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Isolation and characterization of the complete cDNA sequence encoding a putative insulin-like peptide from the androgenic gland of *Penaeus monodon*

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ABSTRACT

Sexual differentiation in male crustaceans is known to be controlled by the androgenic gland (AG), possibly through a peptide hormone. Recently, three freshwater and one marine crustacean decapod genes encoding AG-specific insulin-like peptides were characterized. We report here the molecular cloning of the complete sequence encoding an AG-specific insulin-like peptide (*Pm-IAG*) in the commercially important marine Penaeid prawn, *Penaeus monodon*. The deduced precursor sequence consists of a signal peptide, B chain, C peptide and an A chain. It exhibits the same structural organization as that of previously identified crustacean insulin-like androgenic gland specific peptides (IAGs). The positions of cysteine residues of the putative A and B chains, which govern the folding of the mature peptide via the formation of disulfide bridges, are highly conserved among the prawn and other crustaceans, while the rest of the amino acids show low sequence similarity. Gene expression analysis of *Pm-IAG* in several tissues, including the closely juxtaposed sperm duct and muscle, confirmed that it is specifically expressed in the AG. The findings suggest that with an appropriate intervention, sexual differentiation could be manipulated and thus might be instrumental for the establishment of monosex culture in this bimodally growing shrimp.

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1. Introduction

The androgenic gland (AG) plays an important role in crustacean sexual differentiation and manifestation of male secondary sexual characteristics. Ever since the discovery of the AG (Cronin, 1947), extensive studies have been conducted to define its function. Charniaux-Cotton (1962) was the first to report the AG's influence on male sexual characteristics in the amphipod *Orchestia gammarella*. Subsequently, it was demonstrated that implantation of AGs into young female isopod *Armadillidium vulgare* induced gonadal masculinization (Katakura, 1960; 1961). In the above isopod, an AG extract and later a recombinant peptide hormone were shown to induce male sexual differentiation (Katakura and Hasegawa, 1983; Martin et al., 1999; Okuno et al., 2002.

In decapod crustaceans, the AG has been shown to have wide ranging effects on sexuality. In intersex individuals of the Australian crayfish *Cherax quadricarinatus*, which are phenotypic males but genotypic females (Parnes et al., 2003), AG ablation leads to demasculinization and subsequent vitellogenesis (Khalaila et al., 1999). Further, AG implantation studies in the same species resulted in altered status of the females including the appearance of male secondary sexual characteristics (e.g., red patch along the propodus, plumose setation on pleopods), male-like aggression, courtship behavior and accelerated growth rate comparable to that of males (Barki et al., 2003; Karplus et al., 2003; Manor et al., 2004). In the giant freshwater prawn *Macrobrachium rosenbergii*, AG implantation transformed young females into phenotypic males (Nagamine et al., 1980). In the same species subsequent studies involving andrectomy lead to functional sex reversal into neofemales (Aflalo et al., 2006).

Male crustaceans have a unique assembly of separate gametogenic gonads and an AG, which regulates male sexual differentiation via secretion of sex hormones (Charniaux-Cotton and Payen, 1988). This physical division of two reproduction-related organs provides a unique opportunity to manipulate sex without risking the gonad tissue. The possibility of reversing phenotypic sex through manipulation of the AG, whilst maintaining the original genotype, offers immense scope for manipulating sexual phenotype. Successful sex reversal in crustaceans has significant application in development of a monosex population in species exhibiting dimorphic growth pattern, thus offering a tool to maximize productivity to the farming community (Aflalo et al., 2006). Virtually, all shrimps farmed today

Abbreviations: AG, Androgenic gland; Pm, *Penaeus monodon*; Cq, *Cherax quadricarinatus*; Mr, *Macrobrachium rosenbergii*; Cd, *Cherax destructor*; Pp, *Portunus pelagicus*; Arv, *Armadillidium vulgare*; Pod, *Porcellio dilatatus*; Pos, *Porcellio scaber*; IAG, insulin-like androgenic gland peptide; AGH, androgenic gland hormone; aa, amino acids.

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belong to the family Penaeidae and nearly 80% of those are either *Penaeus monodon* or *Litopenaeus vannamei* (Josupeit, 2004); both species exhibit dimorphic growth patterns. Studies suggest that sex determination in Penaeidae is chromosomal, wherein males are homogametic (ZZ) and females are heterogametic (ZW) (Benzie et al., 2001; Staelens et al., 2008). If indeed implantation of the androgenic gland or administration of a recombinant androgenic gland hormone is effective in reversing the females into functional males (neomales), mating between the neomale (ZW) and a normal female (ZW) should result in genotypes ZZ, ZW and WW, with a male to female ratio of 1:3, assuming that W is the female conferring chromosome and that WW individuals are viable. Additionally, if WW females are fertile, these individuals can be mated with normal males (ZZ) to produce all female progeny (ZW). Given the economic importance of penaeids, studies into sexual plasticity and the role of the AG in the regulation of sexual differentiation are of prime importance.

