CHAPTER 4
RESULTS

1. *Ov*LAP sequence analysis

The full-length DNA of *Ov*LAP was identified. Blastx showed *Ov*LAP shared 97% identity with leucine amniopeptidase of closely related species, and *C. sinensis*, 68% identity with *Paragonimus westermani* and 64% identity with *Schistosoma mansoni*. *Ov*LAP was comprising of 1,698 bp, encoding 566 amino acids, calculated a molecular mass of 60 kDa with predicted PI of 6.21 (Figure 1). Analysis of signal peptide using SignalP-NN and SignalP-HMM prediction indicated that *Ov*LAP was non-secretory protein by the absence of a cleavage site at N-terminus of amino acid sequence (Figure 2). The prediction of *Ov*LAP localization by PSORT (http://www.psort.org) found *Ov*LAP was localized in cytosol with 73.9% probability. The estimated half-life of *Ov*LAP was more than 10 hours in *E. coli* and more than 20 hours in yeast in vivo. *Ov*LAP was classified as a stable protein with 31.11 stability index (II).

The amino acid sequence of *Ov*LAP was compared to LAP of other organisms including flatworms, protozoas, and vertebrates retrieved from GenBank for multiple sequence alignment. *Ov*LAP showed complete conservation of metal binding site and catalytic site through these divergent species. The conserved metal binding site of LAP in these divergent species was Isoleucine, 2 of Glycine and Lysine.The conserved catalytic site was Asparagine, Threonine, Aspartic acid, Alanine, Glutamic acid, Glycine and Arginine (Figure3). The constructed phylogenetic tree using the neighbor-joining method illustrated that *Ov*LAP formed the same clade with other fluke LAP and was closely related with the liver fluke, *C. sinensis*. The tree also clearly showed that *Ov*LAP formed different clade with plants, protozoas, and vertebrates (Figure 4).

