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# Characterization and functional analysis of fatty acid binding protein from the carcinogenic liver fluke, *Opisthorchis viverrini*

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In the present study, the cDNA encoding FABP (*Ov*-FABP) was isolated from the adult stage of *Opisthorchis viverrini* and characterized. The *Ov*-FABP protein sequence (107 amino acids) was predicted to have a molecular mass of 12.26 kDa and an isoelectric point (PI) of 6.82. This sequence had a high identity and similarity to *Cs*-FABP of the related opisthorchid *Clonorchis sinensis*. Multiple sequence alignment with FABPs from other parasitic flatworms and mammals showed a number of conserved amino acids including $Phe^{34}$, $Gly^{37}$, $Glu^{38}$, $Glu^{39}$,$Val^{50}$, $Iso^{62}$, $Gly^{81}$, $Ile^{84}$, $Ser^{87}$ and $Arg^{101}$. In addition, the structure of *Ov*-FABP was predicted to have eleven β-sheets and one α-helix based on the known structures for FABPs from human (hL-FABP), rat and a schistosome. Phylogenetic analysis of amino acid sequence data revealed a close relationship of *Ov*-FABP with *Cs*-FABP and hL-FABP. Reverse transcription-PCR revealed that *Ov*-FABP was transcribed in the egg, metacercaria, juvenile and adult stages. The soluble form of recombinant *Ov*-FABP (r*Ov*-FABP) was shown to specifically bind fatty acids, including oleic acid, palmitic acid and linoleic acid, as shown for other animals. Anti-serum against r*Ov*-FABP (produced in mice) located the protein to parenchyma, egg, sucker musculature, testes and tegument of adult *O. viverrini*. Taken together, the findings suggest key functional roles for *Ov*-FABP in development, reproduction and/or host–parasite interactions.

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## 1. Introduction

The liver fluke *Opisthorchis viverrini* causes a neglected tropical disease of particular medical and economic importance in Southeast Asia. Complicated mechanisms and interactions of *O. viverrini* with the human host during infection are the causes of serious pathological changes that lead to cholangiocarcinoma and a fatal outcome. Several molecules of *O. viverrini* have been shown to be involved in host–parasite interactions. Excretory/secretory (ES) molecules from the fluke have been found to have a direct effect on the human host, while intracellular molecules can have indirect effects [1–4].

Fatty acid binding proteins (FABPs) are intracellular molecules that have been identified in various organisms including human, animals and some helminths [5]. FABPs represent intracellular lipid binding proteins (iLBPs), similar to sterol carrier proteins and retinol binding proteins. FABPs share basic characteristics, including size, structures and/or functions with other members of iLBPs [6]. In general, FABPs are low molecular weight proteins, and share low sequence similarity, but they display similar tertiary structures and similar functions. The key functions of FABPs are the binding of hydrophobic ligands in cytosol and improved intracellular solubilization of such ligands [7]. These functions are important for every organism including helminths.

FABPs are important to helminths that live in an oxygen-deprived environment in the gastrointestinal tract of their host for the biosynthesis of fatty acids and cholesterols. Helminths depend on fatty acids from the host for intracellular lipid oxidation via transportation by helminth FABPs [8]. FABPs have been identified in various species of helminths, including trematodes, cestodes and nematodes. These helminth FABPs are similar in molecular weight (13–15 kDa), have similar tertiary structures, in spite of low primary sequence similarity and likely share a similar function(s) in the transfer of host fatty acids. Although the primary sequences of FABPs vary, some conserved amino acid residues appear to be associated with fatty acid stabilization during binding [5]. Due to their crucial role in lipid oxidation, FABPs are attractive as targets of new anthelminthic drugs or vaccines. The high immunogenicity of FABPs of helminth might allow the distinction of parasitic infections in patients [9]. Given the lack of knowledge in this area, we isolated the cDNA encoding *O. viverrini* FABP (*Ov*-FABP) from the adult stage of this parasite, and cloned and expressed the recombinant protein. We localized the expression of *Ov*-FABP to structures in the adult worm. Based on the findings of this study, we suggest an involvement of *Ov*-FABP in physiological functions of worm in attachment, development, reproduction and/or interaction with their host.

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## 2. Materials and methods

### 2.1. Sequence analysis

The full-length cDNA sequence of *Ov*-FABP (GenBank accession No. 187339) was isolated in a previous study [10]. *Ov*-FABP DNA homology search was performed with FABP of other organisms in the NCBI database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/). The open reading frame (ORF) of *Ov*-FABP was inferred using ORF finder and the putative signal peptide was identified using SignalP 4.0 software. Multiple sequence alignment of *Ov*-FABP with other FABPs from helminths and mammals was performed using the program ClustalW [11]. Phylogenetic analysis was conducted using the neighbor-joining method (1000 bootstraps) employing MEGA software v. 6.0 [12].

