CHAPTER 5
DISCUSSION

The full-length of *O. viverrini* leucineaminopeptidase (*Ov*LAP) was identified. The properties of typical enzymes belonging to M17 metalloprotease were observed in *Ov*LAP. Two regions of metal-binding sites, IGKG and NTDAEGR, were examined in *Ov*LAP. These regions were conserved throughout LAP from other organisms, including plant, helminth, protozoa, and vertebrate. The deduced amino acid sequence of *Ov*LAP was found to highly identity with closely related liver fluke, *C. sinensis* and shared more than 60% identity with lung fluke, *P. westermani* and blood fluke, *S. mansoni*. *Ov*LAP was predicted to be lack of signal peptide, like other trematode LAP such as *C. sinensis* (Deng et al., 2012), *P. westermani* (Song et al., 2008), *F. gigantica* (Changklungmoa et al., 2012) and *S. mansoni* (McCarthy et al., 2004) indicated that *Ov*LAP are function inside cells where the most of chemical reactions of metabolism and protein degradation occur (Hochstrasser, 2009).

*Ov*LAP was classified as M17 exopeptidase that cleaved the N-terminal part of peptide to a single amino group. These amino groups are used in parasite metabolism to promote parasite growth and development (Matsui, Fowler, and Walling, 2006). *Ov*LAP can function in a board pH range and the optimal pH for efficient activity was found to be in slightly alkaline conditions (pH 8.0) similar with *P. westermani* (Song et al., 2008), *F. gigantica* (Changklungmoa et al., 2012) and *S. mansoni* (McCarthy et al., 2004). At this pH, *Ov*LAP can be activated by several inorganic cofactorssuch as Ca^{2+} and Co^{2+}, in contrast to Mg^{2+} that activates a lack of *Ov*LAP activity. Moreover, fully occupied at metal-binding sites was elevated *Ov*LAP activity. We found that high concentrations of metal ions of Ca^{2+} and Co^{2+} (1 mM) increased more catalytic reaction of *Ov*LAP when compared with 0.1 mM metal ions. From this study, the ordering of preference metal ions on *Ov*LAP active site was $Ca^{2+} > Co^{2+} > Mg^{2+}$.

Besides bestatin, the specific inhibitor of leucine aminopeptidase and EDTA, the metal chelator showed a strong inhibitory effect on *Ov*LAP activity. The degree of inhibition of bestatin and EDTA against *Ov*LAP activity was dose-dependent, in contrast to E-64 that lacked an inhibitory effect on *Ov*LAP activity, similar with other known LAPs (Changklungmoa et al., 2012; Kang et al., 2012; McCarthy et al., 2004; Song et al., 2008). The characteristic and biochemical property of *Ov*LAP required divalent cations for

enhancement of their activity and were suppressed by metal chelating; EDTA and LAPs inhibitor; bestatin that was well conserved with M17 peptidase family.

LAPs are crucial enzymes involved in protein degradation by participation in the final step of protein breakdown cascade to the release of a single amino group from N-terminus peptide (Delcroix et al., 2006). This amino group is used as a building block for protein synthesis and intermediate molecule in cytoplasmic metabolisms required for increased development and growth of the parasite. Thus, *Ov*LAP is vital and observed in all developmental stages of *O. viverrini*, such as *S. mansoni* (McCarthy et al., 2004). The high level of *Ov*LAP mRNA transcript was detected in larva, metacercaria, and egg stage of *O. viverrini*, stages that indicated increased requirements of nutrients and energy for maturation. However, the requirement of amino group for development was observed in tissues and organs of the adult stage. Localization to determine *Ov*LAP expression in adult *O. viverrini* using anti-*Ov*LAP antibody found a strong signal reaction in egg shell. This suggested *Ov*LAP plays a role in the egg hatching process by acting as a hatching enzyme to degrade egg shell and then facilitating hatching of miracidium from egg. This finding was similar in *S. mansoni* that showed LAP activity in hatching fluid (McCarthy et al., 2004; Rinaldi et al., 2009). Anti-*Ov*LAP antibody was also recognized in the muscular region of the ventral sucker, the attachment organ which probably requires high energy to maintain adherence to the host bile duct. The strong reaction of anti-*Ov*LAP antibody was also showed in testis indicating the role of *Ov*LAP in germ cell maturation. In addition, localization was also present in tegument and sub-tegumental tissue where *Ov*LAP was suggested to serve as a cell surface remodeling enzyme (Kang et al., 2012; McCarthy et al., 2004; Mulvenna et al.; Song et al., 2008). In addition, localization was found to be distributed on the surface of gut epithelial cell where the final step of protein degradation occurs. From localization, *Ov*LAP was not present in gut lumen where slightly acidic pH environments was reported but possibly present in the apical membrane of gut epithelial cell where the environmental pH suits *Ov*LAP activity. This indicated that *Ov*LAP is not secreted like other protease, but functions intracellulary by generating single amino acid from short peptide that diffuses into gut epithelial cell surrounding the gut lumen (Changklungmoa et al., 2012; Kang et al., 2012). This corresponds with the optimal pH for *Ov*LAP activity that functions efficiently in slightly alkaline pH but less efficiently in slightly acidic pH, like in gut lumen environment (Caffrey et al., 2004; Delcroix et al., 2006; Sajid and McKerrow, 2002). *Ov*LAP was also found in parenchymal cell where organs and muscles are embedded. Due to the lack of body cavity, parenchymal cells are found throughout the fluke body and function as supporting

tissue to anchor the fluke's organs and muscles. This cell could connect and probably transmit some nutrients and amino acids to permeated organs and muscles for fluke living.

From localization, some cross reaction with host hepatocyte and epithelial cell of bile duct was observed. It is possible to have little cross reaction of anti-*Ov*LAP antibodies with the vertebrate host, especially the liver organ where normally found LAPs in cell. In case of a human host, two isoforms of LAPs were identified in host liver and shared 22% identity with *Ov*LAP (Ledeme et al., 1983). *Ov*LAP was qualified as good antigenic molecules since it can evoke high IgG production in mice (data not shown). Moreover, localization was present in the accumulation of *Ov*LAP in essential organs of *O. viverrini* including tegument, reproductive organ, ventral sucker and parenchymal cell. Thus, *Ov*LAP was attractive as a vaccine candidate, but the inhibition of *Ov*LAP activity would not have significant effect on the parasite, due to multi-enzyme cascade functions in intestine of *O. viverrini* for host protein degradation, including cathepsin B (Sripa et al., 2010), cathepsin D (Suttiprapa et al., 2009), cathepsin F (Pinlaor et al., 2009), asparaginyl enopeptidase (Laha et al., 2008) and leucine aminopeptidase. The specific inhibition for these multiple enzymes using multivalent vaccine or RNAi technique may impair these enzymes' function and have an effect on systemic hierarchical events in host protein hydrolysis. The blocking host protein hydrolysis, nutrient uptake, protein synthesis and cytoplasmic metabolism may impair parasite survival.