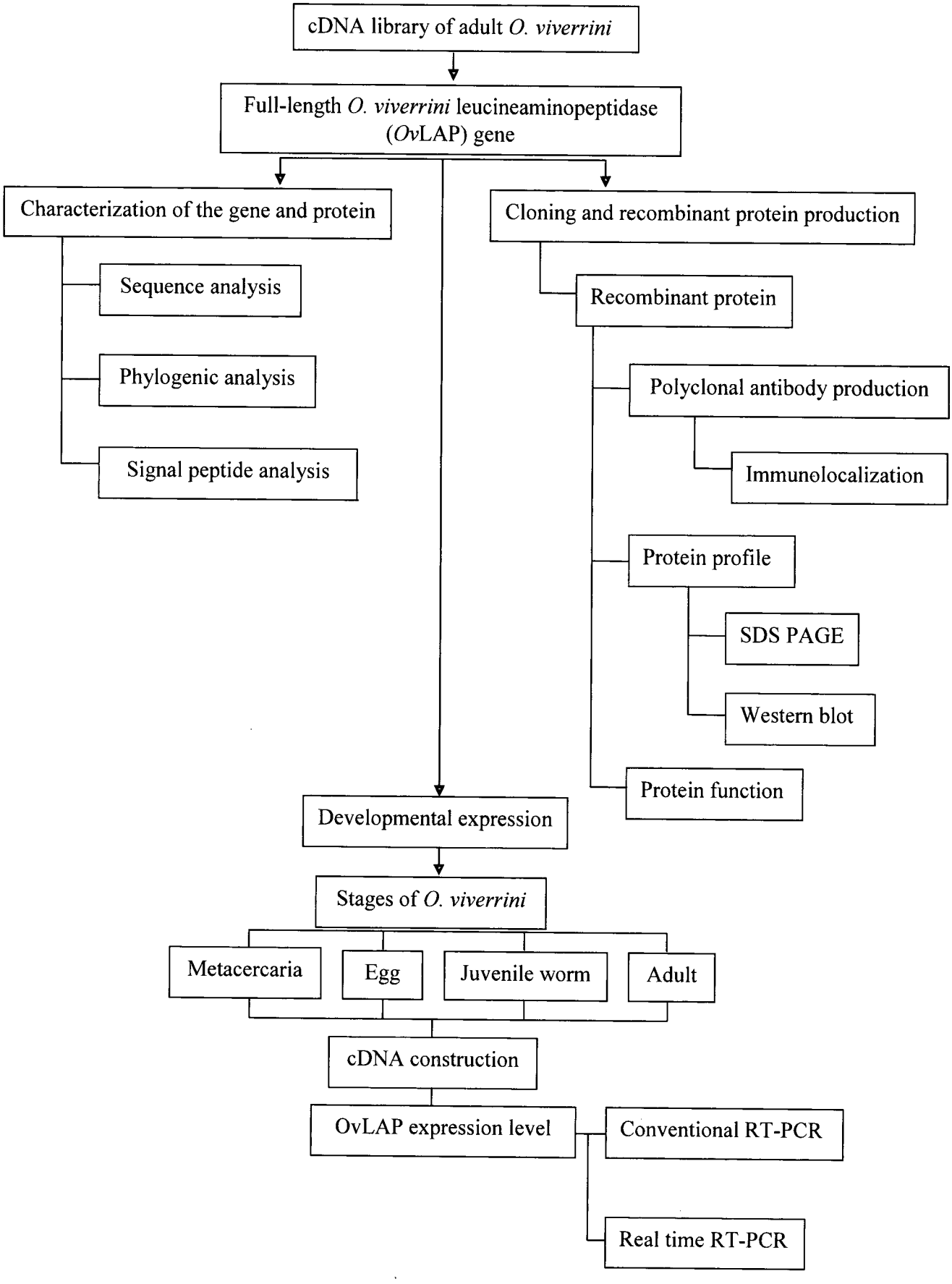
CHAPTER 3
RESEARCH METHODOLOGY

1. Research design

The full length DNA sequence of *O. viverrini* encoded leucineaminopeptidase (LAP) was identified from a previous study (Laha et al., 2007). The studies of other organisms revealed the important of LAP as an essential enzyme for parasite catabolism and attractive as a vaccine candidate molecule. In this study, *O. viverrini* leucineaminopeptidase (*Ov*LAP) will clarified their characteristic including sequence analysis, phylogenic analysis and signal peptide analysis. For production of recombinant protein, the full length sequence of *Ov*LAP will amplified from cDNA library of *O. viverrini* using specific primers and subsequently cloned into T-vector and expression vector, pET15b+. The recombinant DNA will transform into bacteria *Escherichia coli* stain BL21(DE3) and induction for recombinant protein expression with 1 mM IPTG at 26 °C. The *Ov*LAP recombinant protein will determine the function with specific substrates at various pH conditions. Moreover, recombinant *Ov*LAP protein will be used as an antigen to produce polyclonal antibody in mice. Mouse anti-*Ov*LAP antibody will be used as primary antibody to detect the expression of *Ov*LAP in adult stage of *O. viverrini* by immonolocalization technique. For gene expression analysis, conventional RT-PCR and real time RT-PCR will perform to detect transcript of *Ov*LAP gene in various developmental stages of *O. viverrini* including adult, immature worm, metacercaria and egg.

2. Conceptual framework



3. Materials and Methods

1. Sequence analysis

The full length DNA sequence of *Ov*LAP was identified from previous study (Laha et al., 2007). The *Ov*LAP sequence was compared to the homology of LAP of other organisms. The nucleotide sequence of *Ov*LAP was edited using the Bioedit program (Hall, 1999). The edited sequence was translated to protein in the correct open reading frame. The *Ov*LAP sequence was searched for homology with LAP sequences of other organisms in the DNA database using BLAST algorithm including Blastn, Blastxand tblastx at http://www.ncbi.nlm.nih.gov/blast/. Multiple sequences alignment was performed using ClustalW (Thompson, Higgins, and Gibson, 1994) in the Bioedit program.

2. Phylogenetic analysis