Histological and biochemical studies in Crustacea suggest that the AG hormone is proteinaceous in nature (King, 1964; Martin et al., 1996). Investigations into its structure show it to be a double-chain heterodimeric peptide (Martin et al., 1999). Recently in the decapods, *C. quadricarinatus*, *M. rosenbergii and P. pelagicus*, AG-specific insulin-like genes termed *Cq-IAG*, *Mr-IAG* and *Pp-IAG*, respectively, have been identified (Manor et al., 2007; Sroyraya et al., 2010; Ventura et al., 2009) The overall structural organization of pro-insulins consists of a B chain, a C peptide and an A chain. Though their conservation level in terms of amino acid sequence and peptide varied, the pro-insulins found in the AGs of these crustaceans show remarkable structural similarity. The mature peptide is a product of post-translational processing in which removal of the C peptide takes place, resulting in a heterodimer consisting of A and B chains interlinked by disulfide bonds (Martin et al., 1999; Okuno et al., 2002).

Recently, injection of double stranded RNA constructs based on the *Mr-IAG* sequence resulted in decreased spermatogenesis and male sexual characteristics, further confirming *Mr-IAG*'s role in male prawn sexual differentiation (Ventura et al., 2009).

Here we report for the first time the isolation and characterization of the complete *Pm-IAG* transcript sequence, encoding a putative AG-specific insulin-like peptide in the commercially valuable marine Penaeid *P. monodon*.

2. Materials and methods

2.1. Animal husbandry and tissue sampling

Wild caught adult *P. monodon.* males, weighing approximately 90 g, were obtained from a commercial broodstock supplier and maintained as per standard prevailing tiger prawn hatchery practices. They were fed twice a day *ad libitum* on a mixed fresh diet of squid and mussel. Hypertrophication of the AG was achieved by bilateral eyestalk ablation which incorporates the X-organ sinus gland complex (Khalaila et al., 2002). Ten days post-eyestalk ablation, dissections were performed on anaesthetized prawns subjected to cold seawater. AGs, sperm duct, abdominal muscle, eyestalk, antennae, lymphoid organ and hepatapancreas were dissected and preserved in RNAlater (Ambion Inc., USA) until use.

2.2. Construction of AG cDNA library

RNA extraction was performed using RNeasy plus Mini kit (Qiagen, Netherlands). To get a broad representation of transcripts, a total of 8 AGs from 4 individuals were used. AG cDNA, which was synthesized using 1 μg of total RNA, was used for the library construction using Creator SMART cDNA Library Construction Kit (CLONTECH Laboratories Inc., USA). The subsequent PCR products were cloned into pDNR-lib vector. The recombinant plasmids were transformed into α-Select Chemically Competent Cells (BIOLINE, UK). Colony PCR was conducted on 96 distinct colonies with M13F and M13R primers using the following cycling parameters: 95 °C for 5 min, followed by 25 cycles of 94 °C for 20 s, 48 °C for 30 s and 68 °C for 2 min. Sequencing was carried out by Macrogen Inc (South Korea). The sequencing results were analyzed using sequence alignment software Sequencher™ (Gene Codes Corp., USA).

2.3. RT-PCR

Total RNA from AGs, sperm duct, muscle, eyestalk, antennae, lymphoid organ and hepatapancreas was extracted using RNeasy plus Mini kit (Qiagen, Netherlands). Total RNA (1 μg) was used for the synthesis of cDNA using SuperScript III (Invitrogen Inc., USA) according to the kit manufacturer's protocol. Resulting cDNA (1 μl) (which represents 10% of the total cDNA generated from 1 μg RNA) was amplified by PCR (94 °C for 3 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s and 72 °C for 10 min. Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to design gene specific primers (*Pm-IAG* F – 5′-AGCTGCTTGTGGGAATGC-3′ and *Pm-IAG* R – 5′-GGTACTGGCGAG-GAAGCA-3′).

2.4. In situ hybridization

Hypertrophied AGs attached to the vas deference were fixed in 4% paraformaldehyde and processed as described previously (Ventura et al., 2009). Digoxygenin (DIG)-labeled oligonucleotides for antisense and sense probes, corresponding to nucleotides 50–313 of *Pm-IAG* cDNA, were synthesized using the T7 RNA polymerase (New England Biolabs, USA). Hybridization was carried out as described previously (Shechter et al., 2005), with the slight modification of adding 100 μg/ml tRNA to the hybridization solution. The DIG was visualized using colorimetric substrates NBT/BCIP (Roche Diagnostics) according to the manufacturer's instructions and slides were observed under a light microscope.

2.5. Bioinformatics analysis

Multiple sequence alignment was performed using Sequencher™ (Gene Codes Corp., USA). The sequences were trimmed of any poor quality sequences, stripped of primer sequences and any vector contamination followed by sequence assembly. The resulting contigs and singlets were compared to the Uniprot database (Swiss-prot and TrEMBL) and in particular to the AG-specific sequences of decapods (*Cq-IAG* and *Mr-IAG*). The full length of putative *Pm-IAG* was computationally translated with the ExPASy Proteomics Server (http://au.expasy.org/tools/dna.html) and the most likely frame selected depicting the open reading frame. The translated sequence was further analyzed by SMART (http://smart.embl-heidelberg.de/smart) for the prediction of conserved domains and CBS prediction servers (http://www.cbs.dtu.dk/services) for prediction of signal peptide and N-linked glycosylation sites. Potential endoproteinase arginine cleavage sites for removal of C peptide were processed with ExPASy Proteomics Server (http://au.expasy.org/tools/peptidecutter).

