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Characterization and differential expression of a ferritin protein from *Fasciola hepatica*

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Abstract

Ferritins are proteins that play a central role in maintaining intracellular iron balance. A cDNA clone of *Fasciola hepatica* (687 bp long) encoding a putative 228-amino acid polypeptide (FhFtn-1) homologous with ferritins of vertebrates and invertebrates was identified. FhFtn-1 contains a conserved motif of the ferroxidase center typical of vertebrate ferritins. Phylogenetic tree analysis showed that FhFtn-1 clusters with two ferritins of *Paragonimus westermani*, which suggests a common ancestry for the ferritins of these two trematodes. Recombinant FhFtn-1 protein expressed and purified from an *Escherichia coli* system showed iron-uptake ability. Moreover, FhFtn-1 showed strong reactivity with sera from rabbits infected with *F. hepatica* for 2–12 weeks, which suggests that this protein could be a potential antigen for immunodiagnosis of fascioliasis. qPCR analysis demonstrated that FhFtn-1-mRNA is expressed at significantly higher levels in adults and unembryonated eggs than in juveniles or miracidia. These results represent the first characterization of a ferritin protein from the liver fluke *F. hepatica*.

Keywords

fascioliasis; ferritin; liver fluke; developmental expression

1. Introduction

Iron is an essential element for virtually all prokaryotic and eukaryotic organisms. Under physiological conditions, free iron has reversible transitions between two oxidation states: the relatively soluble ferrous state (Fe^{2+}) and the very insoluble ferric state (Fe^{3+}) [1]. This property has enabled organisms to use iron for many essential biochemical reactions, e.g. electron transfer, oxygen transport, energy transduction, nucleic acid synthesis, and detoxification [2]. However, the biological use of iron is constrained by the element's low solubility and because, if not appropriately chelated, it can participate in harmful free-radical reactions via Fenton chemistry [3]. Thus, although essential for life, iron can also be harmful, a paradox which has compelled organisms to evolve efficient mechanisms for iron transport and storage. Ferritins are proteins that play a central role in the maintenance of intracellular iron balance. Mammalian ferritins are 24-subunit proteins [composed of heavy (H) (21kDa) and light (L) (19kDa) subunits] capable of storing up to 4500 Fe (III) ions in a ferrihydrite mineral core [4, 5]. This storage capability suggests that ferritins function as protective proteins, minimizing free-radical reactions and preventing cellular damage by sequestering iron inside the storage cavity. Ferritin proteins have been reported in a wide range of organisms from prokaryotes to eukaryotes, including plants [6, 2, 7, 8, 9]. Ferritin

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proteins have also been characterized in *Schistosoma mansoni* [10], *Schistosoma japonicum* [11, 12, 13], *Taenia saginata* [14], *Echinococcus granulosus* [15], *Paragonimus westermani* [16] and *Clonorchis sinensis* [17]. Also, a recent proteomic study demonstrated the presence of ferritin in soluble egg extracts of the liver fluke *Fasciola hepatica* [18]. However, to date, no ferritin from *F. hepatica* has been purified or characterized.

F. hepatica is a digenetic trematode and the causative agent of fascioliasis in mammals [19, 20, 21], including humans [22, 23]. Following ingestion of metacercariae, the juveniles burrow through the host gut wall and migrate to the liver, where they cause extensive damage before moving into the bile ducts. Finally, the flukes reach the bile-duct walls as mature adults and excrete large amounts of eggs that pass with the host feces to the external environment to continue the life cycle. During development in the mammalian host, *F. hepatica* parasites feed on blood, hepatocytes and bile; therefore, it seems likely that iron compounds are required for parasite nutrition and egg production, congruent with reports on schistosomes [10, 13], *P. westermani* [16], and *C. sinensis* [17]. It is also expected that ferritin molecules in *Fasciola* provide a protective mechanism against the harmful effects of iron. Therefore, ferritins could represent a potential drug target and/or vaccine candidate because of the vital roles they play in iron metabolism. Unregulated degradation of ferritins could potentially lead to cellular toxicity owing to uncontrolled release of iron. The present study reports the molecular biological characterization of a ferritin protein of *F. hepatica* (FhFtn-1) and demonstrates that expression of this protein is developmentally regulated.

