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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ
   จะมีการนำผลงานไปตีพิมพ์ในวารสารระดับนานาชาติ
2. การนำผลงานวิจัยไปใช้ประโยชน์
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Cloning and characterization of *Opisthorchis viverrini* luecine aminopeptidase

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ABSTRACT

Leucine aminopeptidase (LAP), classified as a metalloprotease, was identified from cDNA library of adult *Opisthorchis viverrini*. The contig of nucleotide sequences of *O. viverrini* leucine aminopeptidase (*Ov*LAP) was assembled to the full length DNA that was comprised of 1,698 bp, encoding 566 amino acids, and a calculated molecular mass of 60 kDa with predicted PI of 6.21. Analysis of signal peptide using SignalP-NN and SignalP-HMM prediction indicated that *Ov*LAP was a non-secretory protein by the absence of a cleavage site at the N-terminus of the amino acid sequence. Similar to LAP in other organisms, the *Ov*LAP sequence contains the hallmark of the LAP protein sequence with the conserved metal binding site: Isoleucine, 2 of Glycine and Lysine and catalytic site: Asparagine, Threonine, Aspartic acid, Alanine, Glutamic acid, Glycine and Arginine. Phylogenetic analysis based on the amino acid sequence of *Ov*LAP revealed a close relationship with the liver fluke *Clonorchis sinensis*. Reverse transcriptase-PCR indicated that the *Ov*LAP was expressed in egg, metacercaria, juvenile, and adult stages. Recombinant *Ov*LAP was expressed in bacterial cells as a soluble form and its molecular weight appeared as a single band at 60 kDa. The recombinant protein of *Ov*LAP was used to immunize mice to produce polyclonal antibodies. The immmunohistochemical for tissue localization of *Ov*LAP in adult worms by the use of polyclonal antibodies demonstrated the presence of *Ov*LAP in parenchymal cells, eggs shell in the uterus, tegument, sub-tegumant, testis, Mehlis gland and in ventral suckers of adult worms. *Ov*LAP was required divalent metal ions; Ca^{2+} and Co^{2+} to assist in catalytic activity. The optimal activity of *Ov*LAP was observed at slightly alkaline condition.

Keywords: *Opisthorchis viverrini*, Leucine aminopeptidase, Cloning, Characterization

1. Introduction

Opisthorchis viverrini infection remains a major public health problem in Southeast Asia. This parasite can be pathogenic in its own right and direct risk factor for CCA of the bile duct (1994). The mechanisms by which *O. viverrini* induces CCA in humans are being explored and hypothesized to be a complex processes, involving several mechanisms. There are many secreted, membrane and/or intracellular protein that abundantly expressed in *O. viverrini* adult worm. Some of these proteins are involved in many aspect of parasitism such as food digestion, immune invasion, tissue invasion etc. To the function of food digestion, many of proteases encoded endo- and exoproteases are abundantly represented in *O. viverrini* adult stage (Laha et al., 2007).

Of particular interest is member of the M17 family, Clan MF of metallo proteases, leucine aminopeptidase (LAP) that has been identified from adult stage of *O. viverrini* (Young et al., 2010). LAP is known as exopeptidases that play crucial role in the parasite of protozoa and helminth with broader amidolytic activity beyone leucine hydrolysis. LAP participates in processing/maturation/activation or degradation of substrates that support their participation in vital processes in the parasite life cycle.

In *O. viverrini*, there have been extensively studied in endopeptidases due to their prominent participation in fluke's biology but there have any studied in exopeptidases. Thus there is little known of this molecule in the benefit of *O. viverrini* and harmful affecting to their host. Thereafter, it is important to clarify the biochemistry, the molecular biology and function of the LAP in *O. viverrini* and the evolution of this molecule in general for gaining insight the basic knowledge of the parasite. The knowledge from this study leading to answer the questions about the important of proteases roles in catabolism of essential nutrition for *O. viverrini* and might be useful information to understand the pathogenesis of *O. viverrini* infection. All these information could provide an opportunity for the development of novel anti-parasitic drug, vaccine or serodiagnostic agent for detection of opisthorchiasis.

2. Materials and methods

2.1. Parasites specimens

O. viverrini metacercariae were isolated from naturally infected fish from endemic area by pepsin digestion as described previously (Srisawangwong, Sithithaworn, and Tesana, 1997). *Opisthorchis viverrini* metacercariae were collected under a dissecting microscope and viable cysts were kept at-80 C for RNA extraction and used to infect hamsters. Infected hamsters at 1st, 2nd, 3rd, 4th and 2 month were sacrificed to collected *O. viverrini* worms. The collected *O. viverrini* worms were kept at-80 C for RNA extraction. Some of adult *O. viverrini* worms were maintain at 37 C in RPMI in order to collect *O. viverrini* egg. The collected eggs were also kept at -80 C waiting for RNA extraction.

2.2. Sequence and phylogenetic analyses

The full length DNA sequence of *Ov*LAP was identified from previous study (Laha et al., 2007). The *Ov*LAP sequence was compare the homology with LAP of other organisms in the DNA database using BLAST algorithm including Blastn, Blastx and tblastx at http://www.ncbi.nlm.nih.gov/blast/. Multiple sequence alignments of LAP were employed bootstrap analysis for 1,000 replicas using Seqboot in Phylip package version 3.66 (Felsenstein, 1993). Phylogenetic tree of LAP evolution was constructed with neighbor-joining method using Protdist in Phylip package. Consensus neighbor-joining tree was constructed using consense program in Phylip.

2.3. Cloning and recombinant protein production

The full length of *Ov*LAP was accomplished amplified from *O. viverrini* cDNA library with specific primers. These primers were designed encompass N- and C-terminal regions of *Ov*LAP nucleotide sequence. The forward primer was 5' GCG GCGCAT ATGATG TCT GTG AGC CGT AGC GTC3' and reverse primer was 5' GCG GCGCAT 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 were in bold). The stop codon was added in reverse primer (in bold and underlined).

