Characterization of a profilin-like protein from *Fasciola hepatica*

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*Fasciola hepatica* is the causative agent of fasciolosis, an important disease of humans and livestock around the world. There is an urgent requirement for novel treatments for *F. hepatica* due to increasing reports of drug resistance appearing around the world. The outer body covering of *F. hepatica* is referred to as the tegument membrane which is of crucial importance for the modulation of the host response and parasite survival; therefore, tegument proteins may represent novel drug or vaccine targets. Previous studies have identified a profilin-like protein in the tegument of *F. hepatica*. Profilin is a regulatory component of the actin cytoskeleton in all eukaryotic cells, and in some protozoan parasites, profilin has been shown to drive a potent IL-12 response. This study characterized the identified profilin form *F. hepatica* (termed *Fh*Profilin) for the first time. Recombinant expression of *Fh*Profilin resulted in a protein approximately 14 kDa in size which was determined to be dimeric like other profilins isolated from a range of eukaryotic organisms. *Fh*Profilin was shown to bind poly-L-proline (pLp) and sequester actin monomers which is characteristic of the profilin family; however, there was no binding of *Fh*Profilin to phosphatidylinositol lipids. Despite *Fh*Profilin being a component of the tegument, it was shown not to generate an immune response in experimentally infected sheep or cattle.
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Abstract

Fasciola hepatica is the causative agent of fasciolosis, an important disease of humans and livestock around the world. There is an urgent requirement for novel treatments for F. hepatica due to increasing reports of drug resistance appearing around the world. The outer body covering of F. hepatica is referred to as the tegument membrane which is of crucial importance for the modulation of the host response and parasite survival; therefore, tegument proteins may represent novel drug or vaccine targets. Previous studies have identified a profilin-like protein in the tegument of F. hepatica. Profilin is a regulatory component of the actin cytoskeleton in all eukaryotic cells, and in some protozoan parasites, profilin has been shown to drive a potent IL-12 response. This study characterized the identified profilin form F. hepatica (termed FhProfilin) for the first time. Recombinant expression of FhProfilin resulted in a protein approximately 14 kDa in size which was determined to be dimeric like other profilins isolated from a range of eukaryotic organisms. FhProfilin was shown to bind poly-L-proline (pLp) and sequester actin monomers which is characteristic of the profilin family; however, there was no binding of FhProfilin to phosphatidylinositol lipids. Despite FhProfilin being a component of the tegument, it was shown not to generate an immune response in experimentally infected sheep or cattle.
**Introduction**

Fasciolosis is a worldwide distributed zoonotic infectious disease and constitutes a serious worldwide problem in both humans and livestock (Mas-Coma et al. 2018; Mas-Coma et al. 2019). There are two major pathogens of fasciolosis; *Fasciola hepatica* and *F. gigantica*, which are commonly referred to as liver fluke. The control of fasciolosis has relied upon chemotherapy, predominantly with the drug triclabendazole. However, due to an over-reliance on this drug in recent years, resistance to triclabendazole has developed (Fairweather 2009; Kelley et al. 2016).

As triclabendazole is the only drug that will kill both the juvenile and adult life stages of liver flukes, there is an urgent need for the development of novel treatments. For this reason, vaccine development is seen as a sustainable method for the control of *Fasciola* spp. (Molina-Hernandez et al. 2015; Toet et al. 2014).

The current *F. hepatica* vaccine candidates being investigated have shown only moderate protection against *F. hepatica* infection (Toet et al. 2014). The development of an effective vaccine will require a thorough understanding of the host-parasite interactions (Cwiklinski & Dalton 2018; Cwiklinski et al. 2018). While the excretory-secretory (ES) products of *F. hepatica* have been thoroughly investigated as vaccine antigens with little sustained success, the tegument surface represents the key interface in host-parasite interactions, performing numerous functions for the parasite such as nutrient absorption, sensory input and protection from the host immune response (Halton 2004). Host antibodies have been demonstrated to have an ability to bind to the tegument antigens of *F. hepatica* (Howell and Sandeman 1979; Hanna 1980; Sulaiman et al. 2016; Cameron et al. 2017), suggesting that tegument-directed vaccine candidates warrant further investigation. Despite this, there are few reported cases of tegument-directed vaccines.

Tegument proteins as vaccine targets in other helminth parasites such as *Schistosoma mansoni* show immense promise, with phase 1 clinical trials in progress (Fonseca et al. 2015; Merrifield et al. 2016; Molehin 2020) and we propose that tegument proteins represent potential novel vaccine antigens for *F. hepatica* control (Hanna et al. 1988; Sobhon et al. 1998).