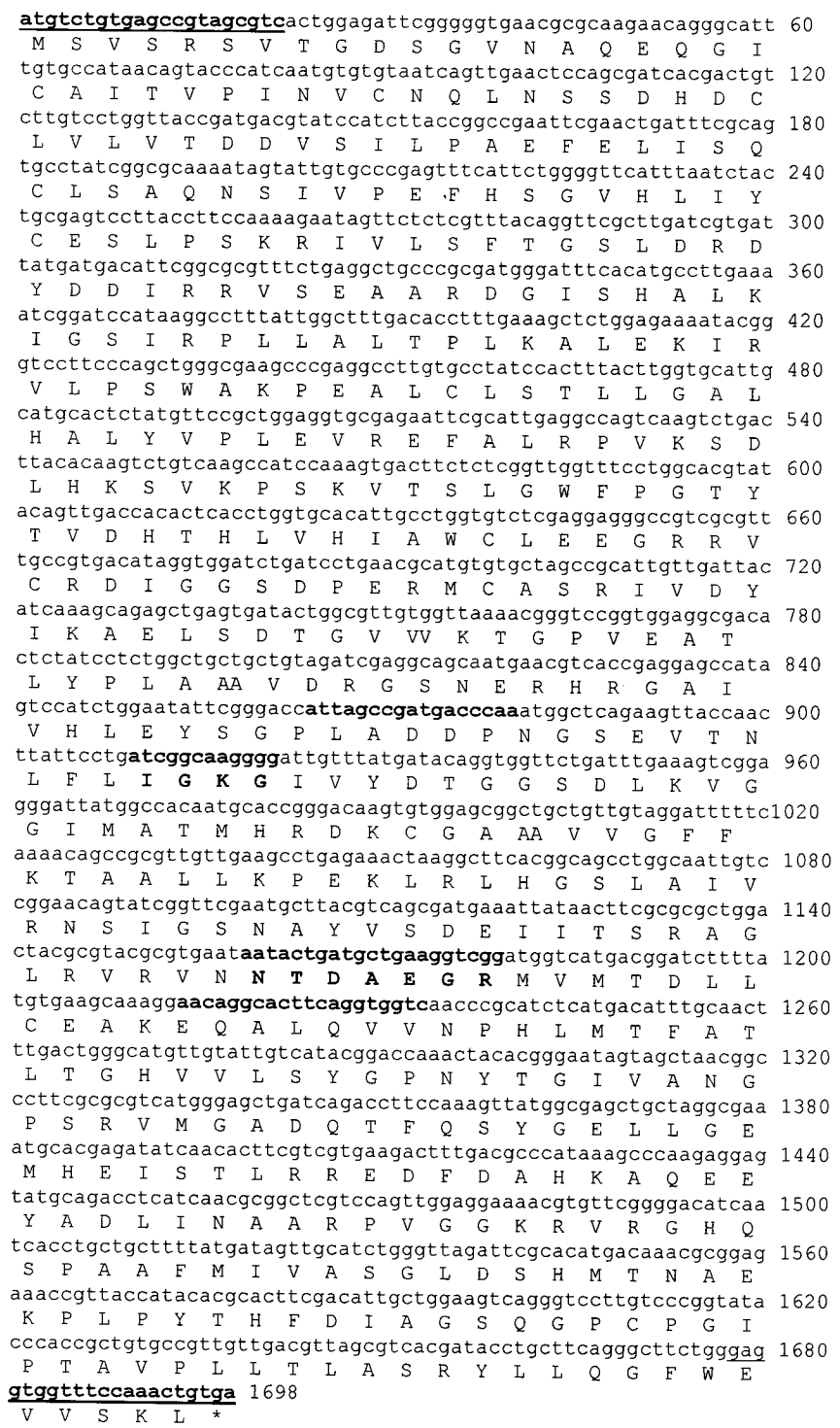

Figure 1 Deduced amino acid sequence of *Ov*LAP. The nucleotide sequence shows in the upper line. The sequence corresponding with forward and reverse primer binding sites are shown as bold letters and underlined. The binding sites for RT-PCR primer are indicated as bold letters. The conserved amino acid sequences including metal binding site; IGKG and catalytic site; NTDAEGR are indicated as bold letters. The termination codon (TGA) is marked with an asterisk.

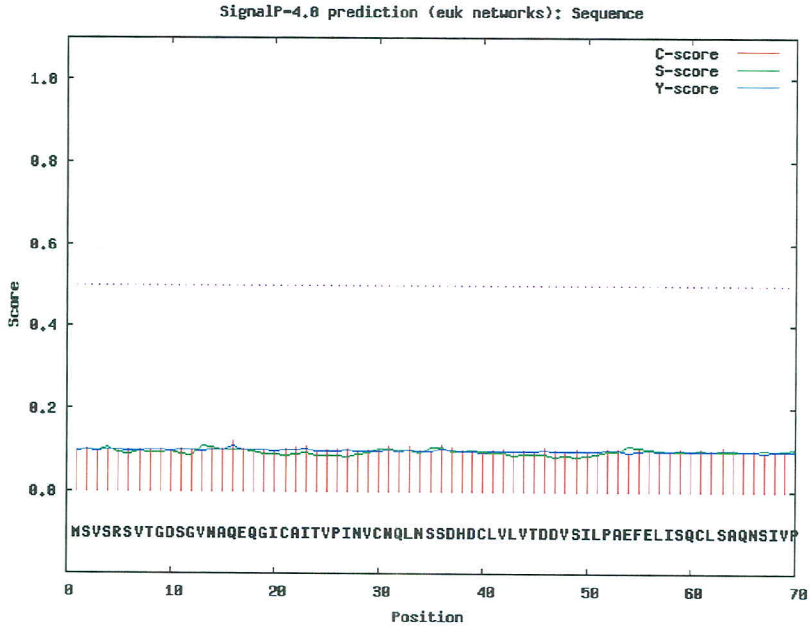

Figure 2 Prediction of *Ov*LAP signal peptide. The cleavage site of *Ov*LAP signal peptide was predicted by using of SignalP-NN prediction of SignalP version 2.0 at http://www.cbs.dtu.dk/services/SignalP/. The absence of a cleavage site for signal peptide in *Ov*LAP amino acid sequence was predicted by this program.