*Ov*LAP amplification reaction was perform with *Phusion Tag* DNA polymerase (Thermoscientific, USA).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 DNA fragment was recovered, purified, cloning into TOPO-T vector (Invitrogen, USA) and then sub-cloning into expression vector, pET 15b+ (Novagen, USA). The recombinant DNA of *Ov*LAP in pET15b+ was prove for correct nucleotide sequence before transformed into competent *E. coli* BL21(DE3) cells by heat shock steps.

The transformants of *Ov*LAP in *E. coli* BL21(DE3) were selected by plating on LB agar containing 100 ug/ml carbenicillin (Gibco®, USA). The single carbenicillin resistance colony was growth overnight at 37 C in 50 ml LB broth with 100 ug/ml carbenicillin (Gibco®, USA) for collect bacterial pellet. The next day, the bacterial pellet was resuspended in 200 ml fresh LB broth containing 100 ug/ml carbenicillin (Gibco®, USA) and induced recombinant *Ov*LAP protein production with 1 mM IPTG. The culture media was incubated at 26 C for 8 hrs with shaking speed of 225 rpm (Non-induced control and induced cells were also collected and analyzed by SDS-PAGE electrophoresis). The bacterial pellet was collected after 8 hrs induction by centrifugation at 3,500 rpm for 15 min at 4 C. The supernatant was discarded and the bacterial pellet was resuspended in 3 ml native condition binding buffer containing 1X cocktailed proteases inhibitor (without EDTA) (Sigma-Aldrich, USA). The resuspend pellet was frozen and thawed at 42 C for 3 times then subsequently to sonication for 5 min at 4 C with 37% of amplitude. The supernatant containing *Ov*LAP recombinant protein was collect for protein purification through Ni-NTA resin column (Thermoscientific, USA) following company instruction. Protein fractions were analyzed with SDS-PAGE observing a single band with coomassie blue and/or protein immunoblot staining using His-Tag antibody. The protein concentration was determined its absorbance at 280 nm (Nanodrop).

2.4. Production of polyclonal anti-OvLAP 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 at tail vein for base line 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 days intervals with incomplete Freund's adjuvant. Before each booster, blood sample was collected at animal tailed 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 evaluated the specificity of serum by Western blotting.

2.5. Immunoblotting assay

Immunoblotting was carried out in detection of the specificity of anti-*Ov*LAP against recombinant *Ov*LAP. In brief, recombinant OvLAP was separated on 10% SDS-PAGE, the electrophoretically-separated proteins was transferred to nitrocellulose membrane using a Mini Trans Blot)Biorad, USA(. Protein was accomplished transferred at 0.5 mA for 1 hr. After complete transferred, immunostaining was performed immediately. Following their removal from the transferred apparatus, the membrane was cut into strips and blocked the non-specific binding with 5% skim milk in PBST for 2 hr. The membrane was first incubated overnight with mouse anti-*Ov*LAP antibody at dilution 1:50 then incubate with IgG goat anti-mouse conjugated with horseradish peroxidase at dilution 1:1,000 for 2 hr. After additional wash with PBST, the strips were incubated with chemiluminescent detection kit, ECL Plus (GE Healthcare) and then visualized with ImageQuant™.

2.6. Immunolocalization of OvLAP 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 heat at 55-60 C in oven for 15-30 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 as following steps; absolute ethanol for 3 times, 5 min each, 95% ethanol for 2 times, 3 min each, 70% ethanol for one time in 3 min and finally rinse the section with PBS for 1-2 min. After that, antigen retrieval was performed in 0.1 M citrate buffer pH 6.0 for 3 min in pressure cooker. After antigen retrieval was complete and the slides were cooling down, wash the slides with 1X PBS for 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 site in the sections also block with normal horse serum in dilution 1:20 for 30 min at room temperature in humidified chamber. The *O. viverrini* sections was probed with mouse anti-*Ov*LAP antibody at dilution of 1:300 in PBS/NaN_3 and incubated overnight at room temperature in humidified chamber. For negative control, the slides were probed with pre-immunized serum. In the next day, the sections were probed for one hour at room temperature with anti-mouse antibody conjugated with horseradish peroxidase at dilution of 1:500 in PBS. Then the slides were washed with PBS for 2 times, 10 min each. To develop color, slides was submerge in freshly prepare diaminobenzidine (DAB) solution for 5 min then, stop reaction 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 in one time, 95% alcohol for 2 times, 3 min each finally dehydrate the slides in absolute alcohol 3 times, 3 min each. The slides were then observed under a light microscope and analyzed using Aperio ScanScope™.

2.7. Enzyme assays

The activity of recombinant *Ov*LAP protein was determined by monitor the increase of p-Nitroanilide releasing from L-Leucinep-Nitroanilide (Sigma-Aldrich, USA), the specific substrate of LAP. The activity was performed at 37 °C in wavelength of 405 nm using VERSAmax Tunable Microplate Reader™. The activity assay of *Ov*LAP was performed accompanied with Leucine Aminopeptidase, microsomal porcine kidney type IV-S (Sigma-Aldrich, USA) based on assay procedure described in Pfleiderer G., 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 range of pH buffer that containing different of metal ions. The assay was carried out in 96-well microtitre plate format in 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). In each buffer was contain 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 were measured the releasing of p-Nitroanilide for 60 min, 3 min interval at 37 °C in 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 was measure the releasing of p-Nitroanilide for 60 min, 3 min interval at 37 °C in wavelength of 405 nm. All reactions were compared to negative control without 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 amount of enzyme liberating 1 umol of p-Nitroanilide per minute at 37 C.

