CHAPTER VI
DISCUSSION

In this study, the dopamine receptor of *P. monodon* (DAR_Pem) was characterized in terms of sequence analysis and function. The possible involvement of DAR_Pem in shrimp reproduction was studied by investigating mRNA and protein expression of DAR_Pem in tissues of female *P. monodon* at different ovarian developmental stages.

6.1 Determination of the full-length DAR_Pem sequence

The full-length nucleotide sequence of DAR_Pem was determined by RACE strategy. Three clones of 3'RACE fragments exhibited nucleotide mismatches at four positions; these may come from a lack of proof-reading activity of taq DNA polymerase used for amplification. However, the deduced amino acid sequences from these clones showed 100% identity when compared with the coding DAR_Pem sequence that was subsequently obtained by *Pfu* DNA polymerase. The polyadenylation signal (AATAAA) was not found at the 3' end of the cDNA, however a few similar sequences were found downstream of the stop codon including a sequence AAGAAA that was previously reported as a polyadenylation signal in human (58). This AAGAAA sequence is probably used as a polyadenylation signal of DAR_Pem because it is located at appropriate distance about 50 nucleotides upstream of the poly A. The deduced amino acid sequence of DAR_Pem was highly similar to DAR type 1 of other arthropods such as *P. interruptus*, *B. mori*, *D. melanogaster*, *A. mellifera* and showed the highest homology at about 70% to $D1_{\beta\_Pan}$ of *P. interrupts* (33). The DAR_Pem contained the conserved seven transmembrane domains and other typical features of G-protein coupled receptors such as DRY sequence, N-link glycosylation site and the cysteines that are involved in structural conformation form by a disulfide bridge between the first and the second extracellular loops. The phosphorylation sites, which is believed to be important for G-protein couple receptors

were found at several positions in the short i3 loop and C-terminal tail. The amino acid residues that are also involved in ligand binding such as aspartic acid (D) residue in TM III, two serine (S) residues in TM V and phenylalanine (F) residue in TM VI domains, which are highly conserved among DAR in other organisms. From the above characteristics, this putative DAR_Pem protein is structurally related to type 1 of DAR.

6.2 Tissues distribution and mRNA expression of DAR_Pem in *P. monodon*

Determination of DAR_Pem expression in tissues of female *P. monodon* showed that DAR was expressed in diverse tissues at all ovarian stages and revealed the highest level of expression in the brain whereas low level was expressed in the ovary. Previous studies demonstrated that dopamine (DA) was mainly localized and played important functions in the brain of organism that was corresponded to its receptor (DAR) expression (9, 39). In addition, DA was also widely expressed in central nervous tissues and involved in controlling movement and emotion in vertebrate (9). DA distribution was reported in crayfish and other crustaceans, in which various functions of DA were demonstrated. For example, DA was detected in the neuropil and several neurons in abdominal ganglia of the lobster, *Homarus gammarus*, the crayfish, *O. limosus* and *Orconectus rusticus* that may play roles in movement and modulation of adaptive behavior (59). In addition, DA in stomatogastric nervous system of *P. interruptus* was demonstrated to control the movement of striated muscles in the gut (60). Therefore, these results suggested that DA was mainly synthesized and operated its function in the brain and central nervous tissues which is correlated with DAR_Pem expression in this study. In addition, DA was reported to be involved in ovarian development in many organisms. For examples, in the European eel, *Anguilla anguilla* and the teleost fish, DA inhibited the secretion of luteinizing hormone (LH) in vitellogenesis process (61-62). Similarly, in the invertebrate, DA was shown to inhibit egg laying in *C.elegan* (63). In crustacean, the levels of DA concentration were measured at each ovarian developmental stage in *M. rosenbergii* by HPLC-ECD method (64). DA levels in the brain increased slightly

from stage I to stage II and then declined at ovarian stage III to stage IV while DA levels in thoracic ganglia showed similar pattern but at the level higher than 6-folds of the brain. In *P. monodon*, the DAR expression in the brain was high from stage I through stage III and then slightly declined in stage IV showing correlation with the profile of DA level in the brain and thoracic ganglia of *M. rosenbergii*. In addition, DAR of *P. monodon* was expressed at low levels in ovary at all stages. This is in contrast to DA level in the ovary of *M. rosenbergii* that was higher than other tissues and showed high concentration in ovarian stage I, then slightly declined at ovarian stage II to stage IV. Localization of DA in *M. rosenbergii* by immunohistochemmistry (65) could detect DA in central nervous tissues and ovary in all ovarian stages, but DA showed intensive expression in thoracic ganglia more than other parts in central nervous tissues. In the ovary of *M. rosenbergii* DA was detected in the cytoplasm of previtellogenic oocyte (stage I) and vitellogenic oocytes (stage II to stage III), especially at stage I and stage II. This supported the hypothesis that, DA may inhibit the release of GSH and stimulates the release of GIH in vitellogenesis process probably by acting through DAR type 1. In addition, DAR_Pem seemed to be highly expressed in eyestalks of shrimp that developed the ovary from stage I to stage III and sharply dropped in stage IV. It is possible that expression DAR_Pem may concern with stimulation of GIH in eyestalks to inhibit the release of GSH in the brain or thoracic ganglia.