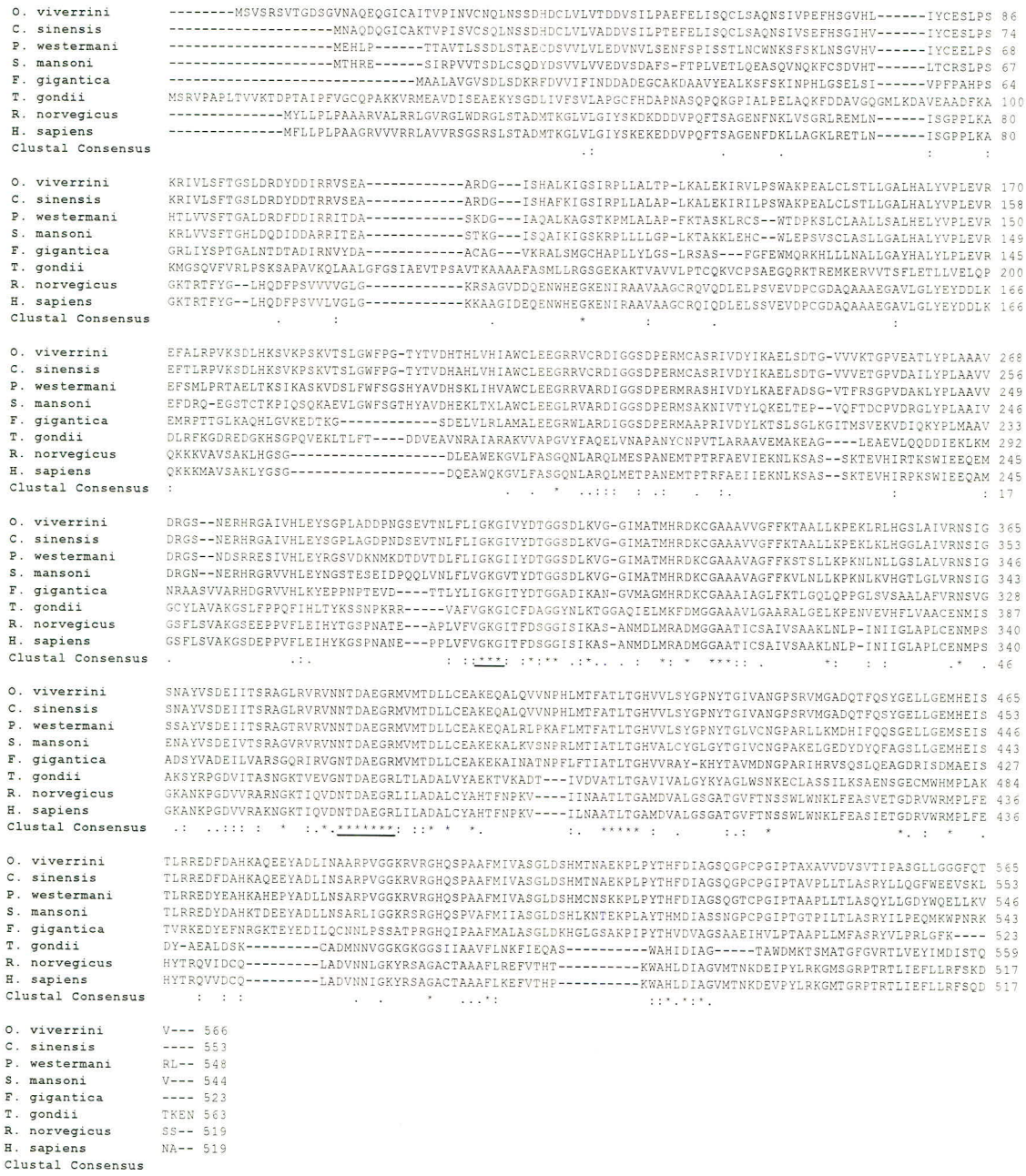

Figure 3 Multiple alignment of amino acid sequences of LAP from *O. viverrini* and other organisms. All sequences were retrieved from GenBank: *C. sinensis* (ACR27084), *Paragonimus westermani* (ABL96624), *S. mansoni* (ACQ77149), *F. gigantica* (ACS94976), *T. gondii* (XP_002368461), *R. norvegicus* (NP_001011910) and *H. sapiens* (AAD17527). The two conserved amino acid sequences are underlined; IGKG is the metal binding site and NTDAEGR is the catalytic site. The identical residues are indicated with (*), the highly and moderately conserved residues are indicated with (:) and (.) respectively.