2. Materials and Methods

2.1. Collection of adult flukes, eggs, miracidia and newly excysted juveniles

F. hepatica adult flukes were recovered from bovine livers at a local abattoir, washed several times with 0.1M phosphate-buffered saline (PBS) pH 7.4 to eliminate all traces of blood and bile and transported to the laboratory in RPMI-1640 medium (Sigma-Aldrich, St. Louis, Missouri). During transportation, flukes release a large number of eggs into the medium, which are allowed to settle. Flukes were removed from the RPMI medium and washed three times with sterile PBS, snap-frozen in liquid nitrogen and stored at -80°C until use. The eggs were removed from the medium, resuspended in fresh double-distilled water and then allowed to settle. After several washes, the eggs were examined at $\times 100$ magnification to confirm the absence of visible contaminants. Eggs were snap-frozen in liquid nitrogen and stored at -80°C until use. Another batch of eggs was allowed to mature by incubation in the dark at 22°C for 9–12 days and then stimulated to hatch by exposing to light for 2h at 25°C . Free-swimming miracidia were collected using a transfer pipette, immediately snap-frozen and stored as described above.

Metacercariae (obtained from Baldwin Aquatic, Inc, Oregon) were washed in distilled water, transferred to watch glasses and pre-incubated in 1.2% sodium bicarbonate, 0.9% sodium chloride, 0.8% sodium tauroglycolate for 30 min at 37°C . Metacercariae were then transferred to watch glasses and allowed to excyst for up to 3 h at 37°C in excystment medium freshly prepared by diluting the pre-incubation solution 1:1 with 0.33% HCl and 0.8% L-cysteine. Newly excysted juveniles (NEJs) were removed from excystment medium and maintained in fresh *Fasciola* saline [FS; Dulbecco's modified Eagle's medium (DMEM) (w/o $\text{NaHPO}_3/\text{PO}^{-3}$) plus 0.5ml/ml distilled water, 2.2mM calcium acetate, 2.7mM MgSO_4 , 61.1mM glucose, 1 μM serotonin, 5 $\mu\text{g/ml}$ gentamicin, 15mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4] at 37°C . NEJs were snap-frozen and stored at -80°C until use.

2.2. Rabbit infection sera

Four New Zealand White rabbits were orally infected with 60 *F. hepatica* metacercariae. Animals were bled before infection and then at biweekly intervals over 12 weeks. Serum samples were stored at -20°C until use.

2.3. FhFtn-1 cDNA isolation

An unamplified cDNA expression library of *F. hepatica* constructed in λ gt11 [24] was screened using pooled sera from rabbits with 4 wk of *F. hepatica* infection using standard procedures [25]. Clones which remained positive after low-density re-screening were selected, subcloned into pCR-Blunt Vector (Invitrogen, Carlsbad, California) and sequenced in both directions using an ABI PRISM BigDye™ Terminator Sequencing Kit (Perkin Elmer, Massachusetts). One clone (FhFtn-1), encoding a protein truncated at the amino terminus, was selected for further analysis because of sequence similarity (E value = $8e^{-26}$) with the yolk ferritin of *P. westermani* (PwYF-1) [16]. Full-length cDNA encoding FhFtn-1 was synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR) using the SuperScript® One-Step RT-PCR System (Invitrogen) with specific primers designed based on PwYF-1 and FhFtn-1 sequences. The forward primer (5'-ATGCACTCTGCACGCATCAAC-3') corresponds to the 5'-end region of the PwYF-1 open reading frame (ORF) and the reverse primer (5'-CCAAAGTGACAATTTGCGCTC-3') corresponds to the 3'-end of the truncated FhFtn-1 ORF. Cycling conditions were: 94°C for 15 sec, 55°C for 30 sec, 68°C for 1 min; 40 cycles, followed by a 5-min extension at 68°C . The obtained product was subcloned into pCR®2.1-TOPO® vector and sequenced in both directions using M13 primers.