Phylogenetic analysis and multiple sequence alignment of the putative B and A chains of *Pm-IAG* (Genbank accession number: GU208677.1) with known sequences of AG-specific insulin-like factors including four decapods (*Cq-IAG* accession number: DQ851163.1, *Mr-IAG* accession number: FJ409645.1, *Cd-IAG* accession number: EU718788.1, *Pp-IAG* accession number: HM459854) and three isopods (*Arv-AGH* accession number: AB029615.1, *Pod-AGH* accession number: AB089811.1, *Pos-AGH* accession number: AB089810.1) was conducted using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). LALIGN server (http://www.ch.embnet.org/software/LALIGN_form.html) was employed to find sequence similarity among various AG-specific insulin-like factor sequences.

Table 1
Sequence identity and number of amino acid (aa) overlap (LALIGN) among various AG-specific insulin-like genes.

Species compared	% Identity	aa overlap
Pm-IAG vs. *Cq-IAG*	40.30	72
Pm-IAG vs. *Cd-IAG*	40.30	72
Pm-IAG vs. *Pp-IAG*	40.30	72
Pm-IAG vs. *Mr-IAG*	36.0	75
Pm-IAG vs. *Arv-AGH*	32.9	73
Pm-IAG vs. *Pod-AGH*	32.9	73
Pm-IAG vs. *Pos-AGH*	31.5	73

The 3D model of *Pm-IAG* was created by the FUGUE web server (http://tardis.nibio.go.jp/fugue/) and processed by Swiss-PdbViewer software (http://spdbv.vital-it.ch/).

3. Results

A *P. monodon* cDNA library was constructed, and subsequent sequencing and assembly of 96 clones resulted in 9 contigs and 50 singlets, thus representing 59 putative genes. Among those was the complete coding sequence of *Pm-IAG* (GenBank accession no. GU208677, *Penaeus monodon* insulin-like androgenic gland hormone precursor, mRNA, complete cds), the identification of which was based on sequence similarity with the *Cq-IAG* and *Mr-IAG* putative peptides. Though there was low sequence similarity among the above IAGs (Table 1), the cysteine backbone conservation, typical of insulin family of genes, provided necessary clues to the identification of *Pm-IAG*.

RT-PCR of sequences corresponding to *Pm-IAG* on the AG, sperm duct, muscle, eyestalk, antennae, lymphoid organ and hepatopancreas from male prawn showed that it is expressed exclusively in the AG (Fig. 1). RNA *in situ* hybridization further confirmed that *Pm-IAG* transcript is localized strictly to the AG and not to neighboring tissues such as the sperm duct, connective tissue and muscle (Fig. 2). A specific signal in the AG cells was clearly observed only when using the antisense probe (Fig. 2, right). No signal was detected when using the sense probe (Fig. 2, middle).

The predicted complete coding sequence encodes for the prepro-peptide of 176 amino acids with a predicted mass of 19.85 kDa. Bioinformatics analysis indicates its structural similarity to insulin-like family of peptides with a signal peptide at its N-terminus (33 aa) and B and A chains (45 and 31 aa). These chains are connected by a predicted C peptide that is assumed to be proteolytically cleaved at potential endoproteinase arginine cutting sites at the N and C termini to form the putative mature heterodimer peptide comprising B and A chains with a predicted mass of 8.6 kDa. The predicted mature peptide has 7 cysteine residues, 6 of which are conserved among the insulin-like family of peptides and a single N-glycosylation site near the N-terminus of the B chain (Fig. 3).

The predicted mature peptide was further analyzed for sequence similarity with published sequences of AG-specific insulin-like peptides. The multiple sequence alignment of *Pm-IAG* with 7 other known AG-specific insulin-like peptides reveals a backbone of 6 conserved cysteine residues which form a typical representation of the insulin family of hormones (Figs. 4A and 5A). The predicted disulfide bonds form two inter-chain bridges across the two chains and one intra-chain bond on the A chain which are suggested to be pivotal for the acquisition of the functional structure of the mature dimeric peptide.

