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Crustacean hyperglycemic hormone family gene silencing in *Penaeus monodon* mediated through dsRNA synthesized *in vitro* from genomic and cDNA

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RNA interference (RNAi) is the phenomenon in which long dsRNA is able to silence cognate gene expression. In the present study, 801 bp crustacean hyperglycemic hormone 1 (CHH1) and 795 bp moult inhibiting hormone 1 (MIH1) specific dsRNAs from genomic DNA, and 316 bp gonad inhibiting hormone (GIH) specific dsRNA from cDNA were constructed *in vitro*. Then the specific dsRNA constructs were administered into adult shrimps (*Penaeus monodon*). The gene expression was studied by semi quantitative RT-PCR and by monitoring haemolymph glucose concentration, duration of moulting and expression of vitellogenin as measures of specific biological activity. The gene silencing of CHH1, MIH1 and GIH genes could be attained within 24 h of dsRNA application. GIH gene silencing was observed up to 60th h. However, a complete silencing of MIH1 and MIH2 continued to 108 h post administration. Physiology of the animals injected with dsRNA of CHH1, MIH1 and GIH corroborated with the silencing of the specific genes resulting in the decrease of haemolymph glucose level, reduction in the days of moulting and expression of vitellogenin gene, respectively in adult shrimp. These results suggest the possibility of using dsRNAs of CHH family hormone genes as molecular tools for silencing inhibitory genes in turn affecting induced maturation in *P. monodon.*

Keywords: CHH family hormone, crustacean hyperglycemic hormone, gonad-inhibiting hormone, dsRNA, moult-inhibiting hormone, RNAi

Introduction

Physiology of crustaceans is partly regulated by diverse neuropeptides synthesized by medulla terminalis of X-organ sinus gland complex located in the eyestalk. The neuropeptide family includes crustacean hyperglycemic hormone (CHH), moult-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[1]. CHH/MIH/GIH family, precisely named as CHH family hormones, is multifunctional despite their similarities in structure[2].

CHH is involved mainly in elevating the glucose level in haemolymph by glycogen degradation in hepatopancreas. However, its function has been demonstrated to be pleiotropic, playing remarkable role in reproduction, moulting, digestion, osmoregulation and lipid metabolism in various species[2]. MIH controls moulting by inhibiting the synthesis of ecdysteroids by Y-organ, the process essential for the development and maturity of decapod crustaceans[3]. GIH controls the gonad maturation playing a highly complex role in reproduction and moulting[4].

Traditionally, shrimp maturation is induced by unilateral ablation of eyestalk, which reduces the CHH family hormone by half[5]. However, being an unethical and destructive process, this necessitates the replacement that would help standardize the maturation process with sustainability. It has been proven that RNA interference (RNAi) mediated gene silencing is operative in whole shrimp[6]. The RNAi has been used for experimental manipulation of gene expression and to prove the function of certain genes at genomic level[7]. Consequently, the technique has revolutionized research in 'reverse genetics' by introducing dsRNA into organisms or cells as it could

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knockdown a gene and produce its phenotypic loss. The approach of recombinant protein and RNAi was used to study the reproductive functions of gonad stimulating hormone in *Metapenaeus ensis*[8]. During the same period, the use of dsRNA to elucidate the function of GIH in *Penaeus monodon*[9] was first reported. They demonstrated the influence of Pem-GIH on vitellogenin (*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 using 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 eyestalk ablation to achieve induced maturation.

Materials and Methods

Amplification of CHH1 and MIH1 Genes from Genomic DNA, and GIH Gene from cDNA

Genomic DNA was extracted from haemocytes of *P. monodon* using the established protocol[10] with slight modifications. CHH1 and MIH1 genes were amplified by PCR using the primers (Table 1) designed from the 5′ and 3′ ends of the Pem-CHH1 (GenBank ID: AF233295.1) and Pem-MIH1 (GenBank ID: AY496454.1) sequences. A hot start PCR was adopted where the reaction mix was heated to 95°C for 5 min and subsequently, 0.5 U of *Taq* polymerase (New England Biolabs, USA) was added. PCR was conducted in a 25 µL reaction volume containing 2.5 µL PCR buffer (10×), 2.5 µL dNTP (2.5 mM), 1 µL each forward and reverse primer (10 pmol/µL), 16 µL milliQ, 1 µL (5 U) *Taq* DNA polymerase and 1 µL (75 ng) DNA template. The PCR amplification protocol consisted 35 cycles of 94°C for 40 sec, 60°C for 45 sec (for CHH1) and 64.1°C for 45 sec (for MIH1), 72°C for 2 min with a final extension at 72°C for 10 min.