2.8. Developmental expression

Real-time PCR and conventional PCR were performed to evaluate transcript of *Ov*LAP in various developmental stage of *O. viverrini*. The developmental stages of *O. viverrini* including adult worm, juvenile, egg and metacercaria were collected. Then, total RNA was extracted using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. Total RNA was treated with Dnase (Thermoscientific, USA) to excluded interference of DNA before prepared cDNA using 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') that designed cover 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 was analyzed on 0.8% agarose gel/EtBrand analyzed with 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 was followed manufacturing 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 increase to 95 C continuously to collected fluorescence signal.

3. Results

3.1. Characteristic of OvLAP and phylogenetic relationships

The full-length DNA of *Ov*LAP was identified. Blastx showed OvLAP shared 97% identity with leucine amniopeptidase of closely related species, *C. sinensis*, 68% identity with *Paragonimus westermani* and 64% identity with *Schistosoma mansoni*. *Ov*LAP comprising of 1,698 bp, encoding 566 amino acids, calculated molecular mass of 60 kDa with predicted PI of 6.21 (Fig. 1). Analysis of signal peptide using SignalP-NN and SignalP-HMM prediction indicated that *Ov*LAP was non-secretory protein by absence of cleavage site at N-terminus of amino acid sequence. 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 stable protein with 31.11 stability index (II).

Amino acid sequence of *Ov*LAP was compared to LAP of other organisms including flatworms, protozoas and vertebrates that retrieved from GenBank for multiple sequence alignment. *Ov*LAP showed complete conserve of metal binding site and catalytic site through these divergent species. The conserve metal binding site of LAP in these divergent species was Isoleucine, 2 of Glycine and Lysine. The conserve catalytic site was Asparagine, Threonine, Aspartic acid, Alanine, Glutamic acid, Glycine and Arginine (Fig. 2). The constructed phylogenetic tree using neighbor-joining method was illustrated that *Ov*LAP formed the same clade with other fluke LAP and closely related with the liver fluke, *C. sinensis*. The tree also clearly showed that *Ov*LAP was formed different clade with plants, protozoas and vertebrates (Fig. 3).

3.2. OvLAP 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), this was a good agreement with the predicted molecular mass (Fig. 4).The expressed protein was recognized by anti-histidine tag antibody and had revealed the *Ov*LAP protein band at molecular weight ~60 kDa (Fig. 4).

3.3. Recognition of OvLAP with polyclonal anti-OvLAP antibody

The recombinant *Ov*LAP produced in bacteria *E. coli* was examined their properties and make antiserum to its localization on adult *O. viverrini* paraffin section. Polyclonal antibody against *Ov*LAP was raised in mouse and confirmed the recognition with the *Ov*LAP protein band (Fig. 5). The recognition of recombinant *Ov*LAP was performed using immunoblotting technique. Polyclonal anti-*Ov*LAP antibody at dilution 1:100 was used as primary antibody. Pre-immunize mice serum at the same dilution was used as negative control. Anti-*Ov*LAP antibody was shown strongly recognition single band of recombinant *Ov*LAP at ~60 kDa. The molecular weight of protein band from immunoblotting analysis was corresponding with the band from Coomasie-blue staining. No band was detected in blot probed with negative control serum (Fig. 5).

3.4. Immunolocalizationof OvLAP on O. viverrini paraffin section

Polyclonal anti-*Ov*LAP antibody produced in mouse was used to investigate the expression of *Ov*LAP in 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. The 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 also observed in some of hepatocyte and bile duct walls. There was no signal detected *O. viverrini* section probed with pre-immunized mouse serum (Fig 6).

3.5. Developmental expression of OvLAP

The expression pattern of *Ov*LAP was determined in various stage of *O.viverrini* by conventional RT-PCR and real time-PCR using primer design cover catalytic site and metal binding site. From real time-PCR, the value of threshold cycle (CT) of *Ov*LAP-mRNA and *Ov*Actin-mRNA at different stage was calculated and quantified as $2^{-\Delta\Delta CT}$. *Ov*LAP was high 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 difference 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 was detected in 2nd week larva that 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).

3.6. Activity of recombinant OvLAP 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 was showed the activity against specific substrate at broad pH range between 6.0 – 8.0. The maximum activity of *Ov*LAP was observed at slightly alkaline pH (pH 8.0) (Fig. 8). At slightly alkaline pH, *Ov*LAP activity was increased in the presence of Ca^{2+} and Co^{2+}. In contrast with the present of Mg^{2+}, that could not activated *Ov*LAP activity. The activity *Ov*LAP was progressive increase 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 inhibitory effect of EDTA against *Ov*LAP activity. In contrast with the protease inhibitor, E-64 that did not showed inhibitory effect on *Ov*LAP activity (Table 2).

4. Discussion

Full-length of *O. viverrini* leucineaminopeptidase (*Ov*LAP) was identified. The properties of typical enzyme that belonging to M17 metalloprotease were observe in *Ov*LAP. Two regions of metal-binding sites, IGKG and NTDAEGR were examined in *Ov*LAP. These regions were conserving throughout LAP from other organisms including plant, helminth, protozoa and vertebrate. Deduce amino acid sequence of *Ov*LAP was found 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 classified as M17 exopeptidase that cleaving the N-terminal part of peptide to single amino group. These amino groups are used in parasite metabolism to promote parasite growth and development (Matsui, Fowler, and Walling, 2006). *Ov*LAP could function in board pH range and the optimal pH for efficient activity was found at slightly alkaline condition (pH 8.0) that 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 could activate by several inorganic cofactor such as Ca^{2+} and Co^{2+}, in contrast of Mg^{2+} that activates lack of *Ov*LAP activity. Moreover, fully occupied at metal-binding sites was elevated *Ov*LAP activity. Moreover, fully occupied at metal-binding sites was elevated *Ov*LAP activity. We found that high concentration of metal ions; Ca^{2+} and Co^{2+} (1 mM) was 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 has show strongly inhibitory effect on *Ov*LAP activity. The degree of inhibition of bestatin and EDTA against *Ov*LAP activity was dose-dependent. In contrast of E-64 that lack of 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 that require divalent cations for enhance their activity and suppressed by metal chelating; EDTA and LAPs inhibitor; bestatin was well conserved with M17 peptidase family.

