NATIVE AND RECOMBINANT FATTY ACID BINDING PROTEIN 3 FROM FASCIOLA HEPATICA AS A POTENTIAL ANTIGEN

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ABSTRACT
Lipid-binding proteins are members of widely distributed protein family. They take part in metabolism of different lipophilic ligands and also have a role in physiological activity, metabolism and disposition of essential hydrophobic compounds (fatty acids, phospholipids, eicosanoids, retinoids, etc.) which are important molecules involved in several cellular processes including gene transcription, immune responses etc. Fatty acid binding proteins (FABPs) are mostly cytosolic but several members are excretory-secretory (E-S) proteins. There is a lot of data that helminthic FABPs from E/S products cause antigenic and allergic reactions. The aim of the present study is to make a detailed investigation of native and produced recombinant FABP3 as a potential antigen for vaccine creation. The native FABP3 from Fasciola hepatica was purified and recombinant one was expressed in E. coli. Polyclonal antibody was produced against recombinant FABP3 and it was tested on the native one. Obtained results give the opportunity to investigate the effectiveness of the native and recombinant FABP3 as an immunotherapeutic agent and if it could be used for immunoprophylactic.

Keywords: Fasciola hepatica, antigens, alternative vaccines, fatty acid binding protein 3

Introduction
Parasitic diseases are huge problem for human and veterinary medicine and for economy, agriculture and wildlife management. One of these diseases is fasciolosis, which is caused by two trematode species, Fasciola hepatica (liver fluke) and Fasciola gigantica. Only F. hepatica is a concern in Europe and Americas but the distribution of both species overlaps in many areas of Africa and Asia (14). Fasciolosis is recognized as an emerging human disease: the World Health Organization (23) has estimated that 2.4 million people are infected with F. hepatica and a further 180 million are at risk of infection (13).

Considering all these facts, searching for alternatives of the antihelminthic drugs and preventive measures against fasciolosis is in view of most researchers in past 20 years. Subjects of interest are helminth-specific potential antigens for vaccine creation (19). Such antigens are usually proteins, part of specific metabolite pathways which differentiate in biochemistry of parasitic helminthes and their hosts. Helminthic metabolite pathways are modified regarding the specific habitat. Their lipid metabolism is highly reduced. Most specimens do not synthesize de novo long chained fatty acids and steroids and there is lack of β-oxidation of lipids (3). Good example is that fatty acid levels in animals are around 1% in species from taxa Trematoda, Cestoda and Nematoda could be from 2% to 15% (2). Basic element of these metabolic processes is the transport and assimilation of lipids by transporter proteins (i.e. fatty acid binding proteins or FABPs). Fatty acid binding proteins are widely distributed, low molecular weight, highly expressed cytosolic proteins that are capable of binding, in noncovalent manner, a broad range of lipophilic ligands (1, 22). It has been defined that the recombinant Fh15 (FABP1) from F. hepatica has immunoprophylactic properties – decreasing of adult forms number and limiting their pathological effect (5). Recombinant Sm14 (from Schistosoma mansoni) has been also used as a vaccine and has showed high activity against cercaria (16, 21). Biochemical activity of FABPs associates their biological role with parasite survival through host immune response modulation and recognition, binding and removal of antihelminthic drugs which are usually hydrophobic compounds often with lipid structure and acts in competitive manner.

From another point of view very important as antigens for vaccine creation are the excretory-secretory (E-S) proteins (15). As it concerns FABPs from F. hepatica the only one found to be E-S protein is FABP3. There is a lot of data that helminthic FABP3s from E-S products cause antigenic and allergic reactions.

Many researchers have studied FABPs from F. hepatica as vaccine candidate (4, 9, 12). That is the reason why this paper reports the investigation of native and recombinant F. hepatica FABP3 and its possible role in chemotherapy and as a diagnostic antigen.

Materials and Methods
Sample collection
Adult liver flukes were isolated from naturally infected sheep from Bulgaria (Sofia). Variable numbers of flukes were isolated from host animals, washed in physiological saline, and stored in 100% ethanol.
RNA extraction and cDNA synthesis
Total RNA from adult liver flukes was obtained by homogenization using TRIzol^® Reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed with 4 μg of RNA, oligo (dT) primer and M-MulLV Reverse Transcriptase (Fermentas).

PCR amplification
PCR reaction was performed from cDNA. Each 50 µl PCR reaction sample contained final concentrations of 1X Pfu PCR buffer (Fermentas), 2mM dNTP, 25mM MgCl₂, 1U Pfu polymerase (Fermentas), 1 mg total DNA and 1 pmol of each primer. Used primer sequences covered the coding ORF of FABP3 and are as follows:

S (sense) primer - 5’GGGAATTCATATGGCC AAT TTT GTG GGT TCG3’ and AS (antisense) primer - 5’CGCGGATCCCTTA_ACA GCC TTC ACG TCC C3’ where the underlined regions contain adapter sequences respectively for NdeI and BamHI restriction sites for direct cloning in expression vector. These primers were designed based on GenBank™ sequence with accession number AJ250098. PCR was accomplished by LittleGenius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation 94°C for 5 min.; 35 cycles (denaturation 94°C for 30 sec.; primer annealing 50°C for 30 sec.; extension 72°C for 1 min.) and final extension 72°C for 10 min. PCR products were visualized on 1% agarose gel with ethidium bromide under UV light. Fragment size was determined using GeneRuler™ 1kb Ladder Plus (Fermentas).