2.4. DNA and peptide sequence analysis

The FhFtn-1 sequence was examined for similarity against the GenBank™ nonredundant protein and nucleic acid databases using the BLAST algorithm [26]. Secondary structure predictions were made using the SOPMA server [27]. All complete FhFtn-1 cDNA and predicted protein sequences were compared by pairwise alignment to determine the percentage of sequence identity using the Genetics Computer Group GAP Program (version 10.0, for Unix) with default settings. Multiple sequence alignments were performed using the Clustal W program, version 1.7 [28], and submitted to the GBlocks server to eliminate poorly aligned positions [29]. The best suitable alignment retained 66% of the positions and was thus utilized for further analysis. Phylogenetic analysis was performed using the Neighbor-joining tree algorithm from the PHYLIP v.3.9 package [30]. SEQBOOT and PROTDIST (Jones-Taylor Thornton matrix) programs were used to create a bootstrapped data set of 1000 replicate samples and to generate protein distance matrices, respectively. The CONSENSE program was used to build an un-rooted consensus tree using an extended majority rule. Phylogenetic tree output files and bootstrapping results were visualized using TreeView version 1.6.6 [31]. The tertiary structure of FhFtn-1 was predicted using the Protein Homology / AnalogY Recognition Engine (PHYRE) server and compared with a representative member of the human H subunit (PDB ID:2fha) [32]. The 3D image was processed with RasMol [33].

2.5. RNA isolation and PCR analyses

Quantitative-PCR (qPCR) was used to assess FhFtn-1 expression in various developmental stages of *F. hepatica*. Total RNA was isolated from adult flukes, NEJs, eggs and miracidia using a PureLink RNA Mini Kit (Invitrogen) followed by treatment with Turbo DNA-free endonuclease (Ambion, Grand Island, New York) to remove contaminating genomic DNA. Total RNA was quantified using a Nanodrop-1000 spectrophotometer (Thermo-Scientific). The real-time qPCR experiments involved cDNA synthesis using the High Capacity RNA-

to-cDNA kit (Applied Biosystems, Carlsbad, California) with 100ng from each RNA sample. qPCR was conducted in triplicate using a StepOne Plus Real-Time PCR system (Applied Biosystems) with cDNA equivalent to 5ng of total RNA, and SYBR green PCR Master Mix (Applied Biosystems). The primers for qPCR were designed so as to amplify approximately 99bp of FhFtn-1 and 176bp of *F. hepatica* GAPDH (GenBank ID: AY005475) as a housekeeping gene. The primer pairs were FhFtn-1: 5'-GTAACGGCCAGGAATTTCTATCG-3' (sense) and 5'-CCAAAAACGCACGAATGCT-3' (anti-sense); and Fh-GAPDH: 5'-GCGCCAATGTTCGTGTTCCGG-3' (sense) and 5'-TGGCCGTGTACGAATGCAC-3' (anti-sense). Primer concentrations were optimized and dissociation curves were generated for each of the FhFtn-1 and Fh-GAPDH primer sets to verify the amplification of a single PCR product. To compare the FhFtn-1 transcript levels between life stages we used the comparative C_T method. The C_T for the target amplicon and the C_T for the internal control were determined. Differences in C_T for the target and C_T for the internal control, called ΔC_T , were calculated to normalize the differences in the amount of total nucleic acid added to each reaction and the efficiency of the reverse transcription step. The ΔC_T for each sample was subtracted from the ΔC_T of the calibrator (NEJ stage). This difference is called the $\Delta\Delta C_T$ value. Finally, the amount of target, normalized to *GAPDH* and relative to the calibrator, was calculated by the $2^{-\Delta\Delta C_T}$ method [34]. Thus, all the experimental samples are expressed as an n -fold change relative to the calibrator.

2.6. Cloning, expression and purification of FhFtn-1

The coding region of the FhFtn-1 cDNA was amplified by PCR using a forward primer that contains a BamHI site (underlined) upstream of the start codon (5'-TGGGGATCCGCCCTTATGCACTCTGCACGC-3', and a reverse primer that contains an EcoRI site downstream of the stop codon (5'-GGATGAATTCCAAAGTGACAATTTGCGCTCAATTC-3'). The PCR product was purified and cloned into the corresponding restriction sites of a prokaryotic expression vector pRSET A (Invitrogen) and the recombinant vector was confirmed by double-enzyme digestion and sequencing. The resulting plasmid construct (pRSET A-FhFtn-1) was transformed into competent *E. coli* BL-21 (DE3) cells (Stratagene, Santa Clara, California). Overexpression of recombinant FhFtn-1 was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM to the culture medium. After induction, bacteria were harvested, suspended in lysis buffer (20mM sodium phosphate, 500mM NaCl, 20mM imidazole, 2% Triton X-100, 0.2mg/ml lysozyme, 1mM PMSF, pH 7.4, DNase (20 μ g/ml) and homogenized by sonication. Unclarified lysate was loaded onto a HisTrap FFTM crude column (GE healthcare) and washed successively with wash buffer (20mM sodium phosphate buffer pH 7.4 + 500mM NaCl + 20mM imidazole). The retained fusion protein was eluted with wash buffer (pH 5.5) containing 500mM imidazole. Eluates were desalted against wash buffer without imidazole using a PD-10 column (Amersham-Biosciences) and protein concentrations were measured using the bicinchoninic acid method [35].