LAPs are crucial enzyme that involved in protein degradation by participate in final step of protein breakdown cascade to the releasing single amino group from N-terminus peptide (Delcroix et al., 2006). This amino group is used as building block for protein synthesis and intermediate molecule in cytoplasmic metabolisms that require for increase development and growth of the parasite. Thus, *Ov*LAP is vital and observe in all developmental stage of *O. viverrini*, like in *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*; the stage that indicated to require more nutrients and energy for maturation. However, the requirement of amino group for development was observed in tissues and organs of adult stage. From localization to determined *Ov*LAP expression in adult *O. viverrini* using anti-*Ov*LAP antibody found that strong signal reaction in egg shell. These suggested *Ov*LAP would play role in egg hatching process by act as hatching enzyme to degrade egg shell and then facilitate hatching of miracidium from egg. This founding was similar in *S. mansoni* that found LAP activity in hatching fluid (McCarthy et al., 2004; Rinaldi et al., 2009). Anti-*Ov*LAP antibody was also recognized at muscular region, ventral sucker; the attachment organ which probably requires high energy to maintain adherence to host bile duct. The strong reaction of anti-*Ov*LAP antibody was also showed in testis indicated the role of *Ov*LAP in germ cell maturation. In addition, localization was also present at tegument and sub-tegumental tissue where *Ov*LAP was suggested to serve as cell surface remodeling enzyme (Kang et al., 2012; McCarthy et al., 2004; Mulvenna et al.; Song et al., 2008). In addition, the localization was found distribution on surface of gut epithelial cell where the final step of protein degradation occurs. From localization, OvLAP was not present in gut lumen where slightly acidic pH environment was reported but possibly present at apical membrane of gut epithelial cell where the environmental pH suite for *Ov*LAP activity. From this supporting indicated that *Ov*LAP is not secreted like other protease, but functions intracellulary by generate single amino acid from short peptide that diffuse into gut epithelial cell surrounding gut lumen (Changklungmoa et al., 2012; Kang et al., 2012). From this indicated was corresponds with the optimal pH for *Ov*LAP activity that function 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 muscle are embedded. Due to the lack of body cavity, thus parenchymal cell are found throughout fluke body and function as supporting tissue to anchor 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 vertebrate host, especially liver organ normally found LAPs in cell. In case of 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 mouse (data not shown). Moreover, localization was present 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 for vaccine candidate, but, the only inhibition of *Ov*LAP activity would not have significant effect on 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 effect on systemic hierarchical event in host protein hydrolysis. The blocking host protein hydrolysis, nutrient uptake, protein synthesis and cytoplasmic metabolism may impair parasite survival.

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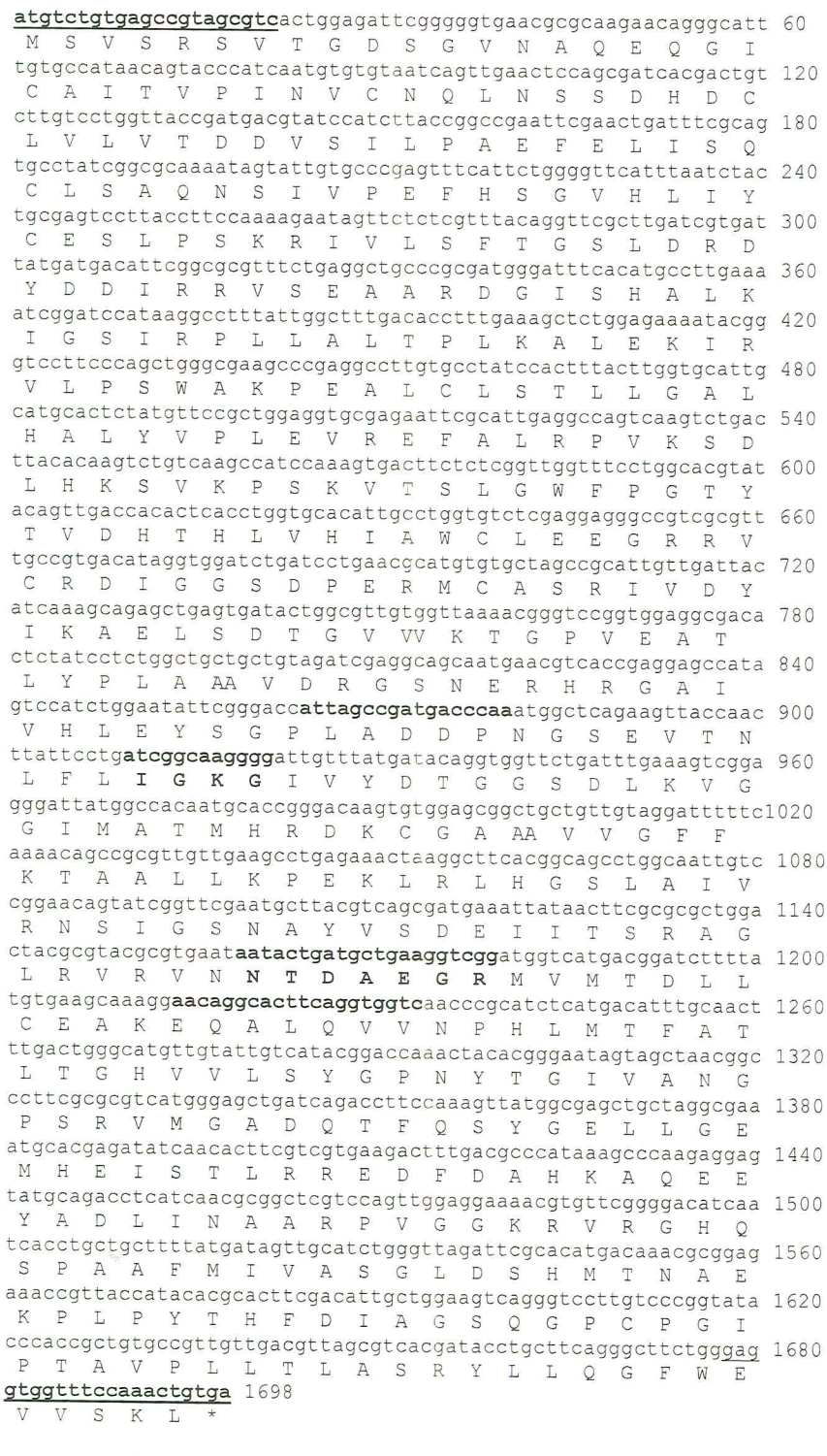


