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

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ABSTRACT

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.

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 AperioScanScope™ (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, 1st, 2nd, 3rd 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-