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Isolation and Characterization of a Molt-Inhibiting Hormone (MIH) from the Eyestalk of Green Tiger Shrimps (*Penaeus semisulcatus*) in Persian Gulf

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ABSTRACT

Penaeid shrimp constitutes the most important resource in fisheries industry. Molting is an important physiological process essential for growth and development in shrimp and other crustacean. This process is inhibited by Molting inhibiting hormone (MIH); a hormone synthesized by a cluster of neurosecretory cells called The X-organ. Green tiger shrimp, *Penaeus semisulcatus*, belongs to Penaeidae family and constitutes large portion of harvests in Persian Gulf. In this study, nucleotide and amino acid sequence of MIH are reported. Total RNA was extracted from X-organ and was used for total cDNA synthesis and 5'RACE procedure. 5'UTR and ORF of mRNA were amplified and sequenced. The results of sequencing showed that MIH mRNA has 399 nucleotides of which 81 nucleotides belong to 5'UTR and 318 to the ORF. The mRNA translation by software yielded a polypeptide with 105 amino acids. For mature MIH in other Penaeus species, a polypeptide with 75-77 amino acids was reported. These hormones have similar amino acid sequences with six conserved Cysteine residues. Comparison of the sequences obtained in this study with those in other reports reveals considerable similarities regarding the level of nucleotides and amino acids.

KEYWORDS: Molt-inhibiting hormone; MIH; *Penaeussemisulcatus*; RACE technique

1. INTRODUCTION

Like other crustaceans, shrimp growth and development are dependent on molting. Molting is triggered by C-27 steroid hormone, ecdysteroids (Sonobe, et. al., 1991). In crustaceans, it is presumed that, at least in part, secretion of molting hormone is regulated by molt inhibiting hormone (MIH). MIH is secreted from X- organ sinus gland complex (XOGT) that is located in the eyestalk (Tsuyoshi, et. al., 2005). MIH belongs to crustacean hyperglycemic hormone (CHH) family. This family includes CHH, MIH, mandibular organ inhibiting hormone (MOIH), and gonad inhibiting hormone (GIH) (Zmora, et. al., 2009). MIH and MIH–like cDNA sequences have been identified in many penaeid shrimps and other decapoda such as *Marsupenaeus japonicus*(Madhyastha & Rangnekar, 1976), *Penaeus monodon*(Benzie, 1997), *Litopenaeus vannamei*(Asunción, et al., 2007), *Fenneropenaeus chinensis* (Wang, et. al., 2003), and *Metapenaeus ensis*(Gu and Chan 1998). These cDNAs encode a polypeptide with primary structure ranging from 90-105 and 74-79 amino acids in immature and mature forms, respectively. *Penaeus semisulcatus* (genus Penaeus and family Penaeidae) is endemic to IRAN and constitutes a large portion of shrimp harvests in Persian Gulf (Niamaimandi et al 2008). Although it is one of the most sought after shrimps in the market of the Persian Gulf region, no report has yet made on its MIH.

In this study, cDNA encoding MIH1 in *Penaeus semisulcatus* was obtained using RT-PCR and RACE protocols. An open reading frame of cDNA encoding MIH1 was translated to amino acids, and theoretical protein parameter was calculated using bioinformatics online software. A gene encoding MIH1 was amplified using genomic DNA as a template and its primary structure was determined. We also tried to use available data and tools for insilico analysis of MIH protein and gene to predict some post translational modifications, tertiary structure, interaction of MIH and their receptor and detect potential regulatory elements in intron, putative microRNA, and repetitive elements. Finally, phylogenic analysis of MIH/MIH like sequences in protein, cDNA (initiation codon to stop codon) and gene (initiation codon to stop codon) level was conducted using MEGA 6.1 (www.megasoftware.net).

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2. MATERIALS AND METHODS

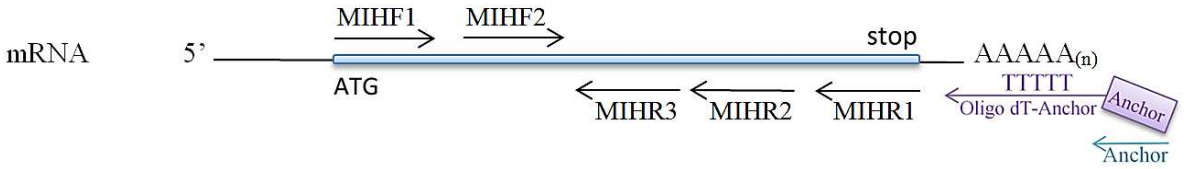
2.1. Primer design

All the primer sequences used in this paper are listed in table 1. The specific primers (MIHF1, MIHF2, MIFR1, MIHR2 and MIHR3) were designed based on conserved sequences of the MIH cDNA of *Litopenaeus vannamei* (AY425615), *Penaeus monodon* (GQ221086), *Fennrepenaeus chinensis* (AF312977) and *Marsupenaeus japonicus* (AB004652). The general primers (OligodT-Anchor, Anchor and Random Hexamer primer) were provided with 5'/3'RACE kit, 2nd Generation and cDNA Synthesis System (Roche).