6.3 Protein expression of DAR_Pem in *P. monodon*

The C-terminal tail region of DAR_Pem was selected for polyclonal antibody production because it contained the most variable sequences across different classes of arthropod type1 DARs. The sensitivity and specificity test by dot blot and western blot analysis revealed that the anti C-tail DAR antibody could detect the lowest amount of 10 ng of recombinant C-tail DAR. The cross-reactivity of anti C-tail DAR antibody with *E. coli* proteins in dot blot was clearly demonstrated to be different proteins from C-tail DAR by western blot analysis. When used to detect DAR in several tissues of female *P. monodon* such as eyestalks, brain, hepatopancrease, nerve cord and thoracic ganglia, the anti C-tail DAR antibody did

not give any positive band in these tissues, although the DAR_Pem transcript could be detected by RT-PCR. This may indicate that the DAR_Pem levels in shrimp tissues examined is lower than the sensitivity (10 ng) of the antibody. The only tissues in which a positive band at the expected size about 49 kDa for DAR_Pem was observed was the ovary, which expressed very low level of DAR_Pem transcript. However, the same band at 49 kDa was also detected with rabbit pre-immunized serum in the ovary. This non-specific band was also detected by anti-serum from the other two rabbits and mouse. Therefore, this 49 kDa band should be the cross-reactivity of rabbit or mouse anti serum to shrimp ovarian proteins rather than the specific DAR_protein. Further investigation should be focused on the detection of DAR_Pem protein in brain and eyestalks that showed high level of mRNA expression. As hypothesized that amount of DAR_Pem in protein samples analyzed in this study may be lower than the sensitivity of the antibody at 1:15,000 dilution. Therefore, the C-tail DAR antibody titer or concentration of proteins from shrimp tissues should be increased in an attempt to detect DAR_Pem protein in shrimp tissues in future experiments.

6.4 Transient expression, localization and functional activity of the DAR_Pem in COS-1 cells

The activity of DAR was previously studied in many cell types. For example, DAR of *P. interuptus* and *B. mori* were heterologously expressed in HEK 293 cells to study the intracellular activity on changes in cAMP levels (33, 45). The activity on cAMP of the DAR of *A. mellifera* was also determined in HEK 293 cell while its pharmacological property was investigated in Sf9 cells and Sf21 cells (39-40). Alternatively, DAR activity of *C. elegans* and *D. melanogaster* was investigated in COS-7 cell (41, 44). Similarly, COS-1 cell was also used for studying the activity of activated DAR in accumulation of intracellular cAMP (66). In this study, COS-1 cell was therefore selected for transient expression of DAR_Pem. Protein expression analysis demonstrated that only the COS-1 cells transfected with DAR_Pem (T-DAR) expression plasmid generated the expected band at 49 kDa, which was not detected in mock and pcDNA 3.1 (+) vector (pC) transfected cells suggesting that DAR_Pem was

successfully expressed in COS-1 cells. The larger bands over 100 kDa may indicate oligomeric form of DAR_Pem or DAR_Pem with putative post-translational modifications (glycosylation or phosphorylation). Alternatively, it may also be possible the recombinant DAR_Pem is not completely separated from the membrane of COS-1 cell structure. In addition, the bands lower than 49 kDa were also found and assume as degraded protein. Before investigating functional activity of DAR_Pem by cAMP assay, the localization in COS-1 cells of the expressed DAR_Pem was investigated by immunofluorescent microscopy. The result showed the DAR_Pem protein expression was visualized around the surface of transfected COS-1 cells indicating that the expressed DAR_Pem exist as a membrane protein.

From the sequencing analysis, DAR_Pem was classified in type 1 of DARs based on sequence similarity with DAR type 1 in other organisms. To verify that the DAR_Pem was also functionally similar to type 1 of DAR, cAMP assay was performed. Before activation of DAR_Pem with biogenic amine neurotransmitters, the forskolin was added to pre-activate adenylyl cyclase activity of the cells (67). In previous study, the activated CHO cells exhibited very low intracellular basal level of cAMP without forskolin. The level of cAMP in activated CHO cell was raised about 200 folds higher than the basal level with 10^{-5} M of forskolin (68). In this experiment, the forskolin was therefore used to facilitate the measurement of intracellular cAMP levels. In addition, phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was also added to prevent cAMP hydrolysis. The result of cAMP assay showed that the T-DAR was activated with DA and resulted in an increased cAMP level which was in agreement with the functional property of DAR type 1. Besides, the cAMP level was also increased when the cells was activated by 5-HT, but the level was lower than DA activation. The activity of DAR type 1 to enhance cAMP level was demonstrated in many organisms. For example; in *A. merifera*, the two isoforms of the DAR type 1 (DOP-1 and DOP-2) showed increasing of cAMP level when activated with DA (38, 40). Similar effect on cAMP level was also shown for BmDopR1 and BmDopR2 in *B. mori* (45), DAR type1 of *D. melanogaster*; DmDOP1 (43-44) and *C. elegan*; CeDOP1 (41). In addition, two isoforms of DAR type 1 in spiny lobster *P. interruptus* ($D1_{\alpha Pan}$ and $D1_{\beta\_Pan}$) were expressed in HEK293 and showed elevation of cAMP level when activated with DA and no effect on parental HEK cell (mock). The cAMP level was

also increased when activated with 5-HT, however the mock also exhibited similar level of cAMP in the present of 5-HT suggesting that the increased in cAMP level may be the result of 5-HT activation on endogenous 5-HT receptor rather than the heterologously expressed $D1_{\alpha Pan}$ and $D1_{\beta\_Pan}$ (33). By contrast, the transfected DAR_Pem in COS-1 cells increased cAMP levels which was significantly different from the levels in parental COS-1 cell (mock), suggesting that response of endogenous 5-HT receptor in COS-1 cells to 5-HT was very low when compared with that in HEK 293 cells. Therefore, the result of cAMP assay provided another evidence that supports the classification of DAR_Pem as type 1 DAR because it exhibited an increase in cAMP levels when exposed to DA. However, further investigation of specific ligand binding using agonist and antagonist to DA is required to confirm the pharmacological property of DAR_Pem.