The tegument surface of *Fasciola* spp. is a dynamic syncytial layer surrounded by a glycocalyx (Hanna 1980; Lammas & Duffus 1983). Various groups in recent times have attempted to characterise the proteome (Hacariz et al. 2012; Ravida et al. 2016; Wilson et al. 2011) and
immunoproteome (Cameron et al. 2017) of *F. hepatica* using mass spectrometry. The proteome of an enriched tegument extract of *F. hepatica* revealed a range of proteins shared with the schistosome tegument including annexins, tetraspanins, carbonic anhydrase and an orthologue of a host protein (CD59) (Wilson et al. 2011). A second study enriched tegument glycoproteins using immobilised lectin chromatography to identify over 369 glycoproteins with a broad range of functions such as proteases, protease inhibitors, paramyosin, venom allergen-like protein II and enolase (Ravida et al. 2016). There is an over-abundance of vaccine antigen candidates from the tegument and to narrow down potential candidates, a recent study used a novel *ex vivo* immunoproteomic technique whereby contact with purified host IgG from infected animals, the flukes will slough (i.e. sheds) its tegument proteins after antibody binding has occurred (Cameron et al. 2017). This immunosloughate identified 38 proteins that could be potential vaccine antigens, (Cameron et al. 2017). Unsurprisingly, all these tegument proteomic studies identified a large number of cytoskeletal elements such as tubulin, actin and profilin that could be potential vaccine/drug targets due to the crucial function of the *Fasciola* tegument.

profilins are small actin-binding proteins that are involved in the regulation of actin polymerization by sequestering actin and ADP/ATP exchange (Krishnan & Moens 2009; Pinto-Costa & Sousa 2020). In addition, they are involved in cell signalling between the cell membrane and cytoskeleton by interacting with polyphosphoinositides (PPI) and proline-rich domain containing proteins (Krishnan & Moens 2009; Pinto-Costa & Sousa 2020). In particular, profilin from apicomplexan protozoan parasites such as *Toxoplasma gondii* have been shown to generate a potent IL-12 response in murine DCs activated through TLR11; as such, profilin from various apicomplexan protozoan parasites have been trialled as vaccine antigens (D'Angelo et al. 2009; Mansilla & Capozzo 2017; Tang et al. 2018; Yarovinsky et al. 2005). Here we describe the identification and biochemical characterization via bioinformatics, phospholipid binding, actin polymerisation and poly-L-proline affinity that a tegument protein from *F. hepatica* can be classified as belong to the profilin family and we describe its potential use as vaccine candidate.

## Materials & Methods

**Cloning and phylogenetic analysis of *Fasciola hepatica* profilin (FhProfilin)**
The native *F. hepatica* profilin sequence (accession number D915_008168) was chemically synthesized and cloned (Bioneer, USA) via *NdeI* and *XbaI* sites into a modified pET-28 vector, resulting in an open reading frame containing an N-terminal hexahistidine tag followed by an HRV 3C protease cleavage site and the *Fh*Profilin sequence. The *Phylogeny.fr* program was used to compare *Fh*Profilin with identified profilins from other parasitic species and construct a phylogenetic tree based on multiple alignments and a neighbour-joining method as well as to estimate the confidence value of the branching patterns (Dereeper et al. 2008).

**Recombinant FhProfilin expression**

Plasmid DNA containing the *Fh*Profilin sequence was transformed into BL21 (DE3) *E. coli* cells and plated onto a Luria–Bertani (LB) agar plate containing kanamycin (50 µg ml\(^{-1}\)). A single colony was inoculated into a starter culture and grown overnight in 10 ml LB medium containing 50 µg ml\(^{-1}\) kanamycin with shaking at 225 rpm. The starter culture was used at a 1:100 dilution to inoculate 400 ml of fresh LB medium containing 50 µg ml\(^{-1}\) kanamycin and grown at 37°C until the optical density at wavelength 600nm (OD600) reached 0.5. Expression of *Fh*Profilin was induced with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and the cells were allowed to grow for a further 4 h at 37°C. The cells were collected by centrifugation at 6000 g for 10 min at 4°C and were stored at -20°C.

**Recombinant FhProfilin purification**

The frozen cell pellet was thawed on ice and resuspended in 2 ml of lysis buffer (50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl and 10 mM imidazole at pH 8.0) per gram of wet cell weight. After thawing, 200 µL of 25 mg/ml lysozyme and 200 µL of 2 mg/ml DNase was added and incubated on ice for 30 minutes. The solution was sonicated with 3mm microprobe using a Sonics Vibracell VCX 130PB at 25-30% amplitude with 30 sec bursts on ice for a total sonication time of three minutes, with 30 seconds of rest in between each burst. The cell debris was removed by centrifugation at 30,000 g for 20 min at 4°C. The supernatant was added to 1 mL of 50% (w/v) Ni-Sepharose resin (Clontech) pre-washed with 5 column volumes of lysis buffer and incubated for 1 h with gentle shaking at 4°C. The lysate-nickel Sepharose mixture was loaded into a gravity flow column and the flow through collected. The column was washed with 2 column volumes of wash buffer (50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl and 20 mM imidazole,
pH 8.0) and eluted with 8 ml of elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl and 250 mM Imidazole, pH 8.0) and collected as 2 ml fractions. Each step of the purification process was validated by visualization of protein fractions using SDS–PAGE.