Table 1: Presents the primers used in this study

Primers	Sequences
MIHF1	**5' CACTCATGTATCGGCTHGCRATG 3'**
MIHF2	**5' GATTGTAGTTGGGACAAG 3'**
MIHR1	**5' CTCACTSWCMRGCRTTCAGRATG 3'**
MIHR2	**5' CGATCTCGTCCTCCCTRTTG 3'**
MIHR3	**5' GAACCATTCGTTGTAGAAGCAC 3'**
OligodT-Anchor	**5'GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT V (A, C or G) 3'**
Anchor	**5' GACCACGCGTATCGATGTCGAC 3'**
Random hexamer	**5' NNNNNN 3'**

Fig1. Schematic representation of the structure of a cDNA encoding, the MIH, and locations of the oligonucleotide primers



2.2. Sample collection and RNA isolation

Live green tiger shrimps, *Penaeus semisulcatus*, were purchased from local fishermen and transported to biotechnology laboratory at Persian Gulf University. Eyestalks were removed from adult shrimps, frozen immediately and were stored in -70˚C or RNAlater (Qiagen) until they were used. Total RNA was extracted from micro-dissected eyestalks of both sexes after grounding in liquid nitrogen using RNeasy Fibrous Tissue Mini Kit (QIAGEN) according to the instructions of the manufacturer. The extracted RNA was immediately used for cDNA synthesis or was stored at -70 ◦C until use.

2.3. cDNA synthesis and RT-PCR reaction

2.3.1. cDNA library synthesis and amplification of cDNA - MIH ORF

Extracted total RNA was used for cDNA library synthesis by cDNA Synthesis System Kit (Roche, Germany). cDNA library was synthesized from 4µl of total RNA in a total volume of 40µl containing of 8µl Buffer RT (5X), 20 pm random hexamer, 2µldNTP mix (10mM), 2µl DDT (0.1M), 1µlRNAase inhibitor and 2µl AMV reverse transcriptase. The MIH coding region (Open Reading Frame (ORF)) was amplified using degenerate primers, MIHF1 and MIHR1, (table 1) and cDNA library as a template. The MIH amplification reaction (50µl) contained 5µl PCR Buffer (10X), 2µldNTP mix (10mM), 3µl MgCl2 (50mM), 2.5 U Taq DNA polymerase, primers MIHF1 (20 Pmol) and MIHR1 (20 Pmol) and 2µl RT product. The PCR reaction was carried out according to the following thermal program: 4 min at 94°C for initial denaturation, followed by 30 cycles with 94°C at 1 min, 59°C at 45 sec, 72°C for 1 min, and a final extension at 72ºC for 5 min. The size and quality of PCR products were visualized on 1% agarose gel. Amplified fragments in the expected size range (~320bp) were excised from the gel and fully sequenced on an Applied Biosystems 3730 Automated Sequencer (Bioneer, Korea) in both strands using the same primer pairs for PCR.

2.3.2. Amplification of the 5' and 3' end of cDNA

5'/3' RACE Kit, 2nd Generation (Roche), was used for the amplification of the 5' and 3' end of MIH cDNA. Experimental protocol for 5' and 3' RACE was carried out according to the protocol of the 5'/3' RACE Kit, 2nd

Generation (Roche) with modifications of the procedures as recommended by the manufacturer. The PCR reaction in the 5′ and 3′ RACE experiments was carried out as described in section 2.3.1, except for the differences in primers and thermal programs.

