CHAPTER V
DISCUSSION

The white shrimp (*L. vannamei*) is one of the popular crustaceans that culture in commercial shrimp farm in Thailand. Viral diseases are remained a major problem in Thailand and worldwide. Virus infection such as yellow head virus (YHV), white spot syndrome virus (WSSV), taura syndrome virus (TSV), Laem-Singh virus (LSNV) or infectious hypodermal and hematopoietic necrosis virus (IHHNV) results in extensive serious production loss of shrimp farming industry. IHHNV caused mortalities in the postlarvae and juveniles of *Penaeus stylirostris* up to 90% in 1983 (1). The virus usually causes runt-deformity syndrome (RDS) and physical abnormalities in *L. vannamei* but no clinical sign in *P. monodon*. There were several approaches to inhibit IHHNV replication in shrimp but the strategy in order to prevent or cure IHHNV infection have still been un-effective. RNAi has been widely used as a powerful strategy to investigate gene function or combat various viral infections. In this study, suppression of LvRab7 by dsRNA-PmRab7 was used to investigate the knockdown effect of dsRNA on IHHNV replication.

The IHHNV both 6×10^{6} and 6×10^{7} particles can completely infect in *L. vannamei* after 5 days IHHNV challenge. This result is similar to the previous recent report. The IHHNV genome expression levels were substantially detected by PCR in the pleopods of all shrimp on day 5 after injection with10^{-2} dilution of their viral stock (7).

There are two approaches to synthesize dsRNA, *in vitro* transcription and *in vivo* bacterial expression. The dsRNA produced *in vivo* in *E. coli* showed a similar knockdown effect to the dsRNA produced by *in vitro* transcription (56). Several advantages of using an *in vivo* bacterial expression system to produce dsRNA are as follows. Production of dsRNA by *in vivo* bacterial expression can be scaled up by

growing up a larger volume of the bacterial culture. The yield of dsRNA production by *in vivo* is 30 – 40 µg/OD cell . However, dsRNA production by using *in vitro* transcription required large amounts of the DNA template (5 µg each sense and antisense DNA template in 100 µl reaction) to produce approximately 0.5 – 1 mg dsRNA. This additional step added up the cost of dsRNA production by *in vitro* transcription. In addition, the reagents used for an *in vitro* transcription are more expensive than *in vivo* expression (47,56). Therefore, the dsRNAs (dsRNA-PmRab7 and dsRNA-GFP) were produced by *in vivo* bacterial expression method in this study. DsRNA-GFP showed a single band of size about 400 bp. However, 3 major bands of size about 400 bp, 300 bp and 100 bp were observed for dsRNA-PmRab7 after RNase A digestion in the purification step (Figure 4-3). This is due to some mismatched bases in the pET-17b-stRab7 that used to express dsRNA-PmRab7. However, these bands can be cleaved by RNaseIII suggesting that the synthesized dsRNA-PmRab7 is really in the form of dsRNA.

The yield of dsRNA-PmRab7 and dsRNA-GFP is 30 and 14 µg/ 1 OD bacterial cell culture, respectively (Figure 4-4). The difference in the yield of dsRNA production was due to the plasmid backbone that used to express dsRNA. DsRNA-GFP was expressed from pET3a-GFP whereas dsRNA-PmRab7 was expressed from pET17b-stRab7 which the ribosome binding site was deleted. Therefore, the transcribed dsRNA was not used for translation. The larger yield of dsRNA was obtained.

Suppression of Rab7 of *L. vannamei* (LvRab7) can be performed by using dsRNA corresponding to Rab7 of *P. monodon* (dsRNA-PmRab7). LvRab7 mRNA was knocked down at 2 and 4 days after injection. The effect of dsRNA-PmRab7 on day 2 and day 4 showed no difference in the silencing effect (Figure 4-5). In the previous report in *P.monodon*, the dsRNA-PmRab7 can specifically knockdown PmRab7 mRNA at 2 days after dsRNA injection. The suppression of PmRab7 was remained at low levels until 9 days after injection (38). It indicated that dsRNA-PmRab7 can be used to suppress both PmRab7 and LvRab7 mRNA.