Fractions containing $Fh$Profilin were pooled and concentrated to 5 ml using Amicon ultracentrifugal filters (3 kDa molecular-weight cutoff; Millipore). The partially purified $Fh$Profilin was further purified by size-exclusion chromatography with a Superdex S75 16/60 gel-filtration column (GE Healthcare Life Sciences) equilibrated in TBS (10 mM Tris-HCL and 300 mM NaCl, pH 8.0) using an AKTA Basic fast protein liquid-chromatography (FPLC) system at 1 ml/min. The molecular weight, purity and identity of the $Fh$Profilin preparation were confirmed by SDS–PAGE and Western blotting.

**Actin affinity assay**

The ability of $Fh$Profilin to bind and sequester actin was investigated. Actin (5 µM) derived from bovine muscle (Sigma, USA) was induced to polymerise with 1 mM MgCl$_2$ and 0.15 M KCl. $Fh$Profilin was added in molar ratios of 1:1, 1:2 and 1:4 molar ratios to the polymerized actin in total volume of 150 µl and incubated at room temperature for 2-3 h. After incubation, the actin-profilin mixtures were centrifuged at 100,000 x g for 30 min at 20°C. Equal amounts of the supernatants and pellets fractions were analysed by SDS-PAGE.

**Phospholipid affinity assay**

PIP Strips™ (Echelon Biosciences Incorporated, USA) were used to assess the specificity of $Fh$Profilin for associating with various phospholipids. Each membrane has been spotted with 15 assorted phospholipids at 100 pmol in each spot. The membrane was blocked with 5 mL of PBS (50 mM Sodium phosphate, 150 mM NaCl, pH 7.4) plus 3% (w/v) skim milk blocking solution and gently shaken for 1 h at room temperature. The blocking solution was then discarded and various concentrations of $Fh$Profilin, starting with an initial concentration of 20 µg/mL up to 500 µg/mL, was added to 10 mL of PBS plus 3% (w/v) skim milk and gently shaken at room temperature for 1 h.
For the positive control, 5 µg/mL of PI(4,5)P$_2$ Grip™ protein in 5 mL of PBS-T (50 mM Sodium phosphate, 150 mM NaCl, 0.05% (v/v) Tween 20 pH 7.4) plus 3% (w/v) BSA was used. The protein solution was discarded, and the membrane was washed three times with 5 mL of PBS with 5 min of gentle shaking for each wash. After washing the test strip, anti-His HRP-conjugated antibody (R&D systems, USA) was diluted to 1: 10,000 in PBS with 3% (w/v) skim milk for FhProfilin and added to the membrane and incubated for 1 h at room temperature with gentle shaking. For the positive control strip, anti-GST-HRP antibody (GenScript, USA) was diluted to 1: 2,000 in PBS-T 3% (w/v) BSA. The antibody solution was discarded, and the membrane washed as previously stated. For both FhProfilin and positive control samples, detection was performed incubating the membrane with 5 ml of Clarity ECL substrate (Bio-Rad Laboratories, USA) for 5 min and imaged using the C-DiGiT blot scanner (Li-Cor).

Poly-L-proline affinity assay

The affinity of FhProfilin for proline-rich domains was investigated. Poly-L-proline (pLp) sepharose was prepared by coupling 50 mg of pLp (Sigma-Aldrich) to 1 g of cyanogen bromide-activated sepharose resin (Sigma-Aldrich). The pLp was dissolved in 4 mL of ice cold deionized water and the sepharose was resuspended in 8 mL of 250 mM sodium carbonate to make a 50% slurry. The pLp was added to the 50% slurry and stirred for 2 h at room temperature. The mixture was transferred to a cold room and stirred at 4°C overnight. The reaction was quenched with 1.2 mL of 10X bead buffer (1 M NaCl, 1 M glycine and 100 mM Tris). The resin was washed in a Buchner funnel with 500 mL of deionized water, dried and stored at 4°C in 1X storage buffer (10 mM Tris at pH 7.5, 50 mM KCl, 1 mM EDTA and 0.002% (w/v) sodium azide). 50 µL aliquots of the 50% pLp sepharose slurry with PBS was incubated with 10 nM of purified FhProfilin for test samples and 10 nM of BSA for control samples. Each sample was incubated at room temperature for 15 min and analysed by SDS PAGE. To quantify the binding effect of FhProfilin with poly-L-proline (pLp), different concentrations of FhProfilin, from 1-10 µg/µL, were incubated with the poly-L-proline sepharose beads for 15 minutes and analyzed by SDS-PAGE.

