CHAPTER IV

DISCUSSION

Isolation and characterization of reproduction-related genes in ovaries of *P. monodon*

Closing life cycle culture of the giant tiger shrimp (*Penaeus monodon*) is crucial to the sustainability of the shrimp industry. However, poor reproductive maturation of captive *P. monodon* females and low quality of spermatozoa of captive males have limited the potential of genetic improvement, which in turn, resulted in remarkably slow domestication and selective breeding programs of *P. monodon* (Withyachumnarnkul et al., 1998; Preechaphol et al., 2007).

Molecular mechanisms and functional involvement of reproduction-related genes and proteins in ovarian development of *P. monodon* is necessary for better understanding of the reproductive maturation of *P. monodon* to resolve the major constraint of this economically important species in captivity (Preechaphol et al., 2007; Klinbunga et al., 2009).

Transcription in germ cells during oogenesis follows carefully regulated programs corresponding to a series of developmental events of oocytes (Grimes, 2004; Qiu and Yamano, 2005; Qui et al., 2005). Recently, genes expressed in the vitellogenic ovaries of *P. monodon* were identified and characterized. A total of 1051 clones from a conventional cDNA library were unidirectionally sequenced from the 5′ terminus. The nucleotide sequences of 743 EST (70.7%) significantly matched known genes previously deposited in GenBank (*E*-value $<10^{-4}$), whereas 308 ESTs (29.3%) were regarded as newly unidentified transcripts (*E*-value $>10^{-4}$). A total of 559 transcripts (87 contigs and 472 singletons) were obtained after sequence assembly. Several reproduction-related genes including *chromobox protein*, *ovarian lipoprotein receptor*, *progestin membrane receptor component 1* and *ubiquitin-specific proteinase 9, X chromosome*, were isolated and characterized (Preechaphol *et al.*, 2007).

Subsequently, suppression subtractive hybridization (SSH) libraries between cDNA in stages I (previtellogenic) and III (cortical rod) ovaries of the giant tiger shrimp (*Penaeus monodon*) were established. In all, 452 ESTs were unidirectionally sequenced. Sequence assembly generated 28 contigs and 201 singletons, 109 of which (48.0%) corresponding to known sequences previously deposited in GenBank. Several reproduction-related transcripts were identified.

In order to provide a further insight into the molecular mechanisms involved in the reproductive development and maturation processes of *P. monodon*, the full length cDNA of *O-methyltransferase* (*PmCOMT* and *PmFAMeT*) and ecdysteroid-responsive genes (*PmBr-cZ1* and PmBr-cZ4) in *P. monodon* were characterized.

The full length cDNA of *O-methyltransferase* (*OMT*) was first identified in *F. chinensis* (ORF of 666 bp encoding a polypeotide of 221 amino acids). Northern blot and *in situ* hybridization analyses demonstrated that the OMT transcripts were constitutively expressed in various tissues and it was up-regulated in hepatopancreas and stomach in shrimp challenged with a mixture of live *Vibrio anguillarum* and *Staphylococcus aureus* suggesting that *OMT* may play multi-functions in physiological processes (Li, D.X. et al., 2006).

In this study, the full length cDNA and genomic DNA of *PmCOMT* was characterized. *PmCOMT* exhibits a relatively high degree of sequence similarity with *catechol-O-methyltransferases domain-containing protein 1* in other species implying the conserved function of this gene family across taxa. Unlike other *P. monodon* genomic DNA sequences (e.g. cyclins A and B, Visudtiphole et al., 2009) previously reported, the exon-intron boundaries of *PmCOMT* did not follow the GT/AG rule. Moreover, the deduced PmCOMT lacks a signal peptide and regarded as non-secretory protein in ovaries. *PmCOMT* and *PmFAMeT* from various species (*Homarus americanus*, *Metapenaeus ensis*, *L. vannamei* and *M. japonicus*) were allocated to be different groups of OMT. Phylogenetic analysis indicated that both *PmCOMT* and *F. chinensis OMT* are members of *catechol-O-methyltransferase*.

