CHAPTER IV
DISCUSSION

In previously study, *Pm*VRP15 was identified from SSH libraries of WSSV infected-shrimp (Vatanavicharn *et al.* 2014). However, *Pm*VRP15 had no significant amino acid sequence similarity to any proteins in the NCBI GenBank database using BLASTX search. From a protein-structural analysis (TMHMM Server v. 2.0) (Krogh *et al.* 2001), this protein contained a transmembrane helix of 23 amino acids. Localization study showed that *Pm*VRP15 located at nuclear membrane (Vatanavicharn *et al.* 2014). Based on these evidences, *Pm*VRP15 may function at least in part as a nuclear membrane or related with membrane protein.

In primary hemocyte cultures, *Pm*VRP15 was highly up-regulated after WSSV infection (Figure 3.2). *Pm*VRP15-silenced hemocytes also showed a reduction of VP28 expression after WSSV infection compared to normal hemocytes (Figure 3.5). In addition, WSSV-infected hemocyte cells were fractionated into cytoplasmic, membrane, soluble nuclear, chromatin-bound and cytoskeletal fractions and probed with anti-*Pm*VRP15 antibody. The result showed that *Pm*VRP15 was found in soluble nucleus and chromatin-bound fractions (Figure 3.8). Based on these results, *Pm*VRP15 is a nuclear localized protein which involves in WSSV propagation.

Previous study showed that *Pm*VRP15 transcript was found in heart, hepatopancreas, lymphoid, gill and intestine but *Pm*VRP15 transcript was mainly expressed in the hemocytes in normal shrimp (Vatanavicharn *et al.* 2014). In shrimp, *Pm*VRP15 mRNA expression was up-regulated in hemocytes at 24, 48 and 72 h after WSSV challenge. *Pm*VRP15 transcript was extremely up-regulated by 9,410.1 fold at 48 h post WSSV infection (Vatanavicharn *et al.* 2014). Knockdown of *Pm*VRP15 caused a reduction of WSSV propagation in shrimp by 4.17 fold (Figure 3.5). Additionally, higher amount of *Pm*VRP15 protein was found in WSSV-infected hemocytes, in comparison to one in uninfected cells. These results imply that *Pm*VRP15 is an important gene in shrimp for WSSV infection. In this study, the silencing of *Pm*VRP15 expression in WSSV-infected *P. monodon* hemocytes cell culture resulted in the reduction of VP28 gene expression as compared to control (GFP). This result is in agreement with *In vivo* study (Vatanavicharn *et al.* 2014).

Many viruses (e.g. influenza and many animal viruses) have viral envelopes covering their protective protein capsids. The envelopes typically are derived from portions of the host cell membranes (phospholipids and proteins), but include

some viral glycoproteins. Functionally, viral envelopes are frequently structural components of the virus that mediate the crucial tasks of receptor recognition and membrane fusion. This occurs by fusion of the envelope with a cellular membrane. The viral envelope then fuses with the host's membrane, allowing the capsid and viral genome to enter and infect the host. Viruses must deliver their genome into the host cells to initiate replication.

Viruses that replicate in the nucleus of nondividing cells with an intact nuclear membrane transfer their genomes through the nuclear pore complex (NPC) into the nucleus. The strategies to target the NPC differ among viruses but invariably involve specific pathways of signaling, endocytosis, access to the cytosol and cytoplasmic transport (Sodeik 2000, Ploubidou *et al.* 2001, Greber 2002, Poranen *et al.* 2002, Meier *et al.* 2003). Nuclear import of incoming viral genomes also depends on viral uncoating and in some cases involves an increase of capsid affinity for the NPC (Greber *et al.* 1994, Whittaker 2000).

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The final steps in the assembly of enveloped viruses occur in the context of a cellular membrane when the nascent particle undergoes a budding reaction that simultaneously generates the viral envelope and releases the free virion. The cellular membrane can be the plasma membrane, leading to virus release directly to the extracellular space, or an intracellular membrane (e.g. the ER, golgi apparatus or endosomal system), in which case the virions are delivered into intracellular vacuoles. In addition, certain viruses and viruslike particles (VLPs) undergo engenous replication and can shuttle their genomes in and out of the nucleus without going through a complete virus assembly process from which they are released to the extracellular space by a secretory-type mechanism.