Immune profile of FhProfilin in sheep and cattle sera
*F. hepatica* were obtained from the abattoir and whole worm extract (WE) was prepared as previously reported (Swan et al. 2019). Native GST was purified as previously described by (Wijffels et al. 1992). 40 µg of WE, purified *Fh*Profilin (3 µg) and native GST (3 µg) were loaded onto an SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, USA) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, USA). After blocking with 5% (w/v) skim milk, pooled sera from experimentally infected Merino sheep (12 animals pooled, 6 weeks post-infection, infected with 200 metacercariae), Indonesian Thin Tailed (ITT) sheep (15 animals pooled, 6 weeks post-infection, infected with 200 metacercariae and cattle (6 animals pooled from 125 days post-infection, infected with 350 metacercariae (kindly donated by Prof. Terry Spithill) were incubated at a 1: 4,000 dilution with the blots in 5% (w/v) skim milk with PBS-T for 1 h. Membranes were washed three times with PBS-T and were then incubated with either anti-sheep HRP-conjugated IgG or anti-bovine HRP-conjugated IgG (Sigma-Aldrich) diluted to 1: 4,000 in 5% (w/v) skim milk in PBS-T for 1 h. Membranes were washed again as above and then were visualized by Clarity ECL substrate (Bio-Rad Laboratories, USA) and C-DiGiT blot scanner (Li-Cor) according to the manufacturer’s instructions.

**Results and Discussion**

**Bioinformatic analysis of the *Fasciola hepatica* profilin gene**

The profilin gene identified (accession number D915_008168; GEVX010111281) in *F. hepatica* (*Fh*Profilin) has an open reading frame (ORF) of 393 bp, encoding a 130 amino acid protein with a predicted molecular mass of 14 kDa and a predicted isoelectric point of 5.35, which was predicted using the “ProtParam” tool from ExPASy Bioinformatics Resource Portal (https://web.expasy.org/protparam/). A domain search using InterPro (http://www.ebi.ac.uk/interpro/), revealed the presence of a conserved profilin domain, containing putative actin binding sites (Fig. 1a). Structural homology modeling revealed that *Fh*Profilin is structural identical to *Saccharomyces cerevisiae* profilin with conservation of residues involved in proline binding however residues in a putative phosphatidylinositol 4,5-bisphosphate (PIP2)-interaction site are not conserved (Fig. 1b; Fig S1).

In a phylogenetic analysis of profilin proteins from other parasite species, it was revealed that *Fh*Profilin clustered in a clade alongside other trematode species with identities ranging from 31.1% (*Schistosoma japonicum*) to 41.5% (*Clonorchis sinensis*), while profilins from apicomplexan parasites clustered in a separate clade (Fig. 2).
Protein expression and purification of *Fh*Profilin

*Fh*Profilin was recombinantly expressed in *E. coli* and purified via immobilized metal-ion affinity chromatography, and when visualized using SDS-PAGE resulted in a single protein with an expected molecular mass of 14 kDa showing that *Fh*Profilin was successfully expressed and purified as a soluble protein (Fig. 2a). To further purify and characterize *Fh*Profilin it was subjected to size-exclusion chromatography. A typical trace (Fig. 2b) revealed that *Fh*Profilin elutes at the volume of approximately 75ml which corresponds to a molecular weight of approximately 30 kDa, suggesting that *Fh*Profilin is a dimer in solution. Several previous studies have reported that profilin from different species such as yeast, birch pollen and humans can form dimers and tetramers in solution (Babich et al. 1996; Mittermann et al. 1998; Wopfner et al. 2002). The oligomeric state of profilin is important for the function of certain profilins, for example, the allergenic potential of birch pollen is higher if profilin is in a dimeric state (Mares-Mejia et al. 2016). In addition, interactions with phosphoinositides and poly-L-proline (pLp) is regulated by an oligomeric form of profilin, with dimers having a weaker affinity to pLp than tetrameric profilin (Korupolu et al. 2009), suggesting the oligomeric state of *Fh*Profilin may regulate its function.

Biochemical characterization of *Fh*Profilin

Profilins are characterized by having three major biochemical functions (Krishnan & Moens 2009; Moreau et al. 2017; Moreau et al. 2020); firstly, profilins bind to and sequester actin monomers, therefore affecting how actin filaments polymerize. Secondly, profilins have an affinity for proline-rich domains contained within their interacting ligands and lastly, they have an affinity for phosphatidylinositol lipids that dissociate the actin:profilin complexes.