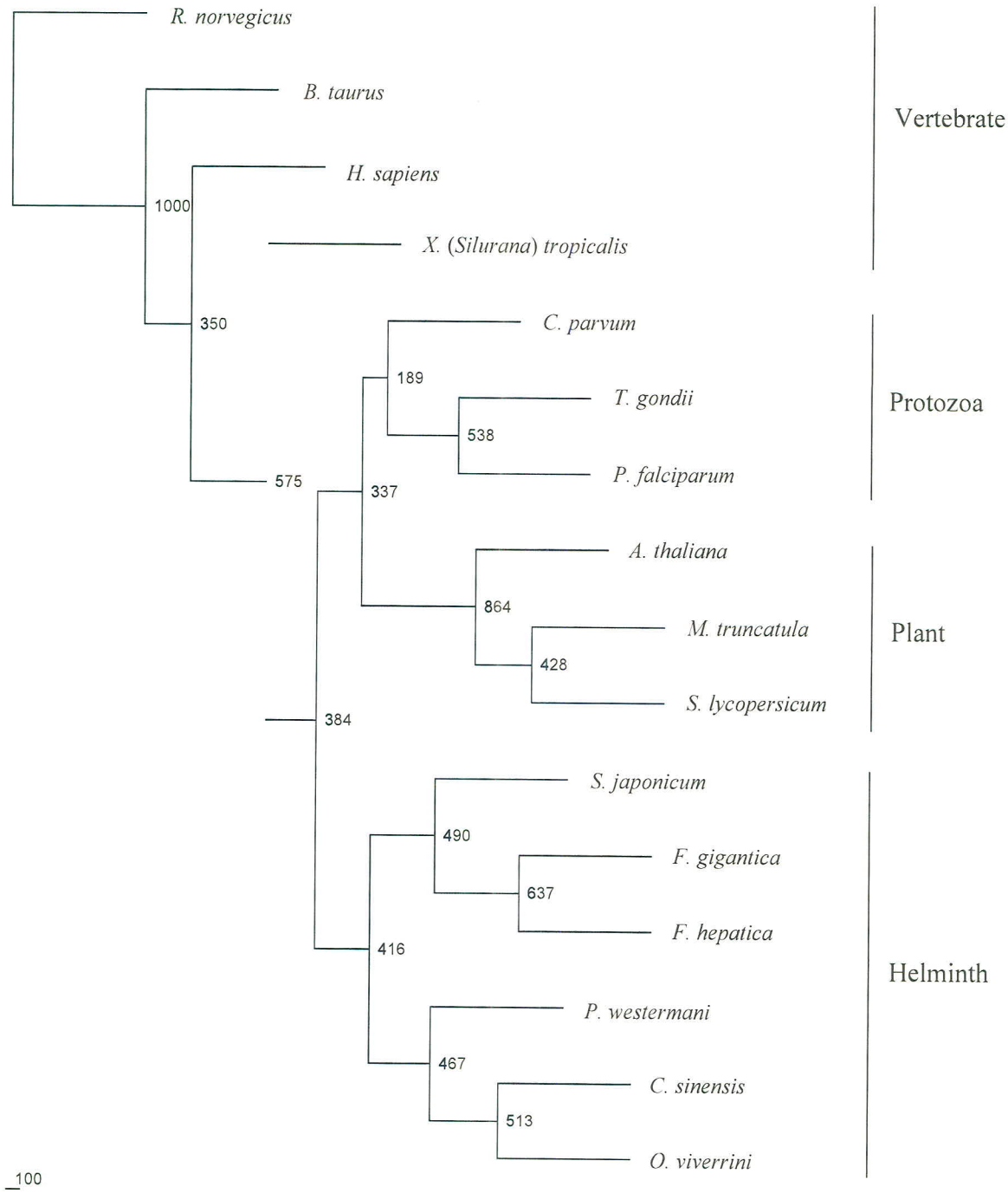

Figure 4 Phylogenetic tree of the evolutionary relationship of *Ov*LAP with other LAP from protozoas, helminths, plants, and vertrebrates. The accession numbers used in the phylogenetic analysis were *C. sinensis* (FJ423547), *F. gigantica* (GQ214329), *F. hepatica* (AY644459), *B. taurus* (NM_174098), *R. norvegicus* (NM_001011910), *X. (Silurana) tropicalis* (001011124), *P. falciparum* (XM_001348577), *P. westermani* (EF155963), *H. sapiens* (AF061738), *S. lycopersicum* (NM_001246955), *M. truncatula* (XM_003610271), *S. japonicum* (AF300423), *S. mansoni* (FJ824843), *T. gondii* (XM_002368420), *C. parvum* (XM_626197) and *A. thaliana* (NM_001202658).

2. *Ov*LAP protein expression in bacteria

The soluble recombinant *Ov*LAP protein fusion with N-terminal six-histidine tag was produced in bacteria *E. coli* stain BL21(DE3) after induction with 1 mM IPTG at 26°C. The protein was purified and analyzed on SDS-PAGE. *Ov*LAP expressed protein appeared as a single band in SDS-PAGE at ~60 kDa (containing His-tag), showing a good agreement with the predicted molecular mass (Figure 5).The expressed protein was recognized by an anti-histidine tag antibody and revealed the *Ov*LAP protein band at molecular weight ~60 kDa (Figure 5).