For 3' RACE reaction, primer OligodT-Anchor was used for priming the reverse transcription reaction of the polyA tail containing total RNA. First, cDNA was synthesized in total volume of 20µl containing 4µl cDNA synthesis buffer (5X), 2µl dNTP mix (10mM), 1µl OligodT-Anchor primer and 1µl Reverse Transcriptase. The reaction mixture was incubated at 55°C for 4 minutes, and then at 54, 53, 52, 51, and 50°C for 2, 2, 2, 2 and 60 minutes, respectively. Eventually, the reaction terminated at 85°C for 5 minutes. 2µl of 3' RACE cDNA was used as a template for amplification of the MIH coding region and 3'UTR by MIHF1 and Anchor primers. The amplified fragments were analyzed on a 1.5% agarose gel. The bands with the expected size were excised from the gel and sequenced as described above. The 3′ RACE cDNA was also used for amplification of MIH ORF by MIHF1 and MIHR1 primers. The PCR products (fragments with ~320bp) were also sequenced as described.

The 5′ RACE was carried out as described above, except that the primer anchor was replaced by primer MIHR1 and the mixture reaction was incubated at 55°C. Then, the first cDNA strand was purified using High Pure PCR Product Purification Kit (Roche), and OligodA (Poly A) Anchor was added to the 3' end of the cDNA. The Poly-A tailed cDNA was used as the template for two rounds of a semi – nested PCR. In the first PCR, tailed cDNA was amplified using an OligodT-Anchor and MIHR2 specific primers under the following PCR conditions: 94°C, 2 min; 6 cycles at 94°C, 15 sec, annealing temperature decreasing 1°C/cycles from 55 to 50°C, 30 sec, 72°C, 55 sec, 30 cycles at 94°C, 15 sec, 50°C, 30 sec, 72°C, 55 sec and the final extension at 72°C for 7 min. The first PCR products were diluted and re-amplified with an anchor and a nested MIHR3 specific reverse primer at the PCR conditions of 94°C, 2 min; 35 cycles at 94°C, 30 sec, 65°C, 30 sec, 72°C, 1 min and the final extension at 72°C for 7 min, after optimization of annealing temperature using temperature gradient. The amplified fragments from the secondary PCR were analyzed on a 1.5% agarose gel. The band with the expected size of 365bp was excised from the gel and sequenced as described above.

2.4. Amplification of MIH encoding gene

Total genomic DNA was extracted from 100-150 mg muscle tissue from the ethanol-preserved samples according to Brandfass and Karlovsky (2008) with some modifications. The tissue was incubated in 700µllysis buffer (100 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2% SDS) containing 7µl of proteinase K (20 mg/ml). The mixture was incubated at 55°C for 6-16 hours and 600 rpm. Subsequently, 5M NaCl (250µl) was added and the samples were mixed before adding 5% CTAB (200µl). The samples were incubated for 10 minutes at 60°C. The DNA was organically extracted with chloroform and precipitated with one volume of cold isopropanol. The DNA pellet was washed with 70% ethanol, air-dried for 10 to 20 minutes, suspended in 50 to 100µl ddH20 and was preserved at 4°C for a short time or -20°C for a long time.

The genomic extracted DNA was used as a template for amplification of Open Reading Frame of MIH gene using MIHF1 and MIHR1 primers. The PCR mixture contained 5µl Mg^{2+} free - PCR buffer (10X) , 3mM MgCl2, 0.4mM of dNTP mix, 20 pico mole of each primer, 2.5 U Taq DNA polymerase (Fermentas), and 300 ngr DNA for a total volume of 50µl. The PCR reaction was carried out according to the following thermal program: 3 min at 94°C for initial denaturation followed by 30 cycles with 94°C for 1 min, 62°C for 45 sec, 72°C for 1.5 min. The final extension was at 72º C for 5 min. The size and quality of PCR products were visualized on 1% agarose gel. The sequencing was performed using ABI 370 automated sequencer (Seq/Tech/California (USA)).

2.5. PCR product sequencing and processing

The sequencing results were obtained in the chromatograms and FASTA format. The primary sequences confirmation and homologies were investigated using Blast (NCBI). Chromatograms of each of the forward and reverse sequences were checked using Chromas Prosoftwares (Technelysium Pty Ltd, Australia). In order to obtain the full-length sequence of each sample, forward and reverse strand sequences were combined using SeqMan software (DNASTAR) with manual adjustment. The exons and introns boundaries of MIH gene were determined by comparing cDNA sequence with genomic sequence and programs of DBGP (http://www.fruitfly.org/seq_tools/splice.html) (Reese, et al., 1997) and SPLM (http://www.softberry.com/berry.phtml?topic=splm&group=programs&subgroup=gfind). The ORF sequence was translated into amino acids using translate tool software (http;//www.expasy.org).