For the preparation of cDNA of GIH, total RNA was extracted from the eyestalk of *P. monodon* using TriReagent (Sigma, USA) and quantified by UV spectrophotometry (Abs_{260} nm). An aliquot of 5 µg total RNA and 4 µL of Oligo d (T) (10 pmol/ µL) were incubated at 65°C for 5 min. The reaction mixture was brought to a volume of 20 µL with M-MuLV reverse transcriptase buffer containing 1 µM each dNTPs, 1U/ µL RNase inhibitor (20 U) and 200 U M-MuLV RTase (New England Biolabs, USA), incubated at 42°C for 60 min and inactivated at 95°C for 5 min. The cDNA generated was used for amplification of GIH gene using primers (Table 1). A hot start PCR was adopted where the reaction mix was heated to 95°C for 5 min and subsequently 0.5 U of *Taq* polymerase (New England Biolabs, USA) was added. PCR was conducted in a 25 µL reaction volume containing 2.5 µL PCR buffer (10×), 2.5 µL dNTP (2.5 mM), 1 µL each forward and reverse primer (10 pmol/µL), 16 µL milliQ, 1 µL (5 U) of *Taq* DNA polymerase and 1 µL DNA template. The PCR amplification protocol consisted 35 cycles of 94°C for 40 sec, 58°C for 45 sec, 72°C for 2 min with a final extension at 72°C for 10 min.

Cloning of CHH1, MIH1 and GIH Genes

The amplified products of CHH1, MIH1 and GIH genes were purified using Sigma Gel elution kit (Sigma, USA) and cloned into pGEM-T Easy vector (Promega, USA). The constructs were transformed into the bacterial strain JM109 (New England Biolabs, USA). The nucleotide sequences were determined by the method of ABI™ Prism Dye termination cycle (Microsynth, GmBH). The putative exon-intron in the CHH1 and MIH1 gene sequences were determined by comparing the sequences of CHH1 (GenBank ID: AF233295.1) and MIH1 (GenBank ID: AY496454.1) with the NCBI database. Since the GIH gene sequence was cDNA based, it consisted of only exon or the coding sequences unlike CHH1 and MIH1, which was compared with NCBI database (GenBank ID: DQ643389.1).

Table 1 — Primers used for generating amplicons from genomic DNA and cDNA.

Primer name	Forward (5′-3′)	Reverse (5′-3′)	Amplicon size from genomic DNA (bp)	Amplicon size from cDNA (bp)
CHH1	cgctcccgatctgcctctactctaa	acgaaagcaacctaataagagtctgg	801	500*
MIH1	cgcgtctccttgggttcattccgtcc	gcgttcgacctcactgaccggcgttc	795	450*
GIH	Atgaaaacatggtgct	ccttctctgaaagcatccca		316
MIH2	cagtgcgtctcgtacatgtcctttc	tctttcctgtacatgctctgcgtgac		420
Vg	ctaaggcaattatcactgctgct	aagcttggcaatgtattccttttt		1200

*Used for semi-quantitative RT-PCR only

***In Vitro* Construction of 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 dsRNAs. 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 *Sac*1 restriction enzyme and gel purified using Wizard SV gel purification kit (Promega, USA). The sense and antisense strands of dsRNA were prepared by carrying out PCR in two separate reactions. The reaction mix (25 µL) included 2.5 µL of RNA polymerase buffer (10×), 2.5 µL of ribonucleotide solution mix (2.5 mM), template(s) (1-4 µg), 1 µL of RNase inhibitor (20 U/µL) and 2-4 µL of RNA polymerase (20 U/µL), and the volume was made up with RNase-free water. The reaction mix of sense strand containing T7 RNA polymerase (20 U/µL) and T7 primer (10 pmol) was incubated at 42°C for 2-4 h. The reaction mix of antisense strand contained SP6 RNA polymerase (20 U/µL) and SP6 primer (10 pmol), and was incubated at 40°C for 2-4 h. The single stranded RNAs were allowed to anneal 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 chloform-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.