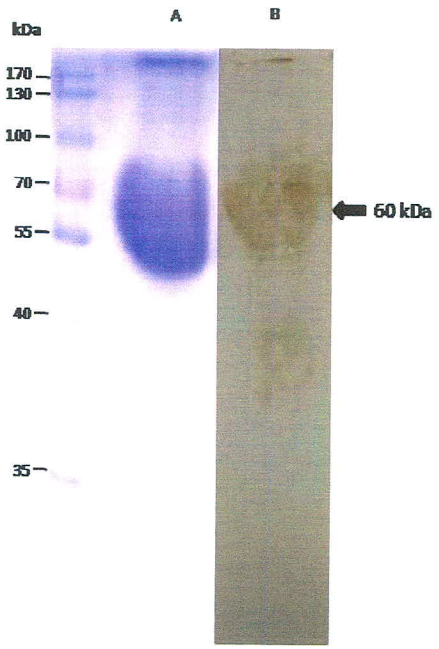

Figure 5 The pattern of recombinant *Ov*LAP protein expression. (A) The Coomassie Blue-stained gel shows a single band of protein at a molecular weight of ~60 kDa. B. The immunoblotting of expressed protein with anti-His tag antibody revealed a strong signal at the same molecular mass.

3. Recognition of *Ov*LAP with polyclonal anti-*Ov*LAP antibody

The recombinant *Ov*LAP produced in bacteria *E. coli* examined their properties and made antiserum to its localization on adult *O. viverrini* paraffin section. Polyclonal antibody against *Ov*LAP was raised in mice and confirmed the recognition with the *Ov*LAP protein band (Figure 6). The recognition of recombinant *Ov*LAP was performed using the immunoblotting technique. Polyclonal anti-*Ov*LAP antibody at a dilution of 1:100 was used as the primary antibody. Pre-immunize mice serum at the same dilution was used as the negative control. An anti-*Ov*LAP antibody showed strong recognition single band of

recombinant *Ov*LAP at ~60 kDa. The molecular weight of the protein band from immunoblotting analysis corresponded with the band from Coomasie-blue staining. No band was detected in the blot probed with the negative control serum (Figure 6).

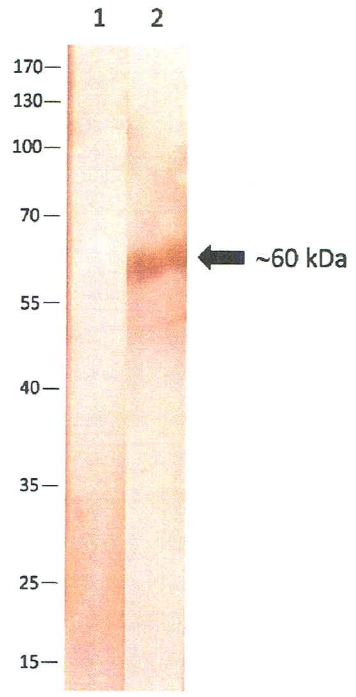

Figure 6 Immunoblotting of purified recombinant *Ov*LAP protein with mouse antiserum against *Ov*LAP protein. The single band of recombinant *Ov*LAP protein at ~60 kDa was strongly recognized with 1:100 of mouse anti-OvLAP serum (2). Mouse pre-immunized serum at a dilution of 1:100 was used as the negative control (1).

4. Immunolocalizationof *Ov*LAP on *O. viverrini* paraffin section

Polyclonal anti-*Ov*LAP antibody produced in mice was used to investigate the expression of *Ov*LAP in the adult stage of *O. viverrini*. The localization *O. viverrini* paraffin section with mouse anti-*Ov*LAP antibody was performed parallel with pre-immunized mouse serum. Strong immunoreactivity was revealed in parenchymal cells, egg shell, Mehlis gland, testis, ventral sucker, tegument, sub-tegument, and gut-epithelial cell of the adult worm. The reaction was also observed in some of hepatocyte and bile duct walls. There was no signal detected in the *O. viverrini* section probed with pre-immunized mouse serum (Figure 6).