2.6. Insilico analysis of MIH 1 gene

The Penaeus semisulcatus MIH 1 gene, was analyzed to approve the presence of repetitive elements in both strands with reference to the data from the web site: http://www.girinst.org/censor/index.php (Kohany et al., 2006).

SPML (http://www.softberry.com/berry.phtml?topic=splm&group=programs&subgroup=gfind) (Solovyev, et al., 1994) was used to find potential splice sites. Detection of potential TFB sites in MIH Introns was carried out using NSITE program (www.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter) (Solovyev, et al., 2010). Computational prediction of putative miRNAs and miRNA like small RNA was performed using Mature Bayes tool (http://mirna.imbb.forth.gr/MatureBayes.html) (Gkirtzou et al., 2010) and find miRNA (www.softberry.com).

2.6.1. Insilico analysis of mature MIH1 protein

The theoretical physico-chemical properties of putative MIH polypeptide were deduced using Expasy Bioinformatics Resource Portal (www.Expasy.org). Signal peptide cleavage sites were predicted using the SignalP4.1 server program (Peterson et al., 2011). Generic phosphorylation sites, Kinase specific phosphorylation sites, N- linked and O- linked glycosylation prediction in Mature MIH, were performed using NetPhos2 server (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 1999), and NetPhosK 1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/) (Blom et al., 2004), respectively. Three-dimensional structure of *Penaeus semisulcatus* MIH was predicted by homology modeling using Swiss-PDB (http://swissmodel.expasy.org/interactive) (Arnold, et al., 2006) and Phyre2 (http://www.sbj.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley and Sternberg 2009) Tools. Software ClusPro (cluspro.bu.edu/login.php) was used to study the interaction between MIH and receptor (Kozakov, et al., 2013).

2.7. Phylogenetic analysis

For a more complex phylogenic evaluation beside the sequence determined in this investigation, the genes and cDNAs MIH ORF and MIH amino acids sequences in some species of crustaceans were retrieved from GenBank database following Blast search. The Sequences were aligned using the multiple–alignment program ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). Sequences distance matrix was calculated using Kimura 2-Parameter and was subjected to construction of Neighbor-joining tree with MEGA6.

3. RESULTS

3.1. Nucleotide sequences and characterization of *P. semisulcatus* MIH1 cDNA

In this study, totally three types of cDNA were synthesized including cDNA library, 3′ and 5′ RACE cDNA. In two distinct PCRs, both cDNA library and 3′ RACE cDNA were used as templates for amplification of open reading frame of cDNA – MIH. For this purpose, primers MIHF1 and MIHR1 were used. The lengths of the open reading frame of cDNA – MIH in both mentioned PCR products were the same and sequencing results showed that open reading frame of cDNA – MIH has 318 nucleotides and is exactly the same in both PCR products. The Blast search results indicated considerable identicalness between *Penaeus semisulcatus* MIH mRNA and other decapoda MIH, Penaeus genus members in particular (Data not shown).

In the other reaction, 3' RACE cDNA (as a template) and Primers MIHF1 and Anchor were used for amplification of 3′ region of cDNA encoding of MIH. Electrophoresis results showed that three fragments (approximately 700, 900 and greater than 1000bp) have been amplified (Fig.3). To ensure the accuracy of 3' RACE PCR product, a semi-nested PCR was performed using mentioned diluted PCR product as template and MIHF2 and Anchor primers. Previous electrophoresis pattern was observed again. After a long time and low voltage electrophoresis, all three fragments were carefully excised from the gel and were fully sequenced. Unfortunately, sequencing results were not satisfactory. Therefore, only the sequences corresponding to the ORF and 5' RACE were used for further analyses. In fourth PCR, a specific DNA fragment of about 365bp (including OligodT-Anchor primer) was obtained from 5′ RACE. A 320bp - section of 5′ RACE PCR products (including OligodT-Anchor primer, 5′ untranslated region and a part of 5′ region) and was well sequenced using MIHR3 primer. The results from ORF and 5′ RACE sequences shared a 189bp overlapping region, which exhibited identical sequences. The data from compilation of nucleotide sequences from 5′ RACE and ORF sequences revealed a 399bp MIH cDNA containing 81bp and 318bp 5′ untranslated region and open reading frame encoding a peptide of 105 amino acids (Accession number KF031444), respectively. Moreover, the *Penaeus semisulcatus* deduced MIH1amino acid showed 100% and 99% to *Penaeus monodon* and *Litopenaeus vannamei* MIH, respectively.

