bacteria are biologically active and suitable for the production of specific antibodies. We chose to generate the specific antibodies against the recombinant protein of CHH1, MIH1 and GIH which were generated from the cDNA of eyestalk. This method is more specific and straightforward to raise an antiserum against the three hormones (CHH, MIH and GIH) of *P.monodon*. The three hormone genes (CHH1, MIH1 and GIH) were cloned in pET32a+ vector to produce fusion protein (rCHH1, rMIH1 and rGIH); the purified protein was used to raise the antiserum in mice. CHH1, MIH1 and GIH sequences have been clearly identified and the protein generated made easily reproducible for standardized hormone quantification studies by applying immunological techniques. The method of chromatographically purified eyestalk neuropeptides requires hundreds of eyestalks and the presence of different isoforms as well as the post translational modifications of the proteins makes the purity and conformation of the protein questionable (Giulianini *et al*., 2002). Furthermore, the method of producing recombinant protein from a specific cDNA sequence makes the generation of specific proteins highly reproducible and the antibodies developed against them to be highly specific for immunological intervention. The hormone specificity was tested by Western blot; antibodies of CHH1, MIH1 and GIH were able to detect the purified and unpurified protein bands specific to rCHH1 (29.47 kDa), rMIH1 (29.85 kDa) and rGIH (32.16 kDa). The sensitivity was proven with 1:50,000 dilutions of the antisera (anti-rCHH1, anti-rMIH1 and anti-rGIH) that could detect 0.05 pg ml^{-1} quantities of the antigens. The cross- reactivity of the antibodies to detect the haemolymph CHH, MIH and GIH level was proven by

replacing the recombinant antigens with that of shrimp haemolymph at various stages of premoult (D0- D4).

The functionality of the antibodies of CHH family hormones could be established by the cross-reactivity assay, the anti-rCHH1, anti-rMIH1 and anti-rGIH could detect the variations in the haemolymph CHH, MIH and GIH level during the different premoult stages (D0-D4). The haemolymph CHH was observed to increase (D0) with a gradual decrease from D1-D4. This result complies with the outcome of glucose level studies in *P.vannamei* (Chan *et al*., 1988). The glucose level shows a gradual decrease from D1-D4 stages of premoult condition in *P. vannamei*. According to Okumura *et al*. (1989), the haemolymph ecdysteroid levels start to increase at the early premoult stage and reduce towards the late premoult. Complying with this, the haemolymph MIH showed a dip during early stage of premoult (D0-D2) and slow rise towards the late premoult (D3-D4). Chan *et al*. (1988) established the similar results in *P. vannamei*, the ecdysteroid titers were low during the moult stages of A- C and rise was shown during the premoult stage D0-D1, a sudden dip from D2-D4. The haemolymph GIH level remained steady with fewer fluctuations as per the stages and no reports were available so far on the level of GIH at different moult stages to compare with.

The antisera of the three hormones of CHH family could specifically recognize the corresponding antigen (0.00005ng ml^{-1} - 50 ng ml^{-1}). When compared, the normal circulating level of neurohormones is much lower. Webster (1996) detected the circulating CHH in crab *C.pagister* as 150-250 pg ml^{-1} using radioimmunoassay (RIA). The sensitivity of RIA is in a range of 0.125 pg ml^{-1}- 0.5 ng ml^{-1}. The circulating CHH hormone level in lobster *H. americanus* was 33.6 pg ml^{-1}; which was detected by double antibody - sandwich-type ELISA. Chang *et al*. (1998) reported that even under stressed conditions like emersion where the lobsters were placed in 2L jars without water and placed in incubator at ambient temperature for 4hrs; the CHH level was only raised up to by 1.4 ng ml^{-1}. Our approach establishes the

reproducibility of the specific recombinant CHH family hormones, and the antibodies developed against these hormones to investigate more precisely the role of specific hormones at various stages of development of *P.monodon*.

3.4.2 Immunolocalization of the CHH family hormone neurosecretory cells in the eyestalk of *P.monodon*

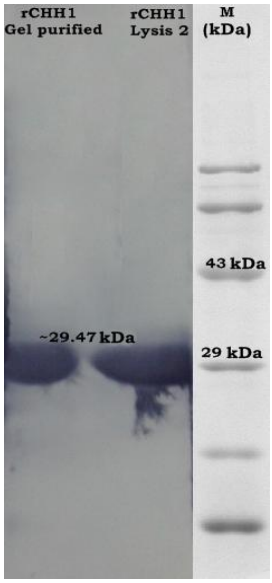
Distinct neurosecretory cells were shown to produce the three hormones of the CHH family in the X-organ and sinus gland. The X-organ-sinus gland complex is the major site located at the medulla terminalis region. The observations we attained with immunofluorescence agreed with the results obtained from the crab, *Carcinus maenas* (Dircksen *et al*., 1988; Klein *et al*., 1993) *Penaeus japonicus* (Shih *et al*., 1998), *Metapenaeus ensis* (Gu *et al*., 2001) and *Nephrops norvegicus* (Edomi *et al*., 2001). The data revealed by these authors showed that neurosecretory cells producing CHH-family peptides were localized in the MTGX (medulla terminalis ganglion X organ). The immunofluorescence showed three kinds of immune-positive neurosecretory cells, which were stained by the three antibodies raised against the rCHH, rMIH and rGIH. It could be observed that in the same cluster, co-localization of two neuropeptides existed. Co-localization of the two neuropeptides has been reported in the *Homarus gammarus* (Rotllant *et al*., 1993) and *Homarus americanus* (De Kleijn *et al*., 1992) by immunohistochemistry and in situ hybridization. Co-localization of MIH and CHH was not reported in *C. maenas* (Klein *et al*., 1993a). These differences in the neurohormone secretory pathway in the XO-SG complex might be species specific or there might exist a possibility of functional divergence of neuropeptides (Gu *et al*., 2001). Thus in *P.monodon*, revealed by our study, there existed two possibilities, either the CHH, MIH and GIH peptides co-existed in some cells, or the antisera (polyclonal) could also recognize other peptides that had not yet been characterized.

3.4.3 Biological activity of anti-rCHH, anti-rMIH and anti-rGIH antisera in *P.monodon*

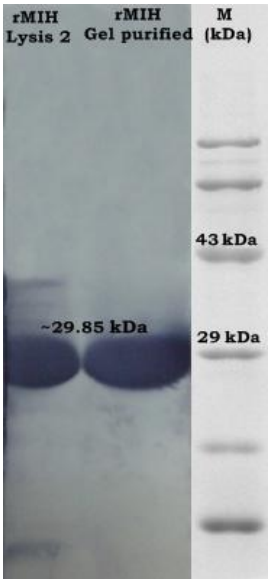
The biological activity (functional aspect) of antibodies against CHH family hormones were observed by *in vivo* administration in *P.monodon*. *In vivo* administration of anti-rCHH was able to reduce the glucose level bringing about hypoglycemic effect, anti-rMIH enhanced the moulting interval and anti-rGIH showed a decrease in haemolymph GIH level. This positively indicated that the antibodies raised in mice against recombinant proteins of CHH family hormones possessed the neurohormonal functions. The *in vivo* injection of 1:500 dilutions of anti-rCHH antisera produced hypoglycaemic effect by 50 % glucose level and 94.76 % haemolymph CHH level, 1.5 hr post administration. The result complied with the research finding of Treerattrakool *et al*. (2006); 30-50% reduction in hyperglycaemic activity was significantly observed from the basal activity of PBS-injected shrimp. This inhibitory effect demonstrated that the antibody could recognize the hormone and inhibit the hormone action *in vivo*.

Anti-rMIH anti-serum (1:500) dilutions possessed the neurohormonal function as the moulting duration was reduced by 28 %, while the haemolymph MIH level was reduced by 21 % upon 24 hrs of administration. Gu *et al*. (2001) had developed the antibody against the recombinant MIH of *M.ensis*, but the *in vivo* study on application of anti-rMIH was lacking. We report here the first studies of the action of anti-rMIH antibody in functioning as a neurohormone in *P.monodon.* The anti-rGIH antiserum (1: 500 dilutions) *in vivo* resulted in 32.94 % reduction in haemolymph GIH levels 24 hrs post administration, a study carried out first time.

In conclusion, the results obtained reveal the specificity and sensitivity of the antibodies (antisera) of the recombinant CHH family peptides. Antiserum specifically recognized the native neurohormones by immunodetection. With the recombinant DNA approach, it is relatively easy to obtain the protein and the antibody for detection and bioassay of hormones. The antibodies developed can be used for future studies to identify the receptors of CHH, MIH and GIH and also to investigate the signal transduction pathway of the CHH neuropeptide family.



(Fig. 1)



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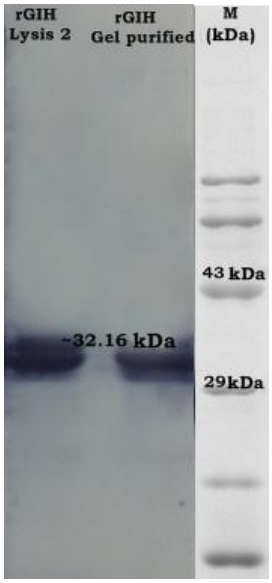


(Fig. 3)

(Fig. 4)

Fig. 1. Western blot of anti-rCHH antiserum detects rCHH1 (29.47 kDa)
Fig. 2. Western blot of anti-rMIH antiserum detects rMIH1 (29.85 kDa)
Fig. 3. Western blot of anti- rGIH antiserum detects rGIH (32.16 kDa)
Fig. 4. Western blot using control serum for detection of rCHH1, rMIH1 and rGIH

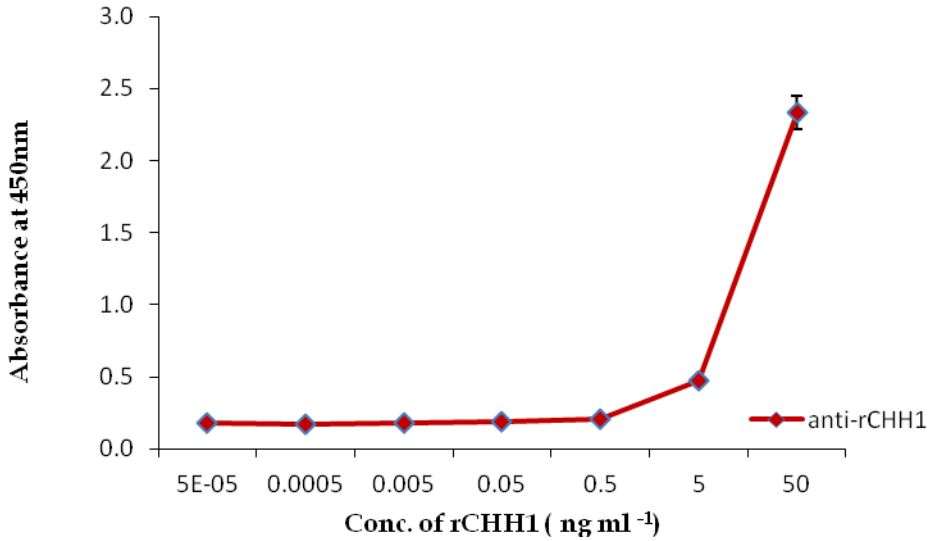


Fig. 5. Indirect ELISA of anti-rCHH1 to detect rCHH1 at various concentrations (ng ml $^{-1}$)

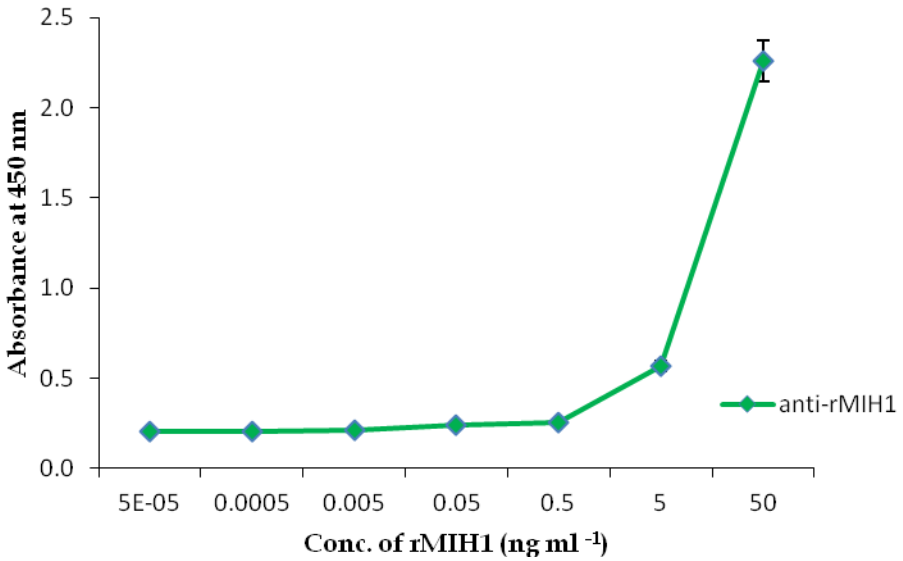


Fig. 6. Indirect ELISA of anti-rMIH1 to detect rMIH1 at various concentrations (ng ml $^{-1}$)

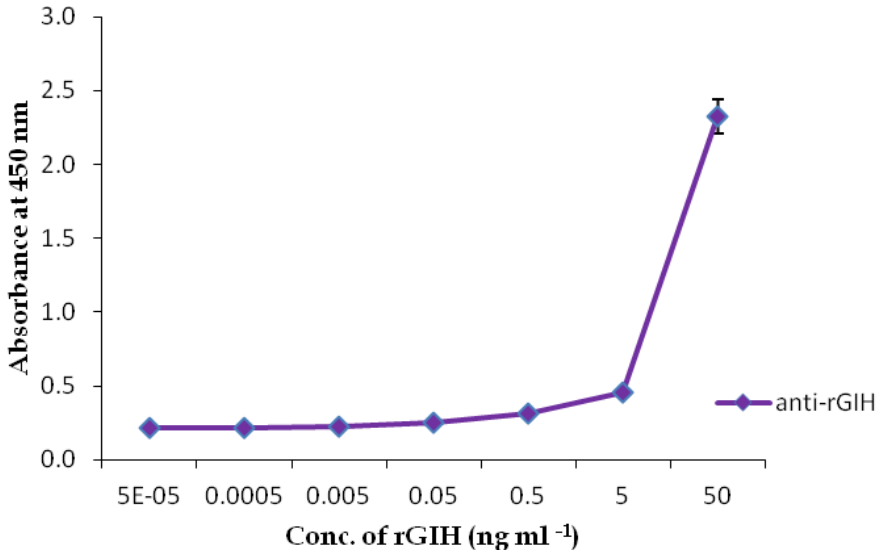


Fig.7. Indirect ELISA of anti-rGIH1 to detect rGIH1 at various concentrations (ng ml $^{-1}$)

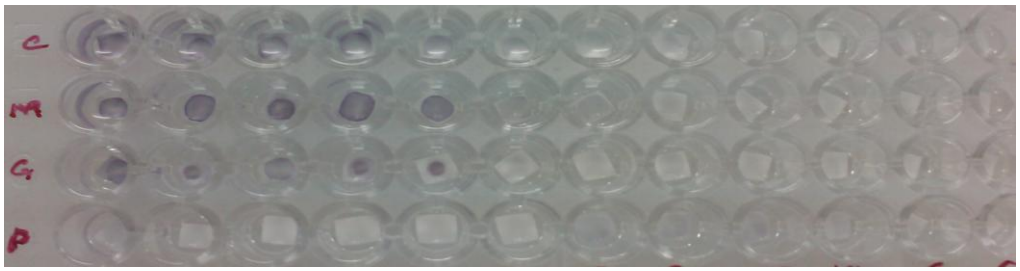


Fig. 8. Dot blot analysis of rCHH1, rMIH1, and PBS rGIH at concentration 0.00005 to 50 ng ml^{-1} **used for indirect ELISA**

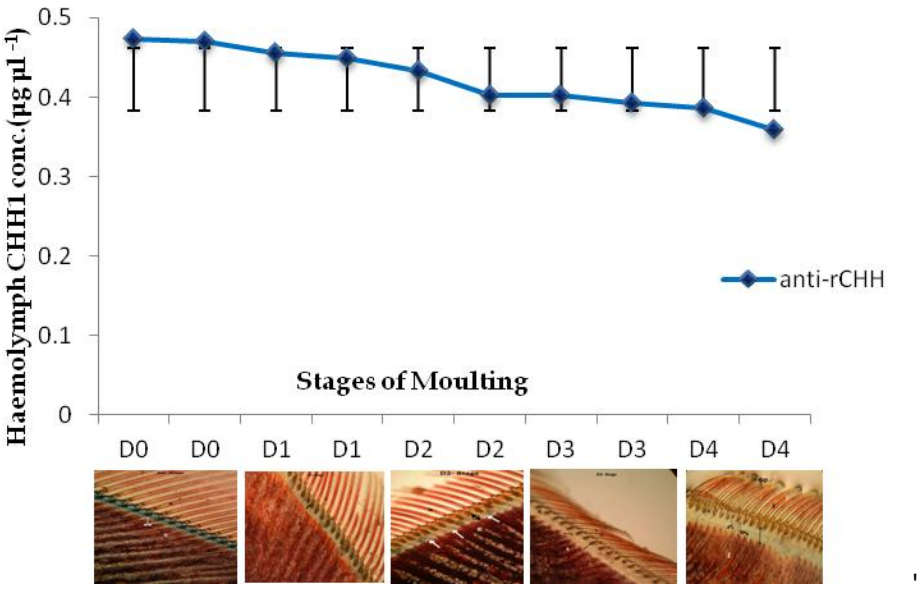


Fig. 9. Relationship of circulating haemolymph CHH levels of female shrimp to various premoult stages (D0-D4)

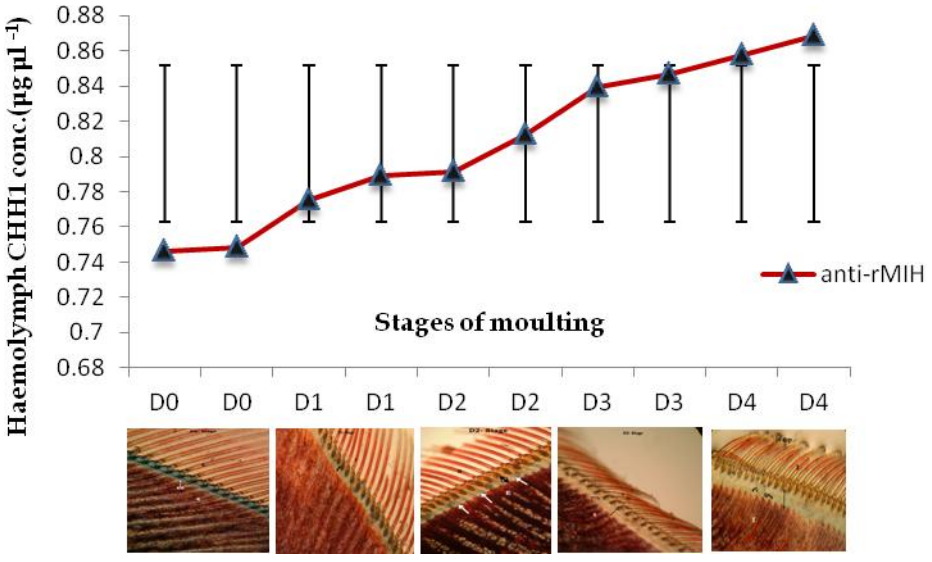


Fig. 10. Relationship of circulating haemolymph MIH levels of female shrimp to various premoult stages (D0- D4)

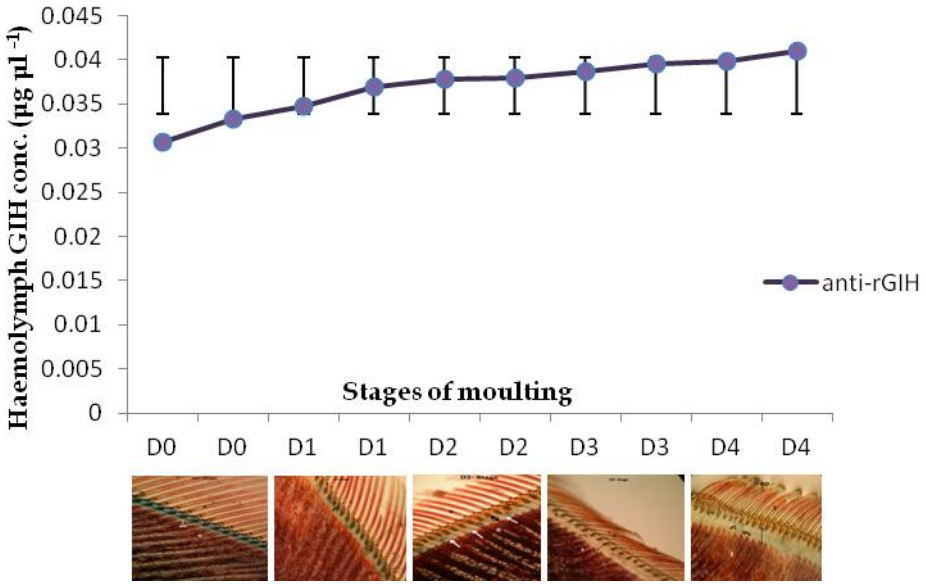
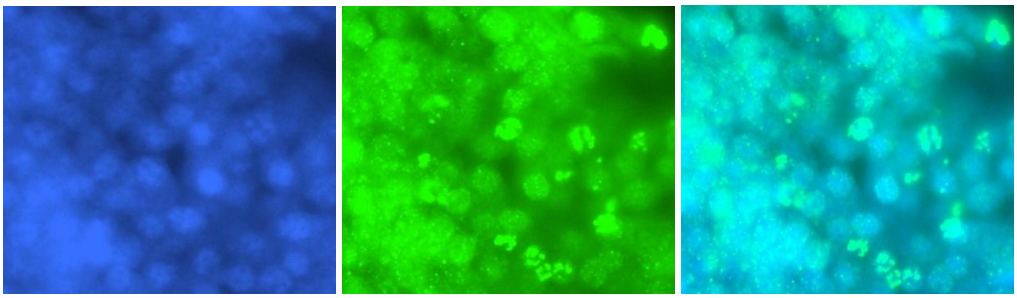
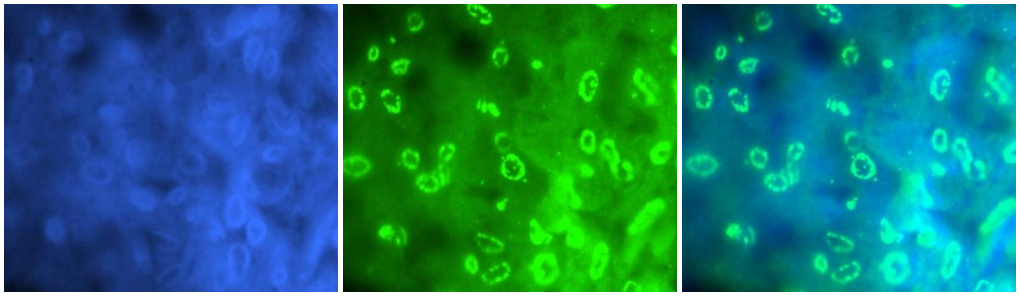


Fig. 11. Relationship of circulating haemolymph GIH levels of female shrimp to various premoult stages (D0-D4)



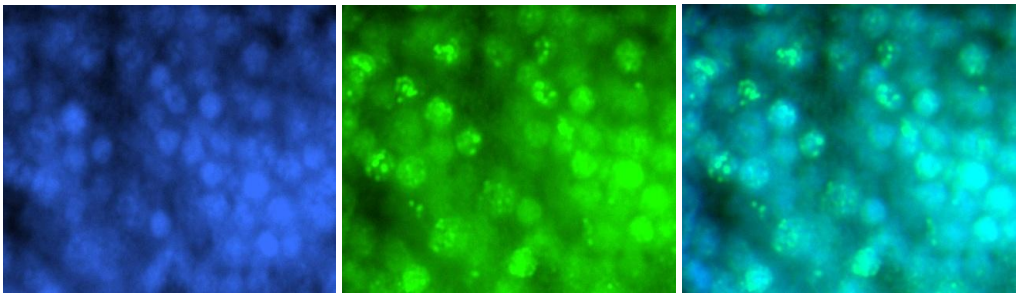
DAPI **FITC** **Merged**

Fig.12. Immunofluorescence of CHH neurosecretory cells in eyestalk using anti-rCHH1