### 2.2. Cloning and protein expression

The full-length cDNA of *Ov*-FABP was amplified from a cDNA library representing adult *O. viverrini* using specific primers (forward primer: 5′-**CACC**ATGTCGGCAGCCACAAATCAC-3′; reverse primer: 5′-TCAGTCCT GTCCGTCATGCC-3′). Four base sequences (CACC) were added to the 5′ end of the forward primer to allow to cloning of PCR products into pET100/D-TOPO® vector using Champion™pET Directional TOPO® Expression Kit (Invitrogen, USA). The blunt-end PCR product representing *Ov*-FABP was amplified using hot-start and proof-reading DNA polymerase (Life Technologies, USA). PCR was performed in a volume of 25 μl containing 25 mM of MgCl, 1× PCR buffer, 10 mM of dNTPs, 5 U Taq polymerase (Life Technologies, USA), 0.2 μM of each primer and 200 ng of total cDNA. The cycling protocol was: 98 °C for 5 min, followed by 35 cycles of 95 °C/30 s, 60 °C/30 s and 72 °C/45 s, with a final extension at 72 °C/10 min.

The blunt-end PCR product representing *Ov*-FABP was cloned into pET100/D-TOPO® (Invitrogen, USA) and fused in frame with epitope tags including poly-histidine tag and Xpress™ epitope. The plasmid DNA of *Ov*-FABP-pET100/D-TOPO® was then propagated in TOP10 *Escherichia coli* (Invitrogen, USA) and induced to produce protein in BL21 Star™ (DE3) (Invitrogen, USA).

The fusion protein representing *Ov*-FABP was produced as an intracellular soluble protein after induction with 1 mM IPTG at 26 °C for 8 h, shaking at 225 rpm. After the incubation time, the bacterial pellet was collected and resuspended in 3 ml of native condition binding buffer containing a cocktail of protease inhibitors (without EDTA) (Sigma-Aldrich, USA). To release the recombinant protein, the resuspend pellet was frozen and thawed at 42 °C and sonicated for 5 min at 4 °C (this procedure was repeated 3 times). For protein purification, the supernatant containing *Ov*-FABP recombinant protein was pass-through a Ni-NTA resin column (Thermoscientific, USA) following the manufacturer's instructions. Protein fractions were analyzed by SDS-PAGE and stained with Coomassie blue and/or protein immunoblot staining using His-Tag antibody.

The fractions of the recombinant protein (*Ov*-FABP) were pooled and concentrated using a concentrator (Eppendrof concentrator 5301, USA). The pooled/concentrated protein was analyzed again by SDS-PAGE, and the final concentration was determined photometrically at 280 nm (Nanodrop, USA).

### 2.3. Polyclonal antibody production and determination

Three male mice were subcutaneously immunized with 100 μg of the purified recombinant protein *Ov*-FABP. The protein was emulsified in an equal volume of Imject™ Alum Adjuvant (Thermoscientific, USA) before immunization. The immunization was repeated 2 times at 2 week-intervals. Before each immunization, blood samples were collected from the tail vein to assess the level of antibody against the recombinant protein. Sera were also collected before immunization (negative controls). All mouse sera were aliquoted and stored at −20 °C until use. All animal procedures and experimental protocols were reviewed and approved by Animal Ethics Committee of Khon Kaen University (Reference No. 0514.1.12.2/5).

The specificity of antibodies produced was determined using purified recombinant *Ov*-FABP protein by Western blot analysis [13]. The SDS-treated purified protein was size separated in 15% SDS-PAGE gel and then electro-transferred to nitrocellulose membrane (Bio-Rad, USA). Then, the membrane was washed in PBS-T (0.5% Tween in PBS) and blocked with 5% skim milk-PBST for 2 h. Subsequently, membranes were incubated in immunized serum (1:100 dilution in 2% skim milk-PBST) for 2 h. The membrane was washed again with PBS-T before being incubated in HRP conjugated goat anti-mouse IgG (1:1000 dilution in PBS) for 1 h. Thereafter, membranes were extensively washed with PBS before detection with diaminobenzidine (DAB) substrate.

### 2.4. Localization of Ov-FABP expression in adult O. viverrini

Paraffin sections of adult *O. viverrini* were obtained from the Pathology laboratory, Department of Pathology, Faculty of Medicine, Khon Kaen University. The sections were mounted on coated glass slides. Before starting other staining steps, the slides were de-parafinized in xylene, to remove the paraffin wax, and hydrated through decreasing concentrations of ethanol. Then, the adult *O. viverrini* sections were treated for 3 min in 1 M citrate buffer pH 6.0 in a pressure cooker. After this "antigen retrieval" was completed, the slides were cooled and washed with PBS. The slides were blocked for non-specific endogenous peroxidase enzyme activities with 30% $H_2O_2$-methanol for 30 min. Then, non-specific binding sites in the sections were blocked with normal horse serum at a dilution of 1:20 for 30 min at room temperature (22–24 °C) in a humidified chamber. To determine *Ov*-FABP expression in adult *O. viverrini*, mouse anti-*Ov*-FABP antibody (at dilution of 1:50 in PBS/$NaN_3$) were probed on to sections and incubated overnight (16 h) at room temperature in a humidified chamber. Some sections were probed with negative control sera. The next day, the sections were probed for one hour at room temperature with anti-mouse IgG antibody conjugated with horseradish peroxidase at a dilution of 1:300 in PBS. The slides were washed with PBS and then developed by submerging them in freshly prepared DAB solution and counter-stained with Mayer hematoxylin. Excess Mayer hematoxylin stain was removed by washing in tap water, and dehydrated in increasing concentrations of ethanol. The slides were then examined under a light microscope and analyzed using ApperioScanScope™ (Leica Biosystems, Singapore).