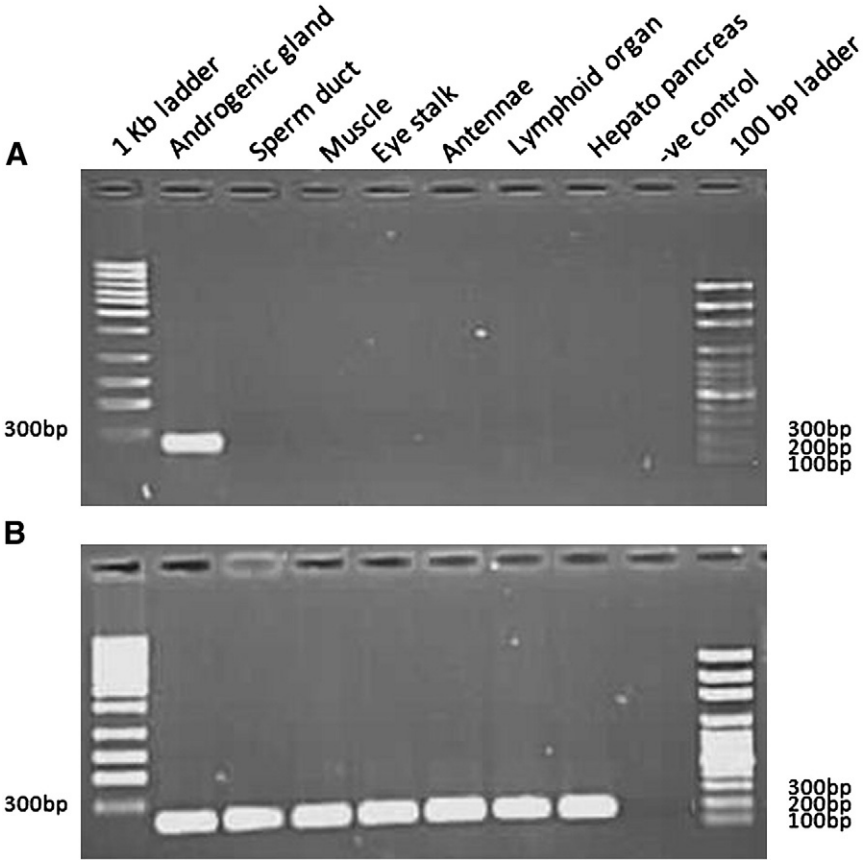

Fig. 1. *Pm-IAG* tissue-specific expression in mature male prawns. (A) As revealed by RT-PCR, *Pm-IAG* amplification is shown only in lanes containing cDNA from the androgenic gland. No amplification was observed in other tissues including sperm duct, muscle, eyestalk, antennae, lymphoid organ and hepatopancreas or in the negative control. (B) A housekeeping gene *β-actin* served as positive control.

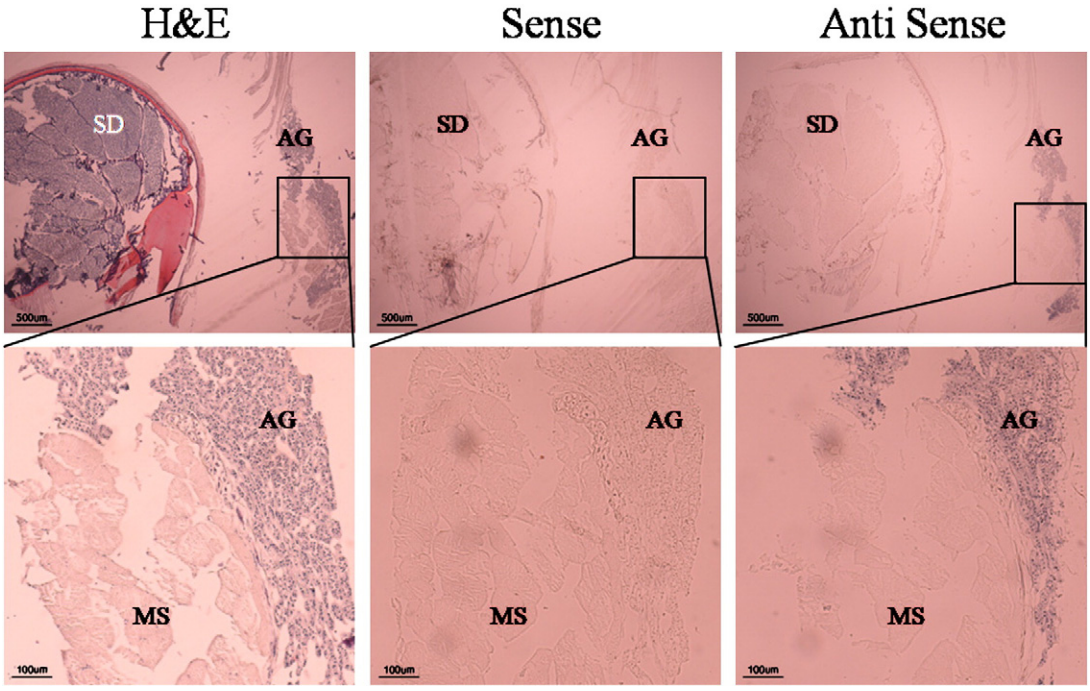

Fig. 2. *Pm-IAG* transcript localization in adult *P. monodon* 6 days post-eyestalk ablation. RNA *in situ* hybridization was performed on consecutive sections (5 μm) of the muscle (MS), sperm duct (SD) and the attached androgenic gland (AG). Sections were probed with either *Pm-IAG* sense (middle) or antisense probes (right). A specific signal restricted to the AG cells was observed only when using the antisense probe (right). No signal was observed when using the sense probe (middle). Standard hematoxylin-eosin (H&E) staining was also performed (left). In bottom row are enlargements of boxed areas in the top row. Top row, bar = 500 μm. Bottom row, bar = 100 μm.