To determine if *Fh*Profilin is able to perform the classic functional profile of other profilins, several in vitro assays were performed. Recombinant *Fh*Profilin was added to polymerizing bovine actin at different ratios and monomeric actin was separated from polymerized actin by centrifugation. At all ratios of actin:profilin, the majority of actin appeared in the soluble supernatant fraction with very little polymerized actin being observed in the pellet fractions (Fig. 3). This indicates that *Fh*Profilin has a strong ability to sequester actin similar to other profilins.
from yeast and humans (Eads et al. 1998; Pinto-Costa & Sousa 2020). A major defense
mechanism of *F. hepatica* is the ability to shed its tegument proteins after binding by antibodies,
which is highly dependent on cytoskeletal rearrangement, thus making *Fh* Profilin an ideal
vaccine candidate or drug target (Hanna 1980). Human and *Plasmodium* profilin have been
explored as possible drug targets and many of the major drugs against *Fasciola* target tubulin,
thus cytoskeletal components present ideal drug targets (Fairweather et al. 2020; Kumpula &
Kursula 2015; Moens & Coumans 2015). In the future, the actin-profilin interaction could be
explored as possible drug target in *Fasciola*.

Profilin interacts with its many ligands via proline-rich sequences (Bjorkegren et al. 1993;
Kursula et al. 2008b; Mahoney et al. 1997). The majority of residues involved in polyproline
binding are conserved in *Fh* Profilin suggesting it interacts with a variety of partner proteins (Fig.
1a). To assess the ability of *Fh* Profilin to interact with polyproline, it was subjected to pulldown
assays using polyproline-sepharose beads (Fig. 4). *Fh* Profilin was successfully detected in the
bound fraction of the assay, whereas BSA was not (Fig. 4), showing that the interaction with
polyproline-sepharose is specific to *Fh* Profilin. Not all *Fh* Profilin was bound to the resin,
suggesting either the interaction was weak, or capacity of the resin was exceeded (Fig. 4). It
appears that *Fh* Profilin may bind to similar physiological substrates as human profilin and
therefore is likely to be involved in other cellular functions such as ribonucleoparticle processing
(Giesemann et al. 1999), mRNA splicing (Skare et al. 2003) and nuclear export (Stuven et al.
2003).

The ability of profilins to bind phosphatidylinositol lipids is important as it can regulate
phosphoinositide metabolism and its ability to move from the membrane to the cytosol where it
can interact with actin or other ligands (Chaudhary et al. 1998; Lambrechts et al. 2002). The
phosphatidylinositol lipid specificity of *Fh* Profilin was assessed using a mini
phosphatidylinositol lipid array (Fig. 5). There was no detectable phosphatidylinositol lipid
binding by *Fh* Profilin even at the highest concentration of 500 µg/ml (Fig. 5b). However, the
positive control protein Grip supplied with the array was positively identified binding to the
appropriate lipid phosphatidylinositol 4, 5-bisphosphate (Fig. 5c) confirming the validity of the
assay and suggesting that *Fh* Profilin has very weak or no association with phosphatidylinositol.
lipids. It is not surprising that \textit{Fh}Profilin does not bind phosphatidylinositol lipids as only two out of five PIP-2 binding sites are conserved (Fig. S1), which are normally seen in other members of the profilin family (Munkhjargal et al. 2016). Human profilin has been shown to bind to PI(3,4)P$_2$, PI(3,4)P$_2$ and PI(3,4,5)P$_3$ which is silimar to bovine profilin that binds PI(3,4,5)P$_3$ and PI(4,5)P$_2$ with some binding to PI(3)P, PI(4)P, and PI(5)P (Kursula et al. 2008a; Lu et al. 1996). This different which has been observed between the more closely related profilins from the apicomplexan parasites \textit{Plasmodium} and \textit{T. gondii} that \textit{Plasmodium} profilin can bind phosphatidylinositol lipids (PI(4)P, and PI(5)P) whereas \textit{T. gondii} profilin cannot (Kucera et al. 2010; Kursula et al. 2008a). The lack of binding of \textit{Fh}Profilin to phosphatidylinositol lipids may suggest the difference in phosphatidylinositol lipid metabolism in \textit{F. hepatica}.

\textbf{Immune profile in cattle and sheep}

Apicomplexan parasitic profilins have the ability to stimulate the immune system via toll-like receptor 11 (TLR11) due to the presence of a parasite-specific surface motif consisting of an acidic loop followed by a long β-hairpin insert (Fig. S1) (Kucera et al. 2010). Due to this immune modulation activity, profilins have been used as potential vaccine candidates and adjuvants, in particular against \textit{T. gondii} and \textit{Eimeria} spp. (Jang et al. 2011a; Jang et al. 2011b; Jang et al. 2011c; Tanaka et al. 2014). To ascertain if \textit{Fh}Profilin was exposed to the host immune system, pooled sera from experimentally \textit{Fasciola} infected animals was tested via Western Blot (Fig. 6). Immune sera from infected animals consisting of the susceptible sheep breed (Merino), the \textit{F. gigantica}-resistant Indonesian Thin Tail (ITT) sheep and cattle did not recognize the recombinant \textit{Fh}Profilin while native \textit{F. hepatica} glutathione S-transferase (n\textit{FhGST}), a major parasite excretory-secretory antigen, was recognized (LaCourse et al. 2012). This suggests that \textit{Fasciola} profilin is not exposed to immune system during infection (Fig. 6). The lack of \textit{Fh}Profilin-specific antibodies from exposed animals is unexpected as profilin from other parasites such \textit{Babesia} spp. (Munkhjargal et al. 2016) and \textit{Schistosoma japonicum} do elicit an immune response post infection (Zhang et al. 2008). Further the use of electron microscopy to perform ultrastructural studies to determine where within the tegument \textit{Fh}Profilin is located and whether this protein is exposed to the host immune system would help to assess the validity of \textit{Fh}Profilin as a vaccine candidate. A lack of direct exposure on the tegument should not exclude...
Profilin as a vaccine candidate, as the “hidden” antigen vaccines are against the nematode *Haemonchus contortus* (LeJambre et al. 2008; Munn 1997) and the tick *Rhipicephalus microplus* (Willadsen & Kemp 1988) demonstrate the commercial viability of targeting antigens of this nature, as long they are essential in function to the parasite. Profilin is essential for the survival of *P. falciparum* (Kursula et al. 2008a) and necessary for virulence of *T. gondii* (Plattner et al. 2008), suggesting that profilin could also be essential for the survival and pathogenesis of *F. hepatica*.