The *PmCOMT* transcripts were expressed in various tissues of broodstock but more abundantly expressed in ovaries and intestine than other tissues. Similar results were found from western blot analysis and a low level of expression was observed in

hemocytes (data not shown). Presumably, PmCOMT may majorly involved in catecholamine degradation of non-steroid producing tissues but contribute in steroidogenesis in ovaries. The actual function of *PmCOMT* in a particular environment needs to be elucidated in shrimp.

Two forms of the full length cDNA of *PmFAMeT* were characterized and reported. *PmFAMeT* exhibits a relatively high degree of sequence similarity with that from other species. Like *FAMeT* in *L. vannamei*, the two isoforms of the deduced PmFAMeT are different according to the presence/absence of a five pentapeptide (Glu-Gly-Arg-Gly-Ser) (Hui et al., 2008). The significance of this pentapeptide insertion within the second CF domain on activity of PmFAMeT-s should be further investigation. Phylogenetic analysis clearly indicated that both *PmFAMeT-l* and *PmFAMeT-s* are closely related to *FAMeT* of other decapod crustaceans and are regarded as members of crustacean *FAMeT* rather than *COMT*.

The deduced PmFAMeT lacks a signal peptide and regarded as non-secretory protein in ovaries. Like, LvFAMeT, tissue distribution analysis revealed constitutive expression of the *PmFAMeT* transcript in various tissues. Western blot analysis revealed a possible post-translational modification of the PmFAMeT protein during ovarian development of *P. monodon* as both the sequence-expected size of 32 kDa and a greater product size of 37 kDa were observed. In addition, the preliminary data on expression of the PmFAMeT protein across various tissues indicated the positive 37 kDa band in pleopods, both 32 and 37 kDa bands in ovaries, eyestalk and epicuticle and only a 32 kDa band in other tissues (data not shown). The wide distribution of PmFAMeT expression may be related to its role in growth and regulation of molting as suggested in *L. vannamei* (Hui et al., 2008).

In addition, two isoforms of the full length cDNA of *PmBr-cZ1* and a single isoform of *PmBr-cZ4* were successfully characterized. The deduced PmBr-cZ1 and PmBr-cZ4 proteins contained BTB domains which are the highly conserved amino-terminal domain and widely distributed the protein-protein interaction motifs found in a family of transcription factors that play critical roles in cellular differentiation, development, and neoplasia (Costoya and Pandolfi, 2001; Lin et al., 2001) and four (for *PmBr cZ1)* or two (for *PmBr cZ4)* ZnF C_2H_2 domains as those of others species. Both *PmBr-cZ1* and *PmBr-cZ4* were more abundantly expressed in ovaries than other

tissues of *P. monodon* broodstock. This indicated that both genes should play an important role on ovarian/oocyte development in *P. monodon*.

Expression of reproduction-related genes during ovarian development of *P. monodon* by semi-quantitative RT-PCR and quantitative real-time PCR

Ovaries are functionally important in reproduction and secretion of hormones for growth and development regulation. Ovarian maturation of *P. monodon* results from rapid synthesis and accumulation of a major yolk protein (vitellin) (Meusy and Payen, 1988; Yano and Hoshino, 2006). Understanding the role of various genes/proteins during ovarian and oocyte development of *P. monodon* may lead to the possible ways to effectively induce ovarian maturation in shrimp.

Quantitative real-time PCR revealed higher expression of *PmCOMT* in ovaries of juveniles than intact wild broodstock suggesting that PmCOMT may be necessary for development of premature ovaries of juvenile *P. monodon* and that *PmCOMT* may play a different role during juveniles. *PmCOMT* did not show differential expression profile in ovaries of intact broodstock. Expression of *PmCOMT* during reproductive maturation of intact and eyestalk-ablated *P. monodon* broodstock indicated that *PmCOMT* mRNA is sufficient for ovarian development but a greater level of this transcript may be required during growth and development of juvenile shrimp. *PmCOMT* was comparably expressed in different ages of domesticated juveniles (6 months old) and broodstock (14 and 18 months old).