Injection of dsRNA of CHH1, MIH1 and GIH into Adult Shrimp

Female *P. monodon* (10 in number; weighing 10-15 g) were maintained in 25 L capacity FRP (fibre reinforced plastic) tanks with seawater (~15 g/L salinity) having a depth of 25-30 cm with constant aeration. The gross physico-chemical properties of the rearing water were: pH 7.5-8, temperature 28-30°C, total alkalinity 60-70 g/L, NH_4-N <1 g/L. Prior to injection of dsRNA, the shrimps were allowed to molt once and on the 3rd d after the first molt, they were injected with dsRNA of the three genes of CHH family hormones individually. Prior to injection, the animals were kept unfed for 18 h[11]. 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). The dsRNA injections were made into abdominal body cavity of the animals in case of CHH1, MIH1 and control, and through the arthrodial membrane of the second walking leg in the case of GIH.

Semi Quantitative RT-PCR Analysis of Expression of CHH1, MIH1 and GIH Genes after Their dsRNAs Administration

The eyestalks from dsRNA injected animals were dissected for RNA extraction during the time period 24, 60 and 108 h of administration. Total RNA was extracted using TriReagent (Sigma, USA) and quantified by measuring the absorbance at 260 nm (Abs_{260} nm), and used for cDNA synthesis. An aliquot of 5 µg of total RNA and 4 µL of Oligo d(T) (10 pmol/µL) were incubated at 65°C for 5 min and placed on ice. The reaction mixture was brought to a volume of 20 µL with M-MuLV reverse transcriptase buffer, 1 µM each dNTP, 1 U/ µL of RNase inhibitor and 200 U of M-MuLV reverse transcriptase (New England Biolabs, USA), incubated at 42°C for 60 min and at 95°C for 5 min. The PCR was carried out with primers (Table 1) using the same conditions as detailed in amplification of CHH1, MIH1 and GIH. The expression of MIH2 gene, an isoform of MIH1, was analysed using the primers given in Table 1. The PCR amplification protocol consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 40 sec, 64.1°C for 45 sec, 72°C for 2 min, with a final extension at 72°C for 10 min.

Detection of Specific Biological Activity

A glucose oxidase diagnostic kit (J D Diagnostics, India) was used to determine glucose concentration in haemolymph of all groups of shrimps including control. Duration of moulting cycle was monitored in the group of shrimps, which was administered with dsRNA of MIH. The expression of *Vg* gene was detected by semi-quantitative RT-PCR using *Vg* gene specific primers in the GIH dsRNA administered shrimps. Total RNA was extracted using TriReagent (Sigma, USA) and quantified by measuring Abs_{260} nm, and used for cDNA synthesis. An aliquot of 5 µg of total RNA and 4 µL of Oligo d(T) (10 pmol/µL) were incubated at 65°C for 5 min and placed on ice. The reaction mixture was brought to a volume of 20 µL with M-MuLV reverse transcriptase buffer, 1 µM each dNTP, 1 U/µL of RNasin and 200 U of M-MuLV RTase (New England Biolabs, USA), incubated at 42°C for 60 min and at 95°C for 5 min. The PCR was carried out using *Vg* gene specific primers (Table 1). A hot start PCR was adopted where the reaction mix was heated to 95°C for 5 min and then 0.5 U of Taq

polymerase (New England Biolabs, USA) was added. PCR was conducted in a 25 µL reaction volume containing: 2.5 µL PCR buffer (10×), 2.5 µL dNTP (2.5 mM), 1 µL each forward and reverse primer (10 pmol/µL), 16 µL milliQ, 1 µL (5 U) of *Taq* DNA polymerase and 1 µL DNA template. The PCR amplification protocol consisted 32 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min, and final extension at 72°C for 10 min.

Results

dsRNA of CHH1, MIH1 and GIH

The dsRNAs (Figs 1a-c) synthesized using PCR were found to have a ratio at Abs_{260}/Abs_{280} of 2.0 and concentrations of 84.30 (CHH1), 243.5 (MIH1) and 40.23 µg (GIH). The 20 µg per animal concentration of dsRNA was used for silencing experiments.

Gene Silencing Demonstrated through Semi-quantitative RT-PCR

Suppression of CHH1, MIH1 and GIH genes during administration of dsRNA was demonstrated through semi-quantitative RT-PCR.

Silencing of CHH1 (~500 bp), MIH1 (450 bp) and GIH (316 bp) genes were conspicuous during the initial phase of 24 h (Fig. 2). Meanwhile, GIH silencing continued to 60th h and that of MIH1 alone to 108th h of administration of the respective dsRNA (Fig. 2). The control showed higher levels of CHH1, MIH1 and GIH transcripts throughout the experiment. On administration of GIH dsRNA, there was no suppression of CHH1 and MIH1 genes and *vice versa* (Fig. 3). All the gene expressions were compared with that of ß-actin.