Fig. 1. Deduce amino acid sequence of *Ov*LAP. The nucleotide sequence showed in upper line. The sequence corresponding with forward and reverse primer binding sites were showed as bold letters and underlined. The binding sites for RT-PCR primer were indicated as bold letters. The conserved amino acid sequences including metal binding site; IGKG and catalytic site; NTDAEGR were indicated as bold letters. The termination codon (TGA) was marked with asterisk.

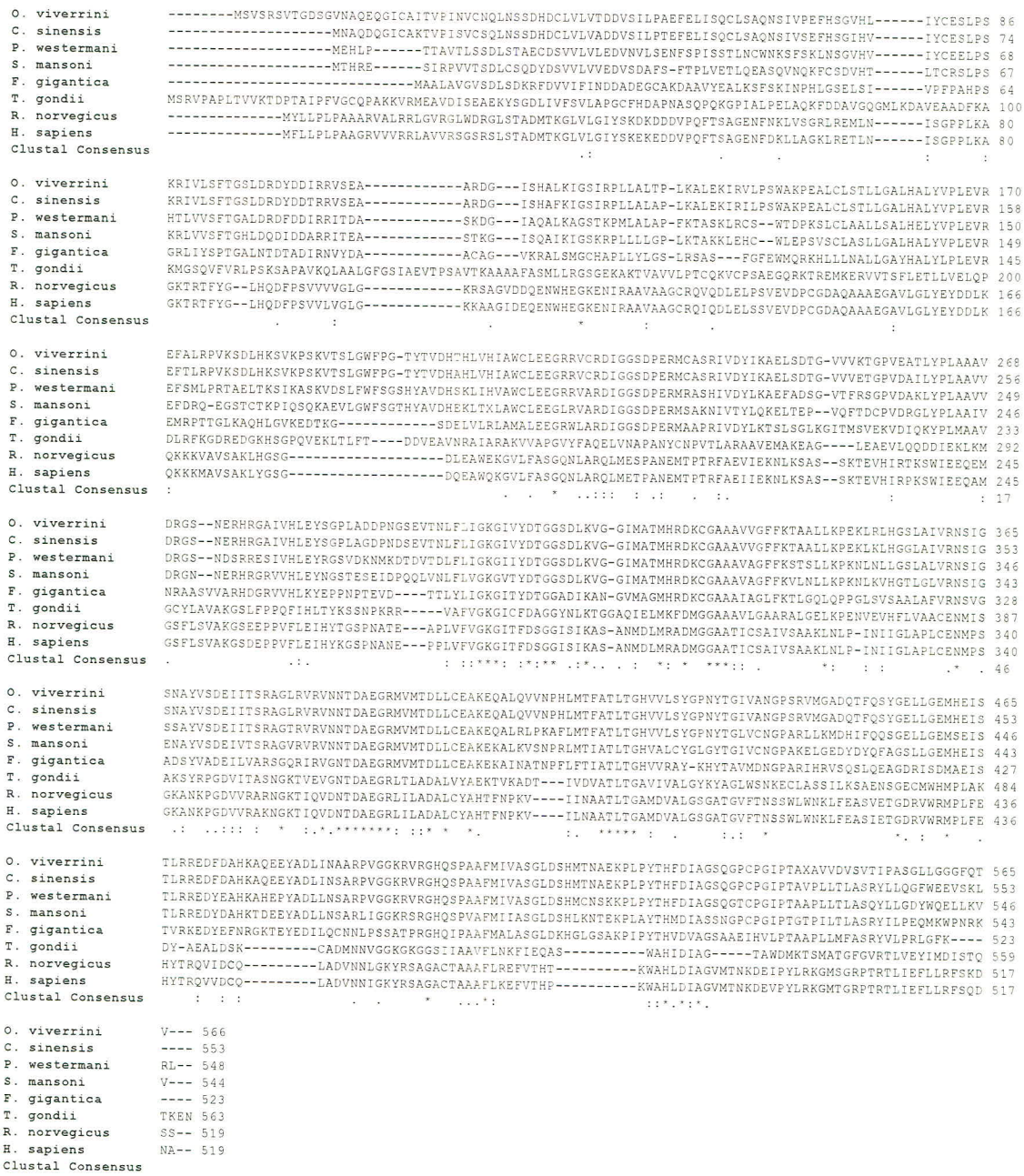


Fig. 2. Multiple alignment of amino acid sequences of LAP from *O. viverrini* and other organisms. All sequences retrieved from GenBank: *C. sinensis* (ACR27084), *Paragonimus westermani* (ABL96624), *S. mansoni* (ACQ77149), *F. gigantica* (ACS94976), *T. gondii* (XP_002368461), *R. norvegicus* (NP_001011910) and *H. sapiens* (AAD17527). The two conserved amino acid sequences were underlined; IGKG was the metal binding site and NTDAEGR was the catalytic site. The identical residues were indicated with (*), the highly and moderately conserved residues were indicated with (:) and (.), respectively.