The mature peptide sequences were further subjected to percentage of sequence similarity with the LALIGN server. *Pm-IAG* showed relatively low sequence similarity when compared with other decapod insulin-like sequences showing 40.30% with *Cq-IAG*, *Cd-IAG and Pp-IAG* over a 72 amino acid (aa) overlap and 36.0% sequence similarity with *Mr-IAG* over a 75 aa overlap (Table 1). This is in contrast to some of the decapods where they share a high sequence similarity of 95.1% (*Cd-IAG and Pp-IAG*) and 91.5% (*Cq-IAG and Pp-IAG*). Both members of the genus *Porcellio*

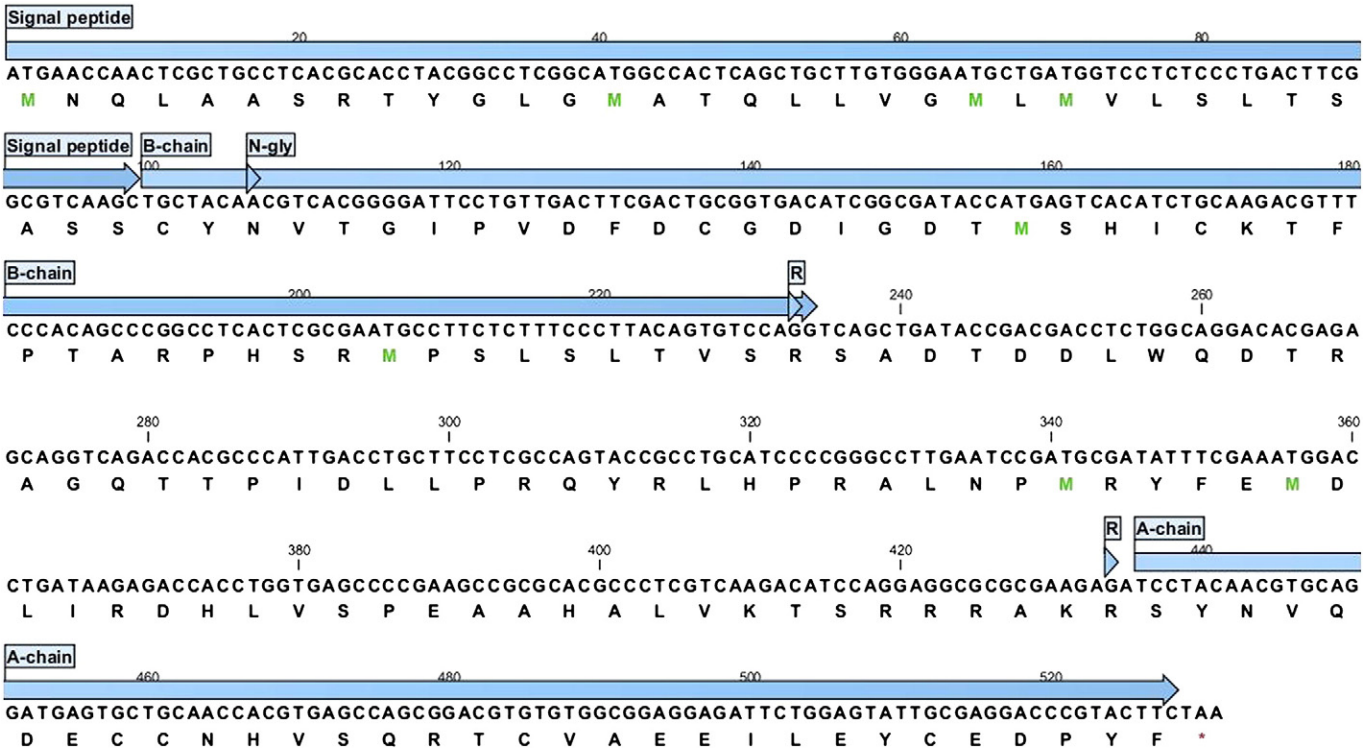

Fig. 3. *Pm-IAG* cDNA sequence and its deduced amino acid sequence. The putative signal peptide is flagged from nucleotides 1 to 99 (33 aa). The putative B chain (100–234 bases, 45 aa) has a single N-glycosylation site at 107th base. A chain is flagged from 436 to 528 bases (31 aa). The potential arginine C-proteinase cleavage sites are flagged with R labels. The putative C peptide is unflagged. The stop codon is marked with an asterisk.