Expression of total *PmFAMeT* rather than that of *PmFAMeT-l* and *PmFAMeT-s* was examined during ovarian development of intact and eyestalk-ablated *P. monodon*. The ovarian *PmFAMeT* transcript was significantly increased at the final stage (IV) of ovarian development in both intact and eyestalk-ablated broodstock.

The expression level of ovarian *PmFAMeT* of domesticated juveniles and broodstock seemed to be gradually decreased following the cultivation period. This suggested reduced reproductive maturation in domesticated shrimp found in our domestication program at present. The information suggested that *FAMeT* gene products play the important role during the maturation stage of *P. monodon* ovaries.

Eyestalk ablation caused an increase in the mRNA levels of *vitellogenin* and *cortical rod protein* in ovaries of *M. japonicus* (Tsutsui et al., 2005; Okumura et al., 2006). Likewise, the increase in mRNA and earlier up-regulation (vitellogenic, early cortical rod and mature stages) of *PmFAMeT* during ovarian development in eyestalk-ablated female broodstock suggests that gonad inhibiting hormone (GIH; Meusy and Payen, 1988) directly affects *PmFAMeT* transcription. Therefore, the mRNA levels of *PmFAMeT* may be used as the biomarker to reveal degrees of reproductive maturation of *P. monodon.*

The expression level of *PmBr-cZ1* was down-regulated in stage II and III before returned to the normal level at stage IV and after spawning in intact broodstock. Its expression level in stages IV (mature ovaries) was significantly greater than that in stage I (previtellogenic) ovaries and II (vitellogenic) in eyestalk-ablated broodstock. The high levels of expression were also observed in domesticated shrimp (6, 14 and 18 months old). This strongly indicated that the expression profiles of *PmBr-cZ1* can be used to monitor the degrees of reproductive maturation following the effects of hormonal administration and/or maturation diets.

Interesting results were observed from expression profiles of *PmBr-cZ4* during different ovarian stages of intact *P. monodon*. The down-regulation of *PmBr-cZ4* at stage IV ovaries in intact broodstock was observed. In addition, eyestalk ablation significantly reduced its expression levels in comparison with intact shrimp. This implied that lower levels of these gene products may be necessary for the development and final maturation of *P. monodon* oocytes. The findings facilitate the possible use of RNA interference (RNAi) for studying their functional involvement in *P. monodon* ovarian development.

Effects of neurotransmitter, progesterone and ecdysteroid administration on expression of reproduction-related genes in ovaries of *P. monodon*

In *P. monodon*, effects of dopamine on ovarian development have not been reported. Nevertheless, simultaneous injections of 5-HT (25 μg/g body weight) and the dopamine antagonist spiperone (1.5 or 5 μg/g body weight) induced ovarian maturation and spawning in wild *F. stylirostris* and pond-reared *L. vannamei* (Alfaro et al., 2004). Results from this thesis indicated that dopamine administration (10^{-6}

M/shrimp) resulted in significant lower expression of *PmCOMT* in ovaries of juvenile shrimp at 24 hpi($P < 0.005$) but significant higher expression of *PmFAMeT* in ovaries of juvenile shrimp at 12 hpi ($P < 0.005$) compared to the treatment at 0 hpi. This was unexpected as dopamine should have stimulated the expression of rather *PmCOMT* than *PmFAMeT*. Accordingly, the experiments should be carefully repeated.

Effects of exogenous 5-HT on the reproductive performance of shrimp were reported (Vaca and Alfaro, 2000). 5-HT induced ovarian development of *P. monodon* (Wongprasert et al., 2006) and *M. rosenbergii* (Meeratana et al., 2006) dose dependently. Adminstration of 5-HT clearly promoted expression of *P. monodon* Ovarian-Specific Transcript (*Pm-OST1*) in ovaries of 5-month-old shrimp. *Pm-OST1* was up-regulated at 12-78 hpi ($P < 0.05$), with the highest expression level observed at 48 hpi ($P < 0.05$) (Klinbunga et al., 2009). Nevertheless, the effects of 5-HT on the expression levels of genes and/or proteins in the ovaries of *P. monodon* broodstock have not been reported.