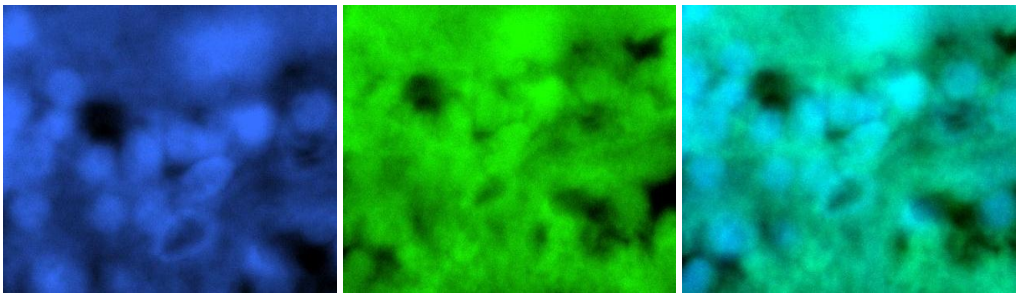
DAPI **FITC** **Merged**

Fig.13. Immunofluorescence of MIH neurosecretory cells in eyestalk using anti-rMIH



DAPI **FITC** **Merged**

Fig.14. Immunofluorescence of GIH neurosecretory cells in eyestalk using anti-rGIH



DAPI **FITC** **Merged**

Fig.15. Immunofluorescence of neurosecretory cells in eyestalk using control serum

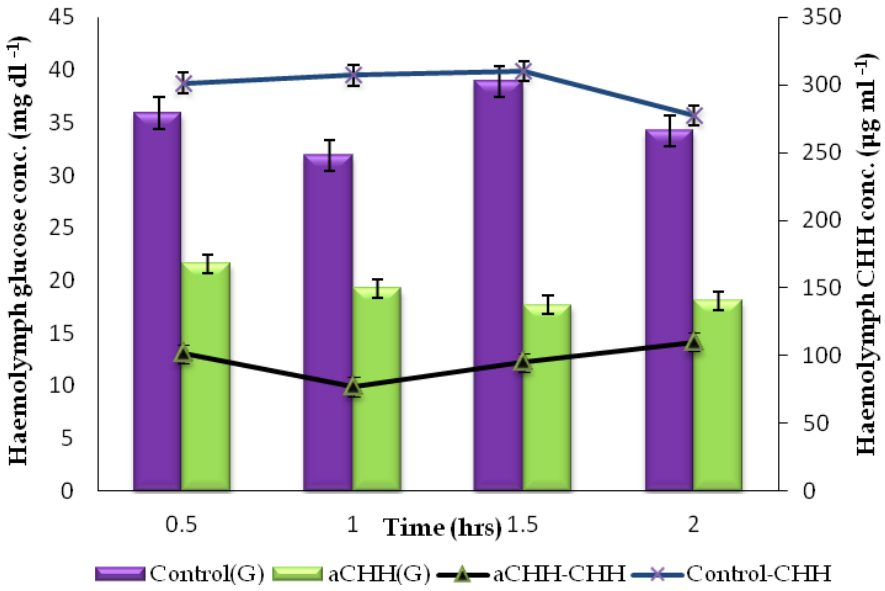


Fig.16. Haemolymph glucose and CHH levels on application of anti-rCHH antiserum (aCHH) in *P.monodon*

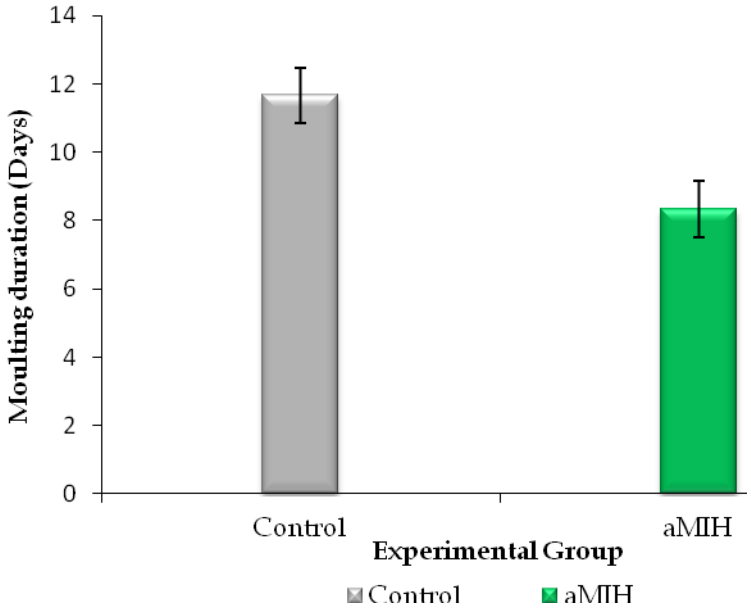


Fig.17. Moulting duration on administration of anti-rMIH antiserum (aMIH) in comparison with control

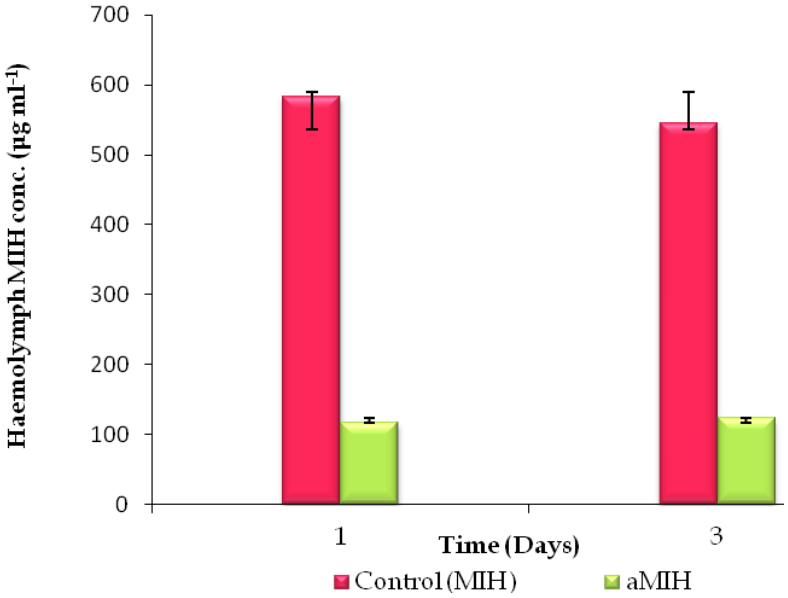


Fig.18. Haemolymph MIH level on application of anti-rMIH antiserum (aMIH) in *P.monodon*

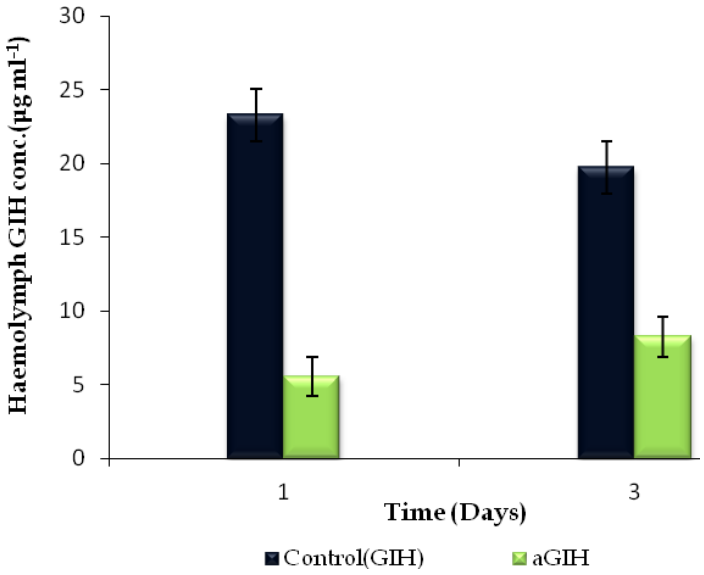


Fig.19. Haemolymph GIH level on application of anti-rGIH antiserum (aGIH) in *P.monodon*

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Chapter

4

IN VITRO GENERATED DOUBLE-STRANDED RNA MEDIATED EYESTALK NEUROPEPTIDE (CHH1, MIH1 AND GIH) GENE KNOCKDOWN IN THE SHRIMP *PENAEUS MONODON*

4.1 Introduction

Physiology of crustaceans is partly regulated by diverse neuropeptides synthesized by medulla terminalis of X-organ sinus gland complex situated in the eye stalk. The neuropeptide family includes hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), gonad- inhibiting hormone (GIH) also called vitellogenesis - inhibiting hormone (VIH) and mandibular organ- inhibiting hormone (MOIH). This unique peptide family in crustaceans is involved in blood sugar regulation, inhibition of ecdysteroid synthesis, regulation of reproduction and growth (Cooke and Sullivan, 1982; Beltz, 1988; Keller, 1992; Fingerman, 1992; Chang, 1993). CHH1/MIH1/GIH family, precisely named as CMG family hormones, is multifunctional despite their similarities in structure (Keller, 1992; Sun, 1994; Chang, 1997; Lacombe *et al*., 1999; Bocking *et al*., 2002).

CHH is involved mainly in elevating the glucose level in haemolymph by glycogen degradation in hepatopancreas (Mettulio *et al*., 2004). However, its function has been demonstrated to be pleiotropic (Chen *et al*., 2004) playing remarkable role in

reproduction (DeKleijn and Van Herp, 1998), moulting (DeKleijn and Van Herp, 1995), digestion (Spanings *et al*., 2000) osmoregulation (Charmantier *et al*., 1994; Serrano *et al*., 2002) and lipid metabolism in various species (Santos *et al*., 1997). MIH controls moulting by inhibiting the synthesis of ecdysteroids by Y-organ, the process essential for the development and maturity of decapod crustaceans (Lachaise *et al*., 1993; Gu *et al*., 2002). GIH controls the gonad maturation playing a highly complex role in reproduction and moulting (Soyez *et al*., 1991).

Traditionally, shrimp maturation is induced by unilateral ablation of eyestalk which reduces the CMG family hormones by half (Browdy and Samocha 1985). However, this being an unethical and destructive process it necessitates replacement that would help standardize the maturation process with sustainability. Recently, it has been proven that RNA interference mediated gene silencing is operative in shrimp cells in culture (Tirasophon *et al*., 2005) and in the whole shrimp organism (Lugo *et al*., 2006). The RNAi has been used for experimental manipulation of gene expression and to prove the function of certain genes at genomic level (Hannon, 2002). Consequently the technique has revolutionized research in 'reverse genetics' by introducing dsRNA to organisms or cells as it could knockdown a gene and produce its phenotypic loss (Amdam *et al*., 2003; Cogoni and Macino, 2000; Blandin *et al*., 2002; Fire *et al*., 1998). Tiu *et al*. (2007) used the approach of recombinant protein and RNA interference to study the reproductive functions of gonad stimulating hormone in Metapenaeus ensis. During the same period Treerattrakool *et al*. (2008) first reported the use of dsRNA to elucidate the function of GIH in *Penaeus monodon*. They demonstrated the influence of Pem-GIH on Vg gene expression and thus implied its role in gonad inhibition. In the present study we demonstrate the ability of dsRNA of CHH1 and MIH1 constructed using genomic DNA and GIH from cDNA, to silence the CHH family hormone genes post transcriptionally in P. monodon. It is hypothesized that these could be employed as molecular tools in place of eye stalk ablation to achieve induced maturation.

4.2 Materials and Methods

4.2.1 Experimental animals

P.monodon (10-15g) obtained from local hatchery were stocked and maintained in laboratory recirculation aquaculture system (RAS) for shrimp integrated with nitrifying bioreactor (Kumar *et al*., 2009, 2011) in sea water having 15g l^{-1} salinity. Water quality was maintained within a narrow range (pH 6.8-7.8; total ammonia < 0.1 mg l^{-1}; nitrite < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness >5000-6000 mg l^{-1}). The animals were fed with commercially available pelleted feed (Higashimaru, India) containing 40 % protein, 3 % fat, 12 % fiber, 18 % ash and 12 % moisture.

4.2.2 Genomic DNA preparation

Apparently healthy *P.monodon* juveniles were used for the DNA extraction. The animals were washed in sterile sea water prior to the collection of haemolymph and other tissues. Haemolymph was drawn using sterile haemolymph collection capillary tube from the rostral sinus and the tissues such as gills and pleopods were removed using sterile forceps and scalpel.

4.2.2.1 *DNA extraction from shrimp tissues*

For DNA extraction 20-100 mg gill/pleopod/muscle tissue and 200 µl of haemolymph were used. The tissue was homogenized in 600 µl extraction buffer (1M Tris-Cl, 0.5M EDTA (pH 8), 10 % SDS, and 20 mg ml^{-1} Proteinase K) using DNase and RNase free plastic pestle and incubated at 55 °C for 1.5 hr followed by centrifugation at 5000 rpm for 10 minutes. The supernatant was treated with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) followed by chloroform: isoamyl alcohol (24:1). The colourless aqueous phase (upper) containing DNA was transferred carefully to a fresh tube. An aliquot of 0.1volume of the ice cold ethanol (100 %) was added incubated at -20 °C for 1hr. The pellet was washed with 70 %

alcohol and air dried at room temperature. The pellet was re-suspended in 50-100 µl TE buffer. The concentration and quality of DNA were determined by measuring absorbance at 260/280 nm using a UV-Visible spectrophotometer (Hitachi, Japan). An aliquot of 5 µl was analyzed by 0.8 % agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

4.2.3 Primer Design

The primers were designed for the genes from the 5' and 3'ends of the Pem-CHH1 (GenBank ID **AF233295.1**) and Pem-MIH1 (GenBank ID **AY496454.1**) based on genomic DNA sequence while for GIH was from the coding sequence (cds) (GenBank ID **DQ643389**) of P. monodon. The sequences were adopted from NCBI database and primers designed using Gene Tool software (Table 4.1).

4.2.4 Amplification of CHH1 and MIH1 from genomic DNA

Genomic DNA extracted from haemocytes of P. monodon was used for the PCR amplification of CHH1 and MIH1. Briefly, PCR was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.5 µl), 1 µl of 10 pmol μl^{-1} of forward and reverse primer (Table.1) and 1.0 µl of 0.5U μl^{-1} of *Taq* DNA polymerase and 1 µl DNA template (75 ng). The PCR amplification programme consisted of heating the mixture to 95 °C for 5 min before addition of *Taq* DNA polymerase enzyme. The following PCR cycle includes initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 60 °C (CHH1), 64.5 °C (MIH1) for 45 sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. Aliquots of 10 µl each PCR products were analyzed by 1 % agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

4.2.5 Total RNA extraction and cDNA synthesis

Total RNA was extracted from eyestalk and cDNA was synthesised using the protocols described previously (See section 2.2.2 in chapter 2).

4.2.5.1 *PCR Amplification of GIH gene from cDNA*

PCR was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.5 µl), 1 µl of 10 pmol μl^{-1} of forward and reverse primer and 1.0 µl of 0.5U μl^{-1} of *Taq* DNA polymerase and 1 µl cDNA template. The PCR amplification programme consisted of heating the mixture to 95 °C for 5 min before addition of *Taq* DNA polymerase enzyme. The PCR cycle included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 58 °C for 45sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. An aliquot of 10 µl PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

4.2.6 TA vector construction of shrimp CHH1, MIH1 and GIH genes in pGEM-T Easy vector

The A tailed PCR products (CHH1, MIH1 and GIH) were cloned into pGEM-T easy vector.

The A tailed PCR product of CHH1, MIH1 and GIH were ligated with pGEM-T easy vector (Promega, USA) by following the manufacturer's instructions. Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T vector (50 ng μl^{-1}), 3.5 µl PCR product, 1 µl ligation buffer (10X), 1 µl ligase enzyme and MilliQ were incubated at 4 °C overnight. This allowed for the ligation of PCR products with pGEM-T easy vector.

4.2.6.1 *Transformation by heat shock*

Transformation was carried out as described previously in Section 2.2.4.2 Chapter 2

4.2.6.2 *PCR confirmation of gene inserts in the selected clones*

The clones were selected and patched on LB/ampicillin (100 µg ml^{-1})/ X-gal (80 µg $ml^{-1)}$ /IPTG (100 mM) plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed, primer could amplify it from either side. The 25 µl PCR reaction mixture containing 2.5 µl 10X buffer, 2.5 µl dNTP (2.5 mM), 1µl *Taq* polymerase (0.5 U $µl^{-1}$), a pinch of colony, 1 µl of T7 Forward and SP6 Reverse primers each (Table 1), and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 45 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

4.2.6.3 *Propagation of confirmed colony and plasmid extraction*

After confirmation, the transformed *E.coli* DH5α containing cloned vectors were propagated in 10 ml LB ampicillin (100 µg $µl^{-1}$) medium with shaking at 37 °C at 220 rpm. Plasmid extraction was carried out as detailed earlier in Chapter 2 (section 2.2.4.5).

4.2.6.4 *DNA Sequencing and sequence analyses*

The nucleotide sequence was determined using ABI PRISM model 3730 DNA ANALYZER (Applied Biosystem) at Microsynth AG - Balgach Switzerland. Homology search was performed using BLAST search algorithm (Altschul et.al., 1997) for the confirmation of the genes sequenced.

4.2.6.5 *Sequence analyses of CHH1, MIH1 and GIH*

A comparison between CHH1, MIH1 chromosomal DNA sequence and complementary DNA sequence of GIH with already recorded P. monodon genes was

made using CLUSTALW multiple alignment analysis. The nucleotide sequences of CHH1, MIH1 and GIH were matched with the GenBank database of nucleotide sequences reported for P. monodon, Pem- CHH1 cds (GenBank **AY346378**), Pem-CHH1 mRNA (GenBank **AF233295.1**), Pem- MIH1 cds (GenBank **AY496454**) and Pem- GIH cds (GenBank **DQ643389.1**) using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997). The span of intron in CHH1 and MIH1 sequences were compared with the Pem-CHH1 and Pem-MIH1. dsRNA of 19-21 nucleotides were constructed manually from CHH1 (NCAAH), MIH1 (NCAAH) and GIH (NCAAH) sequences. Random base mismatches were made in the sequences to confirm the off target phenomenon using BLAST programme.

4.2.7 Construction of *in vitro* dsRNA of CHH1, MIH1 and GIH

The 801 bp CHH1, 795 bp MIH1 and 316 bp GIH cloned into pGEM-T easy vector were used for the construction of dsRNA. Single stranded RNAs were synthesized independently by in vitro transcription, using T7 and SP6 RNA polymerase (New England Biolabs, USA). Prior to *in vitro* transcription, the plasmid DNA was linearised with Sca I restriction enzyme and gel purified using Wizard SV gel purification kit (Promega, USA). The sense and antisense strands of dsRNA were synthesized separately. The reaction mix (25 µl) included 2.5 µl of RNA polymerase buffer (10x), 2.5 µl of ribonucleotide solution mix (2.5mM), template(s) (1-4 µg), 1 µl of RNase Inhibitor (20 units μl^{-1}) and 2-4 µl of RNA polymerase (20 units μl^{-1}) and the volume was made up with RNase-Free Water. The reaction mix of sense strand containing T7 RNA Polymerase (20U μl^{-1}) along with T7 primer (10 pmoles) was incubated at 42 °C for 2-4 hrs. The reaction mix of antisense strand containing SP6 RNA polymerase (20 U μl^{-1}) and SP6 primer (10 pmoles) was incubated at 40 °C for 2-4 hrs. The single stranded RNAs were allowed to anneal (annealing buffer-TE buffer pH 8.0) by mixing equal amounts of each strand, heated to 100 °C for 3 min and cooled gradually to room temperature. The reaction mix was treated with RNase free DNase I (2 U), to remove the DNA template. The dsRNA reaction mix was

extracted once with phenol - chloroform and once with chloroform- isoamyl alcohol. The RNA was precipitated with 2 volumes of 2-propanol and dissolved in RNase free water. The dsRNA was quantified by measuring the absorbance at Abs_{260} nm and verified by running on 1.5 % agarose gel. The concentration of the double stranded RNA was measured by reading the OD at 260 nm and 280 nm respectively. An aliquot of 10µl of the sample was made up to 1 ml using DEPC treated water in the cuvette and the absorbance was measured thereafter. The concentration of RNA was determined using the formula:

Concentration of dsRNA = Absorbance at 260nm x 45 x Dilution factor.

4.2.8 *In vivo* bioassay of dsRNA of CHH1, MIH1 and GIH in *P.monodon*

P.monodon (n=10) were maintained as described in the earlier experimental set up. Prior to injection, the shrimps were allowed to moult once and on the third day after the first moult, they were injected with the specific dsRNAs. Prior to injection the animals were kept unfed for 18 hrs. The control group was injected with the reaction mixture (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, pH 7.3) (Lugo *et al*., 2006). The reaction mix was injected into the abdominal body cavity in case of CHH1, MIH1 and control, whereas in the case of GIH, it was through the arthrodial membrane of the second walking leg.

4.2.9 Functional gene expression: Demonstrated with semi quantitative RT-PCR

The eyestalk tissue was used for the functional gene expression studies. The eyestalks from dsRNA injected animals were extricated; the rigid exoskeleton and retina were removed prior to RNA extraction at a time interval of 24, 60 and 108 hrs post injection. Total RNA and cDNA synthesis were done as detailed in Chapter 2. PCR conditions of CHH1, MIH1 and GIH were as explained in the earlier part of this chapter.

Besides, the GIH gene expression, the functional gene expression of vitellogenin was detected by semi quantitative RT-PCR using vitellogenin gene specific primers (Treerattrakool *et al*., 2008) (Table 1) in the GIH dsRNA administered animals. Total RNA extraction and cDNA synthesis were carried out as detailed in Chapter 2. In MIH dsRNA injected animals the expression of MIH 2 gene was also analyzed. Briefly, PCR was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.5 µl), 1 µl of 10 pmol μl^{-1} of forward and reverse primer and 1.0 µl of 0.5U μl^{-1} of *Taq* DNA polymerase and 1 µl cDNA template. The PCR amplification programme consisted of heating the mixture to 95 °C for 5 min before addition of *Taq* DNA polymerase enzyme. The following PCR cycle included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 52 °C for 45 sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. An aliquot of 10 µl PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

4.2.10 Detection of the specific physiological activity

Haemolymph samples collected using the haemolymph needles were centrifuged at 10,000 xg for 5 min at 4 °C. The haemolymph collected were analyzed for glucose concentration using the glucose oxidase kit (Biolab Diagnostics (I) Pvt. Ltd, India). An aliquot of 5µl of haemolymph was combined with 195 µl of glucose buffer and incubated for 15 min at 37 °C. The glucose level was determined by measuring the absorbance at 500 nm. In the case of MIH group, duration of moulting was monitored and the moult duration was compared with the control.

4.3 Results

4.3.1 pGEM-T vector construction for sequence analyses and in vitro dsRNA synthesis

The genomic DNA prepared from the haemolymph, gill and pleopod tissue gave the ratio (OD_{260}:OD_{280}) of 1.77-1.8 (Fig 1). The DNA from haemolymph was

used for PCR amplifications of CHH1 gene (0.801 Kb) (Fig 2) and MIH1 (0.795 Kb) gene (Fig 3), while GIH (0.316 Kb) (Fig 4) was from cDNA template and the genes cloned in the TA cloning vector (pGEM-T easy). The clones were screened for inserts using the vector primers (T7-F and SP6-R, vector region spanned 178bp/0.178Kb) and the products obtained were CHH1 (~0.979Kb- 0.801+0.178Kb), MIH1 (~0.973 Kb- 0.795 +0.178Kb) and GIH (0.494Kb- 0.316 + 0.178Kb) (Fig 5 and Fig 6). The plasmid (Fig 7) was extracted from the positive clones and the vectors constructed were pGEM-T-CHH1 (Fig 8), pGEM-T-MIH1 (Fig 9) and pGEM-T-GIH (Fig 10).

The sequence analyses of the DNA based nucleotide sequence of CHH1 resulted in 99 % identity with Pem-CHH1 mRNA (AF233295.1) and *P.monodon* CHH1 cds (98%) (AY346378). MIH1 sequence showed 97 % identity with *P.monodon* MIH1 cds (AY496454). The cDNA based nucleotide sequence of GIH had 99% identity to GIH mRNA of *P.monodon* (DQ643389.1). The vectors constructed (pGEM-T-CHH1, pGEM-T-MIH1 and pGEM-T-GIH) were utilized as template for *in vitro* transcription of dsRNA-CHH1, dsRNA-MIH1 and dsRNA-GIH. The dsRNAs (Fig. 11, Fig 12 and Fig 13) synthesized in vitro were found to have a ratio at Abs_{260}/Abs_{280} of around 2.0 and concentrations of 84.30 µg (CHH1), 243.5 µg (MIH1) and 40.23 µg (GIH). The concentration of dsRNA used for silencing experiment was 20 µg per animal.