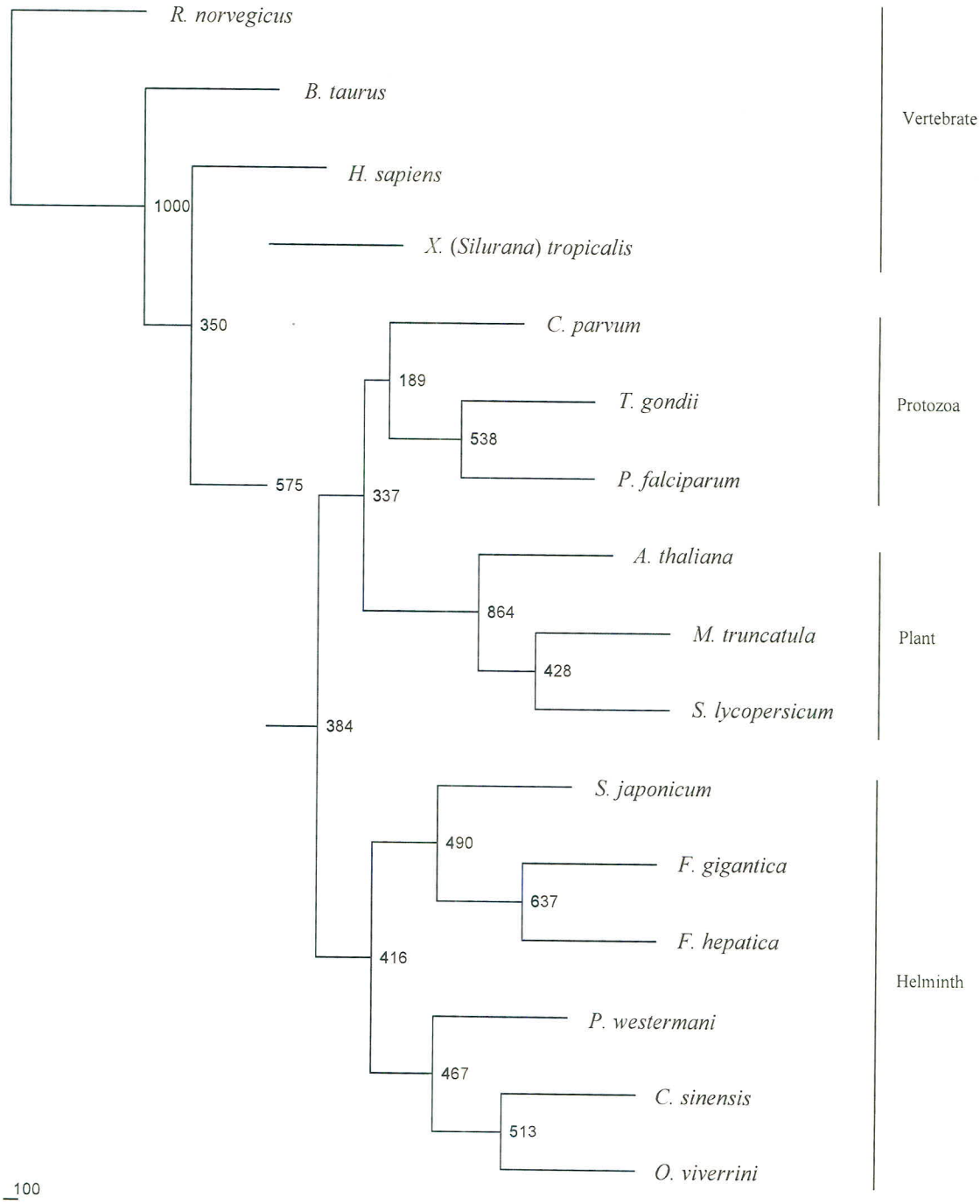


Fig. 3. Phylogenetic tree of the evolutionary relationship of *Ov*LAP with others LAP from protozoas, helminths, plants and vertrebrates. The accession number used in the phylogenetic analysis were *C. sinensis* (FJ423547), *F. gigantica* (GQ214329), *F. hepatica* (AY644459), *B. taurus* (NM_174098), *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).

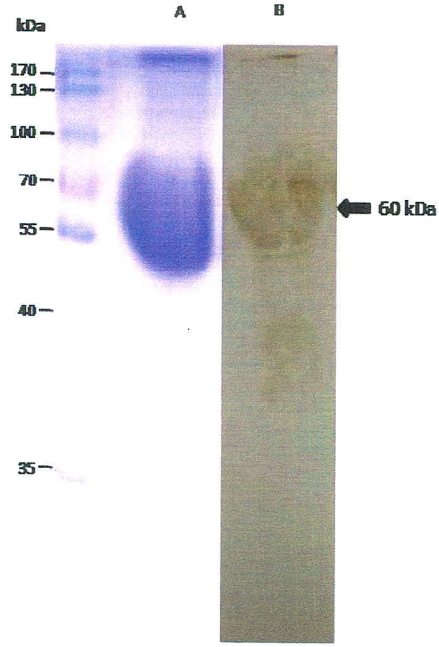


Fig. 4. The pattern of recombinant *Ov*LAP protein expression. (A) The Coomassie Blue-stained gel showed single band of protein at molecular weight of ~60 kDa. B. The immunoblotting of expressed protein with anti-His tag antibody was revealed strongly signal at the same molecular mass.