**Conclusions**

*Fasciola* is a zoonotic infection of worldwide concern which until recently was successfully controlled through the use of triclabendazole; however, the appearance of drug resistant parasites has required the need for the development of a vaccine or new drug targets. We have characterized a putative open reading frame that has homology to the profilin family. Profilin plays an essential role in regulating the actin cytoskeleton in all eukaryotic cells. The recombinant *Fh*Profilin displayed hallmark biochemical features of other profilins by binding to actin and polyproline, however the lack of binding to phosphatidylinositol lipids suggests that phosphatidylinositol lipid metabolism may be different in *Fasciola* compared to other parasite species. Despite recombinant *Fh*Profilin not being recognized by immune sera from infected animals, the use of *Fh*Profilin as a potential vaccine candidate is worth further investigation due to the predicted critical function of this protein to the parasite’s pathogenesis and survivability.

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**References**

Babich M, Foti LR, Sykaluk LL, and Clark CR. 1996. Profilin forms tetramers that bind to G-actin. *Biochem Biophys Res Commun* 218:125-131. 10.1006/bbrc.1996.0022
Bjorkegren C, Rozycki M, Schutt CE, Lindberg U, and Karlsson R. 1993. Mutagenesis of human profilin locates its poly(L-proline)-binding site to a hydrophobic patch of aromatic acids. *FEBS Lett* 333:123-126. 10.1016/0014-5793(93)80388-b

Cameron TC, Cooke I, Faou P, Toet H, Piedrafita D, Young N, Rathinasamy V, Beddoe T, Anderson G, Dempster R, and Spithill TW. 2017. A novel ex vivo immunoproteomic approach characterising Fasciola hepatica tegumental antigens identified using immune antibody from resistant sheep. *Int J Parasitol* 47:555-567. 10.1016/j.ijpara.2017.02.004

Chaudhary A, Chen J, Gu QM, Witke W, Kwiatkowski DJ, and Prestwich GD. 1998. Probing the phosphoinositide 4,5-bisphosphate binding site of human profilin I. *Chem Biol* 5:273-281. 10.1016/s1074-5521(98)90620-2

Cwiklinski K, and Dalton JP. 2018. Advances in Fasciola hepatica research using 'omics' technologies. *Int J Parasitol* 48:321-331. 10.1016/j.ijpara.2017.12.001

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, and Gascuel O. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 36:W465-469. 10.1093/nar/gkn180

Eads JC, Mahoney NM, Vorobiev S, Bresnick AR, Wen KK, Rubenstein PA, Haarer BK, and Almo SC. 1998. Structure determination and characterization of Saccharomyces cerevisiae profilin. *Biochemistry* 37:11171-11181. 10.1021/bi9720033

Fairweather I. 2009. Triclabendazole progress report, 2005-2009: an advancement of learning? *J Helminthol* 83:139-150. 10.1017/S0022149X09321173

Fairweather I, Brennan GP, Hanna REB, Robinson MW, and Skuce PJ. 2020. Drug resistance in liver flukes. *Int J Parasitol Drugs Drug Resist* 12:39-59. 10.1016/j.ijpddr.2019.11.003

Fonseca CT, Oliveira SC, and Alves CC. 2015. Eliminating Schistosomes through Vaccination: What are the Best Immune Weapons? *Front Immunol* 6:95. 10.3389/fimmu.2015.00095

Giesemann T, Rathke-Hartlieb S, Rothkegel M, Bartsch JW, Buchmeier S, Jockusch BM, and Jockusch H. 1999. A role for polyproline motifs in the spinal muscular atrophy protein SMN. Profilins bind to and colocalize with smn in nuclear gems. *J Biol Chem* 274:37908-37914. 10.1074/jbc.274.53.37908