3.2. Primary structure of P. semisulcatus MIH 1 gene and its characterization

A specific DNA fragments, about 720bp, was amplified from genomic DNA using MIHF1/MIHR1 primers pairs. The above PCR product was successfully sequenced in both directions. The sequencing results showed that the amplified MIH gene has exactly 716bp. The Blast search results revealed that *Penaeus semisulcatus* MIH1 gene

exhibits an identicalness over 90 and 80 percent with *Penaeus monodon* and *Litopenaeus vannamei* MIH1 gene, respectively (data not shown). A primary sequence analysis using Blast search and Splice Prediction Tool (Fruit Fly Splice Detector: www.fruitfly.org/seq_tools/splice.html) revealed that MIH1 in *Penaeus semisulcatus* contained three exons and two introns. The *Penaeus semisulcatus*MIH gene was then submitted in GenBank data base (accession no. AB810255). To obtain the primary structure of MIH1 gene, sequences from two the accession numbers (KF031444 and AB810255) were combined with the others (Fig.2).

```
ACAGCATCCACGCCGTCTCAGGGTAGAGGTCCTTCGAGTCGCGTCTCCTTGGGTTCATTC      60
CGTCCCTGCGATTACACACTCATGTATCGGCTTGCGATGGTAAGAGATTAAGAGAGTTTT     120
                      M  Y  R  L  A  M

TGGTAAGTGGATGTTGGTTTGTTAACTCTTTGGTGGCTATCGTATGGTTATTTGTCTGCG     180
GTTTGTCCTGATTTTCATTTATTTTTCTTCTTTTTCACTGCAGAAGACATGGCTGGCGAT     240
                                           K  T  W  L  A  I

AGTGATTGTAGTAGTTGGGACAAGCCTCTTCTTTGACACCGCCTCGGCCAGTTTCATAGA     300
  V  I  V  V  V  G  T  S  L  F  F  D  T  A  S  A  S  F  I  D

CGGCACCTGTCGAGGCGTAATGGGTAATCGTGACATTTACAAGAAGGTAGTGCGTGTGTG     360
  G  T  C  R  G  V  M  G  N  R  D  I  Y  K  K  V  V  R  V  C

TGAGGATTGCACCAATATCTTCCGACTTCCAGGCCTGGATGGCATGTGCAGGTATCCTTG     420
  E  D  C  T  N  I  F  R  L  P  G  L  D  G  M  C  R

CACTTTTCATTTCTAGGGATTTTGACGTATAGAGACAGGCATAGATGGCATATGGATGCG     480
AAATGCCAGTGCTCTTATTATTCAAACAACCCATTTCATTAATCTCTATACTTCCAAATA     540
CACATCACTTGCGATTCAAAATCATTTACTCAAGTGAAATCAATGTTGAGTGAAATTACT     600
CTACGGTAAATACTCTCTGCATTTCACTACATCCTCACTCTCCAGTATAGCCTTTCAAAG     660
CGATATTTTTTCACTACAGAGATCGGTGCTTCTACAACGAATGGTTCCTGATTTGTCTAA     720
                      D  R  C  F  Y  N  E  W  F  L  I  C  L

AGGCTGCCAACAGGGAGGACGAGATCGAAAAATTCAAAGTTTGGATCAGCATCCTGAACG     780
K  A  A  N  R  E  D  E  I  E  K  F  K  V  W  I  S  I  L  N

CCGGACAGTGAG                                                      792
A  G  Q  end
```

Fig.2. The nucleotide and deduced amino acids sequences of the Penaeus semisulcatus MIH gene. Exons, are shown in bold and 5′UTR and Introns also represented in regular. Amino acids are showed in one - letter symbol.

The isolated MIH1 gene in this study consists of 792bp including 5′UTR (nucleotide 1 to 81), exon1 (nucleotide 82 to 99), intron1 (nucleotide 100 to 223), exon2 (nucleotide 224 to 411), intron2 (nucleotide 412 to 679) and exon3 (nucleotide 680 to 791). Like most eukaryotic genes, the splice donor and acceptor site (GT-AG) were detected in exons and introns boundaries.

3.4. Insilico analysis of MIH1 gene sequence

3.4.1. Repetitive elements distribution

Sequence analysis of MIH1 gene has detected 2 repetitive elements; L1MDA6 and Copia – 38 GM-I (table 2). The positions L1MDA6 and Copia – 38 GM-I were in Intron I and Exon II, respectively.

Table2. The repetitive elements detected by the Censor software in *P. semisulcatus* MIH1 gene.