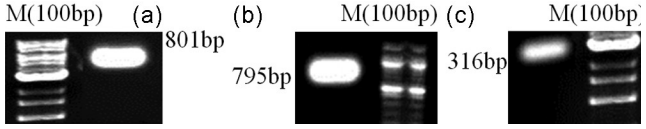

Fig. 1 (a-c) — *In vitro* transcription of dsRNA of CHH1 (a), MIH1 (b) and GIH (c).

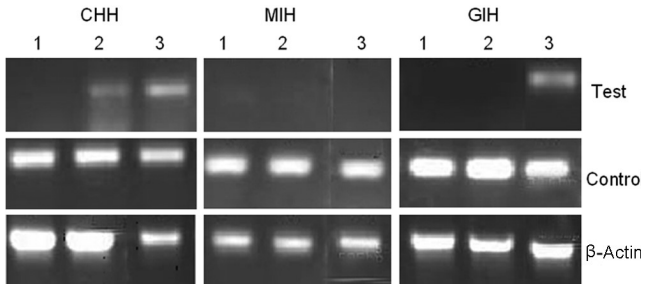

Fig. 2 — Silencing of CHH1, MIH1 and GIH genes were observed during the periods of 24 h (1), 60 h (2) and 108 h (3). [CHH1 expression was suppressed for 24 h post administration of CHH1 specific dsRNA. MIH1 gene was silenced for the period of 108 h post administration of MIH1 specific dsRNA. GIH expression was observed 60 h post administration of GIH specific dsRNA.]

The administration of MIH1 dsRNA was investigated on the expression of MIH2 too, an isoform of MIH1 gene. It was observed that the gene silencing pattern followed that of MIH1 gene. The expression of MIH2 gene silencing was observed to continue to 108th h of post injection. (Fig. 4)

Expression of *Vg* Gene in Response to Administration of dsRNA of GIH

Expression of the *Vg* gene in animals, which received the dsRNA of GIH, was examined through semi-quantitative RT-PCR on the 60th h of administration (Fig. 5). The results clearly showed that the silencing of GIH gene in turn triggered the expression of *Vg* gene, which played a key role in

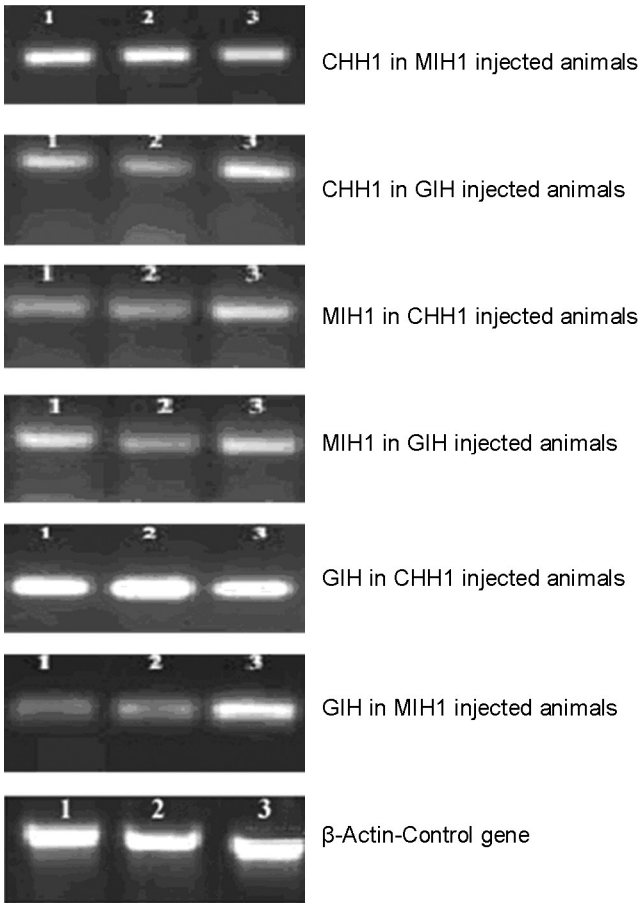

Fig. 3 — Cross checking the expressions of CHH1 in MIH1 and GIH dsRNA administered animals; MIH1 in CHH1 and GIH dsRNA administered animals; & GIH in CHH1 and MIH1 dsRNA administered animals. ß-Actin control at 24 h (1), 60 h (2) & 108 h (3).

maturation. The expression of vitellogenin was not observed in the CHH1 and MIH1 dsRNA injected animals.