2.7. Kinetics of iron uptake

To obtain the apo form of FhFtn-1, the iron was removed by incubation in 1% thioglycolic acid, 0.1M sodium acetate, pH 5.5 for 18 h. To chelate ferrous iron atoms, excess of 2, 2-bipyridine was added to the reaction and the solution was dialyzed exhaustively against 0.1M HEPES pH 7.0 [36]. Apo-FhFtn-1 or apo-horse ferritins (Sigma Aldrich) were incubated in 0.1M HEPES containing 1mM of ammonium ferrous sulfate freshly prepared in 1mM HCl. The iron oxidation was monitored by the increase in absorbance at 310nm, which specifically measures Fe (III) ions, and the measurements were recorded for 10 min [16, 37, 38].

2.8. Protein electrophoresis and immunoblot

Twenty micrograms of recombinant FhFtn-1 protein were separated by 15% SDS-polyacrylamide gel electrophoresis, and electrotransferred onto nitrocellulose (NC) membrane (0.2 μ m; Bio-Rad) at 4°C for 2 h. After blocking for 1 h in PBS containing 0.05% Tween-20 (PBST) and 5% skim milk, the NC membrane was cut into strips, some of which were incubated overnight (O/N) in a mouse anti-Xpress epitope-peroxidase labeled antibody (Invitrogen) diluted 1:5,000. After several washes, the color was developed using DAB as a chromogenic substrate. Other strips were incubated with normal rabbit serum (NRS) or sera from rabbits infected with *F. hepatica* for 2 wk or 12 wk at a dilution of 1:100 in PBST at O/N. After washing, the NC strips were incubated with peroxidase-conjugated anti-rabbit IgG antibody at a dilution of 1:3,000 for 2h at room temperature. A positive brown signal for FhFtn-1 was visualized using DAB as a substrate.

2.9. ELISA

Antigenicity of FhFtn-1 was also evaluated in an ELISA in which the protein was assayed against a panel of sera from rabbits with 0, 2, 4, 6, 8, 10 and 12 wk of *F. hepatica* infection. Polystyrene plates (Costar, Corning, New York) were coated with 20 μ g of FhFtn-1 diluted in 0.05M carbonate buffer (pH 9.6), and sera from rabbits and anti-rabbit peroxidase-conjugated IgG were used at dilutions 1:100 and 1:5,000, respectively, according to an established protocol [39]. Each determination was done in duplicate and the results expressed as the mean absorbance value for each determination.

2.10. Statistical analysis

All data are presented as the mean $\pm$ standard error. Differences between groups were assessed for statistical significance using Student's *t*-test (GraphPad Prism Software, www.graphpad.com). A statistically significant difference for a particular comparison was defined as $P < 0.001$.