Protein sequence of *Ov*LAP was used to study evolutionary the phylogenetic relationship. Protein sequence of *Ov*LAP was aligned with LAP from other organisms using clustalW. Multiple sequence alignments of LAP employed bootstrap analysis for 1,000 replicas using Seqboot in Phylip package version 3.66 (Felsenstein, 1993). A phylogenetic tree of LAP evolution was constructed with the neighbor-joining method using Protdist in Phylip package. A consensus neighbor-joining tree was constructed using the consense program in Phylip.

3. Cloning and recombinant protein production

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3.3.1 Cloning *Ov*LAP gene into T-vector

The full length of *Ov*LAP was amplified from *O. viverrini* cDNA library with specific primers. These primers were designed to encompass N- and C-terminal regions of *Ov*LAP nucleotide sequence. The forward primer was 5' GCG GCG**CAT ATG**ATG TCT GTG AGC CGT AGC GTC3' and reverse primer was 5' GCG GCG**CAT ATGTCA** CAG TTT GGA AAC CAC CTC 3'. For assist cloning into expression vector, forward and reverse primer was incorporated with endonuclease restriction site, *Nde I* (the restriction sites are in bold). The stop codon was added in reverse primer (in bold and underlined).

*Ov*LAP amplification reaction was performed with *Phusion Tag* DNA polymerase (Thermoscientific, USA).The thermocycler condition was denaturation at 98°C for 5 min, 35 cycles of denaturation at 98°C for 1 min, annealing at 60°C for 1 min, extension at 72 °C for 2 min, and a final extention at 72 °C for 10 min. The template for amplified full length *Ov*LAP was *O. viverrini* cDNA library. PCR products were fractionated

on 0.8% agarose gel. The DNA fragment was recovered, purified, and then cloned into TOPO-T vector (Invitrogen, USA). The recombinant DNA in TOPO-T vector was transformed into *E. coli* stain JM109 for propagation overnight at 37 °C in LB broth containing 100 ug/ml carbenicillin (Gibco®, USA). The bacterial pellet was separated from the overnight cultured media by centrifuge at 3,000-3,500 rpm. Plasmid were then extracted from bacterial pellet with GeneJet plasmid purification kit (Thermoscientific, USA) prior to DNA sequencing analysis for proof of the correct nucleotide sequence. Corrected *Ov*LAP sequences without errors such as frame shifts and mutation were selected for sub-cloning into expression vector.