### 2.5. Expression of OvFABP in O. viverrini life cycle stages

Total RNAs from various stages of *O. viverrini*, including metacercariae, eggs, 1$^{st}$, 2$^{nd}$, 3$^{rd}$ week larvae, 4 week- and 2 month-old adult worms were isolated using Trizol® reagent (Life Technologies, USA) following the manufacturer's protocol. One μg of total RNA from each stage was reverse transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit® (Life Technologies, USA). The protocol for reverse transcription followed that of the manufacturer. The *O. viverrini* cDNAs obtained from reverse transcription were aliquoted and stored at −80 °C until use.

The cDNA from each stage of *O. viverrini* was used as a template for conventional and real time RT-PCR using primers specific for the *Ov*-FABP gene. The forward 5′-ATGTCGGCAGCCACAAATCAC-′3 and reverse 5′-GTCCTGTCCGTCATGCCTGG-′3 primers amplified a DNA product of 318 bp. The internal control was included in the experiment to determined RNA integrity using *O. viverrini* β-actin primers (forward 5′-AGCCAACCGAGAGAAGATGA-′3 and reverse 5′-ACCTGACCATCAGGCAGT TC-′3). Negative control reactions were also included in all experiments. For conventional RT-PCR, the PCR reaction (25 μl) containing 5 U of Tag DNA polymerase (Phusion DNA polymerase, Life Technologies, USA), 1× PCR buffer, 50 mM of $MgCl_2$, 10 mM of dNTPs, 0.5 μM of each primer and 200 ng/μl of cDNA was performed in a TPersonal thermal cycler (Biometra, Germany). The cycling protocol for conventional RT-

PCR was: denaturation at 98 °C/5 min, 35 cycles of denaturation at 98 °C/1 min, annealing at 60 °C/1 min, extension at 72 °C/2 min, followed by a final extention at 72 °C/10 min. For real-time RT-PCR, the amplifications were performed in a LightCycler480 II Instrument (Roche, Switzerland) using Maxima SYBR Green/ROX qPCR mastermix (Thermoscientific, USA). The protocol followed the manufacturer's instructions. Briefly, 20 μl of PCR reaction mixture containing of 2 μl of cDNA, 10 μl of SYBR Green qPCR mastermix (2×), 0.4 μl of each of *Ov*-FABP forward and reverse primer (10 pmole each) and 7.2 μl of $H_2O$. The real-time PCR program was 95 °C/30 s, followed by 40 cycles of 95 °C/5 s and 60 °C/20 s. Following amplification, a melting curve analysis was conducted: 95 °C for 30 s, 65 °C for 15 s, followed by an increase to 95 °C continuously to collect a fluorescent signal. Each cDNA sample was run in duplicate. Relative mRNA levels were given in unit values of $2^{[Ct(\beta\text{-actin}) - Ct(Ov\text{-FABP})]}$.

### 2.6. Fatty acid-binding assay

The binding properties and the possible conformational changes of holo- and apo-forms of purified recombinant *Ov*-FABP protein were observed. Six fatty acids purchased from Sigma including stearic acid (C18:0), palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3) and arachidonic acid (C20:4) were selected for the testing of *Ov*-FABP binding properties. The conformations of *Ov*-FABP between holo- and apo-forms were distinguished using clostripain enzyme (ArgC, Sigma-Aldrich, Singapore), which hydrolyses the polypeptide chain of *Ov*-FABP at the C-terminal end of arginine residues. The protein binding properties and conformation changes were examined [14]. In brief, to obtained holo-forms, 2 μg of purified recombinant *Ov*-FABP protein were pre-incubated for 30 min at room temperature with each fatty acid at a ratio of 1:4 in buffer containing 50 mM Tris–HCl, 50 mM DTT, 2 mM EDTA and 5 mM $CaCl_2$. Then, 1 μg/ml of clostripain (ArgC, Sigma) was added to each reaction and incubated further at 37 °C. The reactions were collected at 1 and 12 h after incubation. Then, the reactions were subjected to analysis by SDS-PAGE and stained with Coomassie. As the control, albumin (Sigma-Aldrich, Singapore) was added as a positive control and the clostripain enzyme was omitted in negative control reactions.