Hacariz O, Sayers G, and Baykal AT. 2012. A proteomic approach to investigate the distribution and abundance of surface and internal Fasciola hepatica proteins during the chronic stage of natural liver fluke infection in cattle. *J Proteome Res* 11:3592-3604. 10.1021/pr300015p

Halton DW. 2004. Microscopy and the helminth parasite. *Micron* 35:361-390. 10.1016/j.micron.2003.12.001

Hanna RE. 1980. Fasciola hepatica: glycocalyx replacement in the juvenile as a possible mechanism for protection against host immunity. *Exp Parasitol* 50:103-114. 10.1016/0014-4894(80)90012-0

Hanna RE, Anderson A, and Trudgett AG. 1988. Fasciola hepatica: studies on vaccination of rats and mice with a surface antigen prepared from fluke homogenate by means of a monoclonal antibody. *Res Vet Sci* 44:237-241.

Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Bertrand F, Dupuis L, and Deville S. 2011a. Montanide IMS 1313 N VG PR nanoparticle adjuvant enhances antigen-specific immune
responses to profilin following mucosal vaccination against Eimeria acervulina. Vet Parasitol 182:163-170. 10.1016/j.vetpar.2011.05.019

Jang SI, Lillegohj HS, Lee SH, Lee KW, Lillegohj EP, Bertrand F, Dupuis L, and Deville S. 2011b. Montanide ISA 71 VG adjuvant enhances antibody and cell-mediated immune responses to profilin subunit antigen vaccination and promotes protection against Eimeria acervulina and Eimeria tenella. Exp Parasitol 127:178-183. 10.1016/j.exppara.2010.07.021

Jang SI, Lillegohj HS, Lee SH, Lee KW, Lillegohj EP, Bertrand F, Dupuis L, and Deville S. 2011c. Mucosal immunity against Eimeria acervulina infection in broiler chickens following oral immunization with profilin in Montanide adjuvants. Exp Parasitol 129:36-41. 10.1016/j.exppara.2011.05.021

Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, and Spithill TW. 2016. Current Threat of Triclabendazole Resistance in Fasciola hepatica. Trends Parasitol 32:458-469. 10.1016/j.pt.2016.03.002

Korupolu RV, Achary MS, Aneesa F, Sathish K, Wasia R, Sairam M, Nagarajaram HA, and Singh SS. 2009. Profilin oligomerization and its effect on poly (L-proline) binding and phosphorylation. Int J Biol Macromol 45:265-273. 10.1016/j.ijbiomac.2009.06.001

Krishnan K, and Moens PDJ. 2009. Structure and functions of profilins. Biophys Rev 1:71-81. 10.1007/s12551-009-0010-y

Kucera K, Kobiansky AA, Saunders LP, Frederick KB, De La Cruz EM, Ghosh S, and Modis Y. 2010. Structure-based analysis of Toxoplasma gondii profilin: a parasite-specific motif is required for recognition by Toll-like receptor 11. J Mol Biol 403:616-629. 10.1016/j.jmb.2010.09.022

Kumpula EP, and Kursula I. 2015. Towards a molecular understanding of the apicomplexan actin motor: on a road to novel targets for malaria remedies? Acta Crystallogr F Struct Biol Commun 71:500-513. 10.1107/S2053230X1500391X

Kursula I, Kursula P, Ganter M, Panjikar S, Matuschewski K, and Schuler H. 2008a. Structural basis for parasite-specific functions of the divergent profilin of Plasmodium falciparum. Structure 16:1638-1648. 10.1016/j.str.2008.09.008

Kursula P, Kursula I, Massimi M, Song YH, Downer J, Stanley WA, Witke W, and Wilmanns M. 2008b. High-resolution structural analysis of mammalian profilin 2a complex formation with two physiological ligands: the formin homology 1 domain of mDia1 and the proline-rich domain of VASP. J Mol Biol 375:270-290. 10.1016/j.jmb.2007.10.050

LaCourse EJ, Perally S, Morphew RM, Moxon JV, Prescott M, Dowling DJ, O’Neill SM, Kipar A, Hetzel U, Hoey E, Zafra R, Buffoni L, Perez Arevalo J, and Brophy PM. 2012. The Sigma class glutathione transferase from the liver fluke Fasciola hepatica. PLoS Negl Trop Dis 6:e1666. 10.1371/journal.pntd.0001666

Lambrechts A, Jonckheere V, Dewitte D, Vandenkerckhove J, and Ampe C. 2002. Mutational analysis of human profilin I reveals a second PI(4,5)-P2 binding site neighbouring the poly(L-proline) binding site. BMC Biochem 3:12. 10.1186/1471-2091-3-12

Lammas DA, and Duffus WP. 1983. The shedding of the outer glycocalyx of juvenile Fasciola hepatica. Vet Parasitol 12:165-178. 10.1016/0304-4017(83)90005-5