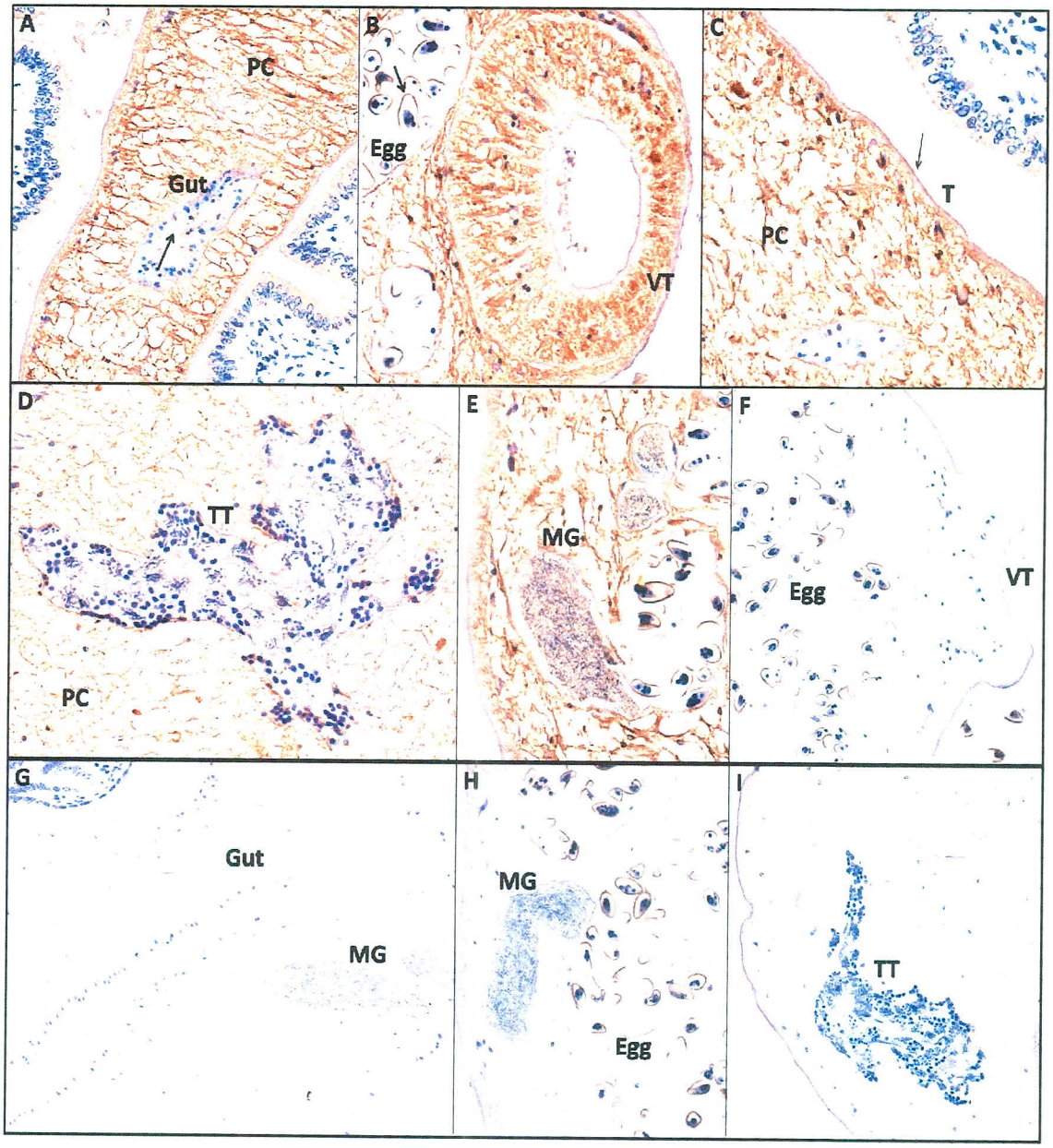

Figure 6 Immunolocalization of *Ov*LAP in adult *O. viverrini* paraffin section with mouse serum against recombinant *Ov*LAP protein. A strong signal of reaction were observed in parenchymal cells (PC), tegument (T), sub-tegument, ventral sucker (VT), gut-epithelial cell (arrow), egg shell (arrow), Mehlis gland (MG) and testis (TT). The signal of reaction was also observed in human hepatocyte and bile duct wall. The signal was also revealed in some of hepatocyte and host bile-duct wall. No staining was observed in sections that were probed with pre-immunized serum (F, G, H and I).

5. Developmental expression of *Ov*LAP

The expression pattern of *Ov*LAP was determined in various stages of *O.viverrini* by conventional RT-PCR and real time-PCR using primer design cover catalytic sites and metal binding sites. From real time-PCR, the value of threshold cycle (CT) of *Ov*LAP-mRNA and *Ov*Actin-mRNA at different stages was calculated and quantified as $2^{-\Delta\Delta CT}$. *Ov*LAP was detected in 2nd week *O. viverrini* larva. The expression level in 2nd week larva was higher than in metacercariae (3.91-fold; $P<0.05$), 1st week larva (8-fold; $P<0.05$) and egg (13.55-fold; $P<0.05$). However, there was no differences of *Ov*LAP expression in metacercariae, egg, 1st week larva, 2nd week larva, 3rd week larva, 4th adult, and 2 month adult worm ($P>0.05$) (Figure 7B). From conventional-PCR, PCR product size of 373 bp (including catalytic site and metal binding site) of *Ov*LAP gene was clearly detected in RNA of metacercaria, egg, 1st week larva, 2nd week larva, 3rd week larva, 4th adult, and 2 month adult of *O. viverrini*. The high expression of *Ov*LAP gene detected in 2nd week larva corresponds with result from real time-PCR. Actin gene was used as a control for cDNA integrity and PCR fidelity. *O. viverrini* RNA was used as templates in negative control (Figure 7A).