## 3. Results

The full-length nucleotide sequence of *Ov*-FABP encoded a protein of 106 amino acids, with a predicted molecular weight of 12.3 kDa and a PI of 6.82. A multiple amino acid sequence alignment of *Ov*-FABP with FABPs from various organisms, including helminths and mammals was performed. Conserved amino acid residues included Phe[34], Gly[37], Glu[38], Glu[39], Val[50], Iso[62], Gly[81], Ile[84], Ser[87] and Arg[101]. The secondary structure of *Ov*-FABP and other FABPs of helminths, including FABPs of *C. sinensis* (*Cs*-FABP) and *F. hepatica* (*Fh*-FABP) were predicted based on known secondary structures of those from humans (hL-FABP), rat (*R. norvegicus* FABP) and *Schistosoma mansoni* (Sm14). The secondary structure had 11 β-sheets and one α-helix, which is conserved for FABPs. In addition, a high degree of sequence similarity (97%) and identities (94%) was found between *Ov*-FABP and *Cs*-FABP (Fig. 1). Phylogenetic analysis revealed two distinct groups that agreed with taxonomic relationships. The first group contained *Ov*-FABP, *Cs*-FABP and hL-FABP, while the second group contained other trematodes, cestodes and mammalian FABPs (Fig. 2).

The soluble form of recombinant *Ov*-FABP protein fusion with 6×-histidine tag and Xpress™ epitope was produced in bacterial system following the induction with 1 mM IPTG at 26 °C. The recombinant protein was partially purified, analyzed on SDS-PAGE and stained with Coomassie blue. After purification, the protein represented a single band at ~16 kDa on stained SDS-PAGE gels, which corresponds to the molecular weight estimated for the mature protein fused to the 6×-histidine tag and Xpress™ epitope (Fig. 3A). The expressed protein was recognized by the anti-histidine tag antibody and estimated the *Ov*-FABP protein band to be ~16 kDa (Fig. 3B).

The expression patterns of *Ov*-FABP gene in various developmental stages of *O. viverrini* were determined using RT-PCR and real time PCR. The PCR product of 318 bp representing the *Ov*-FABP gene was detected in cDNA from metacercariae, eggs, juvenile worms (1, 2 and 3 weeks) and adult worms (4 week- and 2 month-old). The expression of this gene was detected in all developmental stages of *O. viverrini*. Abundant transcription was detected in larva stages, particularly the 2 week-old larvae. However, real time PCR results did not compare well with those obtained using conventional PCR. In real time PCR, detection was more sensitive and reliable in the early exponential phase than endpoint (plateau phase) detection by conventional PCR. The significant increase of PCR products in real time PCR correlates better to initial gene target expression [15]. The actin gene was included in the experiment to control for cDNA integrity and PCR fidelity (Fig. 3C).

The polyclonal antibody against *Ov*-FABP (raised in mice) recognized the *Ov*-FABP protein in Western blot analysis (Fig. 3A). The single band representing recombinant *Ov*-FABP (~16 kDa) was detected following probing with anti-*Ov*-FABP antibody. The molecular weight of the protein band on the blot compared with the band seen following Coomassie blue staining. No band was detected in the blot probed with negative control serum (Fig. 3A). The mouse anti-*Ov*-FABP antibody localized *Ov*-FABP in parenchymal cells, muscle of the oral sucker, eggs, testes and the tegument of adult *O. viverrini*. Some surface membranes of gut and some contents in the worm's gut as well as some of hepatocytes and bile duct walls also bound the antibody. No signal was detected in *O. viverrini* sections probed with pre-immunized mouse serum (Fig. 4).