Suppression of LvRab7 mRNA by using dsRNA-PmRab7 showed inhibition of IHHNV replication in both the injection of dsRNA-PmRab7 2 days before IHHNV challenge and the co-injection (IHHNV and dsRNA-PmRab7). In the preventive effect, suppression of LvRab7 before IHHNV challenge caused inhibition of IHHNV replication (Figure 4-7). The result suggested that LvRab7 involved in IHHNV replication process. IHHNV belonged to the Parvovirus family. The previous report found that canine parvovirus (CPV) enters into host cells by endocytosis pathway that requires acidic endosomal compartment for initiation of infection. CPV vesicles used the motor protein to transport through the microtubule network (57-60). Therefore, it is possible that IHHNV which belonged to the parvovirus family may use endosomal trafficking pathway in shrimp cells. Mammalian Rab7 belonged to a small GTPase protein family and involved in endosomal trafficking pathway to transport cargo from early to late endosome or from late endosome to lysosome. The dominant-negative mutant of Rab7 was used to inhibit Rab7 function and resulted in inhibition of influenza virus infection in Hela cells and HIV-1 infection in polarized human placental cells (61). Furthermore, an over expression of the mutant of Rab7 (Rab7-T22N) showed an accumulation of semliki forest virus in early endosome (34). These results suggested the possible involvement of Rab7 protein in endosomal trafficking of virus inside the cell.

Previous reports showed that suppression an endogenous gene that is required for viral transport or viral replication can inhibit WSSV, YHV, LSNV and TSV (38-40,51). In addition, injection of dsRNA targeting ORF1-2 of IHHNV before IHHNV challenge showed 100% reduction of IHHNV replication at 5 days after viral challenge (7). In other DNA viruses, dsRNA designed corresponding to viral genes can inhibit viral replication. Ds-RNA corresponding to the non-structural protein (ns1) and the structural protein (vp) genes of HPV can inhibit HPV replication in juvenile *P. monodon* (62). Furthermore, Ds-RNA corresponding to rr2 and ie3 of WSSV genes showed more effective on inhibition of WSSV replication (53). In Figure 4-8, suppression of dsRNA-PmRab7 at 2.5 µg/g and 0.63 µg/g presented no significant difference of LvRab7 expression level and resulted in no significant difference of IHHNV inhibition. In this study, an increase in the amount of dsRNA-PmRab7 did not

further reduce IHHNV replication. Dose dependent inhibition by dsRNA was not obtained.

In the study of co-injection dsRNA-PmRab7 and IHHNV, dsRNA can inhibit IHHNV replication. This is probably due to the expression of LvRab7 can be knocked down after 2 days dsRNA injection whereas IHHNV would start to replicate at day 3 after virus challenge. Therefore, suppression of LvRab7 occurred before IHHNV started to replicate in the co-injection experiment (compare with suppression of LvRab7 study in Figure 4-5). So, co-injection of the low dose (0.63 µg dsRNA-PmRab7 per 1 g shrimp) of dsRNA-PmRab7 and IHHNV showed inhibitory effect on IHHNV replication.

In the previous report, injection of dsRNAs designed corresponding to IHHNV gene was performed twice every 3 days after IHHNV 12, 24 and 48 hours challenge. The pleopods were collected on day 3 and 5 after IHHNV challenge. The result showed completely inhibition of IHHNV on day 5 in shrimp injected dsRNA within 24 hours (7). In the therapeutic effect of dsRNA-PmRab7 on IHHNV replication, the low dose (0.63 µg dsRNA-PmRab7 per 1 g shrimp) single injection of dsRNA-PmRab7 1 day after IHHNV challenge can inhibit IHHNV replication. Furthermore, the double injection of dsRNA-PmRab7 (2.5 µg dsRNA-PmRab7 per 1 g shrimp) 1 and 5 day (s) after IHHNV challenge cannot inhibit IHHNV replication on day 10. It is possible that suppression of LvRab7 expression by PmRab7 was occurred before IHHNV replication. These two experiments present the relationship between LvRab7 and IHHNV replication. However, some shrimps that used to perform in the therapeutic effect are natural IHHNV-infected shrimp and were challenged with IHHNV again in the experiment.

The combination of dsRNAs corresponding to a viral gene and an endogenous gene is an effective approach to inhibit viral replication. The recent report found that the combination of dsRNAs demonstrated higher efficacy than the single dsRNA injection dsRNA-YHV and dsRNA-PmRab7 in both preventive and curative of YHV infection in *P. monodon* (63). In contrast, the combination effect by dsRNA-rr2 of WSSV and dsRNA-PmRab7 or dsRNA-rr2 alone showed the same result in which approximately 95% of shrimp still survived on 8 days after viral challenge (53).

Whether the combination of dsRNA targeting IHHNV gene and LvRab7 give a more efficient way to inhibit IHHNV replication remained to be investigated.

DsRNA-GFP showed non-specific inhibition of IHHNV replication in prevention effect, co-injection and low dose single injection experiment (Figure 4-7, 4-9 and 4-11, respectively). It is probably due to any dsRNA can trigger an innate immune response (sequence independent) and resulted in an RNAi like antiviral immunity (64).

Taken together, these results suggested that LvRab7 is involved in IHHNV replication in *L. vannamei*.