Query sequence	From	To	Repetitive elements	Class	Similarity	Score
MIH 1 gene	132	221	L1MDA 5	NonLTR/L1	0.71	256
MIH 1 gene	340	444	Copia – 38 GM- I	LTR/Copia	0.72	241

3.4.2. Exonic and intronic splice sites

The *P. semisulcatus* MIH gene was also examined to track any potential sequences involved in mRNA precursor processing. Total 2 Donor (positions of 123 and 347) and 3 Acceptor (positions of 392, 436 and 645) splicing sites were identified throughout the gene, respectively. The donor splice sites 123 and 347 were positioned in the intron (termed as ISS) and exon (termed as ESS) regions, respectively, while the acceptor splice site 392 was localized in exon, and the remaining ones (436 and 645) were positioned in the intron. Positions 100 and 412 as native donor sites and 223 and 679 as native acceptor splicing sites were also detected by insilico analysis.

3.4.3. Distribution of transcription factor binding (TFB) sites in MIH1 gene introns

Analysis of the MIH gene resulted in detection of 20 and 16 TFB sites in intron 1 and 2, respectively. However, only animal TFB site has been showed here (Table 3).

Table 3. Transcription factor binding sites in MIH1 gene introns

miRNA sequence (5' – 3')	Length	Prediction Tools
CGUAUGGUUAUUUGUCUGCGGU	22	Softberry
UUCUUCUUUUUCACUGCAGAAG	22	Softberry
GGCACCUGUCGAGGCGUAAUGG	22	Softberry
UGUGAGGAUUGCACCAAUAUCU	22	Softberry
UCUCAGGGUAGAGGUCCUUCGA	22	MatureBayes
UGCAUUUCACUACAUCCUCACU	22	MatureBayes

3.4.4. MicroRNA detection in MIH1

Computational prediction represents an effective strategy to identify miRNAs that can further be examined and validated by experimental approaches. Therefore, it is widely utilized to discover tissue-specific miRNAs from various organisms (Thirugnanasambantham, et al,. 2013).

3.5. Insilico analysis of mature MIH 1

3.5.1. Physico–chemical parameters

The theoretical physico–chemical parameters of mature putative MIH were calculated using Expasy (table 4).

Table 4. The theoretical physico–chemical parameters of mature putative MIH

MW (dal)	PI	Extinction coefficients	Instbility index	Alipatic index	GRAVY
8985.4	7.51	14355	31.31	83.51	-0.158

Total numbers of negative (Asp and Glu) and positive (Arg and Lys) amino acids were calculated to be 11 and 12, respectively. The analysis of amino acids showed low contents of Pro (1.3%), and Gln (1.3%) and high contents of Arg (9.1%), Ilu (9.1%), Asp (7.8%), and Cys (7.8%) and Gly (7/8%).

3.5.2. Post-translational modifications

The Generic (non kinase specific) phosphorylation prediction showed that Threonine -10 and Tyrosine -21 were the potential phosphorylation sites. The Threonine -10 and Tyrosine -21 have a score of 0.730 and 0.803, respectively, both of which are above the threshold (0.500). This indicates that the confidence of the fact that these sites are true phosphorylation sites is quite high. Investigation for Kinase specific phosphorylation site showed that Tyrosine-6 and Serine-71 are potential phosphorylation sites by protein Kinase C and protein Kinase A, respectively. Tyrosine -6 has a score of 0.670 indicating a likely phosphorylation site, whereas Serine-71 has a score of only 0.510. The latter score is just slightly above the threshold (0.500) and indicates that the confidence for this site to be a true site for Kinase specific phosphorylation site is quite low.

3.5.3. Penaeus semisulcatus MIH1 tertiary structure

Tertiary structure for mature MIH, which was predicted by Phyr2 software, consists of 4 α-helix (Fig.3), while SWISS- MODEL predicted a tertiary structure with 5 α-helix (data not shown).

3.5.4. Simulation of Interaction of P. semisulcatus mature MIH 1 with receptor

Molecular and Immunocytochemical studies have shown that Guanylylcyclase on Y-organ Cell membrane act as a MIH receptor (Zheng, et al., 2008). Computational simulation of MIH and its receptor improves our understanding of the molting regulation which can further be examined and validated by experimental approaches. The MIH receptor has not been isolated from *Penaeussemisulcatus*; therefore, MIH receptor from *P. clarki* was used for simulation of MIH and receptor interaction. MIH Receptor interaction was mediated by 9 amino acids of both types so that this interaction was mediated through hydrogen bond formation between Asp -4, Arg-8, Tyr-17, lys -18 and Gln -77 of MIH and Arg -329, Asp-348, Asn -108 and Arg -344, respectively. Gly -9, Val -10 and Met -11 of MIH also form hydrogen bonds with Glu-104 of the receptor and Arg-14 of MIH with Asn -107, Asp -114 and Tyr -116.