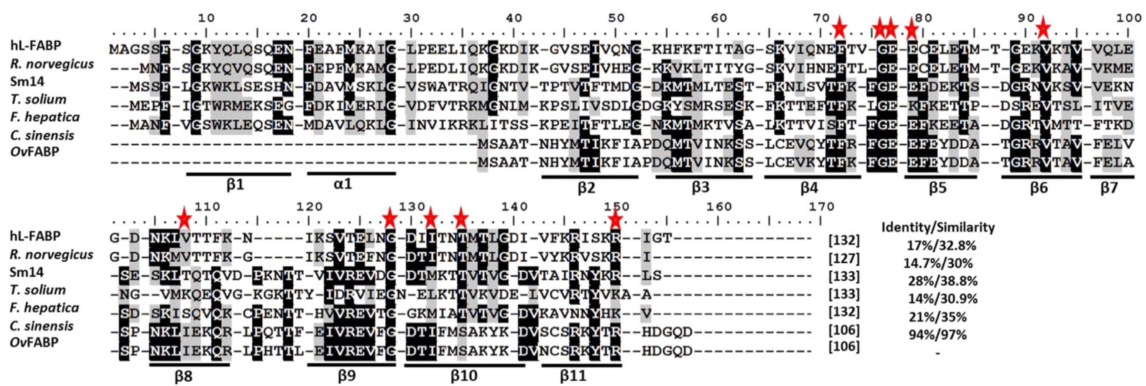


**Fig. 1.** Alignment of selected FABP amino acid sequences. Abbreviations (GenBank accession number): hL-FABP (3STN_A) [35], *R. norvegicus* (2JU3), Sm14 (2POA) [36], *T. solium* (HQ259679.1) [37], *F. hepatica* (CAB65015.1), and *C. sinensis* (GAA49082.1). The identical amino acids were shade in black while the similar amino acids were shade in gray. The secondary structure of one α-helix and eleven β-sheets was estimated based on secondary structure of hL-FABP, *R. norvegicus* FABP and Sm14. The red stars indicate identical and similar properties of each amino acid, which are conserved among the selected FABP amino acid sequences.

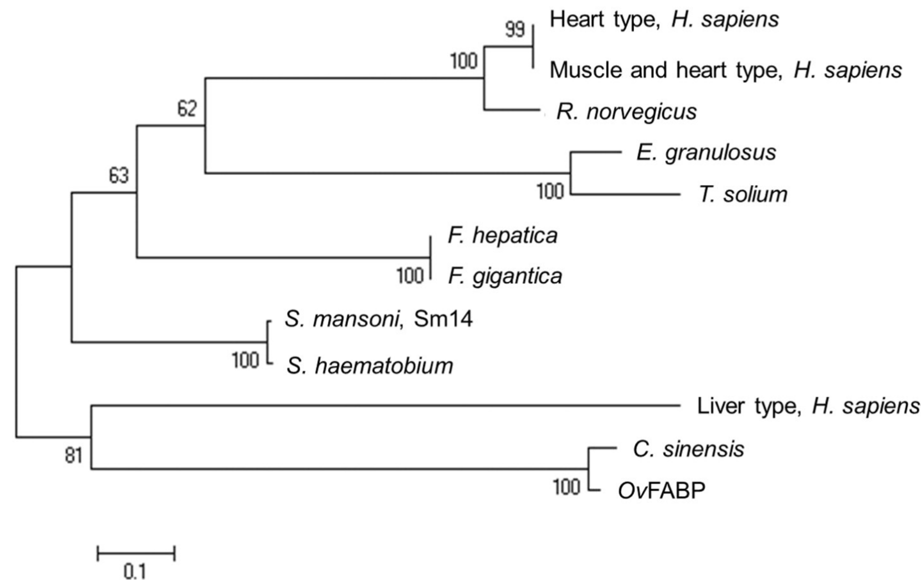


**Fig. 2.** Relationships among fatty acid binding proteins. The scale bar indicates evolutionary distance (0.1 substitution per position). Nodal support is given in percent (1,000 bootstrap replicates). The species names and the GenBank accession numbers of FABPs are as follows: Sm14 (AAA63516), *F. hepatica* (CAB65015), *F. gigantica* (AHA90589), *S. haematobium* (BAF62288), As-p18 (AAA98565), *T. solium* (ABB76135) *E. granulosus* (AAK00579), *H. sapiens*, liver-type (NP_001434), *H. sapiens*, heart-type (NP_004093), *H. sapiens*, muscle and heart-type (EAX07619), *R. norvegicus* (AAA41136) and *C. sinensis* (GAA49082).