LeJambre LF, Windon RG, and Smith WD. 2008. Vaccination against Haemonchus contortus: performance of native parasite gut membrane glycoproteins in Merino lambs grazing contaminated pasture. Vet Parasitol 153:302-312. 10.1016/j.vetpar.2008.01.032

Lu PJ, Shieh WR, Rhee SG, Yin HL, and Chen CS. 1996. Lipid products of phosphoinositide 3-kinase bind human profilin with high affinity. Biochemistry 35:14027-14034. 10.1021/bi961878z

Mahoney NM, Janney PA, and Almo SC. 1997. Structure of the profilin-poly-L-proline complex involved in morphogenesis and cytoskeletal regulation. Nat Struct Biol 4:953-960. 10.1038/nsb1197-953
496 Sobhon P, Anantavara S, Dangprasert T, Viyanant V, Krailas D, Upatham ES, Wanichanon C, and Kusamran T. 1998. Fasciola gigantica: studies of the tegument as a basis for the developments of immunodiagnosis and vaccine. *Southeast Asian J Trop Med Public Health* 29:387-400.

497 Stuven T, Hartmann E, and Gorlich D. 2003. Exportin 6: a novel nuclear export receptor that is specific for profilin:actin complexes. *EMBO J* 22:5928-5940. 10.1093/emboj/cdg565

500 Swan J, Sakhthivel D, Cameron TC, Faou P, Downs R, Rajapaksha H, Piedrafita D, and Beddoe T. 2019. Proteomic identification of galectin-11 and -14 ligands from Fasciola hepatica. *Int J Parasitol* 49:921-932. 10.1016/j.ijpara.2019.06.007

504 Toet H, Piedrafita DM, and Spithill TW. 2014. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. *Int J Parasitol* 44:915-927. 10.1016/j.ijpara.2014.07.011

516 Wilson RA, Wright JM, de Castro-Borges W, Parker-Manuel SJ, Dowle AA, Ashton PD, Young ND, Gasser RB, and Spithill TW. 2011. Exploring the Fasciola hepatica tegument proteome. *Int J Parasitol* 41:1347-1359. 10.1016/j.ijpara.2011.08.003

525 Zhang SM, Lv ZY, Zhou HJ, Zhang LY, Yang LL, Yu X, Zheng H, and Wu ZD. 2008. Characterization of a profilin-like protein from Schistosoma japonicum, a potential new vaccine candidate. *Parasitol Res* 102:1367-1374. 10.1007/s00436-008-0919-2
Figure 1

Bioinformatic analysis of *F. hepatica* profilin (FhProfilin) gene.

A) The amino acid sequence for FhProfilin (accession number D915_008168; GEVX010111281). The red letters indicate the profilin family domain. Black triangles indicate the putative actin-binding sites. B,C) Homology model of FhProfilin showed as a ribbon (B) and the crystal structure of *Saccharomyces cerevisiae* profilin (1YPR) (C). The residues involved in phosphatidylinositol phosphate interaction (light pink) and proline binding (light green). D) Phylogenetic tree of the *F. hepatica* profilin was produced online using Phylogeny.fr (Dereeper et al. 2008). The genebank or UniProtKB accession numbers used to construct the tree appear after each species name. Numbers shown at branch nodes indicate bootstrap values.
Figure 2

Recombinant expression and purification of FhProfilin

A) Cell lysate expressing FhProfilin were applied to a NI-IDA column and washed twice before elution with imidazole. 15 μl of each stage of the purification was resolved by SDS-PAGE and stained with Coomassie blue. B) Size-exclusion chromatography trace of recombinant FhProfilin. Arrows indicate the elution volumes of proteins of known molecular weight. Insert: 15 μl of each fraction was resolved by SDS-PAGE and stained with Coomassie blue.
Figure 3

SDS-PAGE analysis of polymerized actin incubated with FhProfilin.

Different ratios of actin to FhProfilin (1:1, 1:2 and 1:4) were incubated and separated into polymerised or monomeric actin fractions by centrifugation. Control lanes contained no FhProfilin. The pellet (P) and supernatant (S) fractions (15 µL) were resolved by SDS-PAGE and stained with Coomassie blue.
Figure 4

Binding of FhProfilin to phospholipids.

A) The location of the different phospholipids on the membrane (Echelon Biosciences Incorporated, USA). B) FhProfilin and C) GST-Grip respectively binding to phospholipid microarrays detected using an anti-hexhistidine and anti-GST antibody.
Figure 5

Binding of FhProfilin to polyproline sepharose.

Equal amounts of FhProfilin and BSA were passed over polyproline sepharose. Unbound (S) and Bound (B) proteins were visualized by SDS-PAGE and Coomassie staining.
Figure 6

Western blot analysis of FhProfilin with immune sera.

Immune sera from infected ITT sheep (A), Merino sheep (B) and cattle (C) was probed on whole fluke extract (WE), recombinant FhProfilin (P) and native GST (G). The experiment was repeated at least 3 times.