The expression of *Pm*VRP15 transcripts and protein were up-regulated in WSSV-infected *P. monodon* hemocytes (Figure 3.1 and Figure 3.2). *Pm*VRP15 protein was found in all three types of hemocytes including granular, semigranular and hyaline cells. Hemocytes are the major immune cells of shrimps and play an essential role in both the cellular and humoral immune responses. These suggested that *Pm*VRP15 may have a broad immune based function to response virus. From immunofluorescence result, *Pm*VRP15 protein was expressed in all three types of hemocytes at 48 h post WSSV infection. The expression of *Pm*VRP15 protein was located in nuclear membrane of the cell. It corresponds to protein domain prediction of *Pm*VRP15 which contained a transmembrane helix. In WSSV-infected hemocytes, the silencing of *Pm*VRP15 showed the lower protein expression of VP28

than silencing of GFP (control). Thus, *Pm*VRP15 is an essential protein for WSSV propagation.

It has been reported that WSSV mainly occurs in the nuclei of infected lymphoid cells (Wang *et al.* 2000). The GCs and SGCs were found in hemolymph as the targets for WSSV infection by a TEM study. However, clusters of developing virions and mature virions were only found in the nucleus of SGCs (Wang *et al.* 2002). Even the morphogenesis of WSSV in the infected nucleus of heavily infected SGCs was observed. The nucleus was filled with many empty premature nucleocapsids, most of which were surrounded loosely with an envelope, with both the shell and envelope open at the same end (Wang *et al.* 2002). Moreover, Propagation of WSSV was investigated in primary ovarian cultures from the kuruma shrimp *Marsupenaeus japonicas* (Maeda *et al.* 2004). Electron microscope observations clearly showed that the replication of WSSV occurred in nuclei of ovarian cells. Based on these evidence, WSSV replication and assembly within the nucleus are crucial step in WSSV propagation. It was reported that VP35 (nucleocapsid protein of WSSV) contained a nuclear translocation signal which mediate the viral DNA to nucleus of WSSV-infected insect cells for viral replication (Chen *et al.* 2002). It is possible that WSSV use *Pm*VRP15 for enter or exit from the nucleus. It is common for a virus to use the host machinery for its advantage. For example, *Pm*Rab7 is used in sorting and endocytic trafficking of virus in the host cells (Sritunyalucksana *et al.* 2006).

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*Pm*VRP15 may function in nuclear as a part of membrane protein or related with membrane protein. It is possible that *Pm*VRP15 involves in nuclear import/export of WSSV. To test this hypothesis, ratio of WSSV DNA in nuclear and cytoplasmic fractions of normal and *Pm*VRP15-silenced hemocytes were compared. Knockdown of *Pm*VRP15 resulted in a lower ratio of WSSV copy number in nuclear to cytoplasmic fractions by 9.3 fold, comparison to that of control (Table 3.1), indicating that *Pm*VRP15 may involve in nuclear entry of WSSV.

For structural study, a large quantity of purified protein is in need. As a result, r*Pm*VRP15 expression was tested in both *S. cerevisiae* and *E. coli* system. *Pm*VRP15 gene was successfully cloned and transformed into *S. cerevisiae* (FGY217). Expression of r*Pm*VRP15 was induced by addition of 20% galactose (final concentration of 2%) for 22 h. However, no major band of protein of 15 kDa (expected size of r*Pm*VRP15) was detected by SDS-PAGE (Figure 3.12). This indicates that this *S. cerevisiae* system was not suitable for r*Pm*VRP15 production.

Since a protein with transmembrane helice domain is often found to be difficult to crystallize, soluble parts of *Pm*VRP15 were cloned and expressed in *E. coli* C43 (DE3). N-terminal truncated *Pm*VRP15 contained residue 1-112, while C-terminal truncated *Pm*VRP15 consisted of residue 186-414. Western blot analysis showed that both truncated *Pm*VRP15 proteins were expressed in inclusion bodies at 37 °C (Figure 3.16 and Figure 3.17A). Although IPTG induction was carried out at 16 °C, C-terminal truncated *Pm*VRP15 was found in inclusion bodies (Figure 3.17B). This indicated that protein expression at low temperature did not help increase truncated *Pm*VRP15 solubility. Since the truncated *Pm*VRP15 proteins cannot be expressed in soluble form, we then focused on working with full-length r*Pm*VRP15.