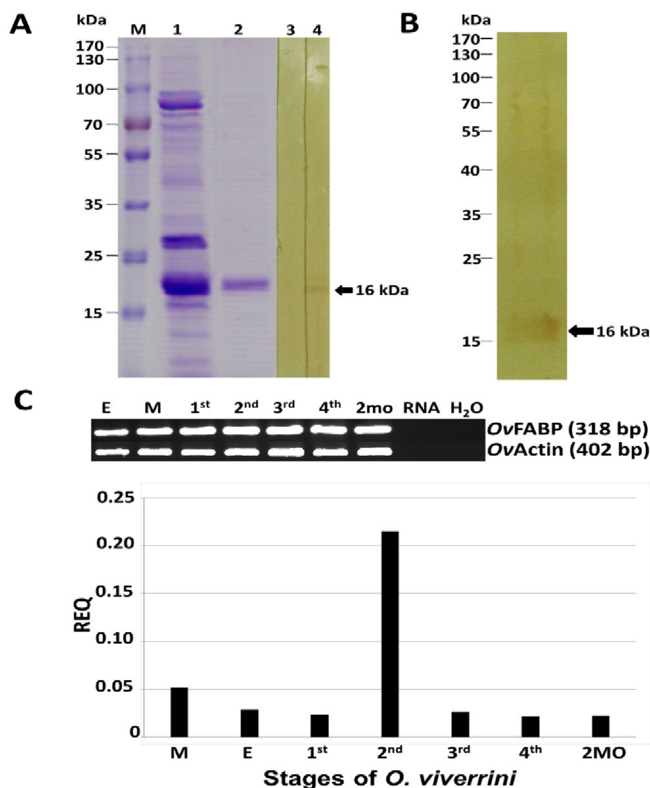


**Fig. 3.** SDS-PAGE and Western blot analysis of the recombinant protein *Ov*-FABP as well as transcription of the *Ov*-FABP gene. A. Recombinant *Ov*-FABP protein and specificity of mouse antiserum against *Ov*-FABP (single band of ~16 kDa). Lane M: protein standards with molecular weights (kDa); lane 1: unpurified recombinant *Ov*-FABP protein; lane 2: partial purified recombinant *Ov*-FABP protein. The probing of recombinant *Ov*-FABP protein with sera from mice before (lane 3) and after (lane 4) immunization. B. The Western blotting of anti-histidine tag against recombinant *Ov*-FABP protein. C. The transcription pattern of *Ov*-FABP gene in different developmental stages of *O. viverrini*. Conventional RT-PCR (upper) and real-time RT-PCR (lower) were performed on cDNA from various developmental stages of *O. viverrini*, including metacercariae (M), eggs (E), juvenile worms (1st, 2nd and 3rd weeks) and adult worms (4th week and 2 months). A high level of *Ov*-FABP mRNA was detected in 2 week-old larvae. A house keeping gene (*Ov*-actin) was used as a positive control. RNA and $H_2O$ were used as negative controls.

The proteolytic patterns of *Ov*-FABP binding with fatty acids after digestion with Clostripain (ArgC) for 1 and 12 h were established by SDS-PAGE. This qualitative detection showed specific binding and differentiation between holo- and apo-forms of the *Ov*-FABP recombinant protein. The apo-forms of recombinant *Ov*-FABP was readily degraded after incubation with or without ArgC at 37 °C. In contrast, the holo-forms of the recombinant protein *Ov*-FABP with oleic acid, palmitic acid and linoleic acid were stable under unsuitable conditions when incubated for 12 h. The ligands bound by specific fatty acids possibly related to changes in conformation or protected the proteolytic site of the recombinant *Ov*-FABP protein from exposure of ArgC and the improper conditions. However, the stability of holo-form was limited. A slow degradation of holo-forms was observed. A similar pattern of proteolysis by ArgC was found for albumin, except the holo-form of albumin with linolenic acid and linoleic acid. Under the same conditions, slow degradation was observed for linolenic acid-bound albumin, while linoleic acid-bound albumin was rapidly degraded. Fatty acid binding increased the stability of recombinant *Ov*-FABP (Fig. 5).

## 4. Discussion

FABPs are important molecules in organisms relying on fatty acid uptake for metabolism in particular tissues. The discovering of *Ov*-FABP and other helminthic FABPs indicate a requirement of fatty acids for energy production or regulate key functions in helminths. Gastrointestinal helminths obtain fatty acids from their hosts.

*Ov*-FABP appears to be a key molecule in *O. viverrini* that interacts directly with host nutrients. This protein likely binds fatty acids from the host bile. Unsaturated and saturated fatty acids present in bile in different amounts are likely the nutrient source in *O. viverrini*. In addition, fatty acids, including oleic acid, palmitic acid and linoleic acid, found at high concentrations in bile bind tightly to *Ov*-FABP [16].

Fatty acids might be taken up by *Ov*-FABP through the *O. viverrini* tegument and/or pass through the mouth of *O. viverrini* before being localized to the gut membrane. *Sj*FABPc of *Scistosoma japonicum* and *Eg*-FABP of *Echinococcus granulosus* were found to be in direct contact or interaction with membranes prior to the uptake of host fatty acids from acceptor membranes prior to translocation into intracellular compartments [17,18]. Although the routes or mechanisms of fatty acid uptake are still unknown, studies have been performed to attempt to clarify