Cloning in expression vector and recombinant FABP3 production
Amplons were purified by QIAquick PCR Purification Kit (QIAGEN) according to manufacturer’s instructions and later used for restrictase reaction. Double restriction of PCR fragments and expression vector was performed with NdeI and BamHI specific endonucleases (Fermentas) according to manufacturer’s protocol. For ligase reaction was used T4 DNA ligase (Fermentas). Utilized expression vector was pJC4086 (6) and with the obtained construct were transformed *Escherichia coli* competent cells, expression strain BL21 (F omp T hsdSb (rB mB) gal dcm (DE3)).

From selected single recombinant colonies plasmid DNA was extracted (GeneJET™ Plasmid Miniprep Kit, Fermentas), sequenced and analyzed for needed fragment confirmation (Macrogen, Inc.).

The expression induction was triggered with final concentration of 1 mM IPTG for 4 hours at 37°C. Cells were lysed through sonification on ice. The soluble fraction was used for purification of recombinant protein.

Recombinant FABP3 was purified by Ni-affinity chromatography with a His Trap kit (GE Healthcare Biosciences) due to the recombinant His-tag adapter from the vector. Protein was visualized by 12% SDS-PAGE after silver or Coomasie-blue (R-250) staining. Fragment size was determined using PageRuler™ Prestained Protein Ladder (Fermentas). The concentration was determined using Bradford assay and A (absorption) at λ=280 nm UV light (Sambrook et al., 1989).

Synthesis of polyclonal antibody against FABP3 and immunoblotting
Five hundred mg of purified recombinant FABP3 were used to raise antibodies against the protein in rabbit (Institute of Experimental Pathology and Parasitology, Bulgaria). The final bleed was used to analyze the native and recombinant protein at a dilution of 1:100 000.

For immunoblotting soluble elution fractions after Ni-affinity chromatography were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (18). Membranes were blocked with 2.5% bovine serum albumin (BSA) (Sigma-Aldrich) in phosphate buffer saline (PBS) and subsequently incubated with the primary antibody at a dilution of 1:100 000. After extensive washing with PBS/Tween, the membranes were incubated with an alkaline phosphatase conjugated ProteinA-secondary antibody (Sigma-Aldrich) in phosphate buffer saline (PBS) and subsequently incubated with the primary antibody at a dilution of 1:100 000. After extensive washing with PBS/Tween, the membranes were incubated with an alkaline phosphatase conjugated ProteinA-secondary antibody (Sigma-Aldrich) and developed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). For Western blotting of native and recombinant FABP3 as primary antibodies both anti-FABP3 polyclonal rabbit serum and monoclonal mouse anti-poly-His antibody (Sigma-Aldrich) were used at dilutions of 1:100 000. As secondary antibodies were used alkaline phosphatase conjugated ProteinA-antibody (Sigma-Aldrich) and alkaline phosphatase conjugated anti-mouse antibody (Sigma-Aldrich) respectively at dilutions of 1:10 000.

Data analysis
Contigs were constructed from the derived sequences with the program ChromasPro 1.5 (Copyright © 2003-2009 Technelysium Pty Ltd.). These were then aligned with the ClustalW2 tool (11). Sequences available in GenBank™ (http://www.ncbi.nlm.nih.gov/) were included in the final alignments for comparison with obtained sequences. The program MEGA 4.0 was used for alignments and BLAST (10).

Results and Discussion
Recombinant FABP3 production
For cDNA synthesis was used mRNA from total RNA extract from adult liver flukes through RT reaction and oligo (dT) primer. The obtained single strand cDNA was amplified with specific S and AS
primers and the amplified product was with length of about 400 bp. That fragment was purified, double digested with NdeI and BamHI and was cloned into the MCS site of linearized with the same restriction enzymes pJC40 expression vector (Fig. 1). The obtained construct was transformed into BL21 E. coli cells.

Fig. 1. Construct preparation of fabp3 in expression vector. 1% agarose electrophoresis; 1. DNA ladder; 2. Circular pJC40; 3. Linearized pJC40; 4. Construct pJC40/fabp3

From the single colonies showed to be recombinant plasmid DNA was extracted. It was sequenced and analyzed. The obtained sequence was 526 bp. That nucleotide sequence correlates to amino acid sequence from 175 amino acid residues.

Both of them were analyzed with BLAST tool and aligned with available in GenBank™ sequences for F. hepatica FABPs to confirm the origin of recombinant clones. Those alignments confirm that the recombinant FABP is identical to FABP3 from liver fluke (Fig. 2).