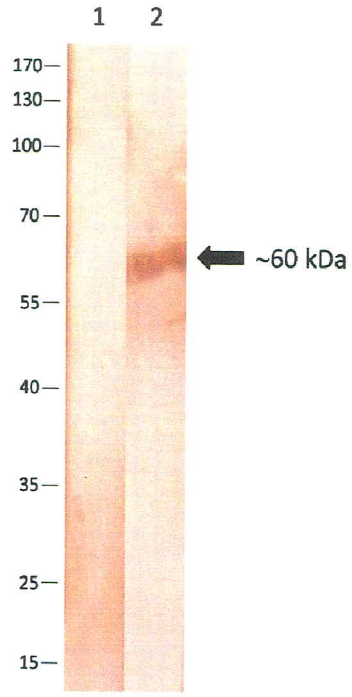
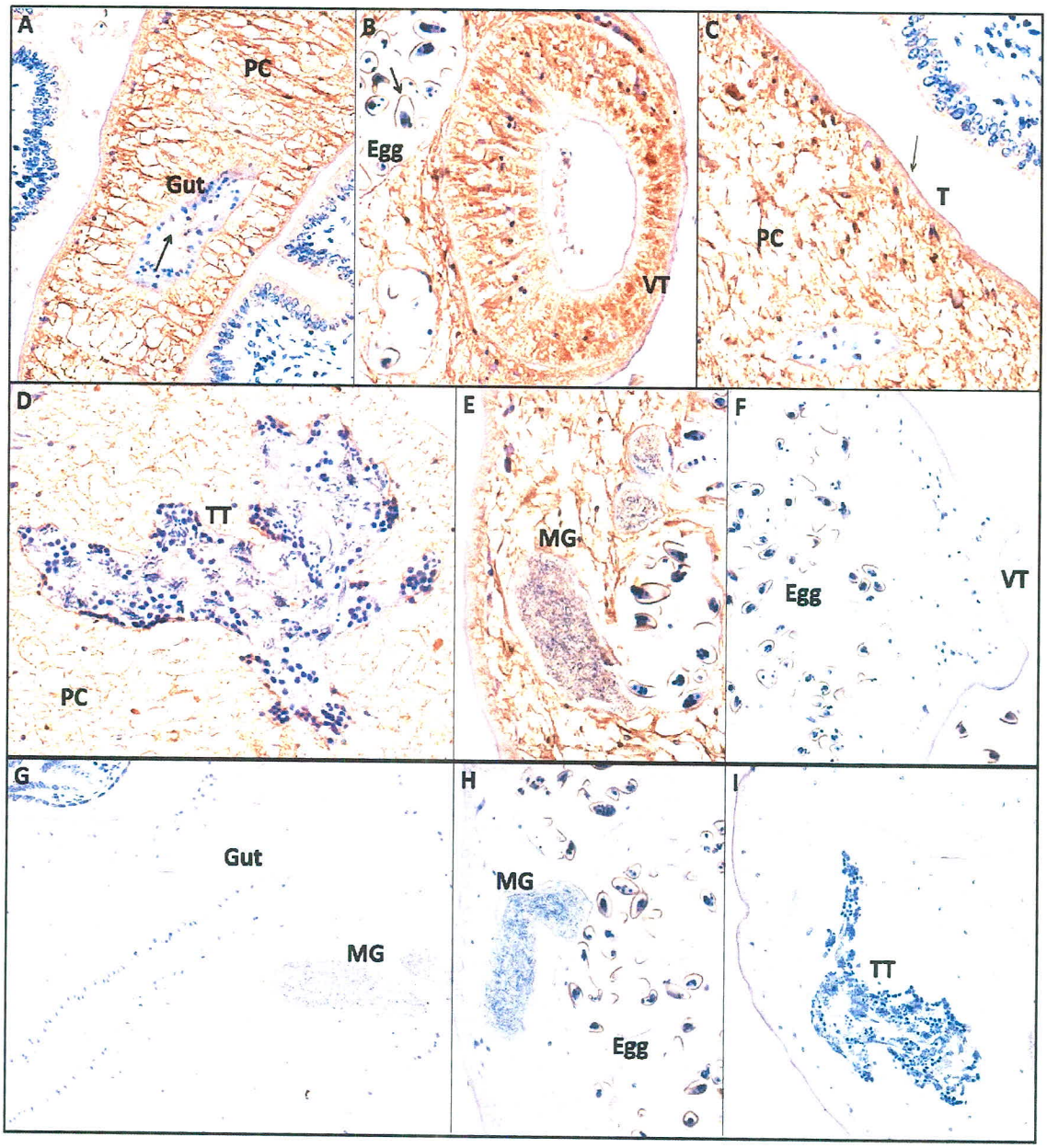


Fig. 5. The immunoblotting of purified recombinant *Ov*LAP protein with mouse antiserum against *Ov*LAP protein. The single band of recombinant *Ov*LAP protein at ~60 kDa was strongly recognized with 1:100 of mouse anti-*Ov*LAP serum (2). Mouse pre-immunized serum at dilution of 1:100 was used as negative control (1).