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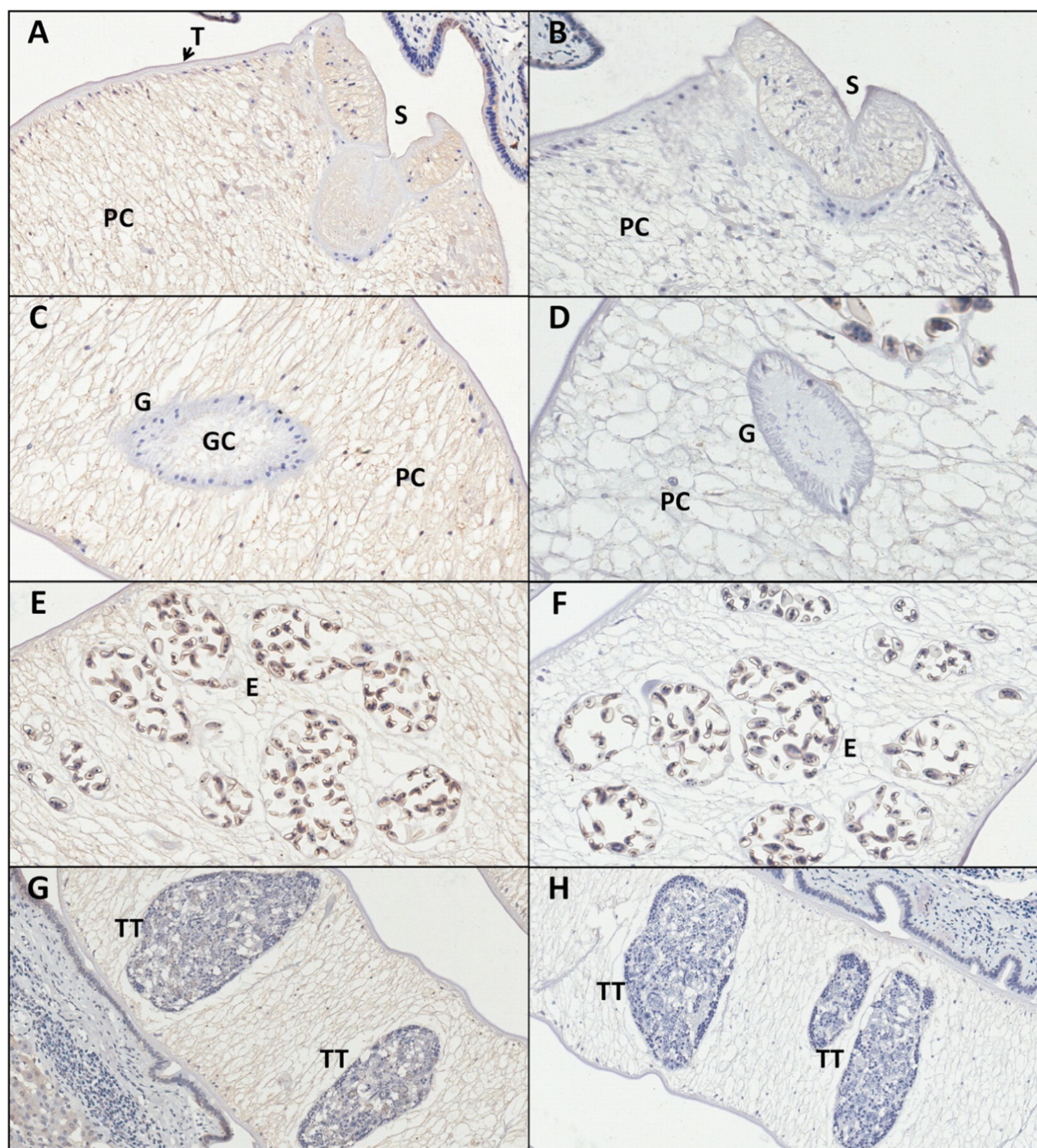


**Fig. 4.** Immuno-histochemical localization of *Ov*-FABP in *O. viverrini* adult worm with using mouse antibody against the recombinant protein. The positive signal was observed in parenchymal cells (PC2), muscle of the oral sucker (S), eggs (E), surface membrane of the gut (G), gut contents (GC), testes (TT) and tegument (T) of adult worm (A, C, E and G). Arrows indicate a positive signal. Immuno-histochemistry using serum from mice prior to immunization (negative control) (B, D, F and H).

them [18–20]. Several membrane proteins are likely involved in facilitating *Ov*-FABP binding and transport of fatty acids. *Ov*-FABP likely takes up fatty acids from acceptors at the cell membrane before translocating into intracellular site or parenchyma, where active lipid metabolism occurs. From this function, *Ov*-FABP also acts as an intracellular and extracellular buffer to regulate fatty acids [20].

Parenchyma, muscle of sucker and reproductive tissues that needed energy from fatty acid oxidation contained relatively large amounts of *Ov*-FABP, which contrasted the situation of the tegument and the gut membrane. The parenchyma, in which organs are embedded, likely contains various types of FABP [21–23]. The quantity of FABP expression can be classified based on the type of parenchyma. *Ov*-FABP was localized to parenchymal cell type 2 (PC2), similar to the finding for *Fg*-FABP of *Fasciola gigantica* [24]. The destination of fatty acids transportation and the storage of fatty acids and cholesterols likely relate to PC2, since an abundance of mitochondria and lipid droplets were found. The energy production in PC2 would supply the metabolic needs in organelles in all types of parenchyma, including PC1, and PC3 [24]. *Ov*-FABP was also localized to eggs in the uterus. Energy from fatty acid oxidation might be essential for egg production, development and survival of eggs following their excretion into the environment. Fatty acids would represent the nutrients that are needed for egg development, in addition to those from vitelline cells [25]. However, *Sj*-FABPc from *S. japonicum* has been detected in vitelline cells, and As-p18 of *Ascaris suum* has been detected in the pre-vitelline fluid of eggs [22,26]. The localization of *Ov*-FABP to the sucker of the adult worm suggests that fatty acid oxidation might provide energy to the sucker to maintain the adhesion of the parasite to the bile duct wall in the host animal.