The additional histidine tail motif (His-tag) in the beginning of recombinant FABP protein is as follow: MGHHHHHHHHHHSSGHIEGRH and it is marked with black on Fig. 2. This motif was used for recombinant protein purification.

Fig. 2. Nucleotide (access.no. AJ250098) and amino acid (access.no. Q9U1G6) sequences alignment between FABP3 from F. hepatica and the produced recombinant FABP. With black – His-tail

Heterological expression of FABP3 in E. coli
The expression is possible to be performed with IPTG induction due to the presence of T7 promoter in pJC40 expression vector (6). Recombinant FABP3 was purified by Ni-affinity chromatography thanks to the His-tag. Presence, purity and relative protein size were determined by SDS-PAGE and silver staining. Expected size of the region identical to FABP3 is about 15 kDa and with His-tag of the recombinant protein the size that we observed on electrophoregrams was about 17 kDa (Fig. 3). The recombinant protein was found only in elution fractions (Fig. 3, line 1 and 2 in ellipse).

Fig. 3. Ni-affinity chromatography purification of recombinant FABP. SDS-PAGE with silver staining; 1, 2. Elution fractions; 3. Wash fraction; 4. Control (cytosolic fraction from not transformed bacterial cells); 5. Protein ladder. Recombinant FABP bands shown in ellipse
The concentration using Bradford assay and A at λ=280 nm UV light was determined to be about 400-500 μg/ml.

For additional recombinant protein evidencing and confirmation Western blot analysis was performed (Fig. 4A).

The immunoblotting with polyclonal rabbit serum supports the statement for identity of *F. hepatica* FABP3 and recombinant FABP and also confirms that the native and recombinant protein has similar surface antigenic conformation (Fig. 4B).

The presence of additional electrophoretic bands visible both on Fig. 3, lane 1 and 2 and on Fig. 4B, lane 2 and 3 is not surprising. That is because native forms of all known FABPs have functional structure as dimmers (most often) or even multimers (7, 20, 24).

For more detailed and complete analysis a database search was performed for nucleotide and amino acid sequences for FABPs from *F. hepatica* in GenBank™. It was found that the available gene and protein data was not rich enough. As it concerns nucleotide database only two available sequences were found under the accession numbers AJ250098 which referred to fabp3 and M95291 (17) which referred to fabp1 (fh15). Only four were the protein sequences found for the liver fluke in the database. They were visible under the accession numbers CAB65015 (undefined FABP), Q7M4G1 (FABP2), Q9U1G6 (FABP3) and Q7M4G0 (FABP1 (Fh15)) (17). The alignments were performed for comparison of these proteins (Fig. 5). The sequence of recombinant FABP from recent study was not included because it is completely identical with FABP3. It appeared that the undefined FABP (CAB65015) is identical with FABP3 as well and it is also not included in alignment.

The homology between FABP1 and FABP2 was found to be 72% and was higher than with FABP3 (65% with FABP2 and 62% with FABP1).

In comparison with mammalian FABPs which are very well studied (24), tissue-specific and cytosolic proteins, the invertebrates FABPs are not tissue-specific but characteristic for particular stages of the life cycle (8). As it concerns helminthes their FABPs are mostly secretory proteins but some of them may be excretory-secretory (E-S) proteins. The E-S products produced by helminthes and in particular by adult liver flukes are key players in understanding the host-parasite interaction and offer targets for chemo- and immunotherapy. Proteomic researches of FABPs from *F. hepatica* showed that only FABP3 is excreted on the surface of the body in vivo (15). That gives the expectation of a commercially feasible vaccine production based on FABP3 as an antigen that might reduce parasite transmission and parasite egg production. The production of recombinant proteins that mimic the levels of protection induced by the native Fasciola antigens is needed. That also could help for alternative drug design acting competitively with lipids in parasitic organism.

**Fig. 4.** Western blot analysis of native and recombinant FABP (in ellipse)  
A. The primary antibody is monoclonal mouse anti-poly-His antibody and the secondary is alkaline phosphatase conjugated anti-mouse antibody. 1. Protein ladder; 2. Soluble protein fraction of heterologous expression; 3. Pellet protein fraction of heterologous expression; B. The primary antibody is anti-FABP3 polyclonal serum and the secondary is alkaline phosphatase conjugated ProteinA antibody. 1. Protein ladder; 2, 3 Elution fractions from Ni-affinity chromatography purification of recombinant protein; 4. Native FABP3 from *F. hepatica*

**Fig. 5.** Protein alignment between FABPs from *F. hepatica* found in GenBank™
Conclusions
Recombinant FABP3 was produced and that could be used as an analog of native one and as an antigen for vaccine creation in future studies. The results of this study could contribute to a better understanding of host-parasite interaction, to offer targets for immunotherapy and could be helpful for antihelminthic drug design.

Acknowledgments
This publication was funded by the European social fund and Republic of Bulgaria, Operational program “Development of human resources” 2007-2013. Grant No BG051PO001-3.3.04/46 from 28.08.2009. The study was supported by the European Union funded DELIVER Project (Contract No.: FOOD-CT-2004-023025).

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