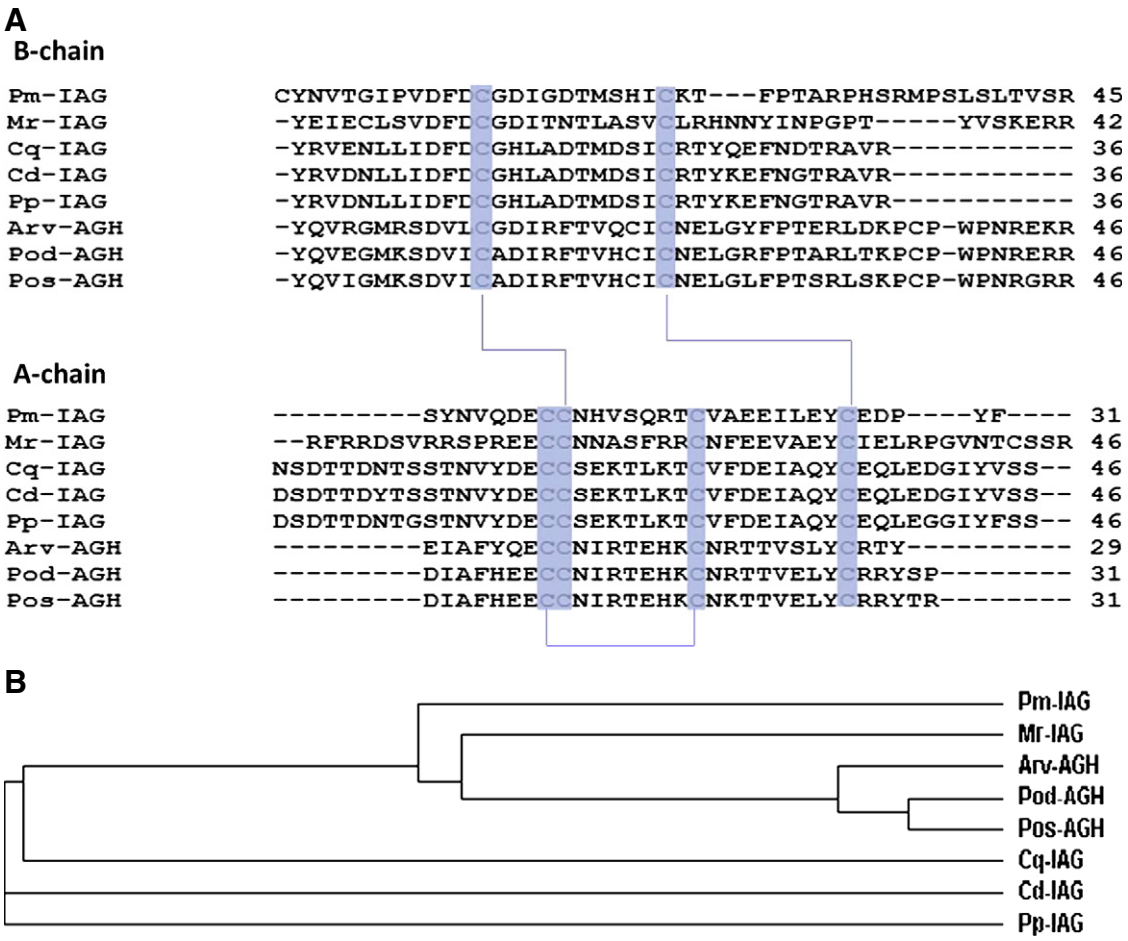

Fig. 4. (A) Multiple sequence alignment of the putative B and A chains of *Pm-IAG* with known sequences of AG-specific insulin-like factors. Multiple sequence alignment of the publicly available sequences of crustacean AG-specific insulin-like factors was obtained using ClustalW. The alignment includes sequences from four decapods: *M. rosenbergii, C. quadricarinatus*, *C. destructor* and *P. pelagicus*; and three sequences from Isopoda: *Armadillidium vulgare*, *Porcellio dilatatus* and *Porcellio scaber*. The conserved cysteine residues are highlighted among the sequences and the predicted disulfide bridges are drawn. (B) Cladogram of AG-specific insulin-like peptide from 5 decapod species including *Pm-IAG* and from 3 isopod species using ClustalW. The *Pm-IAG* forms a common clade along with isopods and *Mr-IAG*, indicating sequence divergence from other decapods.

(accession no. AB089810 and accession no. AB089811) shared high sequence similarity (90.8% in 76 aa overlap), whereas *A. vulgare* (accession no. AB029615) shared a similarity of 81.3% in 75 aa overlap with *P. dilatatus* and 78.7% in 75 aa overlap with *P. scaber* (Table 1).

Sequence similarity results are further reflected in the cladogram of the analyzed sequences. Interestingly, *Pm-IAG* and *Mr-IAG*, though belonging to the order decapod, form a common clade with isopods and are distinctly grouped as a result of their divergent sequences (Fig. 4B).