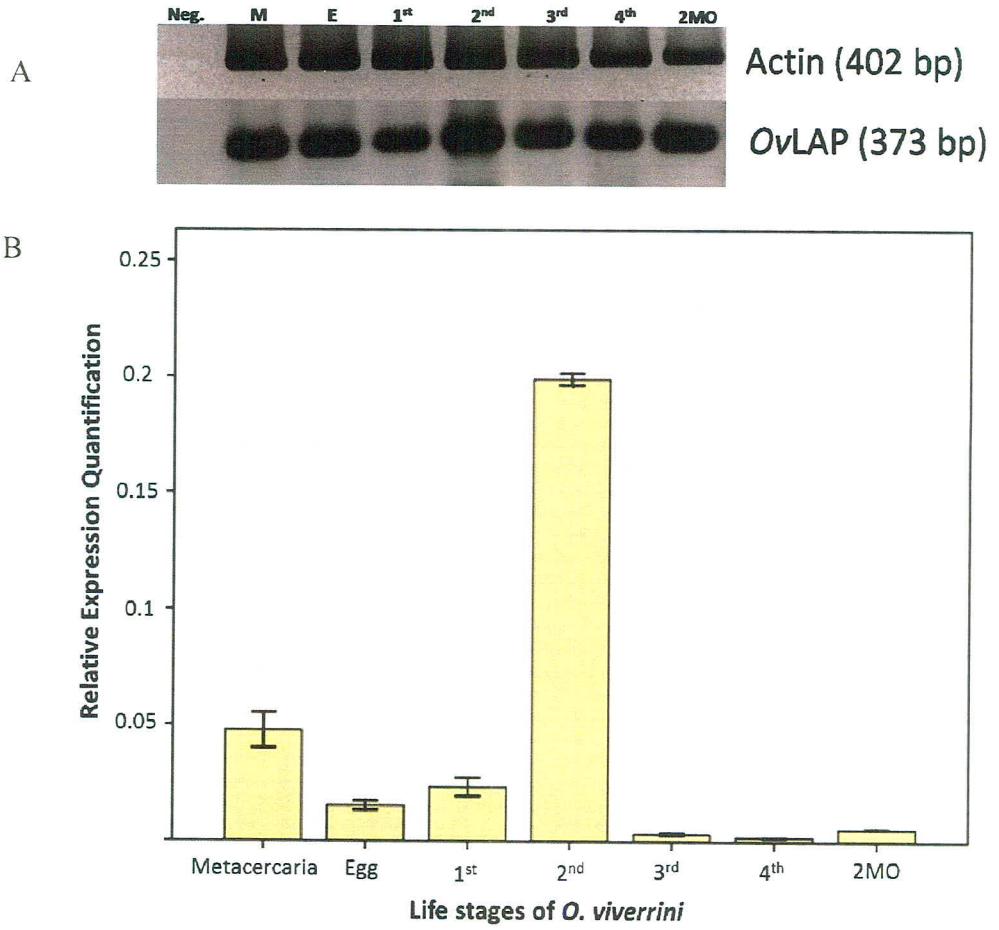

Figure 7 Expression of *Ov*LAP gene in different developmental stages of *O. viverrini*. Conventional RT-PCR was performed on cDNA from various developmental stages in order to determine which stage of *O. viverrini* expressed *Ov*LAP gene. Panel A represents the Conventional RT-PCR products using specific primers for *Ov*LAP. The cDNA template from various developmental stages of *O. viverrini* was used as follows; lane 1, negative controls using RNA as template; lane 2, metacercaria; lane 3, egg; lane 4, 1st week larva; lane 5, 2nd week larva; lane 6, 3rd week larva; lane 7, 4th adult, and lane 8, 2 month adult worm. RT-PCR of the housekeeping gene (Actin) was used as a positive control. Panel B real time-PCR assay of *Ov*LAP was present as quantitative analysis of mRNA level. *Ov*LAP transcripts were detected in metacercariae, egg, 1st week larva, 2nd week larva, 3rd week larva, 4th adult, and 2 month adult worm. A level of *Ov*LAP mRNA was detected in 2nd week larva higher than expression levels in metacercaria (3.91-fold; $P<0.05$), 1st week larva (8-fold; $P<0.05$), and egg (13.55-fold; $P<0.05$). There was no differences of *Ov*LAP expression in metacercariae, egg, 1st week larva, 2nd week larva, 3rd week larva, 4th adult, and 2 month adult worm ($P>0.05$).