3. Results

3.1. FhFtn-1 sequence analysis

Screening of 5×10^7 bacteriophage plaques of a λ gt11 cDNA library with a pool of sera from rabbits with 4 wk of *F. hepatica* infection revealed 13 positive clones. The cDNA fragment inserts of all 13 purified clones were amplified by PCR, subcloned into pCR-Blunt vector and sequenced from both ends. BLAST searches of the nucleotide and amino acid sequences identified one clone with 56.25% identity to PwYF-1, which is a member of the *P. westermani* yolk ferritin family [16]. The sequence determined from this clone (GenBank ID: HQ316639.1) is 533 bp long and contains an ORF of 477bp encoding a putative polypeptide of 158 amino acids. Northern blot analysis, in which a FhFtn-1 cDNA probe was hybridized with RNA species from adult flukes, showed a strong hybridization signal at ~900bp (data not shown). Considering the size of the FhFtn-1 clone (533 bp), this result suggested that the cDNA was not a full-length copy of the corresponding mRNA. A comparison of the deduced amino acid sequence of FhFtn-1 with the sequence of PwYF-1 indicated that at least 70 amino acids were missing from the amino-terminal region of FhFtn-1. Using RT-PCR with specific primers designed on the conserved regions of FhFtn-1 and PwYF-1, a ~900-bp fragment was obtained. Sequencing of the full-length cDNA revealed a 5' untranslated region (UTR) of 113 bp followed by an ORF of 687 bp and a 3' UTR of 100 bp. The ORF was predicted to encode a 228-amino acid polypeptide with a calculated molecular mass of 26.4 kDa and a predicted isoelectric point (*pI*) of 8.75. The amino acid sequence of the full-length FhFtn-1 is rich in hydrophobic (42.5%) and polar (27.2%) amino acids, and also contains 15.4% acidic residues and 14.9% basic residues.

Comparison of the amino acid sequence of FhFtn-1 with homologous sequences in the GenBank database demonstrated that FhFtn-1 is more closely related to a ferritin from *P. westermani* (overall identity = 56%, similarity = 72%) than to ferritins of human, *Schistosoma*, *Clonorchis*, *Taenia* or *Echinococcus* species, which share relatively low identities (25–32%) and similarities (45–52%) with FhFtn-1 (Fig. 1). A phylogenetic comparison with homologous ferritins from all these species demonstrated that the ferritins of *F. hepatica* and *P. westermani* constitute a cluster distant from those of other metazoans, including human ferritins (Fig. 2).

The secondary structure of the FhFtn-1 polypeptide was predicted to have six α -helices connected by loops, according to the SOPMA program. The longest loop was found to be 24 residues long (Arg182-Trp205). The helices were named A, B, C, D, and E in accordance with those of the *P. westermani* yolk-ferritin [16] and the H-subunit of human ferritin [40]. Interestingly, when comparing the ferritins of vertebrates and invertebrates, the FhFtn-1 polypeptide has an extra peptide of approximately 24 amino acids forming an additional helix (helix F) at the C-terminus. The helices of FhFtn-1 are located between residues 10–37 (helix A), 47–70 (helix B), 88–120 (helix C), 127–157 (Helix D), 166–181 (Helix E) and 205–228 (Helix F). FhFtn-1 has five amino acid residues (at positions Tyr23, Asn60, His64, Glu96 and Gln140) which correspond to conserved Fe-binding sites of helices A, B, C and D. The tertiary structure predicted for FhFtn-1 shows characteristics strikingly similar to the global subunit fold of the heavy subunit of human ferritin (HsF-H) (Fig. 3). Helices A–B and C–D are anti-parallel and connected by short loops (AB- and CD-loops). The longest loop observed within the FhFtn-1 structure (i.e. the BC-loop) is consistent with the long loop that connects helices B and C of HsF-H. Short loops that connect helices C–D and D–E of FhFtn-1 are also consistent with those that occur in HsF-H.

3.2. qPCR analysis of FhFtn-1 mRNA expression

To investigate whether FhFtn-1 is developmentally expressed in *F. hepatica* stages other than the adult, expression of FhFtn-1 at the mRNA level was evaluated in adults, eggs, miracidia and NEJs by using qPCR. The data were standardized relative to *F. hepatica* GADPH, and the $2^{-\Delta\Delta C_t}$ method was used to quantify relative FhFtn-1 expression. The relative expression of FhFtn-1 mRNA (C_T values at a total of 40 cycles) was significantly lower in NEJs and miracidia compared to adults or eggs. We therefore used the NEJ stage as calibrator to determine quantitative differences among levels of expression of FhFtn-1. The results demonstrate that FhFtn-1 is upregulated in the egg-laying fluke. We detected the highest levels of FhFtn-1 transcript in eggs (14-fold) and adults (12-fold) ($P < 0.001$) compared to NEJs (Fig. 4). The level of FhFtn-1 in miracidia was significantly lower than that of eggs or adults, but higher than that of NEJs ($P < 0.001$).