In this study, effects of 5-HT on expression of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* in ovaries of 18-month-old *P. monodon* were examined. The injection of 5-HT resulted in increasing of *PmFAMeT* expression for at approximately 50-fold at 1 hpt but it has no effect on expression of other genes. Results clearly suggested that 5-HT plays the upstream effects but not the direct role on stimulation of steroidogenesis in ovaries of *P. monodon*. The molecular effects of 5-HT on *PmFAMeT* gene demonstrate the possible use of 5-HT in place of eyestalk ablation for enhancing ovarian/oocyte development in *P. monodon*.

Interestingly, administration of progesterone had no direct effects on promoting the expression of *PmCOMT*, *PmFAMeT* and *PmBr-cZ1* in ovaries of domesticated 14-month-old *P. monodon* but significantly affects expression of *PmBr-cZ4* at 48 and 72 hpi. The results also indicated that *PmFAMeT* gene products play the upstream effects preceding sex steroid resumption and maturation of ovarian/oocyte development.

Prechaphol et al. (2010) characterized *progestin membrane receptor component 1* (*Pgmrc1*) of *P. monodon*. The full-length cDNA of Pgmrc1 was 2015 bp in length containing an ORF of 573 bp corresponding to a polypeptide of 190 amino

acids. Northern blot analysis revealed a single form of Pgmrc1 in ovaries of P. monodon. Quantitative real-time PCR indicated that the expression level of *Pgmrc1* mRNA in ovaries of both intact and eyestalk-ablated broodstock was greater than that of juveniles ($P < 0.05$). *Pgmrc1* was up-regulated in mature (stage IV) ovaries of intact broodstock ($P < 0.05$). Unilateral eyestalk ablation resulted in an earlier up-regulation of Pgmrc1 since the vitellogenic (II) ovarian stage. Moreover, the expression level of Pgmrc1 in vitellogenic, early cortical rod and mature (II–IV) ovaries of eyestalk-ablated broodstock was greater than that of the same ovarian stages in intact broodstock ($P < 0.05$). *Pgmrc1* mRNA was clearly localized in the cytoplasm of follicular cells, previtellogenic and early vitellogenic oocytes. Immunohistochemistry revealed the positive signals of the Pgmrc1 protein in the follicular layers and cell membrane of follicular cells and various stages of oocytes. Therefore, *Pgmrc1* gene products seem to play the important role on ovarian development and may be used as the bioindicator for monitoring progression of oocyte maturation of *P. monodon*.

Progestin acts as the maturation inducing factor resulting in resuming meiotic maturation of oocyte (Kishimoto, 1999, 2003). The further interesting issue is identification of the appropriate form(s) of progestins that result in the signal transduction pathway in ooocytes of *P. monodon*.

The effects of 20E on expression of these genes in ovaries of juvenile *P. monodon* were carried out. The vehicle control (10% ethanol) seems to affect expression levels of *PmCOMT* and results were difficult to be interpreted. In contrast, the expression level of *PmFAMeT* in ovaries of cultured juveniles was obviously affected at 6-24 hpi while *PmBr-cZ1* and *PmBr-cZ4* were up-regulated at 168 hpi. The molecular effects of 5-HT on this expression should be further examined in both wild and domesticated broodstock to evaluate the use of ecdysteroids in combination with serotonin for enhancing ovarian/oocyte development in *P. monodon*.