4.3.2 Functional gene silencing in eyestalk tissue - demonstrated by semi-quantitative RT-PCR

Functional gene silencing of CHH1 (~500 bp) gene was evident during the initial phase (24 hrs), while MIH1 (~450 bp) expression was weak from 24 hrs and completely silenced by 60^{th} and 108^{th} hrs. GIH functional gene signals were weak from 24 hrs and undetectable by 60^{th} hr. The functional gene expressions were restored by 60^{th} hr in CHH1 and by 108^{th} hr in GIH (Fig 14). The controls evidenced no change in the functional gene expression levels of CHH1, MIH1 and GIH transcripts throughout the experiment. The percentage decrease in the peak intensity of the transcripts with regard to the gel image has been visualized with remarkable alteration on the application of

specific dsRNA. On administration of GIH dsRNA, there was no suppression of CHH1 and MIH1 genes and vice versa (Fig 15). All gene expressions were compared with that of housekeeping gene beta actin.

The administration of MIH1 dsRNA was investigated on the expression of MIH2, an isoform of MIH1 gene. It was observed that the gene silencing pattern followed that of MIH1 gene. The expression of MIH2 was weak from 24 hrs and completely silenced by 60^{th} and 108^{th} hrs (Fig 16).

4.3.3 GIH gene silencing: induction of vitellogenin gene expression

The functional knockdown of GIH expression induced the vitellogenin gene expression in animals which received the dsRNA of GIH. The expression was slightly visible by 60^{th} hr (Fig 17). This clearly showed that the silencing of GIH gene in turn triggered the expression of vitellogenin gene which played a key role in maturation. The expression of vitellogenin was not observed in the controls, CHH1 and MIH1 dsRNA injected animals.

4.3.4 Physiological change induced by dsRNA: Haemolymph glucose concentration

Haemolymph (20 µl) glucose concentrations of shrimp administered with dsRNA of CHH1, MIH1 and GIH were monitored at 24^{th} and 60^{th} hr post injection (Fig. 8 and 9), during which CHH1 dsRNA (20 µg) administered animals showed significant decrease in glucose concentration (23.48 ± 14.88mg dl^{-1}) against the control (95.73 ± 33.40 mg dl^{-1}) ($P \leq 0.01$). The glucose concentration at 60^{th} hr was small and less significant (Fig. 9). However, with respect to dsRNA of MIH1 and GIH (Fig 18 and 19), only a marginal decrease in haemolymph glucose level could be observed (82.52 ± 5.81 mg dl^{-1} and 61.6 ± 31.73mg dl^{-1}, respectively) with that of the control (95.73 ± 33.40 mg dl^{-1}) ($P > 0.05$).

4.3.5 Moulting suppression- Effect of MIH1 dsRNA

The suppression of MIH1 on administering dsRNA was assessed by taking into account the duration required for moulting in comparison with that of the control group. The moulting was suppressed significantly by 18.87 % ($P < 0.005$) (Fig 20).

4.4 Discussion

RNA interference is the phenomenon in which long dsRNA is able to silence cognate gene expression, thereby providing an opportunity to investigate the corresponding protein function (Tiu and Chan 2007). In shrimp, RNAi induced gene silencing has been demonstrated earlier by Yodmuang *et al*. (2006) and Treerattrakool *et al*. (2008). This was preferred as a tool for studying the functional knockdown of CHH1, MIH1 and GIH, which in due course could likely be developed as molecular tools in place of eye stalk ablation for induced maturation. In the present study CHH1- specific dsRNA (801 bp) and MIH1- specific dsRNA (795 bp) were constructed from genomic DNA, meanwhile the GIH-specific dsRNA (316 bp) from cDNA. The CHH1, MIH1 and GIH genes could be silenced within 24 hrs of dsRNA application. However, a complete silencing of GIH continued to the 60^{th} hr while that of MIH1 to 108^{th} hr.

Physiology of the animals injected with dsRNA corroborated with the silencing of the specific gene. The animals injected with dsRNA of CHH1 showed a significant decrease of 75.47 % in the haemolymph glucose concentration compared to that of the control group and the ones administered with dsRNA of MIH1 and GIH genes. The MIH1 transcript signals were weak from 24 hr and undetectable by 60^{th} hr and 108^{th} hr. Simultaneously the moulting pattern also showed a significant change by reducing the days of moulting from 11.5 ± 1.04 days to 9.33 ± 0.816. The MIH2 transcript was also suppressed in the same pattern as that of MIH1, the silencing continued to a period of 108^{th} hr. An assumption, that the combined silencing of two isoforms of MIH1 (MIH1 and MIH2) brought about by the 795 bp length of the

dsRNA might have reduced the moulting duration. Injection of dsRNA of GIH was evidenced with the gene being silenced along with the expression of vitellogenin gene, proving that the silencing of GIH gene in turn stimulated the expression of vitellogenin in adult shrimp *P. monodon*. Treerattrakool *et al*. (2011) reported that the injection of bacterially expressed dsRNA mediated GIH silencing led to ovarian maturation and eventual spawning in both domesticated and wild brood stock.

Various factors like length of target mRNA, length and concentration of dsRNA, the region of homology between the dsRNA and target and other lesser known mechanisms could have triggered the efficiency of interference of RNA *in vivo* (Tiu and Chan 2007). It was demonstrated by Yodmuang *et al*. (2006) that the length and dose of dsRNA determined the potency of gene suppression in shrimp cells in culture, and best of the results were obtained when larger dsRNA with higher dosage was administered. The use of long dsRNA provides the possibility of generating more varieties of effective siRNA (21-23 nucleotides) molecules. The dsRNA can be made from cDNA or genomic DNA templates. In spite of the fact that for RNAi most of the dsRNA corresponds to exon regions, dsRNAs with two or more exon regions interrupted by introns also worked quite well. Further, one can use dsRNAs corresponding to coding sequences and/or untranslated regions (UTRs) as well for better results. One approach has been to use dsRNA corresponding to the 5' or 3' UTR (either UTR can mediate interference) (Carthew, 2003). The introns present in the CHH1 and MIH1 genes which were amplified from genomic DNA could have paved the way for generating longer dsRNA, a pattern hitherto not reported, which in turn might have generated a combination of variety siRNA molecules causing the silencing of CHH1 and MIH1 genes. During the study we could not generate dsRNA from genomic DNA of GIH gene, and there by proceeded with cDNA of the same.

Meanwhile, non-specific silencing, known as off-target phenomenon, can also occur from diverse siRNA products of long dsRNA (Jackson and Linsley, 2004; Quin

et al., 2005). The off target phenomenon checked manually by constructing the dsRNA sequences and using the NCBI BLAST programme proved absence of the off-target phenomenon in the present work. The marginal reduction in glucose level of the animals administered with MIH1 and GIH dsRNA from 95.73 mg dL^{-1} to 82.52 and 61.6 mg dL^{-1} respectively may be attributed to the amino acid sequence similarity which classifies MIH1 and GIH as isoforms under the CHH family (Udomkit *et al*. 2000). At the same time, there was no suppression of CHH1 gene in the MIH1 and GIH dsRNA injected animal and vice versa.

This study contributes to our understanding of CHH1, MIH1 and GIH specific dsRNA mediated temporary knockout of the related gene function in *P.monodon*. The study also confirmed that the silencing of GIH gene with dsRNA administration induced vitellogenin expression signifying the possibility of maturation in adult shrimps as proved earlier by Treerattrakool *et al*. (2011). These findings precisely suggest the possibility of using dsRNA as tool for post transcriptional CHH family hormone gene silencing to achieve maturation in shrimps under captivity. An appropriate delivery system, dosage and frequency of application have to be standardized besides investigating the physiology/ health status of the animals consequently.

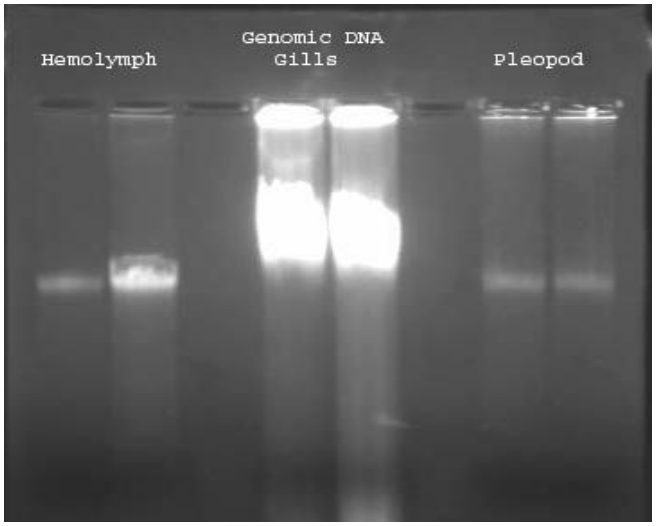
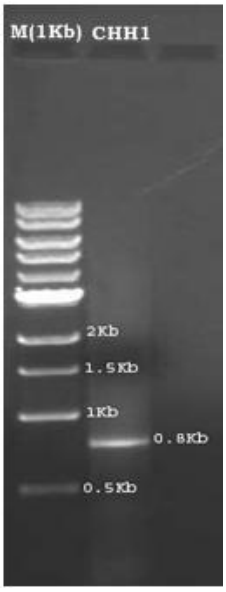
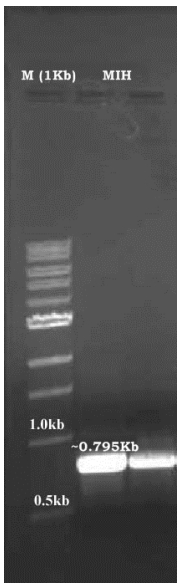


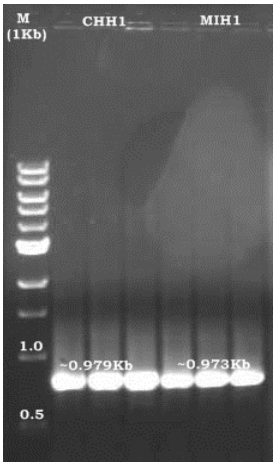
Fig.1. DNA Extraction from Haemolymph, Gills and Pleopod.



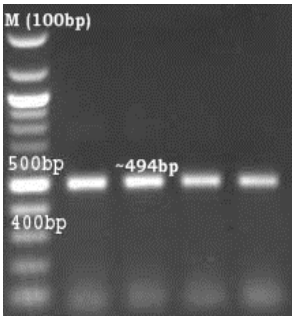


(Fig. 2) **(Fig. 3)** **(Fig. 3)**

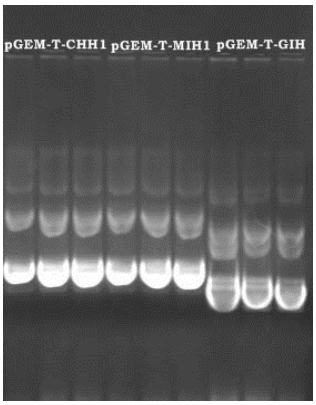
Fig 2. Amplification of CHH1 gene (~0.8Kb) from haemolymph (haemocytes) DNA sample
Fig.3. Amplification of MIH1 gene (~795Kb) from haemolymph (haemocytes) DNA sample
Fig.4. Amplification of GIH gene (316bp) Eyestalk cDNA



(Fig. 5)



(Fig. 6)

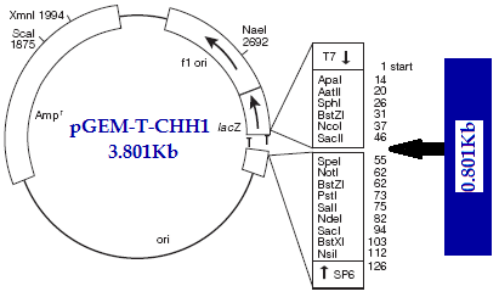


(Fig. 7)

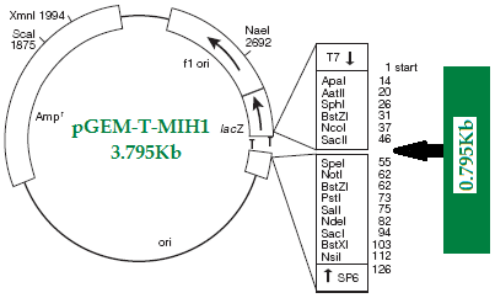
Fig.5. Colony PCR of CHH 1, MIH1 in pGEM-T easy vector with T7 and Sp6 primers

Fig.6. Colony PCR of GIH in pGEM-T easy vector with T7 and Sp6 primers

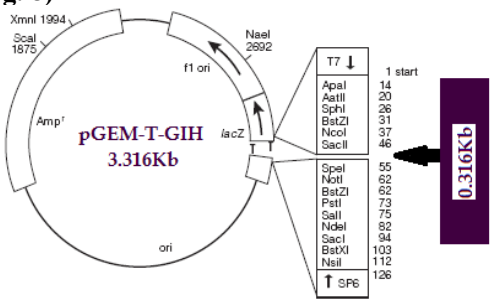
Fig.7. Plasmid extracted from the positive clones of pGEM-T vector constructs



(Fig. 8)



(Fig. 9)



(Fig. 10)

Fig.8. pGEM-T-CHH1 vector (3.801 Kb)
Fig.9. pGEM-T-MIH1 vector (3.795 Kb)
Fig.10. pGEM-T-GIH vector (3.316 Kb)

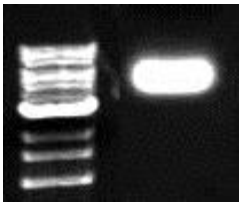


Fig.11. *In vitro* transcription of dsRNA of CHH1

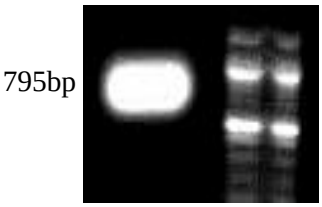


Fig.12. *In vitro* transcription of dsRNA of MIH1

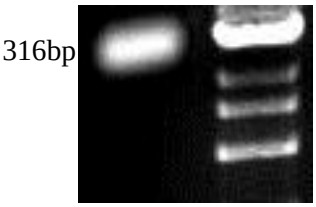


Fig.13. *In vitro* transcription of dsRNA of GIH

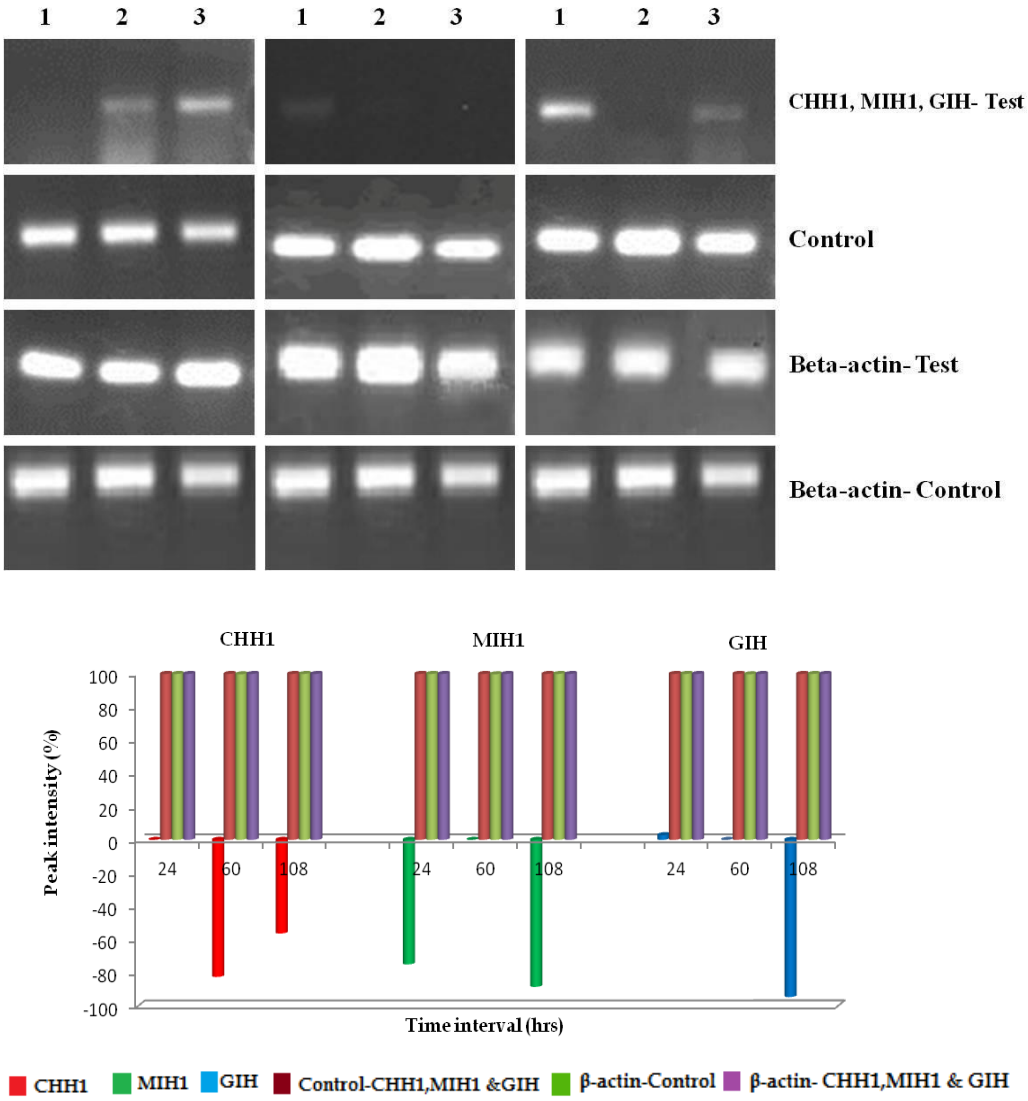


Fig.14. The silencing of CHH1, MIH1 and GIH genes were observed during the periods-1(24hr), 2(60hr) and 3 (108hr). CHH1 expression was suppressed for 24hrs post administration of CHH1 specific dsRNA. MIH1 gene was silenced for the period of 108hrs post administration of MIH1 specific dsRNA while GIH expression was observed 60hrs post administration of GIH specific dsRNA. The graph represents the peak intensity, % increase and decrease of the transcripts as seen in the gel picture.

Fig.15. Cross checking the expressions of (a) CHH1 in MIH1 and GIH dsRNA administered animals, (b) MIH1 in CHH1 and GIH dsRNA administered animals and (c) GIH in CHH1 and MIH1 dsRNA administered animals.

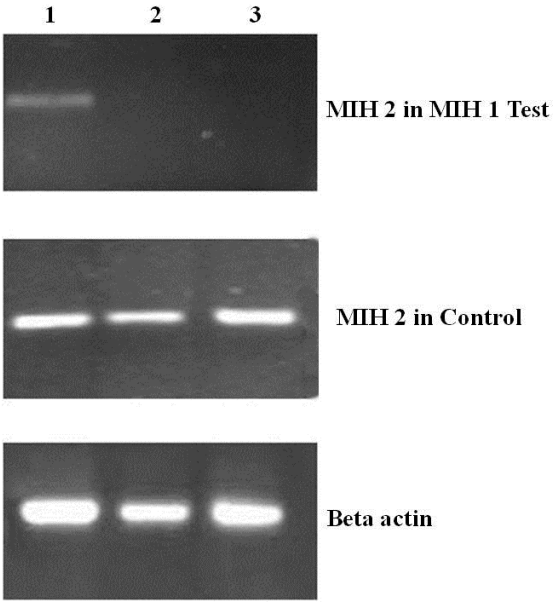


Fig.16. The silencing of MIH2 transcript observed during the periods 24 hr (1), 60 hr (2) and 108 hr (3) in MIH1 dsRNA injected animals, expression of MIH2 in control and Beta-actin as positive control.

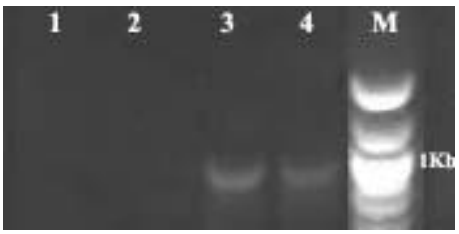


Fig.17. Expression of vitellogenin gene at 60th hr post administration of GIH – specific dsRNA (Lane 3, 4), Control 60th hr Lane 1, 2, M-Marker.

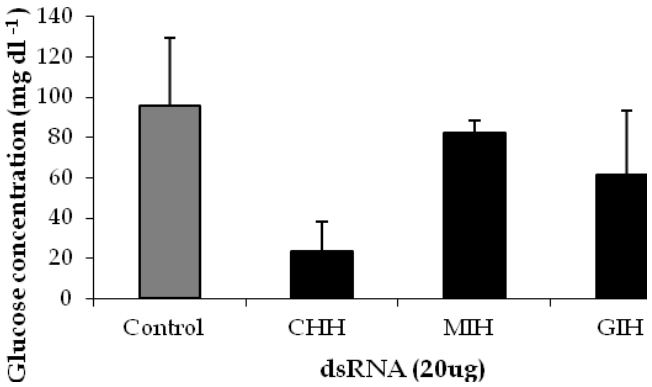


Fig.18. Glucose assay of shrimp haemolymph within 24 hr administration of CHH1, MIH1, GIH dsRNA.

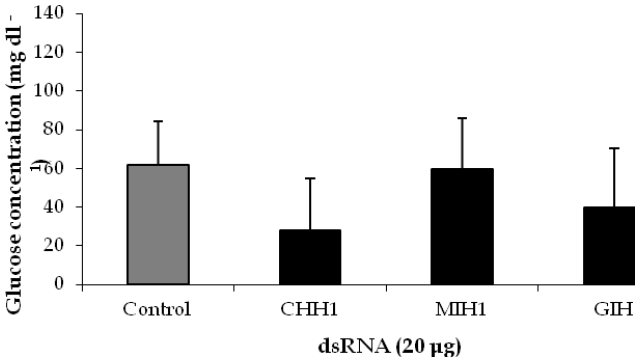


Fig.19. Glucose assay of shrimp haemolymph during the period of 60 hrs post administration of CHH1, MIH1, GIH dsRNA.

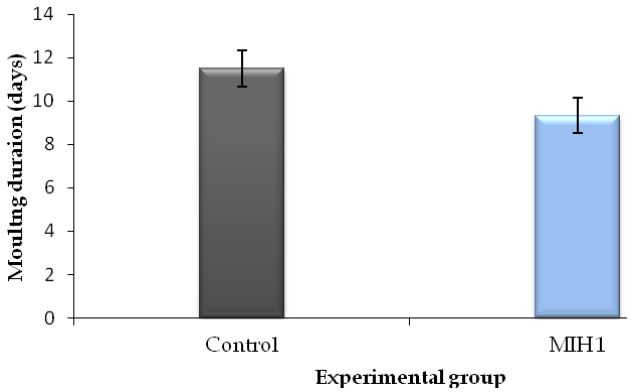


Fig.20. Moulting duration of MIH1- specific dsRNA administered animals in comparison to that of the control group.

Table.1. Primers used for generating amplicons from genomic DNA (gDNA) and cDNA.

Primer	Primer sequence (5'-3')	Tm (°C)	Amplicon size (gDNA)	Amplicon size (cDNA)
CHH1	F- CGCTCCCGATCTGCCTCTACTCTAA R- ACGAAAGCAACCTAATAAGAGTCTGG	60	801bp	500bp
MIH1	F- CGCGTCTCCTTGGGTTCATTCCGTCC R- GCGTTCGACCTCACTGACCGGCGTTC	64.5	795bp	450bp
GIH	F- TCCACAGG CAGCG GCCCCTGCTAC R- CCACGGCCGGCCGGCATTGA	58		316bp
MIH 2	F- CAGTGCGTCTCGTACATGTCCTTTC R- TCTTTCCTGTACATGCTCTGCGTGAC	60		420bp
Vg	F- CTAAGGCAATTATCACTGCTGCT R- AAGCTTGGCAATGTATTCCTTTT	52		1200bp

Table.2. Primers used for single stranded RNA construction

Primer	Primer sequence (5'-3')	Tm (° C)
T7-F	TAATACGACTCACTATAGGG	57
SP6-R	ATTTAGGTGACACTATAG	57

Chapter

5

IN VIVO GENERATED DOUBLE-STRANDED RNA MEDIATED EYESTALK NEUROPEPTIDE (CHH, MIH AND GIH) GENE KNOCKDOWN IN THE SHRIM*P PENAEUS MONODON*

5.1 Introduction

In recent years RNA interference (RNAi) has materialized as a powerful controlling mechanism of post transcriptional gene silencing in animals and plants (Fire, 1999; Finnegan and Matzke, 2003, Su *et al*., 2008). RNA interference is a phenomenon whereby double-stranded RNA triggers a potent and specific inhibition of its homologous mRNA and was discovered by Andrew Fire and colleagues in 1998. In invertebrates, long dsRNA can be efficiently used to silence gene expression without activation of dsRNA-activated protein kinase (PKR) or the interferon response that has been shown to occur in mammalian cell systems. Double-stranded RNA is introduced into animals and cells by injection, electroporation or chemically mediated transfection. The *in vivo* injection studies in animals require highly concentrated quantity of dsRNA, so that a simple and cost effective approach to produce long dsRNA will be useful for RNAi studies, especially in invertebrates. Several approaches have been used to synthesize long

dsRNA using the commercially available kits based on the principle of in vitro transcription of linearized DNA template or PCR generated templates that are widely used. However, it is increasingly expensive to use kits, when one needs to produce large amounts of dsRNA for RNAi studies. The *in vivo* production of dsRNA in E. coli strain HT115, which lacks ribonuclease III (RNase III) activity (Timmon *et al*., 2001) can be used as an alternative approach to produce large amounts of dsRNA at low cost (Tenllado *et al*., 2003; Ongvarrasopone *et al*., 2007). The E. coli strain HT115 was modified to express the T7 RNA polymerase from an IPTG inducible promoter 6. In this study, the effectiveness of CHH1, MIH1 and GIH derived dsRNA produced *in vivo* to silence the CHH family genes in shrimp was considered. The specific silencing effect to knock down CHH family genes was demonstrated, suggesting that dsRNA produced *in vivo* can be used as an alternative method to produce large amounts of dsRNA at low cost.