Administration of dsRNA of CHH1, MIH1 and GIH and Haemolymph Glucose Level

Haemolymph (20 µL) glucose levels of shrimp administered with dsRNA of CHH1, MIH1 and GIH were monitored at 24th and 60th h of injection (Figs 6 & 7). The CHH1 dsRNA (20 µg) administered animals showed significant lower glucose level (23.48±14.88 mg/dL) against the control (95.73±33.40 mg/dL) (P≤0.01) at the 24th h. The glucose concentration at 60th h was also lower in comparison with that of the control (Fig. 7). However, with respect to dsRNA of MIH1 and GIH (Figs 6 & 7), only a marginal decrease in glucose level was observed in the haemolymph (82.52±5.81 & 61.6±31.73 mg/dL, respectively) in comparison to that of the control (95.73±33.40 mg/dL) (P >0.05).

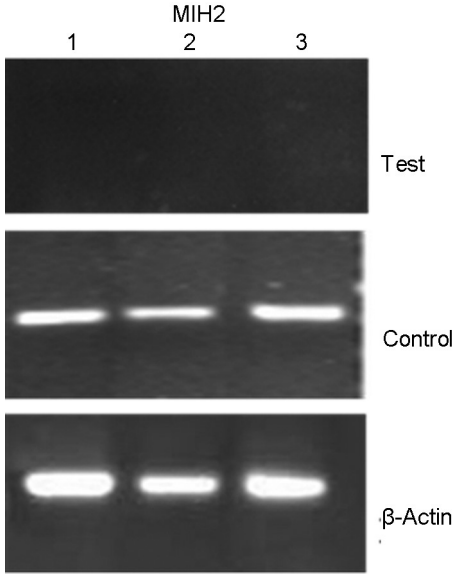

Fig. 4 — Silencing of MIH2 transcript observed during the periods of 24 h (1), 60 h (2) and 108 h (3).

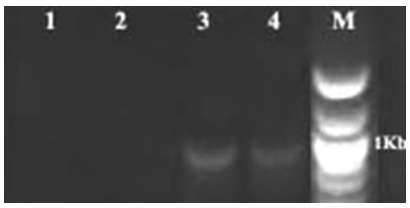

Fig. 5 — Expression of *Vg* gene at 60th h post administration of GIH-specific dsRNA (Lanes 3, 4); Control at 60th h (Lanes 1, 2); & Marker (Lane M).

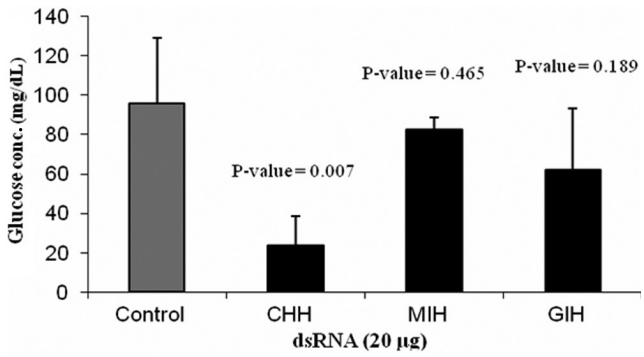

Fig. 6 — Glucose assay of shrimp haemolymph within 24 h of administration of CHH1, MIH1 and GIH dsRNA.

Effect of MIH1 dsRNA on Moulting Period

The suppression of MIH1 on the administration of dsRNA of the MIH1 gene was assessed by taking into account the duration required for moulting in comparison with that of the control group. The control group animals required 11.5±1.04 d to complete one moulting cycle, whereas shrimps injected with MIH1 dsRNA (20 µg) needed only 6.83±1.47 d (P <0.005) (Fig. 8).

Discussion

RNA interference (RNAi) is the phenomenon in which long dsRNA is able to silence cognate gene expression, thereby providing an opportunity to investigate the corresponding protein function. In shrimp (*P. Monodon*), RNAi-induced gene silencing

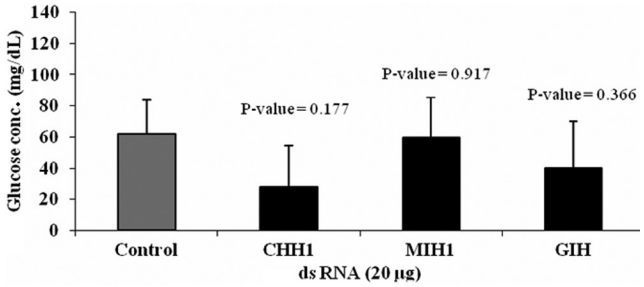

Fig. 7 — Glucose assay of shrimp haemolymph during the period of 60 h post administration of CHH1, MIH1 and GIH dsRNA.