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

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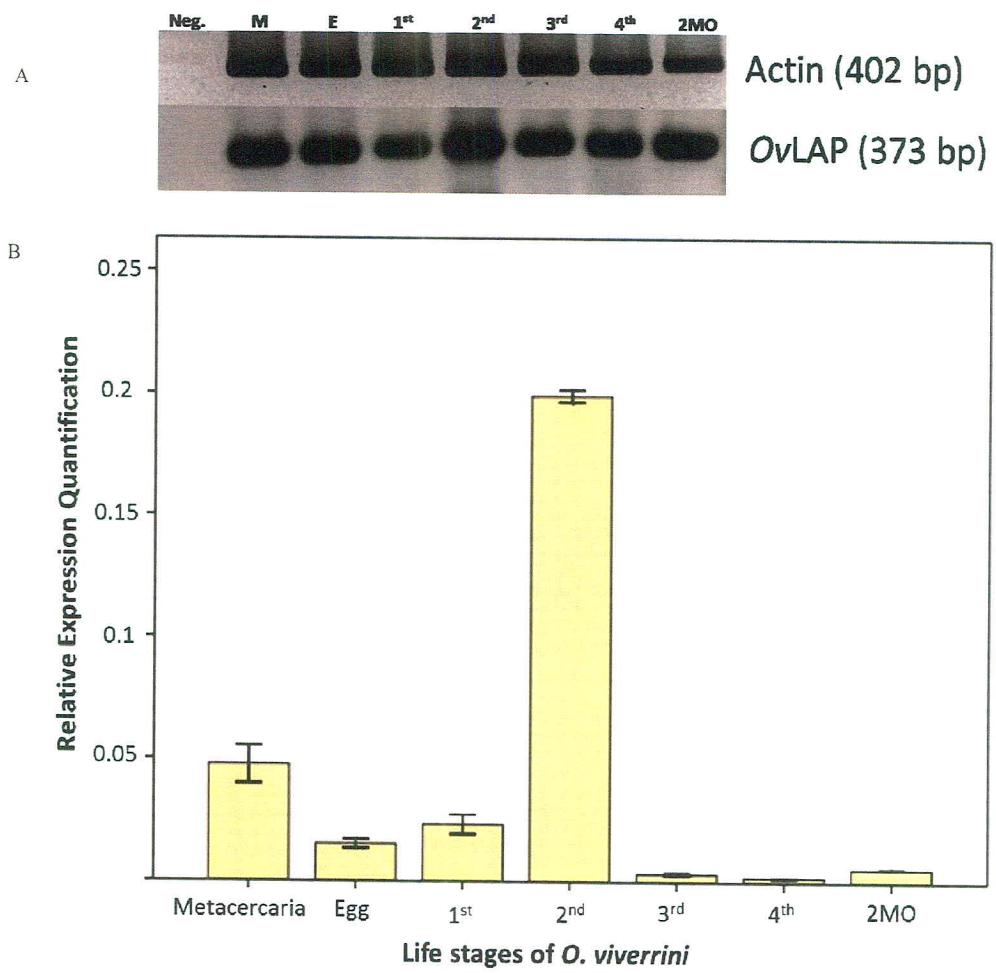

Fig. 7. Expression of *Ov*LAP gene in different developmental stage of *O. viverrini*. Conventional RT-PCR was performed on cDNA from various developmental stages in order to determine which stage of *O. viverrini* was expressed *Ov*LAP gene. Panel A represents the Conventional RT-PCR products using specific primers for *Ov*LAP. The cDNA template from various developmental stage 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 housekeeping gene (Actin) was used as positive control. Panel B real time-PCR assay of *Ov*LAP was present as quantitative analysis of mRNA level. *Ov*LAP transcript was detected in metacercariae, egg, 1st week larva, 2nd week larva, 3rd week larva, 4th adult and 2 month adult worm. High level of *Ov*LAP mRNA was detected in 2nd week larva which higher than expression level 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 difference of *Ov*LAP expression in metacercariae, egg, 1st week larva, 2nd week larva, 3rd week larva, 4th adult and 2 month adult worm ($P>0.05$).

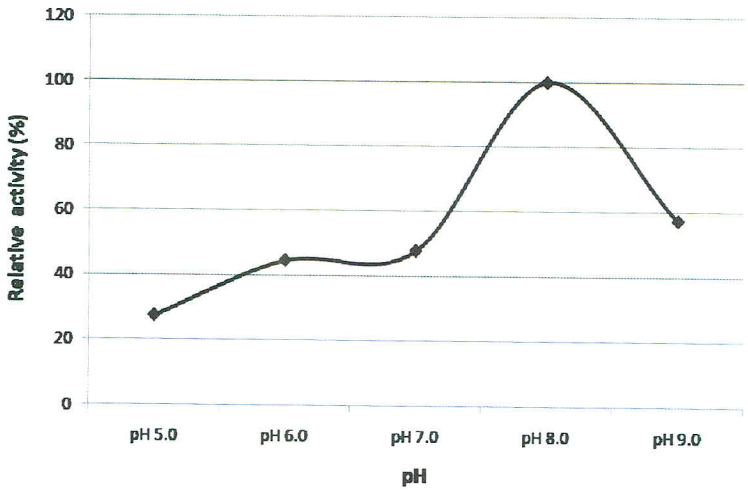

Fig. 8 The optimal pH of *Ov*LAP activity. *Ov*LAP activity was assay in various pH buffers ranging (pH 5.0 – pH 9.0). Broad of optimal pH buffers were observed. Slightly acidic (pH 6.0), neutral (pH 7.0) and slightly alkaline (pH 8.0) conditions were optimal for *Ov*LAP activity. The maximal activity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

598 **Table 1**

599 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	0.1	0.59 ± 0.018	15.86
	1.0	0.69 ± 0.025	18.84
Co	0.1	0.16 ± 0.006	4.30
	1.0	0.02 ± 0.001	5.43
Mg	0.1	0.001 ± 0.001	0.003
	1.0	0.001 ± 0.001	0.0028

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601 **Table 2**

602 The 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

603 Control, without any inhibitor or chelator

604