CHAPTER VI
GENERAL DISCUSSIONS

Peptidases elaborated by helminths are implicated in facilitating nutrition, tissue-migration and evasion of the host immune system (Caffrey et al., 2004). Disruption of peptidase function, therefore, by chemotherapy (Wasilewski et al., 1996) or immunotherapy (Dalton et al., 2003) is detrimental to both parasite survival and fecundity. For most platyhelminthes, clan CA cysteine peptidases (http://merops. sanger.ac.uk/; Rawlings, Tolle, and Barrett, 2004) orthologous to mammalian cathepsins B, F and L predominate in both extracts and excretory-secretory products (Caffrey et al., 2004).

Cathepsin B is a lysosomal cysteine protease which widely distributed in most living organisms. In helminthes, cathepsin B was demonstrated as mediator of protein degradation, processing and activating other enzyme (Sripa et al., 2010b) and highly immunogenic (Law et al., 2003). Cathepsin B has been considered as tool for control parasite infection in multiple ways such as; vaccine target (Jayaraj et al., 2009 ;Chen et al., 2005), anthelminthic target (Robinson, Dalton, and Donnelly, 2008) and tool for serodiagnosis (Sulbaran et al., 2010).

In *O. viverrini*, two isoforms of cathepsin B designated as *Ov*-CB-1 and *Ov*-CB-2 were identified from *O. viverrini* ESTs (Laha et al., 2007; Sripa et al., 2010b). *Ov*-CB-1 comprised a 1,011 bp open reading frame encoding a 337-amino-acid protein (GenBank Accession No. GQ303560). The *Ov*-CB-2 cDNA comprised a 939 bp open reading frame encoding a 313-amino-acid protein (GenBank GQ303559). The deduced *Ov*-CB-1 and -2 proteins contained a putative residue N-terminal signal peptide predicted by SignalP indicated the property of a secreted protein. Sequences analysis and phylogenetic analysis reveal that *O. viverrini* cathepsin B cDNAs showed identity to cathepsin B proteases from other pathogenic trematodes including *Clonorchis sinensis*, *Trichobilharzia regenti* and *Schistosoma japonicum*. These data support for similar functions of cathepsin B in relate organisms.

The *Ov*-CB-1 zymogen was successfully produced in the yeast *P. pastoris* as active zymogen. *Ov*-CB-1 protein encoded the similar function with other helminthic cysteine proteases by showing the abilities in hemoglobin and host tissues (extracellular matrix) digestion that well established in many parasitic helminths (Wilson et al., 1998). This finding was correspond with the pathology in bile ducts of animal model and man that infected with *O. viverrini* by presented of inflammation and biliary epithelium damage (Sripa, 2003).These can assume the role of *O. viverrini* cathepsin B in parasite nutrition and tissue invasion since the worm lying there for ingest several bile constituents such as mucin and even blood as nutrients (Sripa, 2003). Moreover, hemoglobin from vertebrate host may the significant source of nutrition for *O. viverrini* similarly proposed in blood feeding parasite (Baig, Damian, and Peterson, 2002).

From this study also found the abilitiy of native *Ov*-CB-1 protein in trans-activation of *O. viverrini* cathepsin F zymogen by proteolytic removal of its prosegment at pH 5.5, a pH at which the *Ov*-CF-1 zymogen cannot autocatalytically activate. At this acidic condition was common found in gut lumen or vacuoles of intestinal lining cells (Delcroix et al., 2006). From all of these provided clearer understanding about the function of *Ov*-CB-1 in zymogen activation and function of *Ov*-CB-1 as the regulator in protease cascade. From these functions of *Ov*-CB-1 was corresponding with the localization of cathepsin B at the gut lumen and surrounding gastrodermis of the parasites (Sajid et al., 2003).

Besides, *Ov*-CB-1 was predicted as secreted protein with presented of signal peptide at the N-terminal of sequence. Thus it was expected to be found and detected its activity in the ES products of *O. viverrini* similar with *F. hepatica* cathepsin B which exhibited the major biochemical activity of this enzyme in the ES products of the parasites (Wilson et al., 1998) From the study in other liver fluke can assume that the content in ES products of *O. viverrini* may contain some of secreted proteases. These secreted proteases may vomit from the gut lumen of the parasites to perform some functions out side the worm then interact with or may be toxic to the host biliary epithelium. From this reason, some of secreted proteases such as cathepsin B have been found to be highly immunogenic (Noya et al., 2001) and may be induce host immune response.