*Ov*-FABP was shown to be expressed throughout the life cycle stages of *O. viverrini*, which indicates the important role of this fatty acid transporter in energy production in every stage of *O. viverrini*. The bulk of energy from fatty acid oxidation is important for establishment, growth, development and reproduction of *O. viverrini* in their host. After the metacercariae enter the definitive host, the juveniles undergo a high level of growth and development, particularly in the second week.

A high degree of similarity and identity of *Ov*-FABP and *Cs*-FABP indicates that they share a common ancestor. Due to evolutionary relationship of *Ov*-FABP, *Cs*-FABP and hL-FABP, the production of a vaccine

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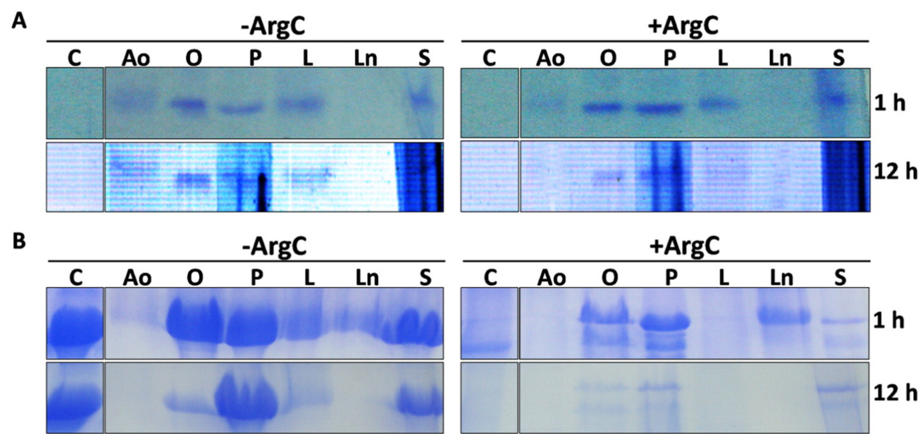


**Fig. 5.** Degradation pattern and stability of apo- and holo-forms of recombinant *Ov*-FABP and albumin after incubation with and without ArgC. Panel A: The degradation pattern and stability of recombinant *Ov*-FABP apo-form (C) and holo-form with arachidonic acid (AO), oleic acid (O), palmitic acid (P), linoleic acid (L), linolenic acid (Ln) and stearic acid (S) after incubation with ArgC (+ArgC) and without ArgC (−ArgC) for 1 h and 12 h at 37 °C. Panel B: Albumin; a standard free fatty acid binding protein, was tested parallel with recombinant *Ov*-FABP protein.

from such a molecule might induce antibodies against hL-FABP. However, some studies have been successful in using FABP as a vaccine molecule in activation immune responses with expected damage in the tegument and parenchyma of helminths [23,27,28]. High immunogenicity of helminthic FABPs indicates that they might have promise as intervention targets. High immunogenicity of *Cs*-FABP has been demonstrated [24]. Following vaccination with plasmid DNA of *Cs*-FABP, a significant reduction of worm burden was seen in rats challenged with *C. sinensis* metacercarial infection [29]. In addition, helminthic FABP was showed a cross-protection against two different parasites. The vaccination with Sm14 (the *S. mansoni* homologe of *Sj*-FABP) cross-protected against *S. mansoni* and *F. hepatica* infections, reflected in less hepatic lesions [30]. Recombinant *Cs*-FABP also showed strong reactivity to antibodies from patients with proven clonorchiasis, fascioliasis or paragonimiasis [9]. Cross-reactivity of recombinant *Cs*-FABP and cross-protection after vaccination with Sm14 suggests a structural similarity of FABPs in different flatworms, which might be advantageous for the development of a broad-spectrum intervention.

FABPs also play roles in transferring non-fatty acid ligands, including lipophilic drugs, to their sites of action [31,32]. *Ov*-FABP may also have a similar role in transferring anthelminthic drugs to their sites of action in the worm. However, unfortunately, the chance of a worm in biliary system to be exposed to metabolized, active praziquantel (PZQ) seems unlikely, since metabolized un-active PZQ is excrete in bile [33,34]. To solve this problem, giving a patient a pro-drug form seems an attractive strategy. The transformation from a pro-drug to an active form following extensive metabolism in the liver might improve drug efficiency and the level of metabolized active drug in bile. In addition, the specific binding of active lipophilic drug to *Ov*-FABP might be considered, to increase drug uptake via *Ov*-FABP into intracellular sites of *O. viverrini*. In conclusion, many fundamental aspects of FABPs remain to be studied, in order to gain insights into the fascinating area of helminth nutrition and metabolism. Clearly, the unique aspects of lipid metabolism in flatworms are exciting and deserve detailed investigations in the future.

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