Full-length r*Pm*VRP15 was expressed in *E. coli* C43 (DE3). The result showed that r*Pm*VRP15 was expressed at 1-3 h after IPTG induction and expressed in the highest level at 2 h post-IPTG induction (Figure 3.20). No *Pm*VRP15 band was found after 3 h induction. This suggested that *Pm*VRP15 may be a toxic protein or an unstable protein. In this research, DM (n-decyl-ß-D-maltopyranoside) was used to solubilize r*Pm*VRP15 to ensure it folded correctly. Membrane protein can be solubilize with DM detergent to study their structure and function. Proteins bound to cell membranes have hydrophobic sites buried within the phospholipid bilayers and hydrophilic sites facing toward the water layer. This detergent can interact with the hydrophobic sites of proteins which are then solubilized in the water layer. Moreover, DM detergent does not interfere the bioactivities of target proteins, denature or inactivate target proteins (Prive 2007). In this study, r*Pm*VRP15 was purified by two steps; Ni-NTA Sepharose^{TM} 6 FF and HiTrap DEAE FF columns, in order to obtain fairly pure protein (Figure 3.24). Molecular mass of purified r*Pm*VRP15 was determined by MALDI-TOF MS. r*Pm*VRP15 has a molecular mass of 15,899.9 Da (Figure 3.23), which is similar to the calculated molecular mass.

Furthermore, Circular Dichroism (CD) was used to predict secondary structure of r*Pm*VRP15. CD spectroscopy is a powerful method in structural biology to examine the structure and conformational changes of proteins, polypeptides, and peptide structures, which by informing on binding and folding properties provides information about their biological functions. It is based on the dependence of the optical activity of the protein in the far ultraviolet (UV) regions (170–240 nm wavelength) with the backbone orientation of the peptide bonds with minor influences from the side chains (Farman, 1996). Different types of secondary structure producing characteristic spectra, the spectrum of a given protein can be used to estimate its percentage content on the major secondary structure types (Greenfield, 2006; Kelly et al., 2005).

CD spectra of r*Pm*VRP15 revealed that the major secondary structure type of r*Pm*VRP15 was alpha-helix, compared to standard protein which known secondary structure. The amount of regular secondary structures (α-helix and β-strand) were estimated by analysis of the CD spectra using K2D3 deconvolution software (Louis-Jeune *et al.* 2011). The result showed that the predicted secondary structure percentages of r*Pm*VRP15 was 48.45% α-helix and 13.57% β-strand (Figure 3.25). The fact that purified *Pm*VRP15 adopted secondary structure indicated that the purification procedure used in this study did not destroy protein secondary structure.

Protein crystallography is used to generate atomic resolution structures of protein molecules. These structures provide information about biological function, mechanism and interaction of a protein with substrates or effectors including DNA, RNA, cofactors or other small molecules, ions and other proteins. This technique can be applied to membrane proteins resident in the membranes of cells. To accomplish this, membrane proteins first need to be either heterologously expressed or purified from a native source. The protein has to be extracted from the lipid membrane with a mild detergent and purified to a stable, homogeneous population that may then be crystallized. Protein crystals are then used for X-ray diffraction to yield atomic resolution structures of the desired membrane protein target (Newby *et al.* 2009).

In this study, appropriate concentration of r*Pm*VRP15 for crystallization screening was determined by PCT™ kit (Hampton research). Using Index™, MembFac™ and Crystal screen 2™ crystallization kit (Hampton research), crystals appeared in 6 conditions. They were (1) Index™ D12 (0.2 M Calcium chloride dihydrate, 0.1 M BIS-TRIS pH 5.5, 45% v/v (+/-)-2-Methyl-2,4-pentanediol); (2) Index™ E1 (0.2 M Calcium chloride dehydrate, 0.2 M BIS-TRIS pH 6.5, 45% v/v (+/-)-2-Methyl-2,4-pentanediol); (3) Index™ E6 (0.05 M Calcium chloride dehydrate, 0.1 M BIS-TRIS pH 6.5, 30% v/v Polyethylene glycol monomethyl ether 550); (4) Index™ H9 (0.05 M Zinc acetate dehydrate, 20% w/v Polyethylene glycol 3,350); (5) MembFac™ A2 (0.1 M Zinc acetate dehydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 12% w/v Polyethylene glycol 4,000); (6) Crystal screen 2™ H3 (0.2 M Magnesium chloride, 0.1 M TRIS-HCl pH 8.5, 2.6 M 1, 6-Hexane diol) (Figure 3.29).

Crystals from Index™ D12, H9 and MembFac™ A2 conditions were subjected to X-ray diffraction test. Crystals from Index™ H9 condition gave strong spots in the diffraction (Figure 3.28), indicating that the crystals are salt. Meanwhile, crystals from MembFac™ A2 condition did not diffract (Figure 3.29). This may be caused by

several factors including small crystals, disorder crystals and inappropriate cryoprotectants.

Crystals appeared in Index^{TM} D12 condition were the most promising one. Their diffraction patterns showed close spots at low resolution (~9 Å), suggesting the presence of proteins (Figure 3.30). Further optimization should be done around this condition, including varying precipitant concentration, additive screening and detergent screening.