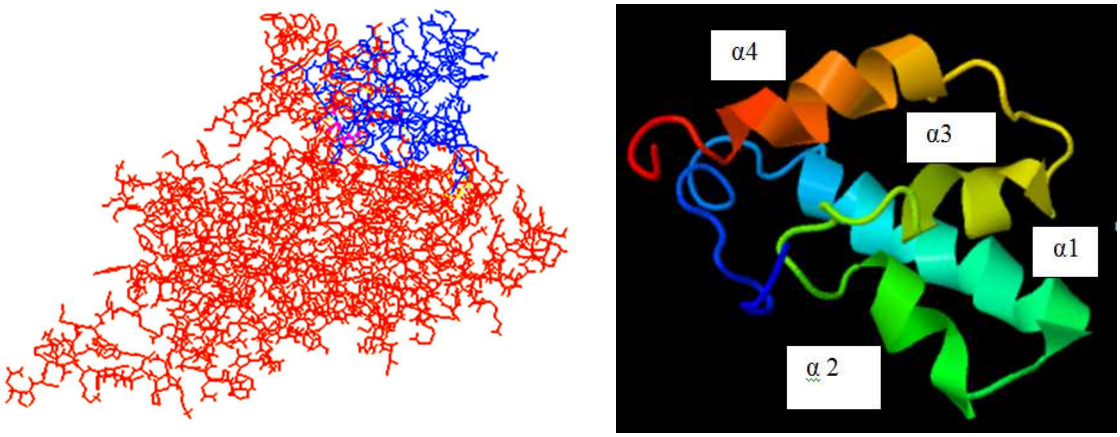


Fig.3. Simulation of MIH – receptor interaction

4. DISCUSSION

In this study, cDNA and gene molt inhibiting hormone (MIH1) of green tiger shrimp *P. semisulcatus* were sequenced and its properties were studied. To isolate the coding region, either 5' and 3' UTR of the cDNA or 5'/3' RACE kit was used. According to kit instructions 3' RACE, cDNA using OligodT- Anchor primer was synthesized at a temperature of 55˚C, but the results showed that this temperature is not suitable for annealing primer to the template. Using the reduced temperature gradient from 55 to 50˚C (similar to Touch Down), 50˚C was diagnosed as the proper temperature. The coding region of cDNA was successfully amplified and sequenced via synthesized 3'RACE technique at the above mentioned temperatures as a template and primers MIHF1/R1. Therefore, we propose the use of a temperature of 50˚C in the case of using the OligodT- Anchor primer. 3'UTR of cDNA was amplified using 3'RACE as a template and MIHF1/anchor primers, and gel electrophoresis of the PCR product determined that three pieces of DNA were replicated. The accuracy of the above PCR using harsher conditions of temperature and Semi-Nested PCR using the first PCR product as a template and the MIHF2/Anchor primers was confirmed, but unfortunately the results of sequencing were not satisfying. Amplification of three cDNA 3'RACE can be attributed to the presence of different isoforms of MIH caused by Alternative Splicing, or striking resemblance of the 5'coding of MIH1 to other CHH family members; however, 5'UTR was successfully amplified and sequenced. Therefore in this study, the coding region and 5'UTR of cDNA-MIH1 were sequenced. Translation of the coding region into the amino acid showed that the MIH1 in *P. semisulcatus* has 105 amino acids, just like MIH1 in *P. monodon* and *L. Vannamei* species, and that its amino acid sequence is exactly identical to *P. monodon* and is different from that of *L. vannamei* only in terms of one amino acid (amino acid No. 26 in Immature MIH1). So *P. semisulcatus* MIH1 has all the characteristics of family members CHH (77 amino acids of the mature form and the 6 amino acids conserved cystein) similar to MIH1 in *P. monodon*. On the other hand, as MIH1 *P. monodon*, the amino acid Glysinat position No. 12 argues that this hormone is type II peptides of this family.