5.2 Materials and Methods

5.2.1 Total RNA extraction and cDNA synthesis

Explained in section 2.2.2 (Chapter 2).

5.2.1.1 *PCR amplification of CHH1, MIH1 and GIH genes*

Section 2.2.3. PCR amplification of CHH family hormone genes (Chapter 2).

5.2.2 Plasmid vectors and *E.coli* strain used for *in vivo* expression of dsRNA

5.2.2.1 *L4440 vector and E.coli HT115*

Timmons and Fire feeding vector (L4440), which is a modified version of Bluescript with a T7promoter on each side of the MCS (Multiple Cloning Site) driving transcription of each DNA strand (Timmons and Fire, 1998) (Fig.1).

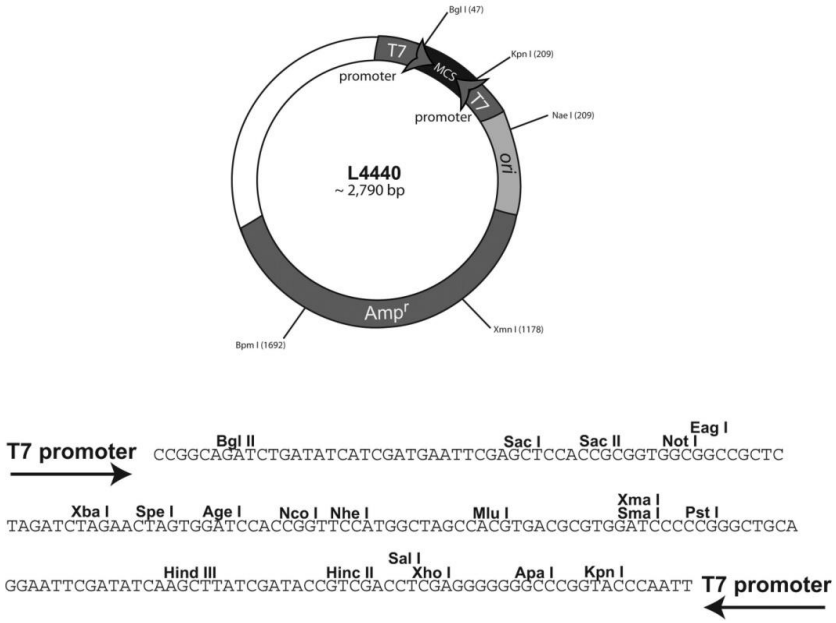


Fig.1. L4440 vector map and MCS features

cDNA cloned into L4440 were transformed into HT115 (DE3), an RNase III-deficient E. coli strain with IPTG-inducible T7 polymerase activity (Timmons *et al*., 2001). The genotype is as follows: F-, mcrA, mcrB, IN (rrnD-rrnE) 1, lambda -, rnc14::Tn10 (DE3 lysogen: lavUV5 promoter –T7 polymerase) (IPTG-inducible T7 polymerase) (RNase III minus) (Fig.2).

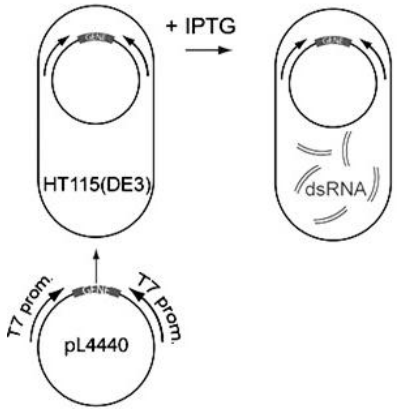


Fig.2. *E. coli* strain with IPTG-inducible T7 polymerase activity

This strain grows on LB or 2xYT plates (and is resistant to tetracycline), and competent cells can be made using standard techniques. The Tn10 transposon interrupting the rnc14 gene carries a tetracycline resistance gene. Therefore, *E.coli* should be subjected to tetracycline selection (12.5 µg ml^{-1}) to maintain the RNase deficiency. The vector L4440 and *E.coli* HT115 were generous gift from Dr. Francis Tenllado, Scientist, Centro de Investigaciones Biológicas Spain.

5.2.2.2 *Propagation of E. coli containing the plasmid vectors*

E. coli DH5α with L4440 were propagated in LB tetracycline (12.5µg ml^{-1}). Cultures were incubated at 37 °C with shaking at 220 rpm. *E.coli* HT115 were grown in LB/ 2XYT with tetracycline 12.5 µg ml^{-1} incubated at 37 °C with shaking at 220 rpm. The plasmid was extracted as mentioned in Chapter 3.

5.2.3 Designing primers with restriction sites

Specific restriction primers were designed for initially cloning CHH family hormone genes in pET32a+ vector. The primers designed for the particular work was part of the trial to express the complete recombinant protein (including signal peptide and mature region) of CHH family hormone gene in pET32a+. *EcoR* I having the sequence GAATTC was added to the 5' end and *Xho* I having the sequence CTCGAG was added to the 3'end of CHH1 and MIH1. The restriction analysis of the GIH sequence showed the presence of *Xho* I restriction site, thus *EcoR* I was added to both the 5'and 3'end of the sequence. The primers designed have the sequence as given in Table below:

Primer	Primer sequence (5'-3)	Annealing Tm (° C)	Product size (bp)
CHH1	F- GAATTCCCTGGAAGTTGCTGACCGTCGCTC	60 °C	432
	R- CTCGAGCTTGCCGAGCCTCTGTAGGGCGG		
MIH1	F- GAATTCCGCGTCTCCTTGGGTTCATTCCGTCC	64.1 °C	357
	R- CTCGAGCTGACCGGCGTTCAGGATGCTGATCC		
GIH	F- GAATTCTCCACAGGCAGCGGCCCCTGC	58 °C	336
	R- GAATTCCCACGGCCGGCCGGCATTG		

5.2.3.1 *PCR amplification of CHH family hormone genes*

CHH1, MIH1 and GIH genes were amplified using the primers with restriction sites (5'-*EcoR* I- 3'- *Xho* I in CHH1 and MIH1, 5' and 3'*EcoR* I in GIH) as specified in Table 2. Briefly, PCR was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.5 µl), 1.0 µl of 10 pmol $\mu l^{-1}$of forward and reverse primers and 1.0 µl of 2.5U μl^{-1} of *Taq* DNA polymerase and 1.0 µl cDNA template. The PCR amplification programme consisted of heating the mixture to 95 °C for 5 min before addition of *Taq* DNA polymerase. The following PCR cycle included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 60 °C (CHH1), 64.1 °C (MIH1) and 58 °C (GIH) for 45 sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. An aliquot of 10 µl PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

5.2.4 Vector construction of shrimp CHH1, MIH1 and GIH gene in pET32a+

5.2.4.1 *Restriction cloning of CHH family hormone genes in pET32a+*

The PCR products, CHH1 and MIH1 were restriction digested with *EcoR* I and *Xho* I enzyme, while GIH with only *EcoR* I (New England Biolabs, UK). An aliquot of 20 µl PCR reaction mixture containing 5 µl of CHH1/MIH1 PCR product, 0.5 µl enzyme (EcoR I and Xho1-10,000 U ml^{-1}) 2 µl reaction buffer, 0.4 µl of 100X BSA and 12.1 µl MilliQ water were incubated for 1 hr at 37 °C and inactivated at 65 °C for 20 min. In the case of GIH, 20 µl PCR reaction mixture containing 5 µl GIH PCR product, 0.5 µl enzyme (EcoR I -10,000 U ml^{-1}) 2 µl reaction buffer and 12.1 µl MilliQ water were incubated for 1 hr at 37 °C and inactivated at 65 °C for 20 min.

The pET32a+ vector was restriction digested with *EcoR* I and *Xho* I enzymes for CHHI and MIHI and only with *EcoR* I enzyme for GIH as explained

above. The restriction digested plasmid was treated with CIP (Calf Intestinal Phosphatase) to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20 µl plasmid, 0.1 µl CIP enzyme and 5 µl buffer was incubated at 37 ºC for 1 hr followed by heat inactivation at 65 ºC for 20 min. The PCR and vector restriction were scaled up to 50 µl and was gel purified using GenElute™ Gel Extraction kit (Sigma, USA) following manufacturer's instructions as detailed in Chapter 3.

The restricted and purified CHH1, MIH1 and GIH PCR products were ligated with pET32a+ vector (Novagen, UK) following the manufacture's instructions. Briefly, 10 µl ligation mixture containing 1.0 µl pET32a+ vector (50 ng μl^{-1}), 3.5 µl PCR product, 1 µl ligation buffer (10X), 1 µl T4 DNA ligase enzyme and MilliQ was incubated at 22 °C overnight. This allowed the ligation of PCR products with pET32a+ translation vector constructs (pET32a+ - CHH1, pET32a+ -MIH1 and pET32a+ -GIH).

5.2.4.2 *Transformation into E. coli DH5α*

The transformation of the pET32a+ translation vector constructs into E. coli was done as detailed in Chapter 2 section 2.2.4.2 'Transformation into E. coli DH5α'.

5.2.4.3 *PCR confirmation of gene insert in the selected clones*

The gene insert in the selected clones were confirmed as explained in Chapter 2 section 2.2.4.3 'PCR confirmation of gene insert in the selected clones'.

5.2.4.4 *Confirmation of insert orientation by PCR*

The orientation of insert in the positive clones were confirmed by PCR using T7-forward (5'-TAATACGACTCACTATAGGG-3') and gene specific reverse primers of CHH1, MIH1 and GIH. PCR mix and conditions were as detailed in section 5.2.3.1, with the exception of colony instead of cDNA as template.

5.2.4.5 *Propagation of confirmed colony and plasmid extraction*

The positively confirmed colonies were propagated and plasmid extraction was carried as detailed in Chapter 2 section 2.2.4.5 'Plasmid Extraction'

5.2.4.6 *Restriction digestion of pET32a+ vector constructs with Bgl II and Hind III (for CHH1 and MIH1 and GIH) -Release of products and their purification*

The purified plasmid vectors containing CHH1 (pET32a+CHH1), MIH1 (pET32a +MIH1) and GIH (pET32a+GIH) were restriction digested with *Bgl* II and *Hind* III enzymes, (New England Biolabs, UK) to release the corresponding products (CHH1, MIH1 and GIH). An aliquot of 20 µl PCR mixture containing 5 µl plasmid (pET32a+-CHH1/pET32a+-MIH1/pET32a+-GIH), 0.5 µl enzyme (*Bgl* II and *Hind* III -10,000 U ml^{-1}) 2 µl reaction buffer and 12.5 µl MilliQ water was incubated for 1 hr at 37 °C and inactivated at 65 °C for 20 min. The restriction digestion was confirmed by 1% agarose gel electrophoresis. The restricted CHH1, MIH1 and GIH were gel purified using GenElute™ gel extraction kit (Sigma, USA) following manufacturer's instructions explained in Chapter 3.

5.2.5 Construction of vector system for dsRNA expression

5.2.5.1 *Restriction digestion of vector L4440 with Bgl II and Hind III and its purifications*

The L4440 vector was restriction digested with *Bgl* II and *Hind* III enzymes as in the methodology explained in the previous section (Chapter 2). Restriction digested plasmids were treated with CIP (Calf Intestinal Phosphatase) to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20 µl plasmid, 0.1 µl CIP enzyme and 5 µl buffer was incubated at 37 ºC for 1 hr followed by heat inactivation at 65 ºC for 20 min. Restriction digested, CIP treated vector was gel purified using GenElute™ gel extraction kit (Sigma, USA) as explained in Chapter 3.

The restricted and purified CHH1, MIH1 and GIH were ligated with L4440 vector using T4 DNA ligase (Fermentas, GmBH, Germany) following the manufacturer's instructions. Briefly, 10 µl ligation mixture containing 1.0 µl

pET32a+ vector (50 ng μl^{-1}), 4.0 µl PCR product, 1.0 µl ligation buffer (10X), 1.0 µl T4 DNA ligase and 3.0 µl MilliQ was incubated at 22 °C overnight. This allowed for the ligation of PCR products with L4440 vector and the constructs (L4440 - CHH1, L4440 -MIH1 and L4440-GIH).

5.2.5.2 *Transformation into E. coli DH5α*

Transformation of the constructs (L4440 - CHH1, L4440 -MIH1 and L4440-GIH) into E. coli was done as detailed in Chapter 2, Section 2.2.4.2 'Transformation into E. coli DH5α.'

5.2.5.3 *PCR confirmation of the gene inserts in the selected clones*

The clones were selected and patched on LB/ampicillin (100 µg μl^{-1}) plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using gene specific primers. The PCR mixture contained 2.5 µl 10x buffer, 2.5 µl dNTPs (2.5 mM), 1µl *Taq* polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of gene specific forward and reverse primers each (CHH1, MIH1 and GIH) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C (CHH1), 64.5 °C(MIH1) and 58 °C (GIH) for 20 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

5.2.5.4 *Propagation of confirmed colony and plasmid extraction*

After confirmation, the transformed *E.coli* DH5α containing recombinant vector constructs were propagated in 10 ml LB ampicillin (100 µg μl^{-1}) medium with shaking at 37 °C at 220 rpm. Plasmid extraction was carried out as detailed earlier in this Chapter. For further screening and dsRNA development, L4440 vector constructs

(L4440 - CHH1, L4440 -MIH1 and L4440-GIH) were extracted and transformed into *E. coli* HT115 (DE3).

5.2.5.5 *E. coli HT115 (DE3) competent cell preparation*

E.coli HT115 (DE3) cells were streaked on LB/tetracycline (12.5 µg ml^{-1}) agar plate for obtaining single colonies. A single colony was inoculated in 10 ml LB medium and grown overnight at 37 °C with shaking at 150 rpm. The overnight culture was inoculated (1:100 dilution) into 25 ml LB and incubated at 37 °C until OD_{595} 0.4. The re-inoculation is necessary to obtain the *E.coli* cells in their log phase. The cells (25 ml) were centrifuged at 3000 rpm for 10 min at 4 °C. All the steps were carried out at 4 °C. The supernatant decanted and cells were resuspended by gentle vortexing with cold, sterile 50 mM CaCl2 (12.5ml). The resuspended cells were incubated on ice for 30 min with intermittent swirling and mixing. The cells were centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was decanted and cell pellet was resuspended in 2.5 ml of 50mM $CaCl_2$. The competent cells formed are stored in -80°C with addition of 10-12 % glycerol.

5.2.5.6 *Transformation into E. coli HT115 (DE3)*

The competent cells (*E.coli* HT115 (DE3) were thawed by placing on ice for 5-10 min, added 1 µl (1-100ng) of positive plasmid constructs (L4440 - CHH1, L4440 -MIH1 and L4440-GIH) to a sterile 15 ml culture tube already on ice, transferred 50-100 µl of competent cells into the 15 ml tubes (containing +ve plasmid construct) on ice, gently flicked the tubes to mix and placed them on ice for 30 min, heat shocked the cells for 1min in a water bath exactly at 37 °C, immediately returned the tubes to ice for 2 min, added 600 µl super optimal broth with catabolite repression (SOC; composition for 10 ml: 0.2 g tryptone ; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose. $MgCl_2$ and glucose were added just before transformation to the tubes containing cells transformed with ligation mixture, incubated for 1 hr at 37 °C with shaking at 220-230 rpm, plated 100 µl of each

transformation culture onto duplicate/triplicate on to LB/ampicillin/ tetracycline plates and incubated the plates overnight (12-16 hrs) at 37 °C.

5.2.5.7 *Induction (IPTG) for dsRNA expression of L4440 - CHH1, L4440 -MIH1 and L4440-GIH constructs*

Single colony each of recombinant L4440 - CHH1, L4440 -MIH1 and L4440-GIH constructs in *E.coli* HT115 was inoculated into 10 ml LB + ampicillin (100 µg ml^{-1}) + tetracycline (12.5 µg ml^{-1}) medium and incubated at 37 °C at 250 rpm overnight. The culture was diluted 1:100 in 2XYT + ampicillin (100µg ml^{-1}) + tetracycline (12.5 µg ml^{-1}) and further incubated at 37 °C until Abs_{595} of 0.4 (optimum Abs for dsRNA expression). The Abs_{595} was monitored frequently during the growth phase of the culture by removing aliquots asceptically. IPTG was added to a concentration of 0.4 mM for induction and incubated further for ~ 4 hrs at 37 °C with shaking at 250 rpm. The culture was spiked with additional antibiotics (100 µg ml^{-1} ampicillin and 12.5 µg $ml^{-}1$ tetracycline) and IPTG (to a final concentration of 0.8 mM). The cells were pelletized at 12000 xg for 2-5min. 1 ml of TriReagent (Sigma, USA) was added and the pellet was pipetted up and down for breaking of cell wall. This pellet was stored at -80 °C until further processing.

Total RNA was extracted following the TriReagent protocol mentioned in detail in Chapter 3. The extracted RNA was subjected to DNase treatment with RNase free DNase 1 (New England Biolabs, UK) by adding 0.2 U of enzyme to 1 µg of RNA and incubated at 37 °C for 10 min followed by 75 °C for 10 min for inactivation. The double stranded RNA extracted was further processed with bovine pancreatic RNaseA (in 0.3 M NaCl and 0.030 M sodium citrate) for the removal of total RNA and single stranded RNA. One-two µl of the products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA). The RNA concentration and quality were determined by absorbency (Abs 260/280 nm) measurement using a UV-Visible spectrophotometer (Hitachi, Japan).

5.2.6 *In vivo* bioassay of dsRNA of CHH1, MIH1 and GIH in *P.monodon*

5.2.6.1 *Injection of dsRNA of CHH1, MIH1 and GIH in P.monodon*

Female *P. monodon* (10 numbers weighing 10-15 g) obtained from a local hatchery were stocked and maintained in the laboratory recirculation aquaculture system (RAS) for shrimp integrated with nitrifying bioreactor (Kumar *et al*., 2009, 2011) in sea water having 15g l^{-1} salinity. Water quality was maintained within a narrow range (pH 6.8-7.8; total ammonia < 0.1 mg l^{-1}; nitrite < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness >5000-6000 mg l^{-1}). The animals were fed with commercially available pelleted feed (Higashimaru, India) containing 40 % protein, 3 % fat, 12 % fiber, 18 % ash and 12 % moisture.

Prior to injection of dsRNA the shrimps were allowed to moult once and on the third day after the first moult, they were injected with dsRNA of the three genes of the CHH family hormones individually. The dsRNA (~3 µg $µl^{-1}$) at 3 µg g^{-1} body weight were injected into the abdominal body cavity in case of CHH1, MIH1, eyestalk extracts (ES), L4440 (total RNA from empty vector induced sample) and control while GIH was through the arthrodial membrane of the second walking leg. The control group was injected with Tris/NaCl (~3 µg g^{-1} body weight). The level of CHH and MIH transcripts in the ganglia were detected by RT-PCR at intervals of 1, 2, 3, 5, and 7 days, while GIH and vitellin (Vg) transcripts in the eyestalk ganglia were detected by RT-PCR at intervals of 1, 3, 5, 7,10,15,19 and 30 days.

5.2.6.2 *Functional gene analyses- Semi quantitative RT-PCR to confirm gene silencing*

The eyestalks from dsRNA injected animals were dissected for RNA extraction during the time interval of days 1, 2, 3, 5, 7, 10 for CHH1, MIH1, ES and L4440 while 1, 3, 5, 7, 10, 15, 19 and 30 days for GIH. Total RNA was extracted using TriReagent (Sigma, USA) and quantified by measuring the absorbance at Abs_{260} nm, and used for cDNA synthesis. cDNA synthesis and PCR conditions were done as explained in earlier part of this chapter.

The expression of vitellogenin gene was detected by semi quantitative RT-PCR using vitellogenin gene specific primers in the GIH dsRNA administered shrimps. Total RNA extraction and cDNA synthesis were done as mentioned in Chapter 2. The PCR was carried out using vitellogenin gene (Vg) specific primers (Treerattrakool *et al*., 2008) (Table 1). Briefly, PCR was conducted in a 25 µl reaction volume containing 1 µl of 10X buffer, 2.5 mM dNTP (2.5 µl), 1 µl of 10 pmol μl^{-1} of forward and reverse primer and 1.0 µl of 0.5U μl^{-1} of *Taq* DNA polymerase and 1 µl cDNA template. The PCR amplification programme consisted of heating the mixture to 95 °C for 5 min before the addition of *Taq* DNA polymerase. The following PCR cycle included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 sec, annealing at 52 °C for 45sec, extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. An aliquot of 10 µl PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA). Images were analyzed with Quantity-one™ image analysis software (Bio-Rad, USA). Data is represented as peak band intensity for each sample.

5.2.6.3 *Physiological and morphological alterations – Detection of glucose, moulting, ecdysteroid and vitellogenin levels*

A glucose oxidase diagnostic kit (J.D.Diagnostics, India) was used to determine glucose concentration in all the groups of shrimps including control. The duration of moulting and moult stages were monitored in the group of shrimp administered with dsRNA of MIH. The determination of moult stages was based on the degree of setae development according to the method described by Chan *et al*. (1988), with modifications. Uropods were observed instead of pleopod (Chan *et al*., 1988). The moult stage determination with uropods gave clear and consistent images in animals (Cesar *et al*., 2006; Promwikorn *et al*., 2004). The uropod morphological pictures were taken with Olympus camera under a light microscope (10X) and were analyzed accordingly. In GIH administered animals, the vitellogenin concentration in the haemolymph were quantified.

5.2.6.4 *Functional hormone echelon- Demonstrated by CHH, MIH and GIH hormone levels in haemolymph*

The functional gene silencing (CHH1, MIH1 and GIH) bringing forth the variation in the functional hormone levels (CHH, MIH and GIH) were demonstrated by measuring the specific hormones in the haemolymph with the polyclonal antibodies of CHH, MIH and GIH through ELISA detection.

5.3 Results

5.3.1 Construction of pET32a+ vector system for sub cloning of CHH family hormone genes in L4440

The *EcoR* I-*Xho* I restricted and purified PCR products of CHH1 and MIH1 and *EcoR* I alone restricted GIH (Fig. 3 and 4) were ligated to pET32a+ vector and the corresponding vector system was constructed.

The inserts were screened using the vector primers (T7-F and T7-R, vector region spans 647 bp) and the product size obtained was CHH1 1079 bp (647+432 bp) (Fig. 5), MIH1 1004 bp (647+357 bp) (Fig. 6) and GIH 983 bp (647+336 bp) (Fig. 7). The orientation of the inserts in the expression vector constructs were confirmed by colony PCR with T7-Forward primer and gene specific reverse primer that resulted in 982 bp CHH1 (vector region; 550+432bp) (Fig. 8), 907 bp MIH1 (Fig. 9) and 886 bp GIH (Fig.10). Further, the insert in the vector constructs were confirmed by sequencing the plasmids isolated (Fig. 11). Finally pET32a+CHH1 (6.332Kb) (Fig.12), pET32a+MIH1 (6.257Kb) (Fig.13) and pET32a+GIH (6.236Kb) (Fig.14) were constructed.

5.3.2 Construction of dsRNA vector system for induced dsRNA expression of CHH family hormone genes

The restricted gene inserts from pET32a+ vector constructs were further cloned into L4440 dsRNA expression vector for the construction of dsRNA expression vectors. The dsRNA expression vector L4440 was restricted using the same enzymes (*Bgl* II and *Hind* III) as that used for restricting the inserts from pET32a+ system (Fig.15).

The inserts were screened using the gene specific primers and the product sizes obtained were CHH1 ~432 bp, MIH1 ~357 bp and GIH ~336 bp (Fig.16). The plasmids extracted from positive clones were subjected to restriction digestion to confirm the presence of the insert (Fig.17). Thus the dsRNA vector constructs generated were L4440+CHH1 (3.222Kb) (Fig.18), L4440+MIH1 (3.147Kb) (Fig.19) and L4440+GIH (3.126Kb) (Fig.20).