*Pm-IAG*h 3D model was based on its organizational similarity to several insulin-like peptides from the following organisms: *Bos Taurus* (PDB 2INS), *Sus scrofa* (PDB 4INS), *Homo sapiens* (PDBs 2GF1, 1IGL and 6RLX) and *Bombyx mori* (PDB 1BON). The peptide backbones of B chain amino acids 37–63 and A chain amino acids 147–171 are presented in the ribbon diagram. Residues 34–36 and 64–78 (found at the N and C termini of the B chain, respectively) and residues 146–147 and 172–176 (found at the N and C termini of A chain, respectively) were not included in the model because of the differences in length between the putative mature *Pm-IAG*h and the consensus structure of the mentioned insulin-like peptides. The side chains of cysteine residues containing disulfide bridges are displayed in yellow (Fig. 5B).

4. Discussion

In this study, we have found and characterized an AG-specific insulin-like gene (*Pm-IAG*) from a member of commercially important family of Penaeidae. Corresponding sequences in other crustaceans have been shown to play a major role in sex differentiation and masculinization (Ventura et al., 2009). *Pm-IAG* shows low sequence similarity to known AG sequences but shares the basal framework, typical of insulin-like genes. Among all the eight AG-specific insulin-like genes compared, the positions of six cysteine residues are conserved (Fig. 4A). *Pm-IAG*h features a typical organization of the insulin family of peptides, with the predicted prepro-hormone organized into signal peptide, B chain, C peptide and an A chain. Unlike the case of decapods, the three isopod AG hormones share two additional cysteine residues, enabling the formation of an additional intra-chain disulfide bond on the B chain (Ohira et al., 2003). Further studies, using recombinant and semisynthetic isopod AGH, reveal that correct pairing of the cysteine residues is essential for conferring biological activity (Katayama et al., 2010). Among the decapods, the deduced Mr-IAG sequence also has two additional cysteine residues, in different locations to that of the isopods: one on the B chain (aa 5) and another on the A chain (aa 43), indicating a possible additional inter-chain disulfide bond (Ventura et al., 2009). The occurrence of a fourth disulfide bond in the family of insulin-like peptides has been reported in the molluscan *Lymnaea stagnalis* (Smit et al., 1996) and in *Caenorhabditis elegans* (Duret et al., 1998). The presence of an additional single cysteine residue in *Pm-IAG*h at the beginning of the N-terminus of the B chain requires further investigation and analysis. *Pm-IAG* has a typical proteolytic cleavage motif of R-X-X-R on the C terminus of the C peptide and a potential proteolytic cleavage site at the C terminus of the B chain represented by a single arginine residue.

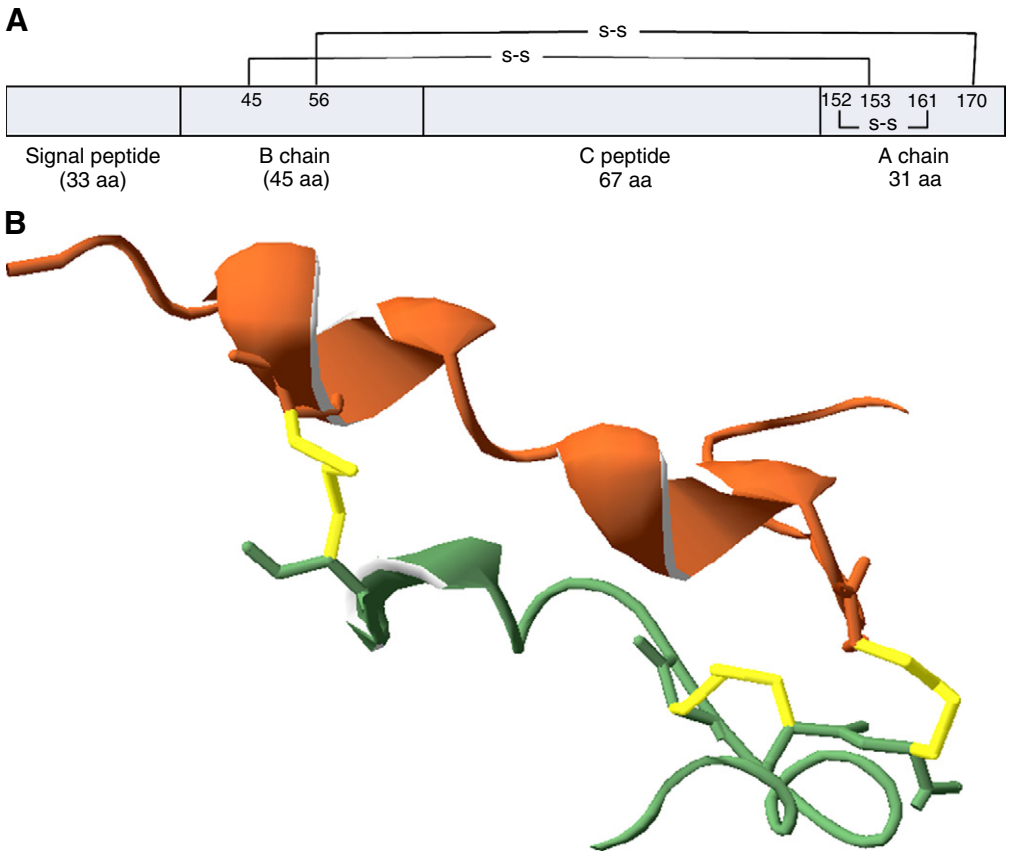

Fig. 5. (A) A schematic diagram of *Pm-IAG*. The model shows classical insulin-like organizational structure with a signal peptide, B chain, C peptide and A chain. Proteolytic cleavage of the C peptide by endoproteinase arginine results in a heterodimeric peptide of B chain and A chain. (B) The predicted 3D model of the suggested mature *Pm-IAG*. The model indicates that *Pm-IAG* demonstrates a unique insulin-like structure. B chain is represented in orange and A chain in green. Disulfide bonds are displayed in yellow.