Calculation of the physicochemical properties showed that the *P. semisulcatus* MIH is an alkaline pH (pH=7.51) while PI for F. chinensis and M. japonicas SGP-V (MIH A) was calculated as 6.14 and 8.27, respectively. PI for MIH1 both in L. vannamei and M. Ensis was 51/7. P. semisulcatus instability index is anticipated to be 31/31 (if instability index is calculated less than 40, protein is stable and if more than 40, the protein is unstable.). Probably all six cysteine amino acids in the protein Participate in disulfide bond formation. The formation of disulfide bonds between cysteine amino acids at positions 7 to 44, 24 to 40 and 27 to 53 were predicted by DiANNA Server (Ferre and Clote 2005). Ironically, the instability index in *M. Japonicus* is calculated to be more than 40 MIH1. Thus MIH A of this species is unstable. While this protein has six cysteine amino acids and DiANNA software predicted three disulfide bonds for this. A probable argument in this context is that in addition to the formation of disulfide bonds, aliphatic index factor is also effective in protein stability. As this factor is larger, protein is more stable. Aliphatic index for MIH1 *P. semisulcatus* was calculated to be 51/83. For species with instability index below 40, aliphatic index is numerically calculated higher than 83, while this index for MIH in *M. japonicus* was calculated as 44/7. Thus the prediction of instability for MIH B *M. japonicus* is due to this reason.

Like other genes of family members CHH/MIH/GIH, the gene encoding MIH1 in the green tiger shrimps has two introns and three exons (Gu, et al., 2002; Yodmuang et al., 2004, Wiwegweaw et al., 2004; Chen et al., 2007). In this study, MIH was studied in three levels of protein, mRNA (coding region and intron) and gene in reptile Decapoda species (non-swimmers) whose nucleotide sequence was reported. The phylogenetic trees drawn on the amino acid sequences of protein and nucleotide sequence of the mRNA were quite similar. Phylogenetic studies in the mRNA sequence suggest that the closest species to the green tiger shrimp is *P. monodon* (MIH1) and then (MIH1) *L. vannamei*. The minimum genetic distance is calculated between the sequences from this study (P. semisulcatus) with P. monodon (MIH1). These findings could be expected in the same gene because the two species P. semisulcatus and P. monodon belong to the same subgenus of the Penaeus. Amino acid sequence of this hormone in the Penaeus semisulcatus, Litopenaeus vannamei (MIH 1) and Penaeus monodon (MIH 1) species are exactly the same.

In this paper, according to the capabilities of the repetitive sequences in the regulation of gene expression and the presence of the green tiger shrimp MIH gene, using the Censer software revealed the presence of two repeated sequences one of which is located in intron I (LiMDAS). The repetitive sequences in introns can be attributed to their role in regulating gene expression. Hence, according to the capabilities of the repetitive sequences in the regulation of gene expression, the transcription factor binding sites in introns I and II were studied by NSite. The results showed that although intron II has more than twice length of the intron I, but the TF binding motifs in the two introns are approximately equal. This result suggests that introns near the promoter are more efficient in regulation of gene expression (Furger, et al., 2002). Although most of these binding sites are specific for human transcription and viral factors, some of these sites have been identified for regulatory elements of Decapoda. For example, the Pit-1 like site (*), FTZ-F1transcription factor binding site in intron I and binding -like site for CREB transcription in intron II. Pit-1 is the most important inpituitary transcription factor that controls the expression of growth hormone and prolactin in the vertebrate. The presence of sequences with similarity to transcription factor binding site in intron I indicates the role of this transcription factor in regulation of MIH gene expression. The position of this transcription factor was also reported in promoter of CHH2 and CHH3 genes of *P. monodon* have (Wiwegweaw et al., 2004). Steroid hormone receptors function as transcription factors. FTZ-F1 is member of Orphan Nuclear Receptor family which acts with ftz transcription starting site and enables the transcription of ftz in Drosophila. This factor also has a role in the development of the nervous system. The transcription factor has also been sequenced in *M. ensis* (Chan and chan, 1999). The presence of transcription factor binding site in intron I in MIH gene in green tiger shrimp indicates a regulatory role in regulating gene expression MIH introns. The presence of binding-like site for CREB in intron II of MIH reveals that cAMP may have a role in regulation of gene expression. In addition to the native situations (100 and 412 to donor site and 223 and 679 to Acceptor site), sites with potential splicing (positions 123 and 347 to the donor site and 392, 436 and 645) were also identified for acceptor site. The sequences with this potential can create new isoforms of expression of gene MIH or produce new MIH genes over thousands of years. Software analyses show the presence of 5 different types of miRNA from genes MIH. We can predict that gene MIH implements part of its roles by miRNA.

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