3.3. Expression and purification of recombinant FhFtn-1 protein

Recombinant FhFtn-1 was produced as a fusion protein with the Tag peptide derived from the expression vector and purified to near homogeneity by a single round of affinity chromatography using a HisTrap FF™ crude column. The molecular mass of recombinant FhFtn-1 protein is 30.7 kDa, including the polyhistidine Tag peptide (Fig. 5).

3.4. Iron-uptake activity of FhFtn-1 protein

We monitored the iron oxidation capacity of FhFtn-1 to determine if the ferroxidase center (FC) is active by measuring the increase in absorbance at 310 nm as a result of the formation of Fe (III). FhFtn-1 was compared with horse spleen ferritin (positive control) and bovine serum albumin (BSA; negative control) in identical reactions (Fig. 6). FhFtn-1 oxidized Fe (II) with kinetics similar to that of horse ferritin, and both proteins oxidized Fe (II) faster than BSA, which does not possess an FC.

3.5. Immunoreactivity of recombinant FhFtn-1 with infection sera

By ELISA, recombinant FhFtn-1 showed 100% reactivity with all the infection sera derived from rabbits infected from 2 to 12 weeks duration. At 2 weeks after infection the mean absorbance value ($A_{492} = 1.36 \pm 0.07$) was significantly higher than that of the negative controls ($P < 0.001$). Thereafter, antibody reactivity to FhFtn-1 fusion protein remained at relatively high levels, with minor fluctuations through 12 weeks of infection (mean $A_{492} = 1.16 \pm 0.06$). By immunoblot, a polypeptide band of the expected size (30.7 kDa) confirmed the antigenic character of the recombinant FhFtn-1 protein. The control sera were all negative, both by ELISA and by immunoblot (Fig. 7).

4. Discussion

Ferritins are the principal iron-storage proteins in most living organisms. Virtually all organisms require iron in order to grow, and the invertebrate *F. hepatica* is no exception. *F. hepatica* is a parasite that lives and develops in the liver where it ingests erythrocytes and hepatocytes as a source of nutrients and absorbs a range of low molecular weight solutes through its membranes [41]. Based on the large amount of iron found in the serum environment, which is essential for parasite development within the mammalian host, it is plausible that ferritins could mediate iron transport and availability during parasite development and maturation.

The putative FhFtn-1 discovered and characterized in this study shows low sequence identity (24.8–31.7%) with human ferritins and ferritins from a group of metazoan parasites that include *S. mansoni*, *S. japonicum*, *C. sinensis*, *E. granulosus* and *T. saginata*. This indicates that conservation of ferritins at the primary-structure level is very low between species, an observation also made by other researchers [16, 17]. In contrast to the relatively low level of conservation of ferritin primary structure, the secondary and tertiary structures are highly conserved among all members of the ferritin family. Therefore, it was not surprising that the predicted secondary structure of FhFtn-1 has high similarity with that of the human ferritin H-subunit. In the case of FhFtn-1, the key residues occur at conserved positions in helices A, B, C and D, as in other helminth and vertebrate ferritins [14, 13, 16, 41]. The long loop that precedes helix C in the FhFtn-1 protein moiety also occurs at the same position in the ferritins of many other vertebrates and invertebrates [14, 13, 16, 41, 17]. Based on the molecular features of FhFtn-1, i.e. its extensive helical structure with six helices and conserved metal-binding sites at residues corresponding to the ferroxidase center, it is not surprising that the predicted tertiary structure of FhFtn-1 is also similar to those of its previously reported homologues [16, 42, 17]. The iron-uptake activity of recombinant FhFtn-1 demonstrates that the protein possesses an active ferroxidase center in the middle of its tertiary structure, as occurs in other members of the ferritin family. Moreover, FhFtn-1 might be classified as an isotype of the ferritin H-subunit because of structural similarities between them.