The dsRNA expression constructs (L4440+CHH1 (3.222Kb), L4440+MIH1 (3.147Kb) and L4440+GIH (3.126Kb) were transformed into *E.coli* HT115 (DE3) and the dsRNA expression was induced with 0.8 mM IPTG. The total RNA extracted was processed with bovine pancreatic RNaseA (in 0.3 M NaCl and 0.030 M sodium citrate) for the removal of total RNA and single stranded RNA, thus purified dsRNA was obtained (Fig. 21). The size of dsRNA on visualization by 2% agarose gel electrophoresis in comparison with L4440 vector alone (Fig. 22 and 23) corresponded to the sizes of CHH1 ~432 bp, MIH1 ~357 bp and GIH ~336 bp. The dsRNA concentrations were measured to be about 5.67 µg μl^{-1} to 7.56µg μl^{-1} from 250 ml preparation.

5.3.3 dsRNA mediated functional gene knockout of CHH1 hormone gene in *P.monodon*

Based on the studies of dsRNA application in shrimp, the animals were injected with 3 µg g^{-1} body weight dsRNA for functional gene knockout. The hypoglycaemic effect was observed within 24 hrs in the group injected with dsRNA of CHH1. There were significant decrease ($p<0.05$) in the haemolymph glucose concentrations (55.09 %, 62.82 % and 28.4%), 24 hrs post injection in comparison with the control 1 (buffer), control 2 (eyestalk extract) and control 3 (L4440). The haemolymph glucose concentration was significantly reduced ($p<0.05$) to 27.6 ± 3.304 mg dl^{-1} in comparison with the control 1 (61.47 ± 28.75 mg dl^{-1}) (Fig. 24) control 2 (74.23 ± 86.66 mg dl^{-1}) (Fig. 25) and control 3 (34.25 ± 4.49 mg dl^{-1}) (Fig. 26). The hypoglycemic effect was evidently demonstrated by measuring the CHH hormone level in the haemolymph

with the antiCHH (aCHH) antibody. The hypoglycemic effect was evidently demonstrated by measuring the CHH hormone level in the haemolymph with the antiCHH (aCHH) antiserum. The CHH level reduction observed was 58.52 %, 78.69 % and 50.4 % in comparison with the control1 (buffer), control 2 (ES) and control 3 (L4440) ($p < 0.05$). It was also observed that among the control groups there were no significant ($p>0.05$) differences either in glucose or CHH concentrations in the haemolymph.

The semi-quantitative reverse – transcription polymerase chain reaction (RT-PCR) demonstrated that the resulting physiological discrepancy was caused by CHH dsRNA silencing at molecular level. The RT-PCR showed a drastic knockdown in the CHH mRNA, 24 hrs post injection, compared to the three controls. The CHH expression level was observed to be reduced even 48 hrs post injection (Fig. 27). The glucose levels of CHH dsRNA injected animals during the 48 hrs period in comparison with the three controls (control1 (buffer), control 2 (eyestalk extract) and control 3 (L4440) were 27.05 %, 54.25 % and 17.9 % , while the CHH levels were 47.85 %, 51.22 % and 53.34 % respectively. The peak intensity of CHH1 transcript expression calculated using the quantity one software (BioRad, USA) confirmed the effect of silencing with dsRNA. The band analysis showed zero fold decrease (highly significant reduction, $p <1.63x\ 10^{-11}$) during the 24 hrs post injection of CHH1 dsRNA, while a fivefold decrease ($p < 4.54 \times 10^{-7}$) was observed 48 hrs post injection (Fig. 28). There was a positive correlation between the physiological and molecular levels. Positive correlation coefficients of 0.9, 0.95 and 0.97 in terms of glucose concentration, haemolymph CHH level and peak intensity of CHH1 transcript expression were obtained. The CHH1 transcript expressions in the dsRNA injected animals were compared to the expression of CHH1 in the three controls and to that of β-actin (house- keeping gene) (Fig. 28).

5.3.4 dsRNA mediated functional gene knockout of MIH1 hormone gene in *P.monodon*

During the intermoult stage (C-stage) the MIH levels are high as the ecdysteroid level decreases (Nakatsuji and Sonobe, 2003); this stage was chosen to be the appropriate time for silencing of the MIH1 gene. The moult inhibiting effect was observed from the initial phase of 24 hrs, the reduction in MIH levels were observed to be reduced by 41.4 % in comparison with the controls. By 48 hrs (2^{nd} day), the MIH levels were significantly reduced to 61 %. The MIH levels on the 3^{rd} day (72 hrs) and 5^{th} day (96hrs) were also significantly lowered by 56.44 % and 47.52 %. The statistical analysis (Student's t- test) evidently proved the significance ($p < 0.05$) in the lowering of MIH levels in MIH dsRNA group in comparison to control group1 (buffer) (Fig. 29). To confirm the hypothesis earlier proved by Nakatsuji and Sonobe (2003) that the increase in MIH levels decreased the ecdysteroid level and vice versa; the ecdysteroid levels were measured during the same time period in the MIH dsRNA injected animals and control. Highly significant rise ($p<0.001$) in the ecdsyeroid levels were evident in MIH dsRNA (65.526 ng ml^{-1}) injected group in comparison with the control (2.17 ng ml^{-1}). The MIH levels of dsRNA group were also compared with two more controls, the eyestalk extract injected group (ES) (Fig. 30) and the L4440 (Fig. 31) injected group. The observations were proved with Student's T-test and a significant difference ($p<0.05$) was observed in the MIH levels of dsRNA injected group compared to the two controls. A steady decrease in the MIH levels were proved from day1 (24 hrs) to 3^{rd} day (72 hrs) in the MIH dsRNA injected group with that of two controls.

According to Chan *et al*. (1988), in *Penaeus vannamei* the C- stage duration occupied 10-15 days (35-40%) of the intermoult period. The MIH dsRNA application reduced the C- stage and the total moulting period by 32.43 %. The moulting days of MIH dsRNA injected animals (n= 20) were observed to be 12.5 ± 0.71 days, while that of control injected with buffer was 18.5 ± 0.7 days

(Fig. 32). The Student's T-test confirmed a significant ($p < 0.05$) difference between the moulting period of MIH test and control.

An experiment was also carried out to test the efficiency of moulting on application of dsRNA of MIH in the D2-D3 stage animals. According to Chan *et al*. (1988), D3 stage duration (10-15g animals) was for 1-2days (3-4%); based on this data available we observed that upon application of dsRNA of MIH the animals moulted immediately without any delay of 1-2 days. The animals in the D2 stage showed an advancement of moult stages from D2-D3. The average moults observed per day for a period of 10 days (n= 12) in the experimental groups were CHH1 (0.4 ± 0.7), MIH1 (0.8 ± 1.3), GIH (0.8 ± 1.2), control (B) (0.3 ± 0.5) ES (0.1 ± 0.3) and L4440 (0) (Fig. 33). The MIH and GIH experimental groups were continued for a period of 32 days and the average moults observed were surprising; the silencing of MIH showed an average moult per day of 0.53 ± 0.7 ($p < 0.01$), and GIH exhibited somewhat similar result (0.468 ± 0.6; $p < 0.01$). The controls showed an average moult of 0.125 ± 0.3 and 0.0625 ± 0.2 per day (Fig. 34).

To demonstrate the whole physiological disparity due to the silencing of the MIH gene, RT-PCR was used for validation. RT-PCR results of MIH1 transcript expression demonstrated complete gene silencing within 48 hr (2^{nd} day) of MIH1 dsRNA application *in vivo*. The silencing effect started within 24 hrs (1^{st} day), but complete silencing was by 48 hrs (2^{nd} day). The expression level of the transcript was observed to be low during the 72 hrs (3^{rd} day) too and a slow gain was from day 5 (Fig. 35 and Fig. 36). The expression level of the MIH1 transcript was proved with the peak intensity calculation using Quantity One software (BioRad, USA). The four fold decrease (22.43 %) ($p < 0.05$) in the peak intensity of MIH1 transcript within 24 hrs proved the beginning of the initial silencing effect, while the zero fold decrease (significant reduction, $p < 2.71 \times 10^{-11}$) within 48 hrs was ultimate silencing of MIH1 gene. The slow gain of two fold increase (41.58 %) ($p < 0.05$) from 72 hrs showed the diminishing effect of the MIH1 silencing. A correlation of the three parameters

(haemolymph MIH level, ecdysteroid level and peak intensity of MIH1 transcript) was carried out. It was observed that the haemolymph MIH level gave a negative correlation (- 0.77) with the ecdysteroid level, while a positive correlation with the peak intensity (0.88). The ecdysteroid level gave a negative correlation with that of peak intensity of MIH1 transcript expression (- 0.84). The MIH1 transcript expressions in the dsRNA injected animals were compared to the expression of MIH1 in the three controls and to that of β-actin (house- keeping gene) (Fig. 35)

5.3.5 dsRNA mediated functional gene knockout of GIH hormone gene in *P.monodon*

Injection of dsRNA of GIH was carried out in previtellogenic animals (n= 20, 15-20 g) with the variations that were able to be measured in the haemolymph as GIH concentration with antiGIH antibody and the vitellin levels with anti-vitellin antibody. Gonad inhibiting effect was observed to have initiated from 24 hrs with slight reduction in the haemolymph GIH level. In comparison with the controls, the haemolymph GIH levels were significantly ($p < 0.005$) depleted by the 3rd day. Reduction of 60 % haemolymph GIH level was observed and a continuous but steady (60-25%) low level in haemolymph GIH was found maintained throughout the experiment. In order to know the effect of GIH suppression, the haemolymph vitellin levels were measured as another physiological change which was found to be significantly higher ($p < 0.05$) than the control 1, control 2 and control 3 with 15.36 %, 15.35 % and 15.62 % increase in the haemolymph vitellin levels (Fig. 37, 38, 39).

The physiological variations were due to silencing was proved by the semi-quantitative RT-PCR analyses and calculating the peak intensity of the GIH transcript expression. The RT-PCR results proved the silencing of GIH transcript by the 3rd day of the injection. It was also observed that the GIH transcript level was steadily diminished throughout the experiment by GIH dsRNA. During the initial 24 hrs (day 1) a one fold decrease and by the 3rd day total silencing (i.e., zero fold decrease, $p < 4.08 \times 10^{-15}$) was observed (Fig. 40). The peak intensity of GIH transcript

expression level showed a decrease of twenty fold by 5th day ($p < 1.55 \times 10^{-10}$) and a steady state maintained (increase of 4-7 fold, $p < 1.04 \times 10^{-6}$) throughout the experiment (Fig. 41). The silencing of GIH gene induced the expression of Vg gene from day 3. The expression of Vg gene was observed to be present throughout the time period of the experiment in comparison with the controls (Fig. 42). A correlation analysis of the haemolymph GIH level, vitellin level and peak intensity of GIH transcripts was carried out. It was observed that the haemolymph GIH level gave a negative correlation with the haemolymph vitellin levels (-0.38). The GIH transcript expressions in the dsRNA injected animals were compared to the expression of GIH in the three controls and to that of β-actin (house- keeping gene) (Fig. 40) to compare the level of expression. The level of knockdown of GIH transcript expression was proved by the above comparison.

5.3.6 Physiological and gene transcript variations induced by dsRNAs: Non specific interactions

The physiological and gene transcript variations i.e., CHH1 level and glucose concentrations were observed in MIH1 and GIH dsRNA injected groups. Similarly, MIH1 levels were evidenced in CHH1 and GIH injected groups, while GIH levels and vitellin concentrations were taken into consideration in CHH1 and MIH1 injected groups.

In the CHH1 dsRNA injected group, the only minor significance observed ($p = 0.04$) was in the haemolymph GIH levels in comparison with the control group 1 (buffer injected) (Fig. 43). There was no significant dissimilarity in the haemolymph MIH levels or the vitellin concentrations when compared to other controls. There were no characteristic peak intensity variations of MIH1 and GIH transcripts.

In MIH1 dsRNA injected animals, slight significant difference ($p = 0.04$) was observed in the glucose concentration in comparison with the control group 2 (eyestalk extract injected) (Fig. 44). There were no significant differences ($p > 0.05$) evident with the control group1 (buffer injected) and control group 3 (L4440 injected). A noticing

variation was the significant difference ($p < 0.05$) of haemolymph CHH1 level in MIH injected group compared to the control group1 (buffer injected), control group 2 (ES injected) and control group 3 (L4440 injected) (Fig. 45) . Highly significant variation ($p = 0.01$) was seen in the haemolymph GIH concentration of MIH injected group with that of control 1, control 2 and control 3 groups (Fig. 46), but no significant differences in vitellin concentrations. No significant ($p > 0.05$) peak intensity distinction of CHH1 and GIH transcript expression was observed in the MIH1 dsRNA injected group.

In GIH dsRNA injected group, significant ($p < 0.05$) decrease was seen in the glucose concentration in comparison to the control group 2 (ES injected) (Fig. 47). No significant differences were evident with the other groups (CHH1, MIH1, control group 1 and control group 2). There were no significant differences in the haemolymph MIH1 and CHH1 levels of all the groups. There was no significant characteristic peak intensity seen in CHH1 and MIH1 transcript expression in the MIH1 dsRNA injected group.

5.4 Discussion

5.4.1 Construction of dsRNA expression vectors of CHH1, MIH1 and GIH for *in vivo* generation of dsRNA in *E.coli* HT115 (DE3)

RNA interference with double stranded RNA activates an effective and definite inhibition of its cognate mRNA. Earlier, the dsRNA was produced by in vitro transcription using genomic DNA and cDNA as template for generating long dsRNA of CHH1, MIH1 and GIH (Chapter 4). Here, we generated dsRNA by *in vivo* expression in *E.coli*. For obtaining more number of possible small interfering RNAs (siRNA) for post transcriptional gene silencing, long double stranded RNA were prepared using the complete open reading frames (ORF) of the three genes CHH1, MIH1 and GIH respectively. Three dsRNA expression vectors (L4440+CHH1 (3.222Kb), L4440+MIH1 (3.147Kb) and L4440+GIH (3.126Kb) were successfully constructed for the *in vivo* generation of specific dsRNA. The dsRNA

for silencing experiments *in vivo* in animals are required in large amounts, so a simple and relatively low cost approach is appropriate. The simple approach is to produce large amount of dsRNA in *E.coli* strain HT115 that lacks ribonuclease III, an enzyme that normally degrades dsRNA. The dsRNA production can be induced by adding IPTG, which induces the expression of T7 RNA polymerase in *E.coli*. Using this approach we were able to obtain approximately 1.5-2 mg of dsRNA from a 50 ml bacterial culture after digestion with RNase A. The production of dsRNA can be scaled by simply growing a larger amount of the bacterial culture.

Lugo *et al*. (2006) synthesized in vitro dsRNA of CHH (216 bp) of *Litopenaeus schmitti* and brought about *in vivo* CHH gene suppression. Tiu *et al*. (2007) had in vitro transcribed dsRNA Me-MIH-B of Metapenaeus ensis and the *in vivo* application caused a decrease in MeMIH-B transcript level. Treerattrakool *et al*. (2011) had produced the GIH dsRNA as hairpin RNA precursor in *E.coli* HT115 and knocked down the GIH transcript. In the present study, we were able to generate dsRNA of CHH1 (432 bp), MIH1 (357 bp) and GIH (336 bp) *in vivo* in bacteria (*E.coli* HT115 (DE3)). Compared to in vitro transcription, *in vivo* generation of dsRNA was more practical and cost effective, mainly for *in vivo* experiments in animals with regard to the functional gene studies.

5.4.2 RNAi as tool for biological assays

The application of dsRNA mediated RNAi studies of CHH1 and MIH1 in *P.monodon* has not been previously reported. dsRNA mediated GIH gene silencing, maturation and spawning in *P.monodon* have been worked out by Treerattrakool *et al*. (2011). The limitation of the work in the field of dsRNA application in *P.monodon*, *Litopenaeus schmitti* (Lugo *et al*., 2006) and Metapenaeus ensis (Tiu *et al*., 2007) was the insufficient information with regard to the CHH1, MIH1, GIH and vitellin hormone levels in the haemolymph. In the present work we give a detailed picture on silencing of CHH family hormone genes and the physiological variations brought in.

The physiological variations detailed are the haemolymph hormone levels of CHH1, MIH1, GIH and vitellin on *in vivo* application of dsRNA specific to CHH1, MIH1 and GIH.

dsRNA mediated silencing of CHH1 had the hypoglycaemic effect within 24 hr of application *in vivo*. The glucose level got significantly decreased by 55.09 % and, concentration reduced to 27.6 ± 3.304 mg dl^{-1} in comparison with the control 1 (61.47 ± 28.75 mg dl^{-1}). The hypoglycemic effect was evident in the haemolymph CHH hormone level, the decrease was 58.52 %, 78.69 % and 50.4 % in comparison with the control1 (buffer), control 2 (ES) and control 3 (L4440). Lugo *et al*. (2006) showed that *L. schmitti* injected with 20 µg of CHH dsRNA into the abdominal cavity significantly reduced the haemolymph glucose concentration by 43 % in 24 hrs.

The MIH 1 gene knockdown effects at the physiological level with regard to haemolymph MIH level has not been reported so far in *P.monodon*, *L. schmitti* and *M. ensis*. The understanding of the changes in the physiological level with regard to the specific hormones gives a better perception on the actual hormone regulation. In the intermoult stage (C-stage) the MIH levels are high as the ecdysteroid level decreases (Nakatsuji and Sonobe, 2003); this stage was chosen to be the appropriate time for silencing of the MIH1 gene. The moult inhibiting effect was observed from the initial phase of 24 hrs, the reduction in MIH levels were observed to be reduced by 41.4 % in comparison with the controls. By 48 hrs (2^{nd} day), the MIH levels were significantly reduced to 61 %. The MIH levels on the 3^{rd} day (72 hrs) and 5^{th} day (96hrs) were also significantly lowered by 56.44 % and 47.52 %. The MIH dsRNA application reduced the C- stage and the total moulting period by 32.43 %. The moulting days of MIH dsRNA injected animals (n= 20) were observed to be 12.5 ± 0.71 days, while that of control injected with buffer was 18.5 ± 0.7 days. On application of dsRNA of MIH in the D2-D3 stage animals, an advancement of moult stages from D2-D3 was observed and the D3 stage moulted immediately without any delay. The modest effect of moult

cycle shortening can also be assumed to be brought by the limitations of other factors involved in moulting. Initially it may be related to duration of the gene silencing effect. The time course study showed that the MIH1 gene knockdown effect lasted only for approximately 2 days and restored on the 3rd day. This restoration of MIH1 reduced the suppression of MIH on moulting. Besides the silencing of MIH1 gene, the MIH neuropeptide already present in the animal might have had initial effect on the moult cycle duration, so that the shortening of moult cycle by RNAi became less obvious (Gu *et al*., 2001). This is the first report on application of dsRNA of MIH1 in attempting to shorten the moult cycle in *P.monodon.*

Treerattrakool *et al*. (2011) showed that injection of 3 µg GIH dsRNA per gram bodyweight in *P.monodon* could silence the GIH gene for a period of 30 days. Their study proved that depletion of GIH expression by GIH dsRNA also increased the Vg mRNA level in the ovary of *P.monodon* (Treerattrakool *et al*., 2008). Reproductive maturation was also achieved in GIH dsRNA injected shrimp; the wild shrimp injected with dsRNA had the spawning rate comparable to that of eyestalk ablated group (Treerattrakool *et al*., 2008). This study could not determine the haemolymph GIH levels due to the unavailability of anti-Pem- GIH antibody. However, in the present work with dsRNA of GIH, we were able to determine the haemolymph GIH levels as well as the haemolymph vitellin concentration. The haemolymph GIH levels were significantly lowered by the 3rd day by 60 % in comparison to control 1. It has to be pointed out that 60-25% % reduced GIH levels could be maintained throughout the experimental period (30 days). GIH is the negative factor in the eyestalk inhibiting the synthesis of Vg (Aguilar *et al*., 1992; Ohira *et al*., 2006). Eyestalk ablation results in increased Vg level in haemolymph, which could be stimulated through gonad stimulating hormone (GSH) released from the thoracic ganglion (Yano, 1992, 1993; Yano *et al*., 1988). This implies that GIH and GSH exert antagonistic functions in controlling Vg synthesis and ovarian maturation. The inhibition or silencing of GIH could have initiated the stimulation

of GSH, which in turn increased the Vg levels in haemolymph. The haemolymph vitellin levels were measured to be significantly higher; a 15.36 % increase in the haemolymph vitellin level was observed.

5.4.3 Functional gene silencing of CHH family hormone genes

The physiological variations observed in the CHH family hormones due to the dsRNA was finally demonstrated at molecular level with semi-quantitative reverse – transcription polymerase chain reaction (RT-PCR). Lugo *et al*. (2006) showed that dsRNA of mature CHH injected into the adult shrimp of *L. schimitti* silenced the specific gene 24 hrs after injection; this was proved with semi-quantitative RT-PCR and northern blot. In the present work 3 μg CHH1 dsRNA per gram bodyweight knocked down CHH1 gene, 24 hrs post injection. The CHH1 expression level was observed to be reduced even 48 hrs post injection. The peak intensity of CHH1 transcript showed a zero expression (significant reduction) during the 24 hrs and a fivefold decrease 48 hrs post injection.

The semi-quantitative RT-PCR results of MIH1 transcript expression demonstrated the effect of silencing beginning from 24 hrs (1st day), with complete silencing by 48 hrs (2nd day). The expression level of the transcript was observed to be low during the 72 hrs (3rd day) and a slow gain from day 5. The expression of MIH1 transcript proved with the peak intensity, showed a fourfold decrease (22.43 %) within 24 hrs and zero expression (significant reduction) by 48 hrs, a definitive silencing of MIH1 gene. The slow gain of two fold increase (41.58 %) from 72 hrs showed the diminishing effect of the MIH1 silencing.

The GIH gene silencing was proved from day 3 with the RT-PCR results. A complete silencing of GIH gene was observed, and the transcript level steadily diminished throughout the experiment. The peak intensity of GIH transcript was reduced by one fold within 24 hrs and zero expression on 3rd day, while a steady decrease of four to seven fold was maintained through the entire experiment. Treerattrakool *et al*. (2008), proved the GIH dsRNA effect in *P.monodon* to be effective

till 5th day of GIH dsRNA administration. In 2011 Treerattrakool *et al*. proved the induced maturation of *P.monodon* with GIH dsRNA application. This unequivocally proves the practicality of using the dsRNA of GIH in maturation and spawning of *P. monodon* under controlled conditions and for its subsequent domestication.

The Vg expression was induced with GIH knockdown in previtellogenic adult *P. monodon* markedly indicated the inhibitory function of Pem-GIH on Vg gene expression in the ovary. The results of Treerattrakool *et al*. (2011) are in concurrence with an increase in Vg expression after eyestalk ablation in *M. japonicus* (Okumura *et al*., 2006, Okumura, 2007) and *L. vannamei* (Raviv *et al*., 2006). It could be proved that GIH dsRNA silencing indeed induced the Vg gene expression.

5.4.4 Correlation: Physiological and molecular aspects

In all the earlier dsRNA studies carried with regard to *P. monodon*, there were no detailed analyses correlating various physiological parameters with those at molecular level. In this study such an attempt has been made.

A positive correlation was obtained between glucose concentration, haemolymph CHH level and peak intensity of CHH1 transcript expression. In the case of MIH1, positive correlation was determined between peak intensity of MIH transcript expression and haemolymph MIH level, but a negative correlation was evident between haemolymph MIH level and haemolymph ecdysteroid concentration. The haemolymph ecdysteroid concentration showed a negative correlation with the MIH transcript expression. Nakatsuji and Sonobe (2003) proved the hypothesis that the increase in MIH levels decreased the ecdysteroid level and vice versa, the negative correlation mentioned between the haemolymph MIH levels and ecdysteroid in the present study once again proved the hypothesis. Haemolymph GIH level, haemolymph vitellin level and peak intensity of GIH transcripts could be correlated. Haemolymph GIH level gave a negative correlation with the haemolymph vitellin

levels. The negatively correlated haemolymph GIH level with haemolymph vitellin concentrations were in accordance with the proven hypothesis that Vg expression increased with eyestalk ablation in *M. japonicus* (Okumura *et al*., 2006, Okumura, 2007).

The physiological and functional response of the animal to the application of dsRNA of respective gene gives a broader picture of the expression of CHH family hormone genes in *P. monodon*. The various parameters dealt with in this chapter are essential for understanding the regulation of CHH family hormone genes in view of its practical application. Another important feature of this piece of work is the *in vivo* production of dsRNA using bacterial system (*E.coli* HT115) that can be efficiently employed to produce large amounts of dsRNA with relatively low cost for RNAi studies. The large amounts required for silencing the specific gene *in vivo* can be effectively produced in this system. The cost for production of large amount of dsRNA can be reduced to one- third in comparison with that in vitro. The generation of dsRNA *in vivo* in bacteria is relatively simple, thus cost effective.