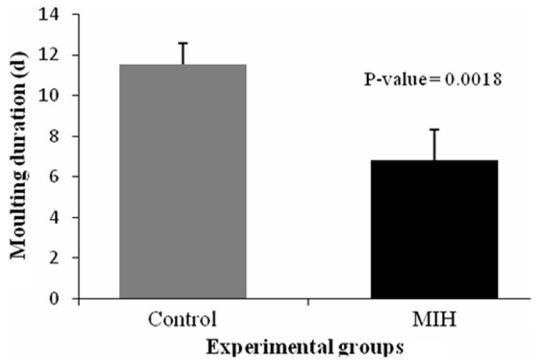

Fig. 8 — Moulting duration of MIH1 specific dsRNA administered animals in comparison to that of the control group.

has been demonstrated in GIH gene[11] and Yellow Head viral replication[12]. RNAi was preferred as a tool for studying the functional knockdown of CHH1, MIH1 and GIH, which in due course could likely to be developed as molecular tool in place of eyestalk ablation for induced maturation. In the present study, CHH1-specific dsRNA (801bp) and MIH1-specific dsRNA (795bp) were constructed from genomic DNA, while GIH-specific dsRNA (316bp) from the coding region of mature GIH. The silencing of CHH1, MIH1 and GIH genes could be attained within 24 h of dsRNA application. However, complete silencing of GIH continued to the 60th h, while that of MIH1 to 108th h.

The physiology of animals injected with dsRNA corroborated with the silencing of the specific gene. The animals injected with dsRNA of CHH1 showed a significant decrease in the haemolymph glucose level compared to that of the control group, and marginal decrease with those administered with dsRNA of MIH1 and GIH genes. The MIH1 transcript was silenced within a period of 24 h and continued to 108th h. Simultaneously, the moulting pattern also showed a significant change by reducing the days of moulting from 11.5±1.04 to 6.83±1.47 d. The MIH2 transcript was also suppressed in the same pattern as that of MIH1 and the silencing continued to a period of 108 h. The combined silencing of two isoforms of MIH (MIH1 & MIH2) might have reduced the moulting duration. Injection of dsRNA of GIH showed the silencing of the gene alongwith the expression of *Vg* gene, suggesting that the silencing of GIH gene in turn stimulated the expression of vitellogenin in adult shrimp, *P. monodon*. Recently, it has been reported that injection of bacterially expressed dsRNA mediated GIH silencing lead to ovarian maturation and eventual spawning in both domesticated and wild broodstocks[11].

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 trigger the efficiency of interference of RNA *in vivo*. It was demonstrated 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[12]. The use of long dsRNA provides the possibility of generating more varieties of effective small-interfering RNA (siRNA; 21-23 nucleotides) molecules. The dsRNA can be made from cDNA or genomic DNA templates. Inspite of the fact that most of the dsRNA for RNAi 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. Another approach has been to use dsRNA corresponding to the 5' or 3' UTR (either UTR can mediate interference)[13]. 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 therefore proceeded with cDNA of the same.

Also, non-specific silencing, known as off-target phenomenon, can occur from diverse siRNA products of long dsRNA[14,15]. The off target phenomenon checked manually by constructing the dsRNA sequences and using the NCBI BLAST programme proved the absence of off-target phenomenon in the present work. The marginal reduction in glucose levels of the animals administered with MIH1 and GIH dsRNA from 95.73 to 82.52 and 61.6 mg dL^{-1}, respectively may be attributed to the amino acid sequence similarity that classifies MIH1 and GIH as isoforms under type II class of CHH family neuropeptides. At the same time, there was no suppression of CHH1 gene in the MIH1 and GIH dsRNA injected animal and *vice versa*.

The present study contributes to our understanding of CHH1, MIH1 and GIH specific dsRNA mediated temporary knockout of the related gene function in *P. monodon*. Further, the study showed that the silencing of GIH gene with dsRNA administration induced vitellogenin expression, signifying stimulation of maturation in adult shrimps. 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 and health status of the animals consequently.

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