3.3.2 Cloning *Ov*LAP gene into expression vector, pET-15b

The plasmid pET-15b (Novagen, USA) was used as an expression vector for the *Ov*LAP gene. To clone the *Ov*LAP gene into pET-15b vector, recombinant *Ov*LAP in TOPO vector was cleaved with *NdeI* endonuclease (Thermoscientific, USA) prior to fractionate on 0.8% agarosegel electrophoresis. The recovered DNA fragment was purified from agarose gel by Silica bead DNA gel extraction kit (Thermoscientific, USA). The expression vector, pET-15b vector, was also digested with the *NdeI* endonuclease enzyme, fractionated on 0.8% agarose gel electrophoresis, and purified from agarose gel.

The digested *Ov*LAP was ligated with digested pET-15b vector using T4-ligase enzyme (Fermentas, USA), then incubated at 16 °C for overnight and 4 °C for another 24 hrs. The ligated recombinant DNA of *Ov*LAP gene in pET-15b vector was transformed into *E. coli* stain JM109 competent cells for propagation. The transformants were plated on LB agar containing 100 ug/ml carbenicillin (Gibco®, USA) then incubated overnight at 37 °C. The single colony was picked to PCR using primer T7 promoter and T7 terminator to confirm the presence of recombinant *Ov*LAP DNA. The colony containing recombinant *Ov*LAP DNA was inoculated in LB broth containing 100 ug/ml carbenicillin(Gibco®, USA) and grown overnight at 37 °C for preparation as a glycerol stock for long-term storage.

3.3.3 Production of recombinant *Ov*LAP protein

In this study, recombinant *Ov*LAP protein was produced as soluble 6X His-tag fusion protein in a bacterial system. The competent *E. coli* BL21(DE3) cells (Novagen, USA) were used as host cells for *Ov*LAP protein production. The recombinant DNA of *Ov*LAP in pET15b+ was transformed into competent *E. coli* BL21(DE3) cells by heat shock steps following the transformation protocol of the company's instructions. The

transformants were selected by plating on LB agar containing 100 ug/ml carbenicillin (Gibco®, USA). The single carbenicillin resistance colony was grown overnight at 37°C in 50 ml LB broth with 100 ug/ml carbenicillin (Gibco®, USA). To collect the bacterial pellet, the overnight cultured media was centrifuged the next day. The bacterial pellet was re-suspended in 200 ml fresh LB broth containing 100 ug/ml carbenicillin (Gibco®, USA). To induce recombinant *Ov*LAP protein production, IPTG was added to the culture media in a final concentration of 1 mM. The culture media was incubated at 26 °C for 8 hrs at a shaking speed of 225 rpm (non-induced control and induced cells were also collected and analyzed by SDS-PAGE electrophoresis). To purify the recombinant proteins, the bacterial pellet was collected by centrifugation at 3,500 rpm for 15 min at 4°C. Then, the supernatant was discarded and the bacterial pellet was re-suspended in 3 ml native condition binding buffer containing 1X cocktailed proteases inhibitor (without EDTA) (Sigma-Aldrich, USA). The re-suspended pellet was frozen and thawed at 42°C 3 times, then subsequently to sonication for 5 min at 4°C with 37% of amplitude. After that, the pellet debris was removed by centrifugation twice at 3,500 rpm for 15 min. The supernatant containing *Ov*LAP recombinant protein was collect for protein purification through Ni-NTA resin column (Thermoscientific, USA) following the company's instructions. Protein fractions were analyzed with SDS-PAGE observing a single band with coomassie blue and/or protein immunoblot staining using His-Tag antibody.

The fractions of *Ov*LAP recombinant protein were pooled and concentrated by concentrator (Eppendrof concentrator 5301). The concentrated protein was analyzed by SDS-PAGE and the protein concentration determined its absorbance at 280 nm (Nanodrop).