These recognition sites are assumed to be proteolytically cleaved in the removal of the C peptide and consequently the formation of the functional heterodimeric form (comprising B chain and A chain).

Pm-IAG has a single putative N-linked glycosylation motif (N-X-S/T) on the B chain (aa 3). This is in agreement with the single putative N-linked glycosylation sites found on AG hormones of other crustaceans considered for this study, with the exception of *Cq-IAG*, which is reported to have two putative N-linked glycosylation sites. Molecular characterization of native AGH in *A. vulgare* identified that it is an N-linked glycosylated hormone (Martin et al., 1999; Okuno et al., 1999). Glycosylation of peptides is generally known to facilitate proper folding of the proteins, receptor–ligand recognition and also confers stability. In a study involving synthesis of recombinant AGH with and without glycan moiety using baculovirus-infected Sf9 cells and *E. coli* expression systems, respectively, it was found that only the glycosylated heterodimer showed hormonal activity (Okuno et al., 2002). In the same study, it was also shown that in addition to glycosylation, proteolytic cleavage of the C peptide was an important post-translational modification for attaining hormone activity. This formation of a heterodimeric peptide formed upon cleavage to obtain functionality is a typical feature of the insulin family.

Although the structural organization of *Pm-IAG* conforms to insulin-like peptides, it shows low sequence similarity to other crustacean IAGs. *Pm-IAG* shares low sequence identity with reference to isopods and even among decapods, possibly indicating the evolutionary divergence of Penaeid order. This can explain why extensive efforts to identify the orthologous AG-specific insulin-like gene in *P. monodon* through the use of degenerate primers designed on the basis of the *Cq-IAG* sequence yielded no results. Such similar negative results were experienced when attempting to identify *Cq-IAG* with degenerate primers designed on the basis of isopod sequences (Manor et al., 2007). In this context, attention is drawn to the narrow species-specificity found with heterospecific implantation trials of the AG in isopods (Martin and Juchault, 1999).

Wide-ranging morphological, physiological and behavioral effects have been observed in response to AG implantation, including masculinization, courtship behavior and an increase in growth (Barki et al., 2006; Khalaila et al., 2001; Nagamine et al., 1980; Sagi et al., 1997). In *M. rosenbergii and C. quadricarinatus*, males display higher growth rates. The effect of the AG in promoting growth of the above two species was demonstrated by implantations of respective AGs into females which resulted in enhanced growth rates, comparable to those of males (Manor et al., 2004; Sagi et al., 1997). Questions have been raised as to whether such diverse biological effects could be attributed to a single factor, such as the AG hormone (Sagi and Khalaila, 2001).

The AG hormone belongs to the insulin superfamily of peptides, which includes known potent growth enhancers such as insulin-like growth factors found in various organisms (Lelbach et al., 2005). The AG hormone therefore seems to be an attractive candidate for being a key factor contributing to the observed AG induced growth enhancement. This is further supported by RNAi experiments in which *Mr-IAG* silencing reduced growth rates in male prawns (Ventura et al., 2009). However, the inverse dimorphic growth pattern observed in *P. monodon* (Hansford and Hewitt, 1994), where females grow significantly faster than males, suggests factors other than the AG to be involved in growth regulation. We suggest that the universal role of the AG is primarily related to sexual differentiation, specifically inducing masculinity. Various associated phenomena observed post-AG implantation, such as enhanced growth rates, behavioral changes and morphological changes, have variable levels of manifestations in a species-specific manner, and are influenced by the sexual plasticity of crustaceans and their amenability to endocrine manipulation. In this perspective, the

direct or indirect role of the AG hormone in the context of growth remains ambiguous and needs further investigation.

5. Conclusion

This study has enabled us to characterize *Pm-IAG*, the first AG-specific gene in Penaeid family. Given the commercial importance of Penaeids, the fundamental understanding of the gene and its known role in sex differentiation and sex reversal can potentially lead to the development of all female monosex culture technologies based on the use of recombinant *Pm-IAG*h to produce neo males that will be crossed with normal females. Characterization of *Pm-IAG* has also added to the growing list of insulin superfamily of genes and their diversified roles, thus strengthening the notion that the insulin gene family might have evolved from a primary role in sex differentiation to their present metabolic and growth promoting 'portfolios' in higher vertebrates. Since *P. monodon* females have advantageous growth relative to males, the role of *Pm-IAG* could be mainly in male sexual differentiation rather than having a direct growth stimulating influence.

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