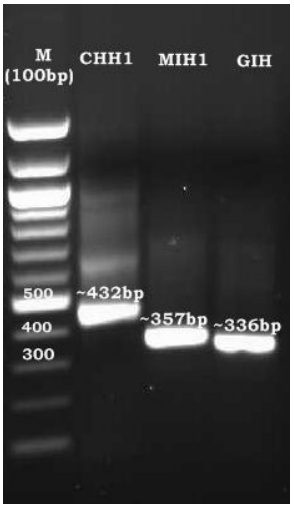


Fig. 3. *EcoR* I-*Xho* I restricted CHH1 and MIH1, *EcoR* I alone restricted GIH

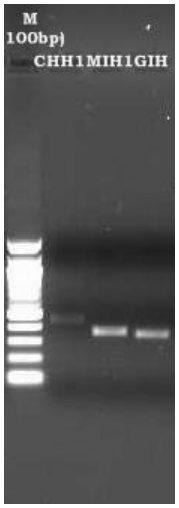


Fig .4. Gel purified CHH1, MIH1 and GIH gene products

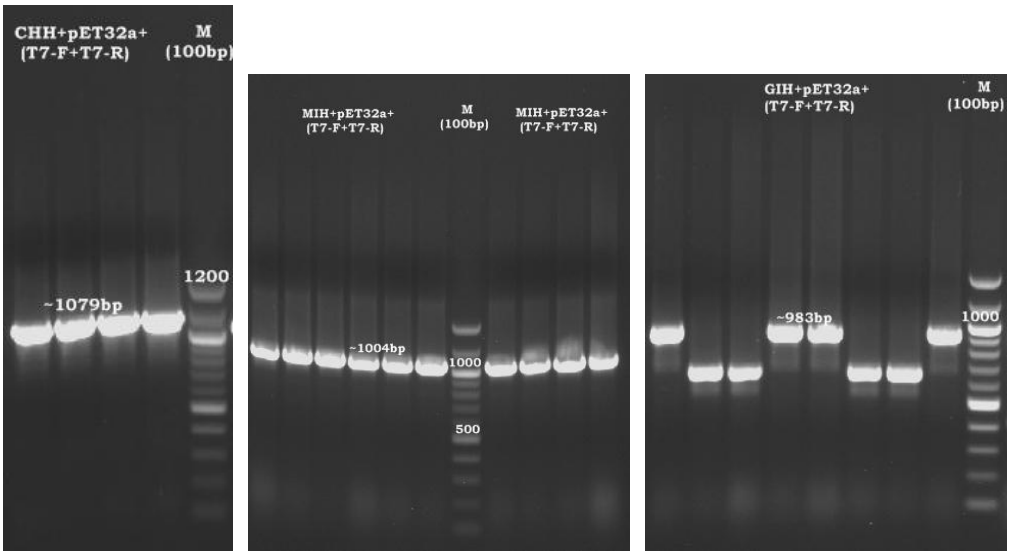


Fig. 5, 6, 7. Colony PCR using vector primers CHH1 (1079 bp), MIH1 (1004 bp) and GIH (983 bp)

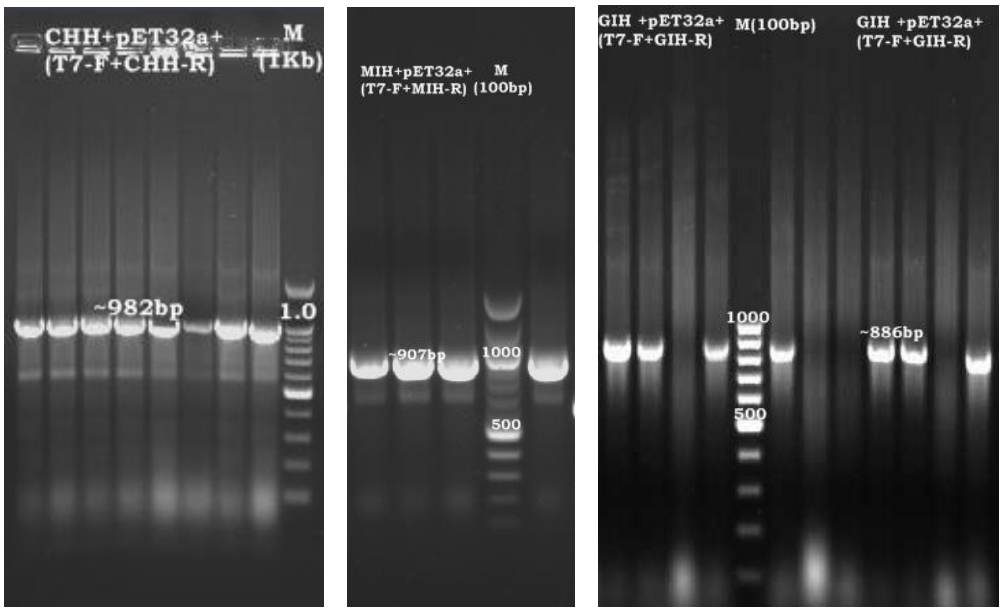


Fig. 8, 9, 10. Insert orientation PCR using forward vector primer and gene specific reverse CHH1 (982 bp), MIH1 (907 bp) and GIH (886 bp).

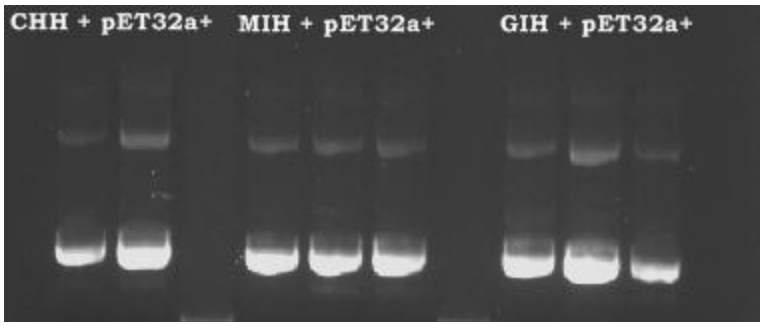


Fig. 11. Positive plasmids of CHH1, MIH1 and GIH in pET32a+

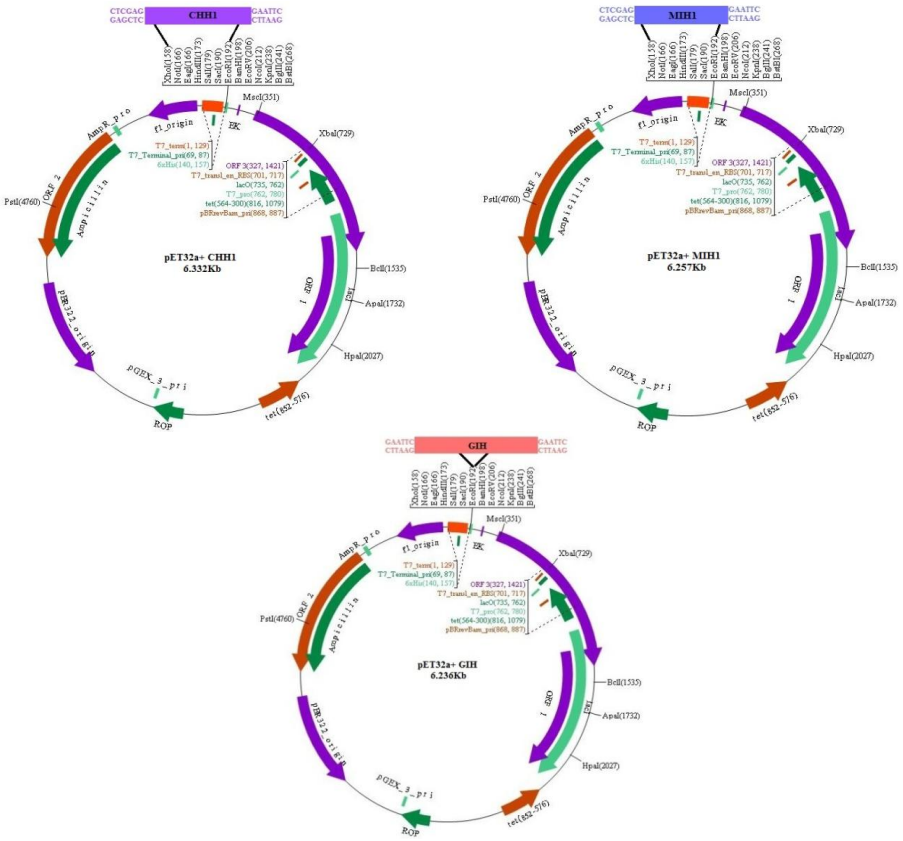
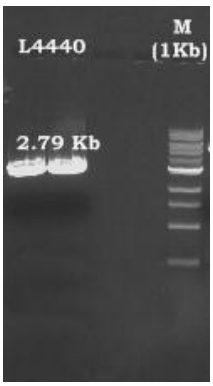
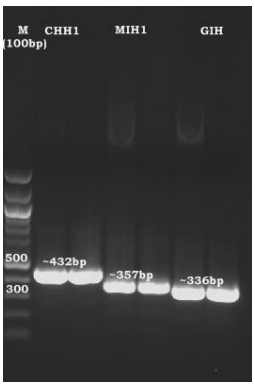
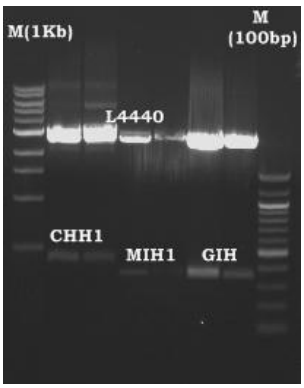


Fig. 12, 13, 14. pET32a+CHH1 (6.332Kb), pET32a+MIH1 (6.257Kb) and pET32a+GIH (6.236Kb)







(Fig. 15) **(Fig. 16)** **(Fig. 17)**

Fig. 15. L4440 restricted *Bgl* II- *Hind* III)

Fig. 16. Colony PCR of CHH, MIH GIH with gene specific primers

Fig. 17. Restriction digestion to and confirm the presence of the insert

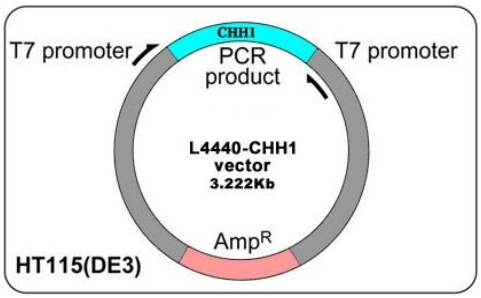


Fig. 18. dsRNA vector constructs were L4440+CHH1 (3.222Kb)

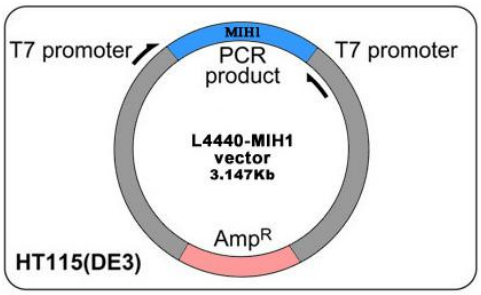


Fig .19. L4440+MIH1 (3.147Kb)

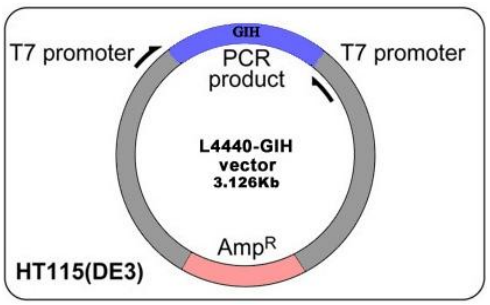


Fig. 20. L4440+GIH (3.126Kb)

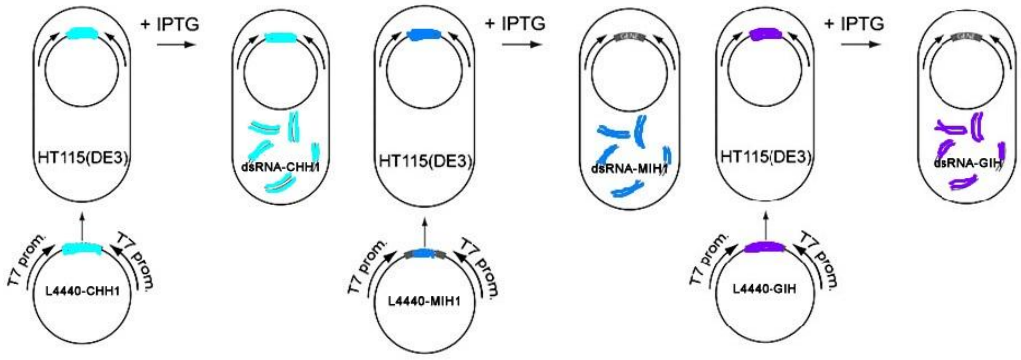


Fig. 21. Induction of L4440 constructs for dsRNA expression of CHH family hormone genes- dsRNA of CHH, MIH and GIH.

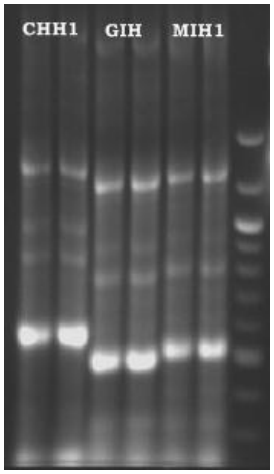


Fig. 22. Total RNA in 2% agarose gel electrophoresis (Untreated)

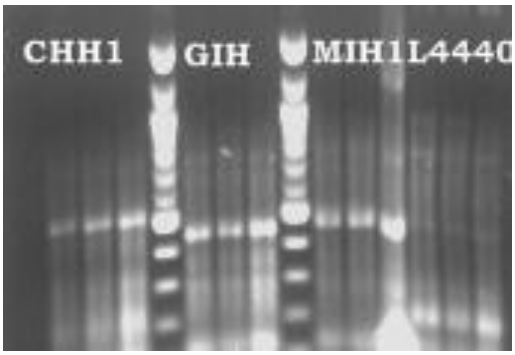


Fig. 23. Total RNA in 2% agarose gel electrophoresis(DNase and RNase treated)

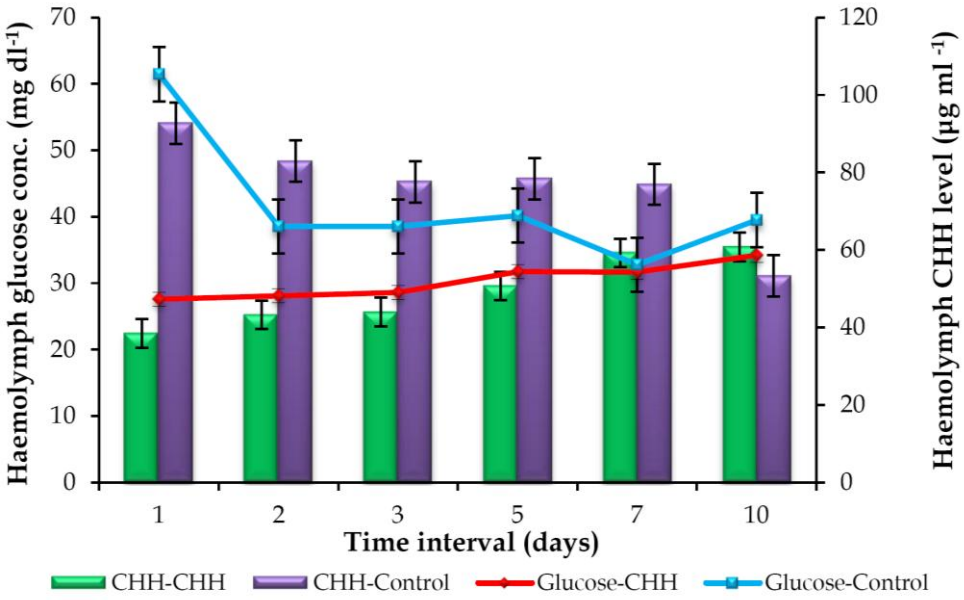


Fig. 24. CHH dsRNA injected group compared with control 1 (buffer) - Comparison of haemolymph glucose and CHH levels

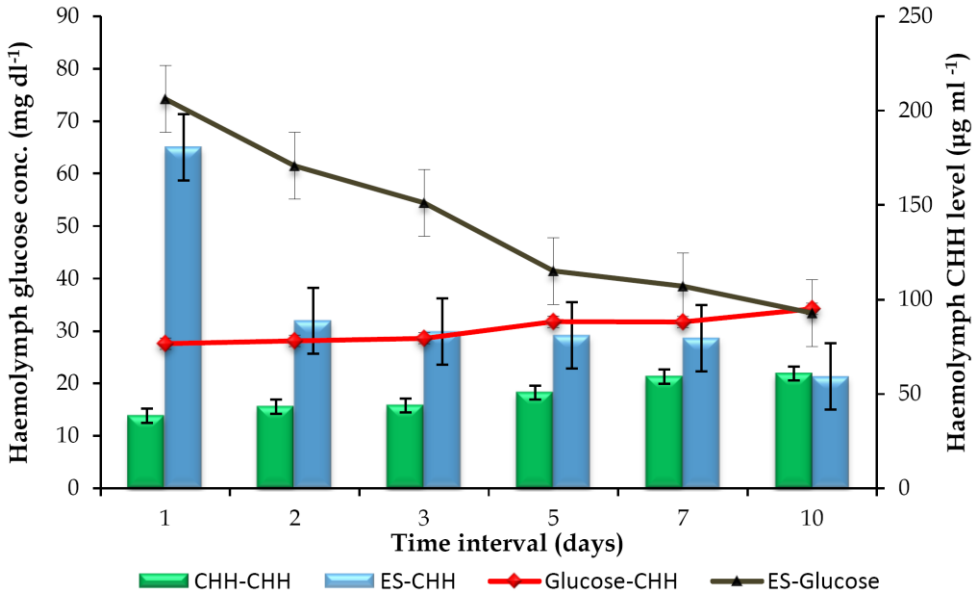


Fig. 25. CHH dsRNA injected group compared with control 2 (ES) - Comparison of haemolymph glucose and CHH levels

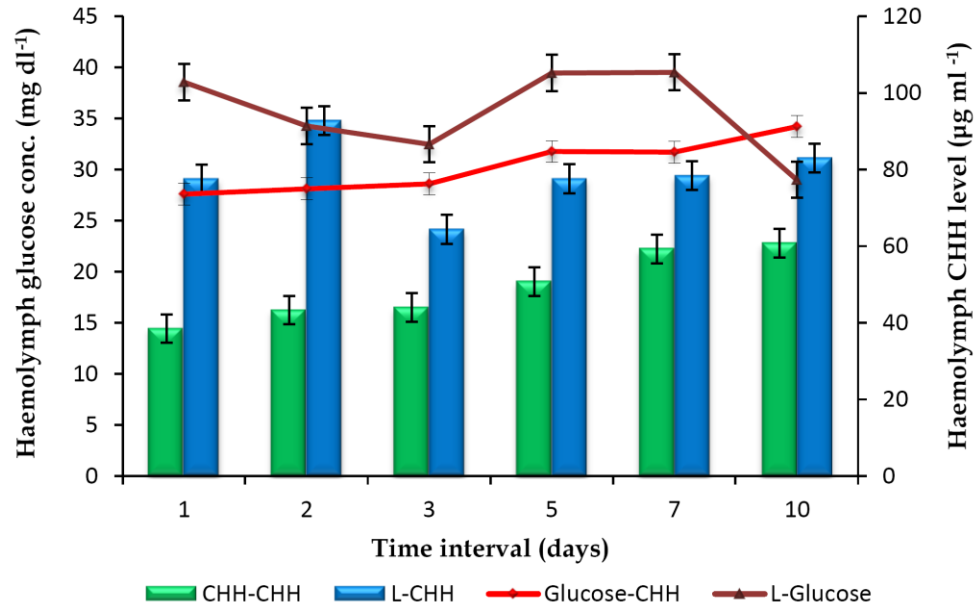


Fig. 26. CHH dsRNA injected group compared with control 2 (L4440) - Comparison of haemolymph glucose and CHH levels

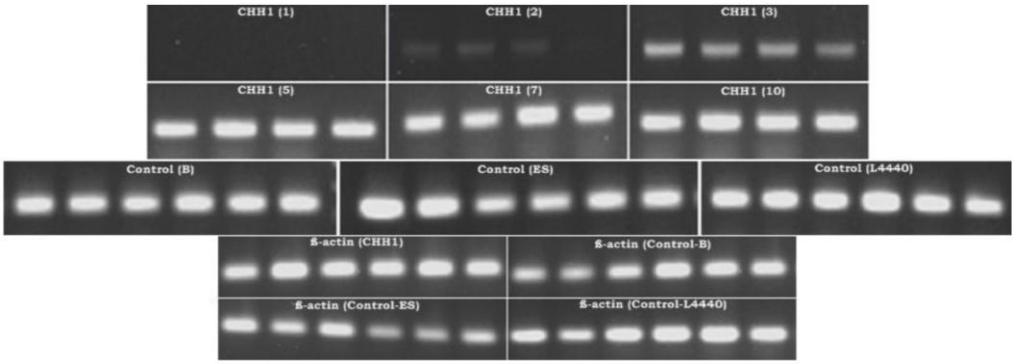


Fig. 27. RT-PCR results of CHH1 silencing

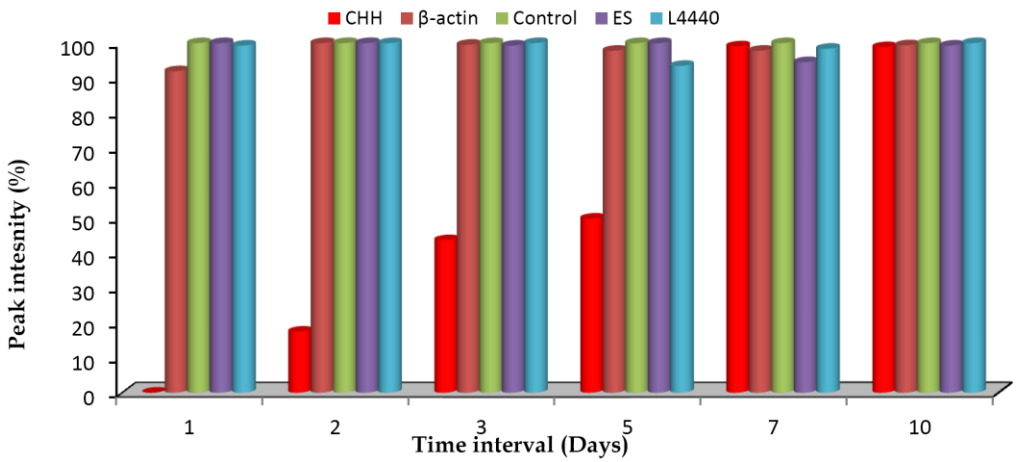


Fig. 28. Peak intensity of CHH1 transcript expression

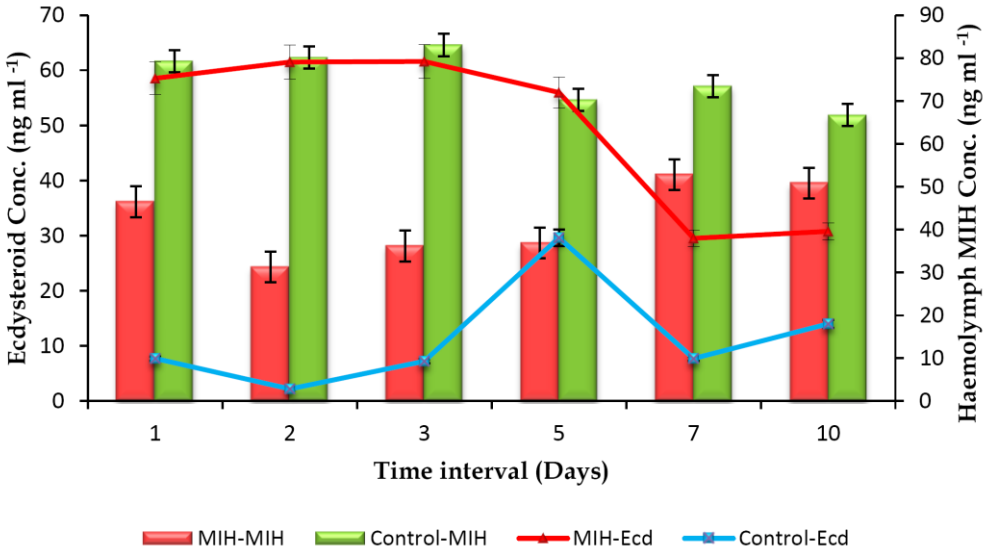


Fig. 29. Haemolymph MIH level of MIH (dsRNA injected) and control 1 (buffer) including ecdysteroid levels.