Since cathepsin Bs are proven as highly antigenic, *Ov*-CB-1 protein was attractive for immunodiagnostic tool for opisthorchiasis and as vaccine candidate against *O. viverrini*. From this study, *Ov*-CB-1 showed the potential of serodiagnostic antigen for detection of *O. viverrini* infection in endemic area by using indirect ELISA. Sera from egg-positive *O. viverrini* infections produced a strong IgG antibody response to r*Ov*-CB-1 both in ELISA and immunoblot analysis. The sensitivity and specificity of the ELISA test was 67% and 81%, respectively. Cross-reactivity of r*Ov*-CB-1 was observed with other parasitic infection including minute intestinal flukes, echinostomes, *G. lamblia*, hookworm, *S. stercoralis* and *Taenia.* Moreover this test was showed good consistent with the intensity for *O. viverrini* EPG. This indicated that r*Ov*-CB-1 showed good efficiency for diagnosis opisthorchiasis in endemic area. However, this test can not discriminate between past and active infections and needed to increase sensitivity and specificity.

With highly immunogenicity of *Ov*-CB-1, this molecule was also attractive for vaccine candidate against *O. viverrini*. r*Ov*-CB-1 vaccination was induced highly humoral immune response in hamsters. This immune response was present some of protective immunity by reduced number of worm burden (18.08%, $P>0.05$) but it was not significant reduction. Moreover, this immune response was not effect to *O. viverrini* reproductive organs because there was not different in the number of egg count when compared with unvaccinated group.

As *Ov*-CB-1 play role in host proteins digestion so the specific gene function need to be investigated. In this study, we have been developing an approach to study function of *Ov*-CB-1 using RNA interference technique by target at *Ov*-CB-1 transcript. The efficiency of this technique was determined by introduced negative control; Cy3-Silencer siRNA using square wave electroporation employing a single pulse for 20 ms of 125 V in the presence of 50 µg/ ml of Cy3-siRNA. Cy3-fluorescence was evident throughout the adult flukes with strong fluorescence signal at the oral sucker, ventral sucker, uterus and vitelline glands. The results confirmed that utility of square wave electroporation to introduce exogenous nucleic acid probes into this liver fluke and the utility of Cy3-RNA to indicate location of the reporter siRNA in the transduced worms. The worms were treated using the same electroporation settings with dsRNA targeting an endogenous papain-like cysteine

protease, *Ov*-CB-1. The suppression of *Ov*-CB-1 transcript was observed more than 90% in three days after introduction and the suppression was maintained for nine day after dsRNA introduction. The suppression of *Ov*-CB-1 transcription is associated with the reduction of *Ov*-CB-1 activity against the diagnostic peptide, Z-Arg-Arg-AMC. This finding not only indicates the presence of active RNAi machinery in *O. viverrini* but also provides an opportunity to gene function study in this pathogen.

In addition this study demonstrates specific knockdown of *Ov*-CB-1 also suppressed the expression of the orthologue gene, *Ov*-CB-2. However, the suppression of *Ov*-CB-2 was presented in two days after exposure *Ov*-CB-1 dsRNA and was decline after that. Meanwhile, *Ov*-CB-1 was suppressed persistently until day 9 and there have no suppression effect to actin and irrelevant gene, GRN. There have no effect was seen on *Ov*-CB-1, *Ov*-CB-2 or *actin* levels after introduced *Ov-grn-1* into adult worms. These can conclude that off-target effects of RNAi may not be a widespread problem in functional genomics analysis in *O. viverrini*.

r*Ov*-CB-1 at concentration 1, 5 and 10 ug/ml were tested for the ability to stimulate human cancer cell growth *in vitro* at 24, 48 and 72 h time-points and the results revealed unsignificant of cell proliferation in every concentrations of protein at all time points. Although, *Ov*-CB-1 was exist in ES products of *O. viverrivi* and have great chance to contact with host tissue but at low concentration of single molecule may not have effect on cancer cell line. Higher concentration of single recombinant protein should be exhibit clearer proliferation of cell line. To observe the ability in cell proliferation should be observe in normal cell line which have certainly growth pattern.