6. Activity of recombinant *Ov*LAP protein

The optimal activity of recombinant *Ov*LAP protein was determined against specific colorimetric substrate of leucineaminopeptidase, L-Leucine p-nitroanilide. The activity of *Ov*LAP was investigated at 37°C. *Ov*LAP showed the activity against specific substrate at a broad pH range between 6.0 and 8.0. The maximum activity of *Ov*LAP was observed at slightly alkaline pH (pH 8.0) (Figure 8). At slight alkaline pH, *Ov*LAP activity increased in the presence of Ca^{2+} and Co^{2+} in contrast with the presence of Mg^{2+} that could not activate *Ov*LAP activity. The activity of *Ov*LAP progressively increased in accordance with the concentration of metal ions, $Ca^{2+}$and Co^{2+} (Table 1). The activity of *Ov*LAP was highly inhibited by bestatin, the specific inhibitor of leucineaminopeptidase. The inhibitory effect of bestatin was dose-dependent, similar with the inhibitory effect of EDTA against *Ov*LAP activity. This was in contrast with the protease inhibitor, E-64 that did not showed an inhibitory effect on *Ov*LAP activity (Table 2).

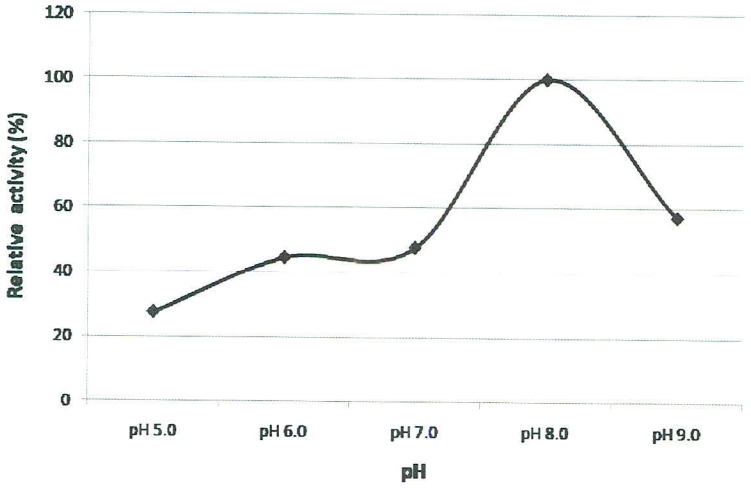

Figure 8 Optimal pH of *Ov*LAP activity. *Ov*LAP activity was assayed in various pH buffers ranging from 5.0 to 9.0. Broad of optimal pH buffers were observed. Slightly acidic (pH 6.0), neutral (pH 7.0), and slightly alkaline (pH 8.0) conditions were optimal for OvLAP activity. The maximal activity of *Ov*LAP was observed at pH 8.0. The highest activity was shown as 100%.

Table 1 Effect of divalent metal ions on *Ov*LAP activity

Metal ion	Concentration (mM)	Activity (umol/min)	Relative activity (U/ul)
Control (no metal ion)	-	0.001± 0.001	0.003
Ca^{2+}	0.1	0.59 ± 0.018	15.86
	1.0	0.69 ± 0.025	18.84
Co^{2+}	0.1	0.16 ± 0.006	4.30
	1.0	0.02 ± 0.001	5.43
Mg^{2+}	0.1	0.001 ± 0.001	0.003
	1.0	0.001 ± 0.001	0.0028

Table 2 Inhibitory effect of protease inhibitors and metal chelator on *Ov*LAP activity

Inhibitor	Concentration (mM)	Activity (umol/min)	Relative activity (U/ul)	Residual activity (%)
Control	-	0.048 ± 0.011	1.31	100
E-64	1.0	0.042 ± 0.013	1.134	86.56
	2.0	0.045 ± 0.018	1.215	92.75
EDTA	1.0	0.027 ± 0.014	0.74	56.49
	2.0	0.007 ± 0.003	0.20	15.27
	3.0	0.003 ± 0.002	0.09	6.87
Bestatin	0.1	0.036 ± 0.009	0.97	74.05
	1.0	0.012 ± 0.007	0.32	24.43

Control without any inhibitor or chelator