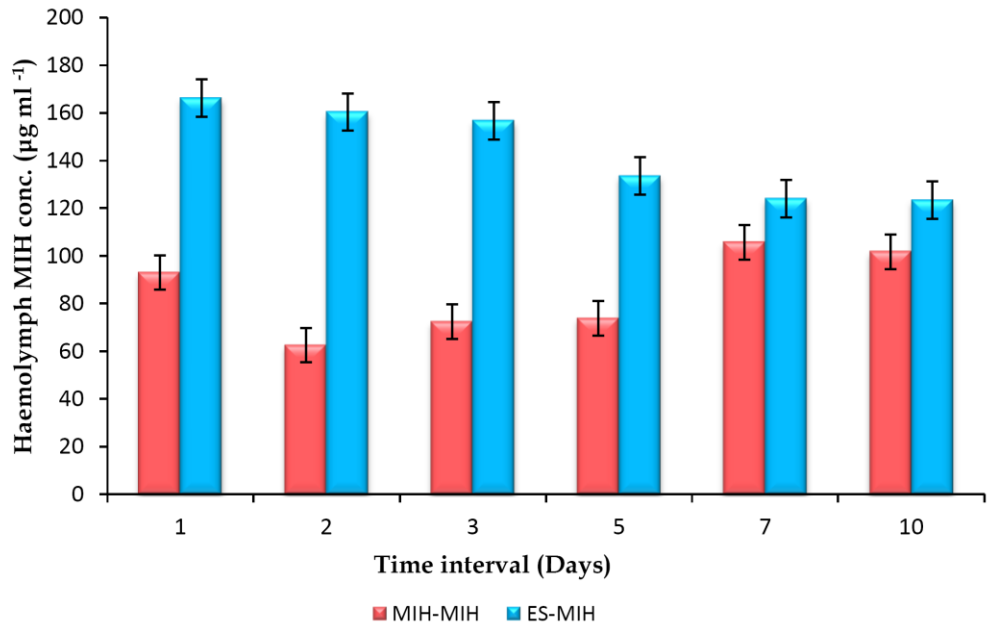


Fig. 30. Haemolymph MIH level of MIH (dsRNA injected) and control ES

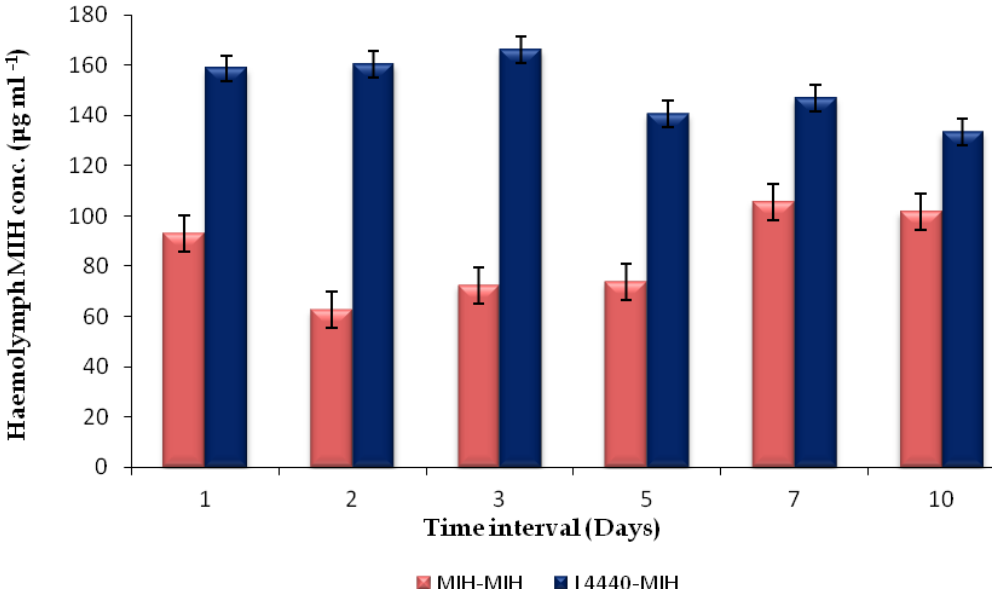


Fig. 31. Haemolymph MIH level of MIH (dsRNA injected) and control L4440

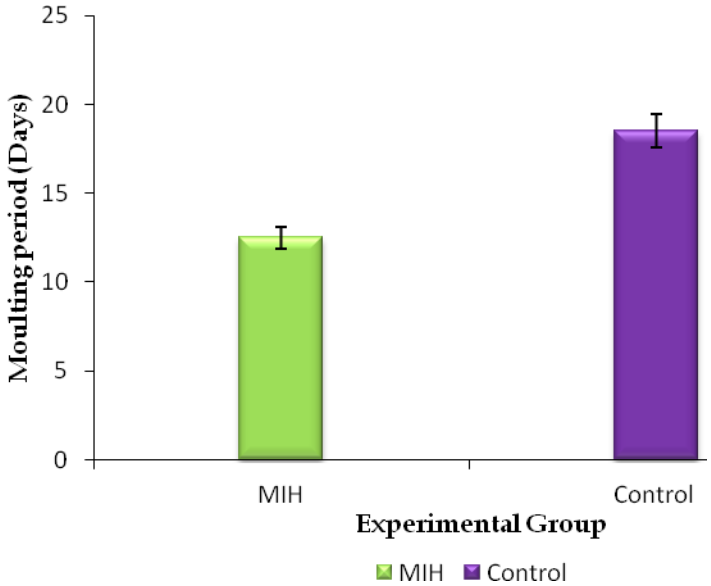


Fig. 32. Moulting duration of MIH (dsRNA injected) and control 1(buffer)

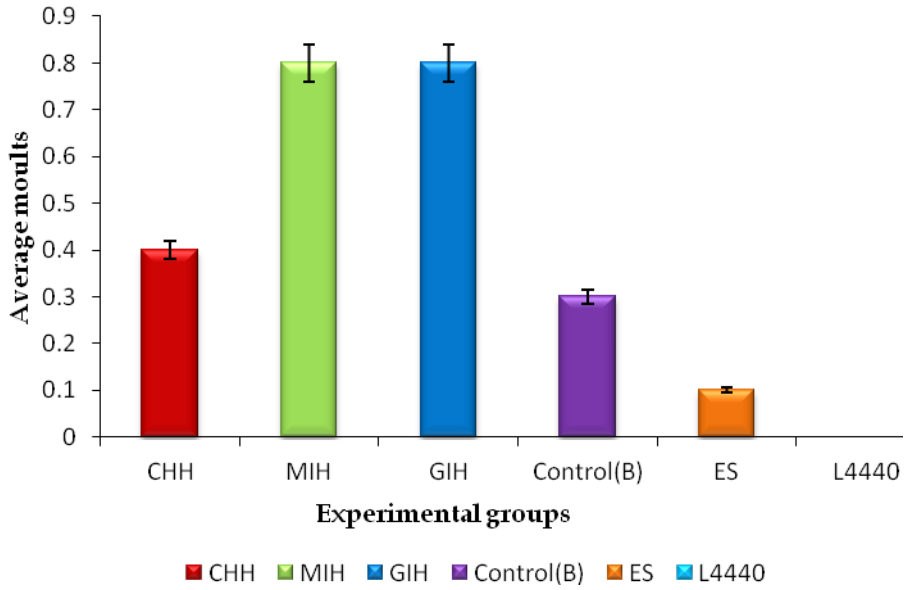


Fig. 33. Average moults of all groups for a period of 10days

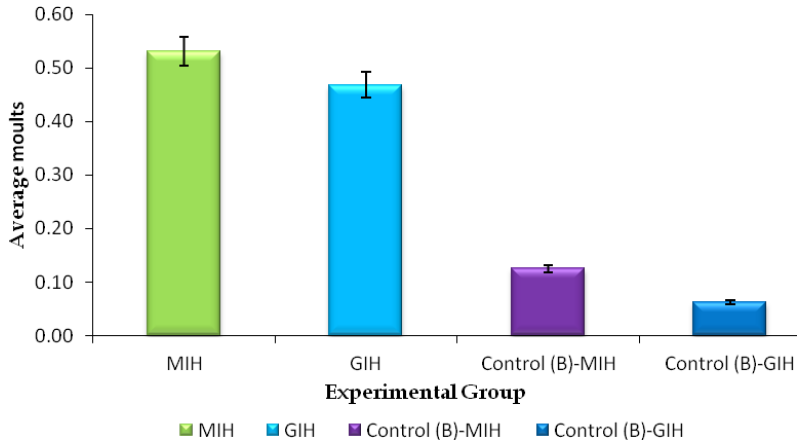


Fig. 34. Average moults per day of MIH and GIH groups in comparison with controls for a period of 32 days

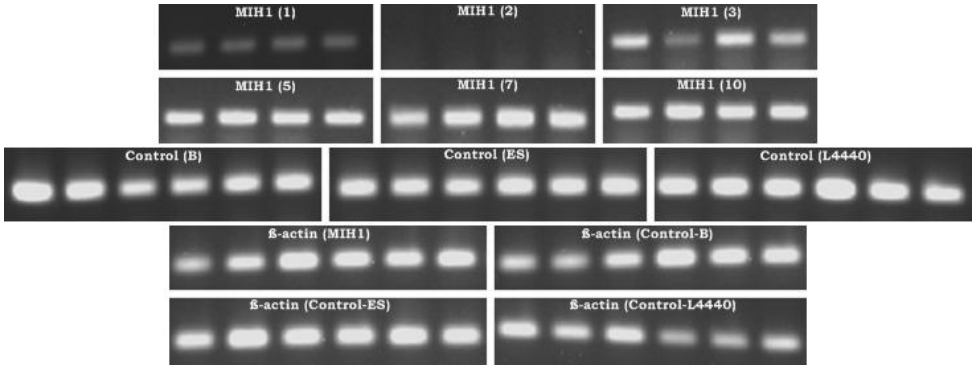


Fig. 35. RT-PCR results of MIH1 silencing

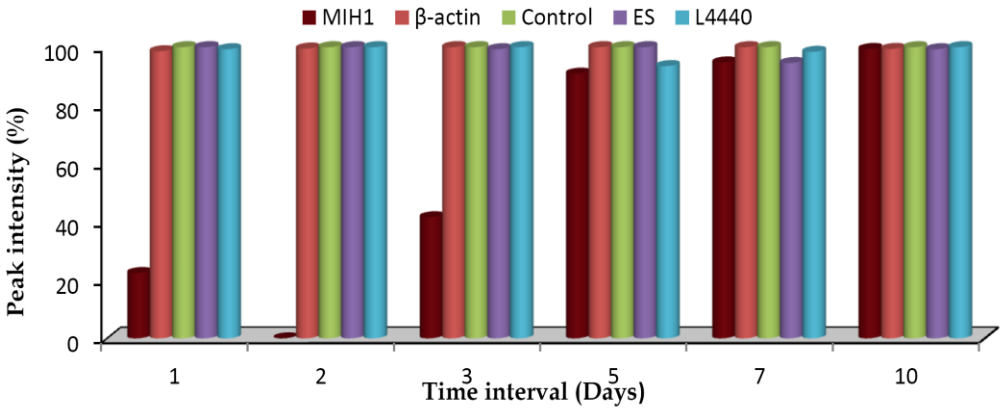


Fig. 36. Peak intensity of MIH1 transcript expression

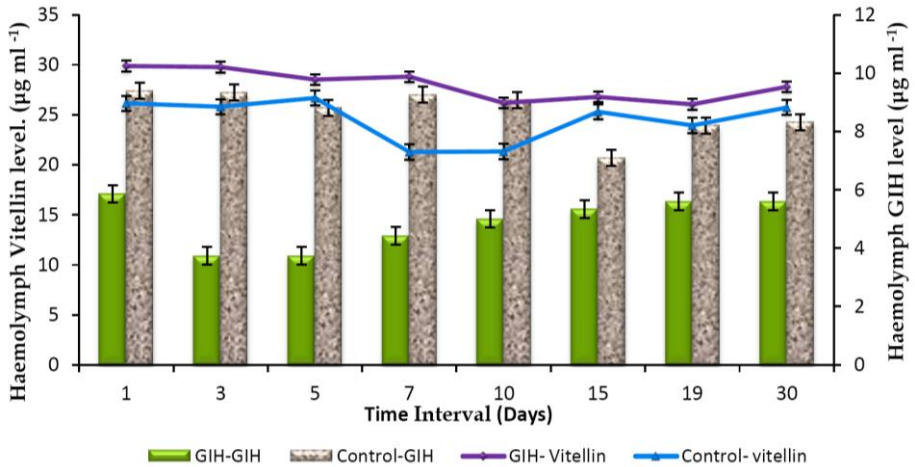


Fig. 37. Haemolymph GIH level of GIH (dsRNA injected) and control 1(buffer) including vitellin levels.

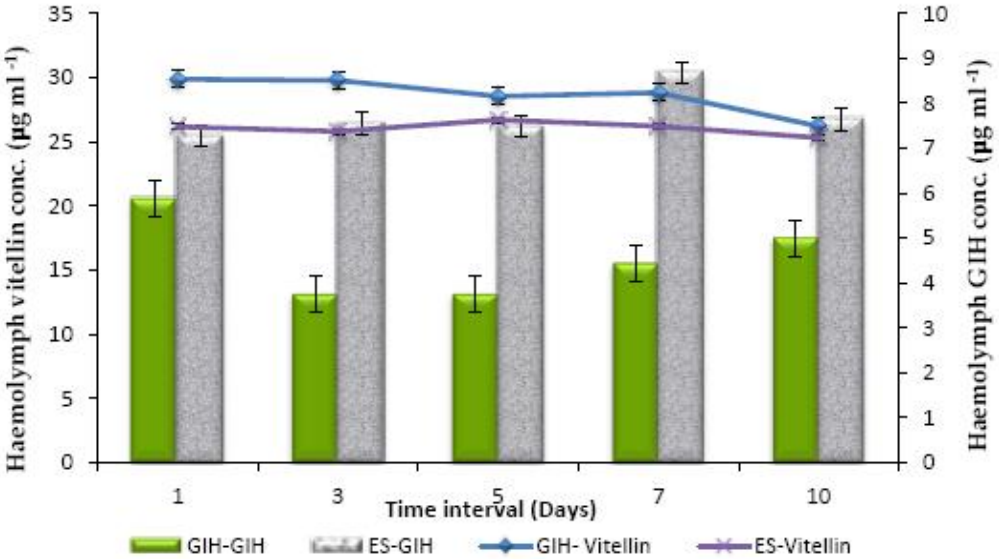


Fig. 38. Haemolymph GIH level of GIH (dsRNA injected) and control ES including vitellin levels.

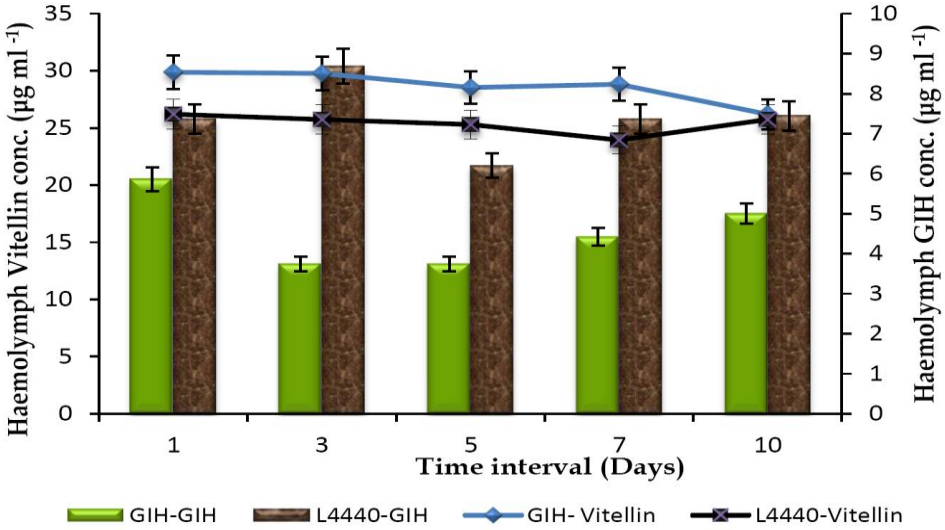


Fig. 39. Haemolymph GIH level of GIH (dsRNA injected) and control L4440 including vitellin levels.

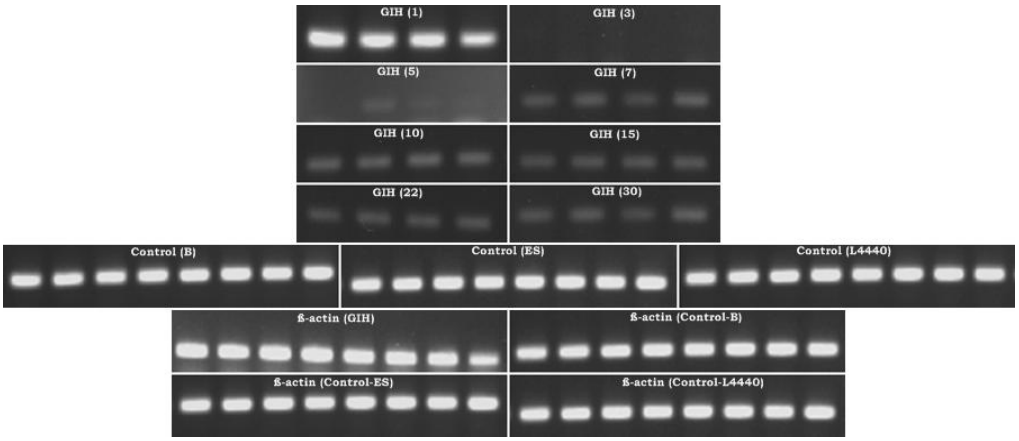


Fig. 40. RT-PCR results of GIH silencing

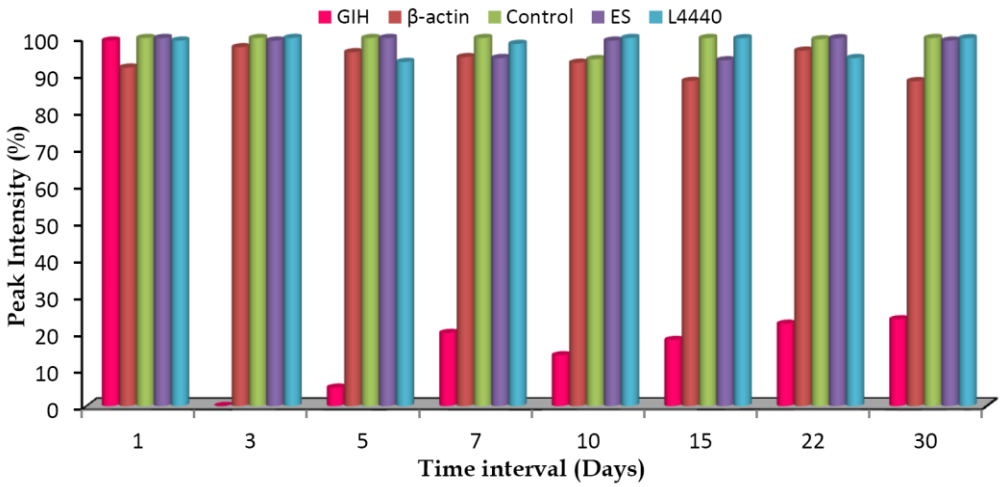


Fig. 41. Peak intensity of GIH transcript expression

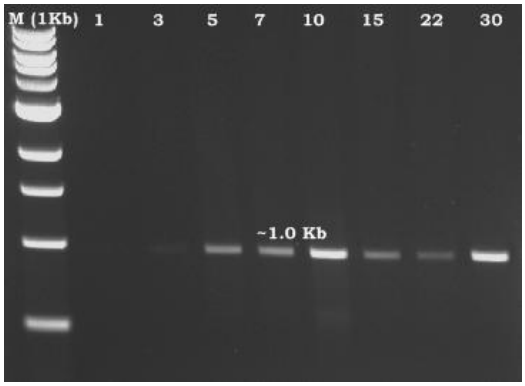


Fig.42. Expression of Vg gene from day 3- day 30

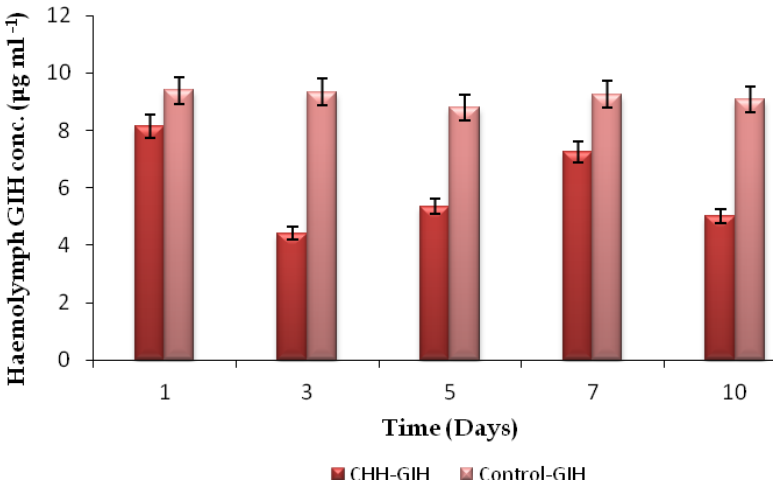


Fig. 43. Haemolymph GIH conc. (µg ml $^{-1}$) in CHH dsRNA injected group in comparison with control 1(Buffer injected)

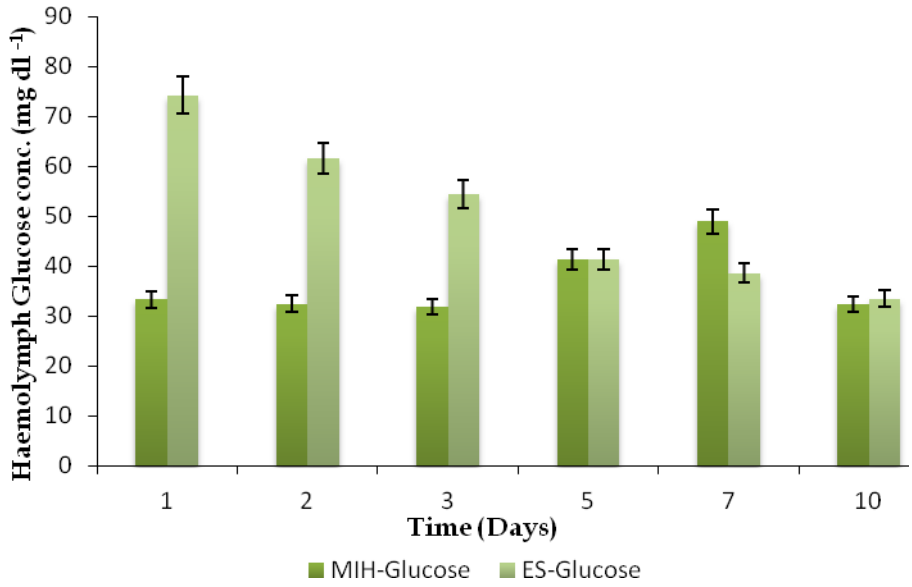


Fig. 44. Haemolymph glucose conc. (mg dl $^{-1}$) in MIH dsRNA injected group in comparison with control 2 (ES injected)

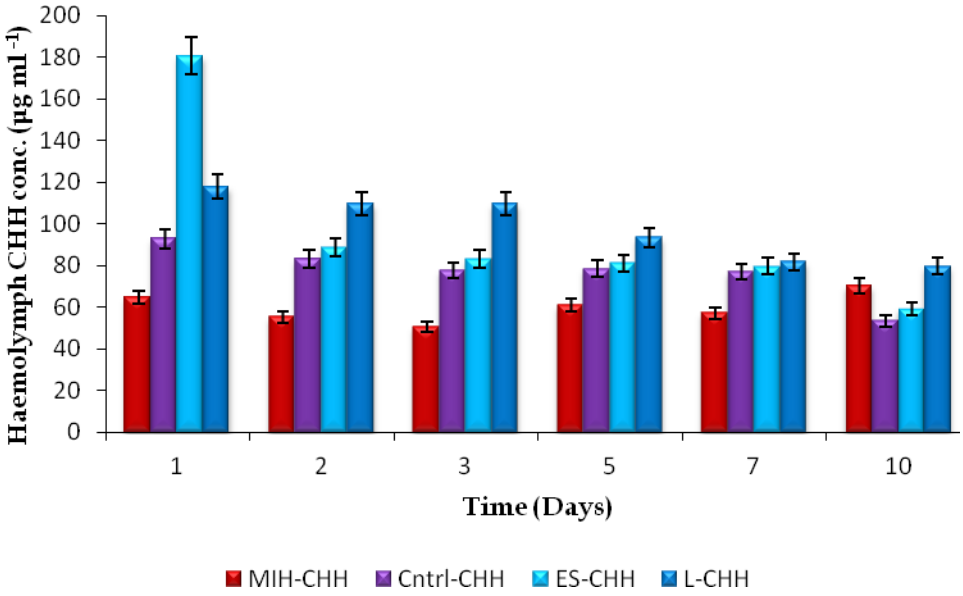


Fig. 45. Haemolymph CHH conc. (µg ml $^{-1}$) in MIH dsRNA injected group in comparison with control 1(buffer injected), control 2 (ES injected), control 3(L4440 injected).