4. Production of polyclonal anti-*Ov*LAP antibodies in mice

Polyclonal IgG antibodies against purified *Ov*LAP protein were produced by hyperimmunization of male mice with subcutaneous injections. Before immunization, pre-immune serum was collected from the tail vein for a baseline of antibody titer. For the first injection, 100 ug of purified *Ov*LAP protein emulsified in equal volume of complete Freund's adjuvant was injected subcutaneously. Immunization was repeated three times at 7 day intervals with incomplete Freund's adjuvant. Before each booster, a blood sample was collected from the animal's tail vein to determine the antibody titer. The whole blood was allowed to clot at room temperature for 30 min. The clotted blood was removed by centrifugation at 3,000×g for 10 min and the serum was carefully collected to store in 20 ul aliquots at -20°C. The antibody titer of serum was assessed against purified recombinant

*Ov*LAP proteins by enzyme link immunosorbant assay (ELISA) and evaluation of the specificity of serum by Western blotting was completed.

5. Immunolocalization of *Ov*LAP in adult *O. viverrini*

Adult *O. viverrini* in hamster liver were embedded in Sakura Tissue-Tek® TEC™ embedding center. *O. viverrini* paraffin embedded was cut in to sections with cryostat microtome. The sections were mounted on coated glass slides and heated at 55-60°C in an oven for 15 to 30 min before deparafinized. Before staining with mouse anti-*Ov*LAP antibody, the sections were deparafinized in xylene 3 times for 5 min each to remove the paraffin wax. After deparaffinization, slides were dehydrated by decreasing concentrations of ethanol according to the following steps; absolute ethanol 3 times, 5 min each, 95% ethanol 2 times, 3 min each, 70% ethanol one time for 3 min, and finally rinse the section with PBS for 1 to 2 min. After that, antigen retrieval was performed in 0.1 M citrate buffer pH 6.0 for 3 min in a pressure cooker. After antigen retrieval was completed and the slides were cooling down, the slides were washed with 1X PBS 2 times, 5 min each. Then non-specific endogenous peroxidase enzyme activities in the sections were blocked with 30% H_2O_2-methanol for 30 min. Non-specific binding sites in the sections also were blocked with normal horse serum in dilution 1:20 for 30 min at room temperature in a humidified chamber. The *O. viverrini* sections were probed with mouse anti-*Ov*LAP antibody at dilution of 1:300 in PBS/NaN_3 and incubated overnight at room temperature in a humidified chamber. For negative control, the slides were probed with pre-immunized serum. On the next day, the sections were probed for one hour at room temperature with anti-mouse antibody conjugated with horseradish peroxidase at a dilution of 1:500 in PBS. Then the slides were washed with PBS 2 times, 10 min each. To develop color, slides were submerged in freshly prepared diaminobenzidine (DAB) solution for 5 min, then, the reaction was stopped with tap water, and counterstained with Mayer hematoxylin for 1 min. The slides were washed the color of Mayer hematoxylin with tap water and dehydrated in 70% alcohol for 3 min one time, 95% alcohol 2 times, 3 min each, and the slides were finally dehydrated in absolute alcohol 3 times, 3 min each. The slides were then observed under a light microscope and analyzed using Aperio ScanScope™.

6. Immunoblotting assay

Immunoblotting was carried out in the detection of the specificity of anti-*Ov*LAP against recombinant *Ov*LAP. In brief, recombinant *Ov*LAP was separated on 10% SDS-PAGE and the electrophoretically-separated proteins were transferred to nitrocellulose membrane using a Mini Trans Blot (Biorad, USA). Protein was transferred at 0.5 mA for 1

hr. After complete transference, immunostaining was performed immediately. Following their removal from the transferred apparatus, the membrane was cut into strips and the non-specific binding was blocked with 5% skim milk in PBST for 2 hr. The membrane was first incubated overnight with mouse anti-*Ov*LAP antibody at a dilution of 1:50 then incubated with IgG goat anti-mouse conjugated with horseradish peroxidase at a dilution of 1:1,000 for 2 hr. After additional washing with PBST, the strips were incubated with a chemiluminescent detection kit, ECL Plus (GE Healthcare) and then visualized with ImageQuant™.