Localization of *PmCOMT*, *PmFAMeT*, *PmBr-cZ1* and *PmBr-cZ4* transcripts and PmCOMT and PmFAMeT proteins in oocytes and ovaries of *P. monodon*

In situ hybridization was used to determine the ovarian localization of the mRNAs of reproduction-related genes in this study. *PmCOMT*, *PmFaMeT*, *PmBr-cZ1*

and *PmBr-cZ4* were localized in ooplasm of previtellogenic oocytes in all ovarian stages of intact and eyestalk-ablated broodstock. Generally, more intense signals were observed in ovaries of eyestalk-ablated broodstocl than intact broodstock. This simply revealed the effects of eyestalk ablation of transcript of examined genes. The finding suggested that these genes should be involved in oogenesis and ovarian development of *P. monodon*. Disappearance of hybridization signals of various genes in ooplasm of more mature stages (vitellogenic, early cortical rod and mature; stages II-IV) of oocytes may be due to significantly increasing oocytes sizes as oogenesis proceeded and low sensitivity of *in situ* hybridization on detecting gene expression per se (Klinbunga et al., 2009).

In situ hybridization signals of *PmFAMeT, PmBr-cZ1* and *PmBr-cZ4* were not observed in follicular cells and more mature (vitellogenic cortical rod and mature) stages of oocytes. This further indicated cell-type specific expression of these transcripts in ovaries of *P. monodon* broodstock.

Immunohistochemistry gave the interesting issues on the possible translocation of PmCOMT and PmFAMeT proteins during oogenesis in *P. monodon*. The positive immunohistological signals of PmCOMT were detected in cytoplasm of previtellogenic and vitellogenic oocytes. Nevertheless, the positive signals were observed in cortical rods of oocytes in both intact and eyestalk-ablated broodstock. Similarly, the positive signals of PmFAMeT were detected in cortical rods of stages III and IV oocytes in both intact and eyestalk-ablated broodstock of *P. monodon*. It is hypothesized that PmCOMT and PmFAMeT were translated and initially localized in cytoplasm of earlier stages of oocytes before translocated into the cortical rods at the later stages of oogenesis.

In vitro expression of rPmFAMeT-l and rPmFAMeT-s proteins and Western blot analysis

In this study, rPmCOMT, rPmFAMeT-s and rPmFAMeT-l were successfully expressed as the insoluble proteins in *E. coli*. The polyclonal antibodies against these recombinant proteins were successfully produced in rabbit. After affinity-chromatographic purification, anti-PmCOMT PcAb, anti-PmFAMeT-l PcAb and anti-PmFAMeT-s PcAb was used to study expression profiles and localization of a

particular protein by western blot analysis and immunohistochemistry (see above), respectively.

The expression profiles of *PmCOMT* mRNA and protein were slightly different. At the transcriptional level, the *PmCOMT* mRNA was stably transcribed but more intense signals of the PmCOMT protein were observed in previtellogenic and vitellogenic ovaries than those in cortical rod and mature ovaries of intact *P. monodon* broodstock. This implied that *PmCOMT* should be earlier translated since the early stages of ovarian development. The reduction of the translated protein in early cortical rod and mature ovaries suggested that the accumulated *PmCOMT* mRNA in oocytes is probably sufficient to be translated during the late stages of ovarian development in *P. monodon*.

Likewise, the expression profiles of *PmFAMeT* mRNA and protein were clearly different. The PmFAMeT transcripts were more abundantly transcribed during the final maturation of ovarian development. Nevertheless, the PmFAMeT protein was found in juveniles and stages I and II but not in stages III and IV ovaries of broodstock. The disappearance of this protein in more mature stages of ovaries of suggested more rapid translation of the PmFAMeT protein during previtellogenic and vitellogenic stages of ovarian development in *P. monodon*.

In the present study, the reproductive related genes of *P. monodon* were identified. The expression profile of various genes in ovaries of intact and eyestalk-ablated *P. monodon* broodstock implied that several key genes (from the same and/or different pathways) may contribute on ovarian development of *P. monodon*. Functionally analysis of genes and proteins involving ovarian development can be further carried out for better understanding of the reproductive maturation of female *P. monodon* in captivity.