Another typical characteristic of H-subunit ferritins is that they contain four subunits that form a 4-fold axis interface with helix E from each subunit and conglomerate with 24 subunits to form a hollow spherical shell. This 4-fold axis is lined with 12 leucine residues from the H-subunit and four histidines at the cavity side, also reported for the L subunit [43]. The FhFtn-1 polypeptide contains three leucine residues within helix E. Moreover, the unique helix F of FhFtn-1 contains an additional leucine and histidine residue. This helix might function during assembly of the hollow spherical shell described above, concurring with previous reports [43]. One can also speculate that the long C-terminal extension of FhFtn-1, including the helices E and F, could stabilize the protein conformation, similar to functions of the human ferritin H-subunit [39]. An additional peptide identified in the yolk ferritin of *Lymnaea stagnalis* is believed to function as a ligand for receptors on the surface

of the oocyst [44]. Recently, the ferritin structure for *Mycobacterium tuberculosis* was resolved and it also has an extended C-terminus. This extra region was implicated in facilitating iron entry toward the ferroxidase center as well as the exit of stored iron from the protein's cavity [45]. Based on these observations, we suggest that the extended C-terminal peptide of FhFtn-1 may also have a role in ferroxidase activity and iron release in addition to providing protein stability during tissue localization in *F. hepatica*.

The finding that FhFtn-1 was immune-reactive with sera from rabbits with 2 to 12 weeks of *F. hepatica* infection indicates that this protein is highly antigenic and is expressed at either early or late developmental stages of *F. hepatica*. The finding of immune reactivity with 4-week infection sera was not unexpected given that FhFtn-1 was primarily identified by immunoscreening of a cDNA library with 4-week infection sera. However, our data seem to disagree with those of Moxon et al. (2010) [18], who, by a proteomic approach, identified ferritin in soluble *F. hepatica* egg extracts and observed immune reactivity only with 9-week bovine infection sera. Their ferritin was identified by peptide homology to an uncharacterized truncated protein sequence of 176 amino acids reported in GenBank (GenBank ID: ABC74792) that is 90% identical to our FhFtn-1 (data not shown). This observation supports the notion of several isoforms of FhFtn-1 with different expression patterns and possibly with redundant functions, as has been reported for *Schistosoma* species [11].

The low conservation of FhFtn-1 with ferritins of schistosomes and cestodes suggests that FhFtn-1 could be used as a specific serodiagnostic antigen. However, based on the high homology between FhFtn-1 and PwYF-1, cross-reactivity may be expected between these two species. Interestingly, a previous study in which recombinant PwYF-1 was assayed by ELISA with a large battery of human infection sera demonstrated that *P. westermani* ferritin is specific, able to be detected by paragonimiasis but not fascioliasis sera [46]. Based on this antecedent we anticipate that FhFtn-1 might also be specific like its homologue in *P. westermani*, but this is an issue that needs to be addressed in further studies.

Our qPCR data demonstrated that expression of FhFtn-1 is low but detectable in NEJs and reaches a high level when the parasite reaches maturity and initiates egg excretion. This indicates that expression of FhFtn-1 increases with parasite development and is consistent with the observed reactivity of FhFtn-1 with 2- and 4-week infection sera. By this time the juvenile parasite is actively migrating through liver parenchyma and feeding on blood and hepatocytes and thereby obtaining iron compounds that are necessary for its nutrition. This developmental regulation of FhFtn-1 is consistent with observations reported on *P. westermani* [16], *C. sinensis* [17] and *S. mansoni* [47], in which their ferritins localized to the vitelline follicles and intra-uterine eggs of adult flukes. The finding of FhFtn-1 in non-embryonated eggs supports the theory that FhFtn-1 could be a component of yolk platelets of vitelline cells (eggshell precursors and possibly yolk cells), which has also been reported for some ferritin isoforms of *S. japonicum* [11]. Interestingly, FhFtn-1 mRNA levels decreased dramatically in NEJs compared to other stages, suggesting a lack of requirement for FhFtn-1 immediately after infection of the mammalian host. However, as the infection in the mammalian host proceeds, this protein reassumes an important role, as suggested by the observed upregulation of the mRNA transcript in adults. These results suggest that, while differentially expressed in adults and eggs, FhFtn-1 is ubiquitously expressed.

In conclusion, this paper reports the characterization of the first member of the *F. hepatica* ferritin protein family. Based on the upregulation of FhFtn-1 mRNA in the *F. hepatica* adult and egg stages, we suggest that FhFtn-1 likely is a yolk protein with a possible function during egg formation. However, this assumption needs to be addressed in future studies. This finding contributes to our knowledge concerning nutrient acquisition in this parasite

and identifies FhFtn-1 as a novel, potential target for therapeutic intervention. Moreover, FhFtn-1 is highly reactive with sera obtained from rabbits with acute and chronic *F. hepatica* infection and, therefore, this molecule could constitute a good antigen for immunodiagnosis of fascioliasis.