7. Enzyme assays

The activity of recombinant *Ov*LAP protein was determined by monitoring the increase of p-Nitroanilide released from L-Leucinep-Nitroanilide (Sigma-Aldrich, USA), the specific substrate of LAP. The activity was performed at 37°C in a wavelength of 405 nm using VERSAmax Tunable Microplate Reader™. The activity assay of *Ov*LAP was accompanied with Leucine Aminopeptidase, microsomal porcine kidney type IV-S (Sigma-Aldrich, USA) based on the assay procedure described by Pfleiderer (1970). To identify the optimal pH and stability of *Ov*LAP activity and effects of metal ions against *Ov*LAP, the assay was performed in different ranges of pH buffer that contained different metal ions. The assay was carried out in 96-well microtitre plate format in a final volume of 100 ul. Briefly, 10 ug of recombinant *Ov*LAP protein was incubated in a range of 50 mM buffers: phosphate citrate (pH 5.0), phosphate citrate (pH6.0), sodium phosphate (pH 7.0), sodium phosphate (pH 8.0), and glycine-NaOH (pH 9.0). Each buffer contained one of following 1 mM metal ion; $MgCl_2$, $CaCl_2$, $CoCl_2$. Then, 0.1 mM of L-Leucinep-Nitroanilide was added into the reactions and pre-incubated at 37°C for 20 min. After that, the reactions measured the release of p-Nitroanilide for 60 min at 3 min intervals at 37°C in a wavelength of 405 nm. All reactions were compared to the control without any metal ion.

To observe the effect of protease inhibitors or metal chelator on *Ov*LAP activity, the assay was performed as above. Briefly, 10 ug of recombinant *Ov*LAP protein was pre-incubated at 37 °C for 20 min in 50 mM sodium phosphate (pH 8.0) containing 0.1 mM of L-Leucinep-Nitroanilide, 1 mM of $CaCl_2$, and 1 mM of various protease inhibitors; E64 (Sigma-Aldrich, USA), leucineaminopeptidase inhibitors; bestatin (Sigma-Aldrich, USA), and metal chelator, EDTA (Sigma-Aldrich, USA). Then, the enzyme activity measured the release of p-Nitroanilide for 60 min at 3 min interval at 37 °C in a wavelength of 405 nm. All reactions were compared to the negative control without an inhibitor. The concentration of p-Nitroanilide was determined by using its molar absorptivity of 9450 M^{-}

$^{1}cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme liberating 1 umol of p-Nitroanilide per minute at 37°C.

8. Developmental expression

Real-time PCR and conventional PCR were performed to evaluate transcripts of *Ov*LAP in various developmental stages of *O. viverrini*. The developmental stages of *O. viverrini* including adult worm, juvenile, egg and metacercaria were collected. Then, the total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The total RNA was treated with Dnase (Thermoscientific, USA) to exclude the interference of DNA before prepared cDNA using a cDNA construction kit (Thermoscientific, USA). Each life-cycle stage cDNA of *O. viverrini* was used as a template in real-time and conventional PCR using *Ov*LAP RT-PCR primer (forward primer: 5'-ACC ATT AGC CGA TGA CCC AAA TG-3' and reverse primer: 5'-GAC CAC CTG AAG TGC CTG TT-3') whose design covered both active site and metal binding region of *Ov*LAP with PCR product size of 373 bp. The internal control reaction was also included using *O. viverrini* actin gene. The negative control reaction was included using RNA of *O. viverrini* and deionized water as templates. To perform conventional PCR, 200 ng/ul was subjected to amplification reaction and amplified with *Phusion Tag* DNA polymerase (Thermoscientific, USA) in TPersonal Thermocycler (Biometra, Germany). The thermocycler condition was denaturation at 98°C for 5 min, 35 cycles of denaturation at 98°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min and a final extention at 72°C for 10 min. PCR products were analyzed on 0.8% agarose gel/EtBrand analyzed with a gel documentation system.

To perform real-time PCR, *Ov*LAP fragment was amplified in LightCycler480 II ®Instrument (Roche, Switzerland) and detected with Maxima SYBR Green/ROX qPCR mastermix (Thermoscientific, USA). The protocol followed manufacturers' instructions. Briefly, 20 ul of PCR reaction mixture containing of 2 ul of cDNA, 10 ul of SYBR Green qPCR mastermix (2X), 0.4 ul each of *Ov*LAP forward and reverse primer (10 pmole) and 7.2 ul of ddH_2O. The real-time PCR program was 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 20 sec. After amplification, a melting curve was constructed using the program as follows: 95°C for 30 sec, 65°C for 15 sec, followed by an increase to 95°C continuously to collect a fluorescent signal.