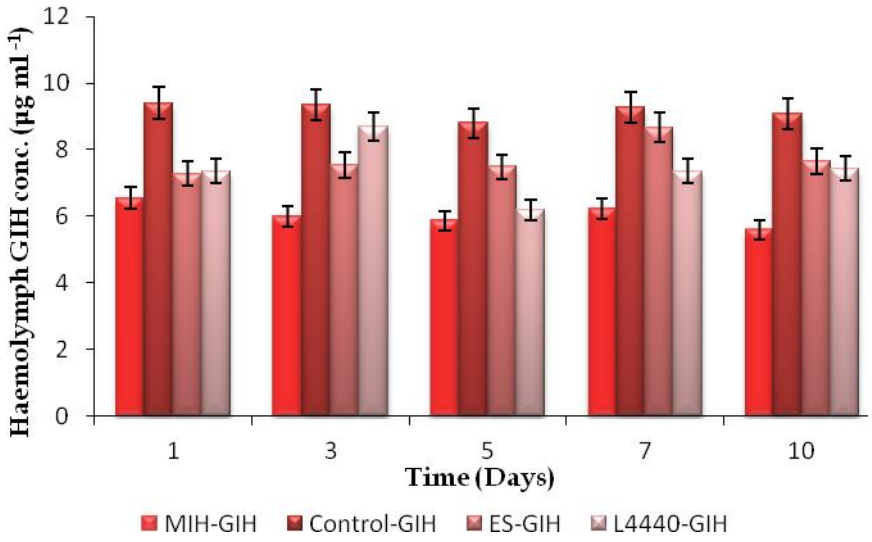


Fig. 46. Haemolymph GIH conc. (µg ml $^{-1}$) in MIH dsRNA injected group in comparison with control 1 (buffer injected), control 2 (ES injected) and control 3(L4440 injected).

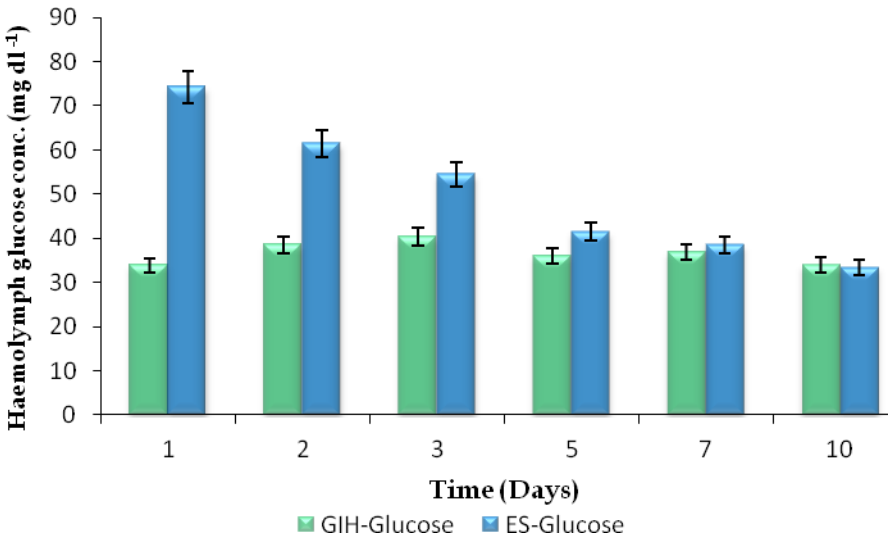


Fig. 47. Haemolymph glucose conc. (mg dl $^{-1}$) in GIH dsRNA injected group in comparison with control 2 (ES injected).

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Chapter

6

SUMMARY AND FUTURE PROSPECTS FOR RESEARCH

About 80 years ago, the neurosecretory eyestalk structures and their role in endocrine regulation was recognized in crustaceans. After the recognition it took half a century to identify the first peptide hormone. Till date a large number of homologous peptides of crustacean hyperglycaemic hormone and moult-inhibiting hormone have been identified, consequently they are called the CHH family hormones. This family comprises of highly multifunctional peptides which according to sequences and precursor structures can be divided into two subfamilies, type-I (CHH/ITP) and II (MIH, MOIH, VIH/GIH) (Webster *et al*., 2012). The XO-SG complex has been the major site of the two subfamilies. The advent of molecular techniques resulted in the characterization of different precursors of CHH, MIH and GIH; these hormones consist of a signal peptide, but only the preprohormone of CHHs contain a precursor- related peptide (CPRP) located between the signal and the mature hormone (Weidemann *et al*., 1989; Klein *et al*., 1993b; De Kleijn and Van Herp, 1995). The essentialities of the gene structure comply with the functions of the CHH family hormones. The CHH family hormone functions are inhibitory as well as stimulatory in the process of reproduction and maturation.

Crustaceans in general have a very complex physiology due to the overlapping influence of multiple processes and multiple actions of hormones, mainly the CHH family (Fanjul-Moles, 2006). In chapter 1, a broad picture of crustacean physiology and endocrine functions from the available scattered research findings has been

provided. Efforts have been made to give an insight into the crustacean hyperglycaemic hormone (CHH) structure, functions and role in the physiology of crustaceans in general. Likewise moult-inhibiting hormone (MIH) and gonad inhibiting hormone (GIH) were given equal importance. The review revealed that there were insufficient research findings with regard to the gonad inhibiting hormone and its role in the physiology of crustaceans. It could be established that the structurally related neuropeptides of the CHH family controlled a diverse array of physiological processes in the crustaceans including carbohydrate metabolism, moulting, reproduction and hydromineral balance. Besides establishing the facts of neurohormones in the crustacean physiology, still little is known of the exact physiological relevance of these neurohormones and their neurochemistry.

Molecular techniques are the most important tool in the field of characterization, structural relativity and expression studies with regard to crustacean neurohormones, in particular the CHH family genes. Efforts were made to understand the regulatory mechanisms of three hormones of the CHH family with the application of various molecular tools as described in Chapter 2. Considering molecular aspects involved in sequence analysis and homology modelling, primers were designed and cloning strategy in pGEM-T easy vector as an attempt to identify the gene structure and the conservation of the sequence homology between the three genes (CHH, MIH and GIH) have been built. The structural similarity of the CHH family genes and the presence of small quantity of the neurohormone make it difficult for the isolation of purified CHH family neuropeptides from eyestalk; lead to the use of recombinant DNA technology. Recombinant DNA technology, a simple technique of fusion of the target gene fragment to the translation vector to express large fusion proteins, saves hundreds of thousands of eyestalks required for the isolation of purified CHH family hormones. The mature peptide region of the CHH family hormones were successfully cloned in to recombinant translation expression vector (pET32a+) for producing large quantities of fusion proteins (rCHH1, rMIH1 and rGIH) in *E.coli* (DE3) BL21 pLysS.

Expression of bacteria is the simplest and fastest method to acquire these proteins. The functional roles of the recombinant CHH family hormones have been proved by the *in vivo* studies in *P. monodon*. .

Hormone expression study of the three inhibitory hormones of CHH family at the various stages of the developmental cycle (both life and reproductive) can give an insight into the neurological and endocrinological regulations. This is promising only with the availability of specific antibodies of the CHH family hormones. In chapter 3 the development of polyclonal antisera raised against the three recombinant hormones of CHH family expressed in a bacterial system and the development of ELISA have been detailed. Besides the hormone expression study, the antibodies (anti- rCHH1, anti - rMIH1 and anti - rGIH) are useful in locating the peptides (CHH, MIH and GIH) in the eyestalk of *P.monodon*. With the three antisera of CHH family hormones, immunostaining revealed strong signals of CHH and MIH, while faint signals were observed in GIH in the eyestalk of *P. monodon*. The functional action of the antibodies of CHH family was proved by the *in vivo* administration in *P.monodon*. We were able to develop an ELISA successfully for the three hormones of the CHH family.

Another phase of molecular intervention which was undertaken was the RNAi in *P. monodon*. In Chapter 4 study of the application of *in vitro* generated dsRNA of CHH, MIH and GIH have been detailed. It could be proved that CHH and MIH dsRNA generated from the genomic DNA was equally functional to that of dsRNA of GIH generated using cDNA. The functional gene silencing and physiological characteristics covered in Chapter 4 proved the effects of *in vitro* generated dsRNA. In chapter 5, to reduce the expenses of developing *in vitro* dsRNA in large amounts for RNAi studies, a cost effective way of *in vivo* generation of dsRNA in *E.coli* system has been described. The *in vivo* generated dsRNA is definitely an alternative to produce large amounts of dsRNA for *in vivo* injection studies at low cost in *P.monodon*. Precisely, it could be able to achieve

the recombinant proteins of CHH family hormone genes and their antagonists as well as in *vitro* and *in vivo* generated dsRNA and its application. Conclusively the thesis has been divided into the following chapters under the headings:

1) Recombinant/fusion Protein expression of CHH family hormone genes: Application, detection and *in vivo* bioassay

2) Antagonists (polyclonal) against the mature recombinant protein of CHH family hormone genes: *In vivo* bioassay and ELISA tool development

3) *In vitro* double-stranded RNA mediated eyestalk neuropeptide (CHH1, MIH1 and GIH) gene knockdown in the shrimp *Penaeus monodon*

4) *In vivo* generated Double-stranded RNA mediated eyestalk neuropeptide (CHH, MIH and GIH) gene knockdown in the shrimp *Penaeus monodon*.

The concise description of the overall achievements is as given below:

- The homology structures modelled with 1J0T as template showed considerable sequence identity and similarity, 33.78 % (CHH1), 96.15 % (MIH1) and 70.51 % (GIH).
- The six cysteine residues are relatively at the same positions (Cys8 and Cys45, Cys25 and Cys41 and Cys28 and Cys54) allowing the formation of the three intramolecular disulphide bonds that provide strong stabilization of the 3-D structure.
- The three inhibitory hormone genes (CHH1, MIH1 and GIH) were cloned and sequenced in expression translation vector pET32a+. Three

expression translation vector constructs could be built successfully for the production of recombinant/fusion proteins *viz*., rCHH1 (29.47 Kda), rMIH1 (29.85Kda) and rGIH (32.16Kda) in *E.coli* BL21 (DE3) pLysS system by IPTG induction.

- Injection of rCHH1 could elevate the glucose level in the haemolymph, rMIH retarded the moulting duration and rGIH slightly enhanced the levels of GIH. These results indicated that the recombinant proteins developed possessed the neurohormonal functions.
- The injection of 4 µg (140.5 pmoles) rCHH produced hyperglycaemic effect similar to that of the eyestalk extract. The hyperglycaemic effect was taking into action a little from 0.5 hrs (59%) of injection and reached maximum at 1.5hr (~100%) and the effect drained off slowly after 2 hrs.
- In the shrimps injected with rMIH, the moult cycle was significantly delayed 16.67 ± 1.03 days in comparison to the control (11.67 ± 1.03 days). As observed the inhibition in moulting at a concentration of 1.30x 10^{-6}M (rMIH1) was 42%.
- Development of recombinant GIH (0.425x 10^{-6}M) of *P. monodon* has been accomplished for the first time in a bacterial system. The rGIH developed could significantly increase the level of GIH. The haemolymph GIH was increased by 10% in comparison with the control. The concentration of rGIH applied was 50 ng ml^{-1}.
- The titre of the polyclonal antisera of CHH1, MIH1 and GIH obtained was 1:4, 09,600.
- The specificity of the antibody was tested by Western blot analysis: the anti CHH1, anti MIH1 and anti GIH antibodies recognized the antigen rCHH1, rMIH1 and rGIH. The Western blot results confirmed the detection of purified recombinant protein and the same in the

supernatant of lysis 2 extracts of the induced *E.coli* BL21 (DE3) pLySs.

- The sensitivity of anti-CHH1, anti-MIH1 and anti-GIH was determined by indirect ELISA and dot blot analysis where 0.00005 ng ml $^{-1}$ to 50 ng ml $^{-1}$ of rCHH1, rMIH1 and rGIH could be detected by 1:50,000 dilutions of the antibodies from the mice antisera.

- 1:500 dilution of antiCHH1 antibody decreased the haemolymph glucose level 0.5 hr post injection. The glucose level was observed to decrease from 21.60 ±5.52 mg dl^{-1} (0.5hr) and continued to diminish and reached at 17.65 ± 3.49 mg dl^{-1} (1.5hr). Meanwhile,in the controls injected with the serum of mice (PBS injected) the glucose concentration remained at 38.91 ± 5.83 mg dl^{-1} (1.5hr) showing a significant (<0.05) hypoglycemic effect of anti-CHH1.

- The shrimps injected with MIH antibody (1: 500 dilutions) exhibited a moult duration that was significantly reduced to 8.33 ± 0.82 days compared to 11.67 ± 1.03 days of control. The MIH concentration in the haemolymph showed a significant decrease (1.8 %) in comparison with the control.

- A significant decrease of 30 % was observed in the haemolymph GIH level on administering anti- GIH (1: 500 dilutions).

- The three antibodies of CHH family hormones upon immunostaining revealed strong signals of anti-CHH and anti-MIH, while faint signals were observed in anti-GIH in the eyestalk of *P.monodon.*

- The DNA from haemolymph was used for PCR amplifications of CHH1 gene (0.801 Kb) and MIH1 (0.795 Kb) gene**,** while GIH (0.316 Kb) was from cDNA template and the genes were cloned in TA cloning vector (pGEM-T easy).

- The sequence analyses of the DNA based nucleotide sequence of CHH1 resulted in 99 % identity with Pem-CHH1 mRNA (AF233295.1) and *P.monodon* CHH1 cds (98%) (AY346378). MIH1 sequence showed 97 % identity with *P.monodon* MIH1 cds (AY496454). The cDNA based nucleotide sequence of GIH had 99% identity to GIH mRNA of *P.monodon* (DQ643389.1).
- Functional gene silencing of CHH1 (~500 bp) gene was evident during the initial phase (24 hrs), while MIH1 (~450 bp) expression was weak from 24 hrs and completely silenced by 60^{th} and 108^{th} hours. GIH functional gene signals were weak from 24 hrs and undetectable by 60^{th} hour.
- The administration of MIH1 dsRNA was investigated on the expression of MIH2, an isoform of MIH1 gene. It was observed that the gene silencing pattern followed that of MIH1 gene. The expression of MIH2 was weak from 24 hrs and completely silenced by 60^{th} and 108^{th} hours.
- The functional knockdown of GIH expression induced the vitellogenin gene expression in animals which received the dsRNA of GIH. The expression was slightly visible by 60^{th} hour.
- CHH1 dsRNA (20 µg) administered animals showed significant decrease in glucose concentration (23.48 ± 14.88mg dl^{-1}) against the control (95.73 ± 33.40 mg dl^{-1}) ($P \leq 0.01$).
- The moulting was suppressed significantly by 18.87 % ($P < 0.005$) by MIH1 dsRNA.
- For the *in vivo* generation of specific dsRNA, three dsRNA expression vectors (L4440+CHH1 (3.222Kb), L4440+MIH1 (3.147Kb) and L4440+GIH (3.126Kb) were successfully constructed.

- Using this approach approximately 1.5-2 mg of dsRNA from a 50 ml bacterial culture after digestion with RNase A could be obtained.
- dsRNA mediated silencing of CHH1 had the hypoglycemic effect within 24 hr of application *in vivo*. The glucose level was significantly decreased by 55.09 %.
- The hypoglycemic effect was evident in the haemolymph CHH hormone level, the CHH concentration obtained was 38.47 μg ml^{-1} compared to 92.74 μg ml^{-1}.
- A quantity of 3 μg CHH1 dsRNA per gram bodyweight knocked down CHH1 gene, 24 hrs post injection. The peak intensity of CHH1 transcript showed a zero fold expression (significant reduction) during the 24 hr post injection.
- The MIH dsRNA application reduced the C- stage and the total moulting period by 32.43 %.
- The moult inhibiting effect was observed from the initial phase of 24 hrs, the reduction in MIH levels were 46.47 ng ml^{-1} in comparison with the controls (79.30 ng ml^{-1}). By 48hrs (2^{nd} day), the MIH levels were significantly reduced to 31.27 ng ml^{-1}, while the controls remained at 80.16 ng ml^{-1}.
- The semi-quantitative RT-PCR results of MIH1 transcript expression demonstrated the effect of silencing beginning from 24hrs (1^{st} day), with complete silencing by 48 hr (2^{nd} day).
- The expression of MIH1 transcript proved with the peak intensity, showed a four fold decrease (22.43 %) within 24 hrs and zero fold expression (significant reduction) by 48 hr, a definitive silencing of MIH1 gene.

- The haemolymph GIH levels were significantly lowered by the 3rd day (3.74 µg /ml^{-1}) in comparison to control (29.77 µg ml^{-1}), recording a 27.06% reduction in the haemolymph GIH level. A reduction of 20-22% GIH was maintained throughout the experimental period (30days).
- The GIH gene silencing was proved from day 3 with the RT-PCR results. A complete silencing of GIH gene was observed, and the transcript level steadily diminished throughout the experiment.
- The peak intensity of GIH transcript was reduced by one fold within 24hrs and zero expression on 3rd day. A steady decrease of four to seven folds was maintained through the entire experiment.
- The Vg expression was induced with GIH knockdown in previtellogenic adult *P. monodon* markedly indicating the inhibitory function of Pem-GIH on Vg gene expression in the ovary.

Scope for future studies of molecular approach in crustacean endocrinology

- The ELISA developed for the antagonists (antibody) of CHH family neurohormone can be utilized for investigating the hormone levels at various stages of the life cycle of *P.monodon* and also other decapod crustaceans.
- The low cost dsRNA generated *in vivo* in bacteria can be applied in brooders for induced maturation and spawning at a large scale.
- The antibodies developed can be used to study the CHH family hormone levels in white spot infected or other disease infected prawns; this will give an idea of hormonal variations at the time of viral or bacterial infections.
- A precise technology of induced maturation and domestication of *P. monodon* can be accomplished applying the molecular tools such as

recombinant hormone antagonists and the dsRNA of the CHH family hormone genes.

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GENBANK SUBMISSIONS

Penaeus monodon crustacean hyperglycemic hormone 1 (CHH1) mRNA, complete cds

GenBank: GQ221085.1

LOCUS GQ221085 479 bp mRNA linear INV 01-JUL-2009

DEFINITION Penaeus monodon crustacean hyperglycemic hormone 1 (CHH1) mRNA, complete cds.
ACCESSION GQ221085 REGION: 1..479
VERSION GQ221085.1 GI: 24219978
SOURCE Penaeus monodon (black tiger shrimp)
ORGANISM Penaeus monodon
Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Crustacea; Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Penaeus.
REFERENCE 1 (bases 1 to 479)
AUTHORS Vrinda,S., Seena,J., Rosamma,P. and Bright Singh,I.S.
TITLE Cloning, expression and antagonists of crustacean hyperglycemic hormone (CHH1) of Penaeus monodon
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REFERENCE 2 (bases 1 to 479)
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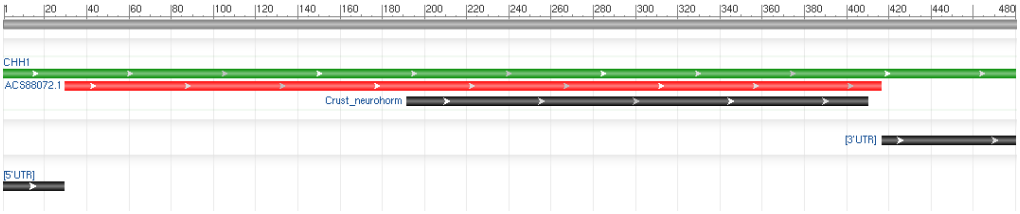


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      361 tccggcctcg tcaaaggaac cagtaccggg ccgccctaca gaggctcggc aagtaggtgg
      421 ttcctcttca gccagacctc gccatgcgac tcccaagacg accagactct tattaggtt
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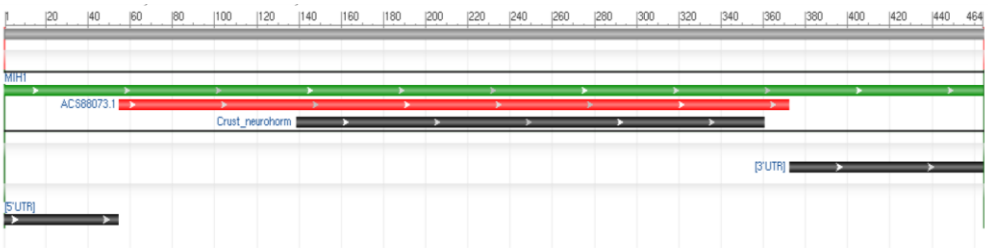
Penaeus monodon molt-inhibiting hormone 1 (MIH1) mRNA, complete cds
GenBank: GQ221086.1

LOCUS	GQ221086 464 bp mRNA linear INV 01-JUL-2009
DEFINITION	Penaeus monodon molt-inhibiting hormone 1 (MIH1) mRNA, complete cds.
ACCESSION	GQ221086
VERSION	GQ221086.1 GI:242199783
KEYWORDS	.
SOURCE	Penaeus monodon (black tiger shrimp)
ORGANISM	Penaeus monodon Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Crustacea; Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Penaeus.
REFERENCE	1 (bases 1 to 464)
AUTHORS	Vrinda,S., Seena,J., Rosamma,P. and Bright Singh,I.S.
TITLE	Cloning, expression and antagonists development of molt-inhibiting hormone 1 of Penaeus monodon
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 464)
AUTHORS	Vrinda,S., Seena,J., Rosamma,P. and Bright Singh,I.S.
TITLE	Direct Submission
JOURNAL	Submitted (24-MAY-2009) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

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      121 tttgacaccg cctcggccag tttcatagac ggcacttgtc gaggcgtaat gggcaatcgt
      181 gacatttaca agaaggtagt gcgtgtgtgt gaggattgca ccaatatctt ccgacttcca
      241 ggcttggacg gcatgtgcag agatcggtgc ttctacaacg aatggttcct gatttgtcta
      301 aaggctgcca acagggagga cgagatcgaa aaattcaaag tttggatcag catcctgaac
      361 gccggtcagt gaggtcgaac gcgacaggac tccttccact tgcaaggcct cgctcccgca
      421 ggccagtacc gacgacactt ggcgactaat gaaccataga ccca
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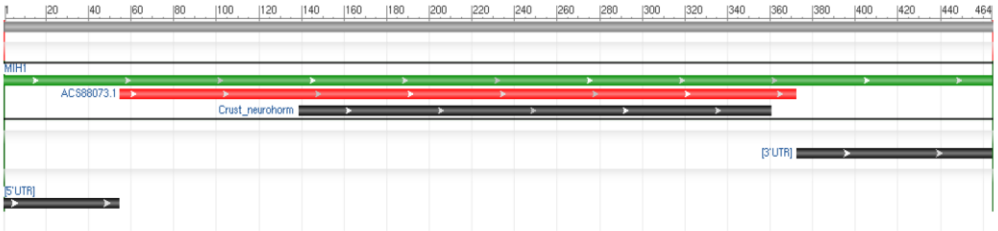
Penaeus monodon gonad inhibiting hormone (GIH) mRNA, complete cds

GenBank: GQ228835.1

LOCUS	GQ228835 322 bp mRNA linear INV 18-JUL-2009
DEFINITION	Penaeus monodon gonad inhibiting hormone (GIH) mRNA, complete cds.
ACCESSION	GQ228835
VERSION	GQ228835.1 GI:253750635
KEYWORDS	.
SOURCE	Penaeus monodon (black tiger shrimp)
ORGANISM	Penaeus monodon Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Crustacea; Malacostraca; Eumalacostraca; Eucarida; Decapoda; Dendrobranchiata; Penaeoidea; Penaeidae; Penaeus.
REFERENCE	1 (bases 1 to 322)
AUTHORS	Vrinda,S., Seena,J., Rosamma,P., Reynold,P., Vijayan,K.K. and Bright Singh,I.S.
TITLE	Cloning and expression of gonad inhibiting hormone (GIH) of Penaeus monodon in Pichia pastoris
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 322)
AUTHORS	Vrinda,S., Seena,J., Rosamma,P., Reynold,P., Vijayan,K.K. and Bright Singh,I.S.
TITLE	Direct Submission
JOURNAL	Submitted (02-JUN-2009) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

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      121 gtttgcgagg actgcaccaa tatctaccgg ttaccacagc tggatggctt gtgcaaaaat
      181 cgatgcttca ataaccagtg gttcctgatg tgcctccact cggccaagcg cgaggccgaa
      241 ctcgagcatt tcagactctg gatcagcatc ctcaatgccg gccggccgtg gtgatccttc
      301 cttctctgaa agcatcccaa aa
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