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Figure 1.

CLUSTAL alignment of amino acid sequences of ferritins from vertebrates and invertebrates. FhFtn-1 (*F. hepatica* ID: ADP24191.1); PwF-1 (*P. westermani* ID: AAG17056.1); SmF1 (*S. mansoni* ID: XP_002576082.1); SmF2 (*S. mansoni* ferritin light chain ID: AAA29881.1); CsFtn (*C. sinensis* ID: AAS92978.1); SjF (*S. japonicum* ID: CAX75606.1); TgF (*T. saginata* ID: CAA65097.1); EgF (*E. granulosus* ID: CAA83506.1); HsF-H (*H. sapiens* heavy subunit ID: AAH66341.1); HsF-L (*H. sapiens* light subunit ID: AAH13928.1). Alpha helical regions (A to F) are indicated with solid black bars. Conserved iron-binding residues are indicated by triangles.

Figure 2.

A neighbor-joining unrooted phylogenetic tree was constructed using the PHYLIP package. Bootstrap index values are indicated at the internal nodes. FhFtn-1 (*F. hepatica* ID: ADP24191.1); PwF-1 (*P. westermani* ID: AAG17056.1); SmF1 (*S. mansoni* ID: XP_002576082.1); SmF2 (*S. mansoni* ferritin light chain ID: AAA29881.1); CsFtn (*C. sinensis* ID: AAS92978.1); SjF (*S. japonicum* ID: CAX75606.1); TgF (*T. saginata* ID: CAA65097.1); EgF (*E. granulosus* ID: CAA83506.1); HsF-H (*H. sapiens* heavy subunit ID: AAH66341.1); HsF-L (*H. sapiens* light subunit ID: AAH13928.1). The *F. hepatica* / *P. westermani* ferritin cluster is boxed.

Figure 3.

Predicted tertiary structure of FhFtn-1 generated by structural alignment of the FhFtn-1 sequence with the human H-subunit (PDB ID: 2fha) using the PHYRE server. Helix A: orange; Helix B: green; Helix C: blue; Helix D: yellow; and Helix E: red. The 3D structure includes only amino acids 1 -181, thus excluding helix F from FhFtn-1. The conserved Fe-binding residues of the FhFtn-1 structure are represented as ball-and-stick models.

Figure 4. Relative FhFtn-1-mRNA levels at different stages of the life cycle of *F. hepatica* as determined by quantitative PCR (qPCR). Results are shown as the fold change in expression relative to that of NEJs and are the mean $\pm$ SEM of a minimum of three experiments, each in triplicate.

Figure 5. Recombinant FhFtn-1 protein was purified from unclarified *E. coli* lysate using an His-Trap FF crude affinity column. Bacterial lysate before induction (lane-1), after induction (lane-2), unbound proteins (wash-out) (lane-3) and purified FhFtn-1 (lane-4) was analyzed by 15% SDS-PAGE and silver stained. Proteins were electrotransferred to nitrocellulose membrane and incubated with a commercial monoclonal antibody raised against the X-press epitope (Invitrogen), which is located at the amino terminus of the recombinant fusion protein between the His-tag and the first methionine residue of FhFtn-1 (lane-5). The 30.7 kDa polypeptide band typical of the FhFtn-1 fusion protein is shown.

Figure 6.

Progression plots of the iron uptake activity of FhFtn-1. Reactions contained 20 μ g/ml of protein, 1mM ferrous ammonium sulfate and 0.1M HEPES pH 7.0 (n=3). The oxidation of Fe (II) to Fe (III) was monitored by an increase in the absorbance at 310 nm during the course of the reaction.

Figure 7.

FhFtn-1 fusion protein was tested with *F. hepatica* infection sera. **(A)** Proteins were probed with sera from four rabbits with 2, 4, 6, 8, 10 and 12 weeks of *F. hepatica* infection. Results represent the mean absorbance value ($A_{492} \pm SD$) for each determination at each week of infection. **(B)** Immunoblot analysis corroborated the ELISA analysis. The presence of the typical polypeptide band of 30.7 kDa was detected in the sera from rabbits with 2 and 12 weeks of infection, but not in the pre-immune sera.