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FASCIOLA HEPATICA AND SCHISTOSOMA MANSONI: IDENTIFICATION OF COMMON PROTEINS BY COMPARATIVE PROTEOMIC ANALYSIS

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Abstract

It is not unusual to find common molecules among parasites of different species, genera, or phyla. When those molecules are antigenic, they may be used for developing drugs or vaccines that simultaneously target different species or genera of parasite. In the present study, we used a proteomic-based approach to identify proteins that are common to adult *Fasciola hepatica* and *Schistosoma mansoni*. Whole-worm extracts from each parasite were separated by 2-dimensional electrophoresis (2-DE), and digital images of both proteomes were superimposed using imaging software to identify proteins with identical isoelectric points and molecular weights. Protein identities were determined by mass spectrometry. Imaging and immunoblot analyses identified 28 immunoreactive proteins that are common to both parasites. Among these molecules are antioxidant proteins (thioredoxin and glutathione-S-transferase), glycolytic enzymes (glyceraldehyde 6-phosphate dehydrogenase and enolase), proteolytic enzymes (cathepsin-L and -D), inhibitors (Kunitz-type, Stefin-1), proteins with chaperone activity (heat shock protein 70 and fatty acid-binding protein), and structural proteins (calcium-binding protein, actin, and myosin). Some of the identified proteins could be used to develop drugs and vaccines against fascioliasis and schistosomiasis.

It is not unusual to find common molecules between species of various genera, families, or phyla. The sharing of molecules able to elicit immune responses between different species of various genera is known as antigenic community, and it is responsible for antigenic cross-reactivity (Losada et al., 2005). The sharing of molecules among organisms is an expected finding because there are many molecules, such as enzymes, hormones, receptors, etc., that have been conserved during evolution. This has special relevance for the identification of molecules with potential for drug or vaccine development effective against different species or genera of organisms.

Fasciola hepatica and *Schistosoma mansoni* are digenetic trematodes that have a major detrimental impact on animal and human health worldwide (Chen and Mott, 1990; Savioli et al., 2002; Mas-Coma, 2005). Worldwide losses in agriculture due to fascioliasis are estimated at over \$2 billion dollars per year due to an increase in animal mortality and a reduction in productivity (Spithill and Dalton, 1998). Moreover, more than 17 million persons are infected and 180 million are at risk of infection in endemic areas of America and

Africa. Currently, there are 210 million persons infected with *S. mansoni* (Berriman et al., 2009) and up to 779 million people at risk of acquiring the infection (Hotez et al., 2009). There are no vaccines against fascioliasis or schistosomiasis. The global strategy for the control of both diseases is the use of chemotherapy. However, only 2 drugs are currently available, i.e., triclabendazole against fascioliasis and praziquantel for schistosomiasis (Keiser and Utzinger, 2004; Keiser et al., 2005). Hence, there is a need to develop novel drugs or vaccines to control both diseases in view of growing concern about resistance developing to existing drugs (Keiser et al., 2005; Melman et al., 2009). The identification of proteins common to *F. hepatica* and *S. mansoni* could provide targets for developing drugs or vaccines that can be simultaneously effective against both organisms. To date, the fatty acid-binding proteins (FABP-Fh15 and Sm14) have been the only *F. hepatica*/*S. mansoni*-common proteins characterized and exploited as a potential dual-vaccine (Ramajo et al., 2001; Vilar et al., 2003; Ramos et al., 2009). However, because *F. hepatica* and *S. mansoni* are parasites with great antigenic complexity, it is expected that they possess common proteins other than FABPs that might contribute to serological cross-reactivity.

The aim of the present study was to identify common proteins of the 2 parasites through analysis of adult worm crude extracts by a proteomic-based approach. After optimizing the separation of both protein extracts by 2-dimensional electrophoresis (2-DE), digital images of the proteomes were superimposed, and protein spots with identical isoelectric points and molecular weights (MWs) were selected for analysis by MALDI-TOF MS/MS. This strategy led us to identify 28 common proteins between *F. hepatica* and *S. mansoni* adult worms, the cross-reactivity of which was confirmed by Western blot.

MATERIALS AND METHODS

Parasites

Adult *Fasciola hepatica* flukes were collected from the bile ducts of infected cattle killed at a local slaughterhouse. Flukes were washed repeatedly with 0.1 M phosphate-buffered saline (PBS), pH 7.2, to eliminate all traces of blood and bile, and then specimens were stored at -20°C until use.

Twenty Swiss mice infected with 150 *S. mansoni* cercaria via skin penetration were obtained from the Biomedical Research Institute, Rockville, Maryland, and necropsied 56 days after infection to recover adult worms by perfusion from the portal system. Worms were washed repeatedly with PBS and then stored at -20°C until use. Mice were kept under conventional germ-free conditions in the animal care facility of the School of Medicine, University of Puerto Rico, and treated according to regulations for the care of laboratory animals.

Preparation of *F. hepatica* and *S. mansoni* whole-worm extracts

Parasites collected as described in the previous paragraphs were repeatedly washed with PBS supplemented with 5 mM EDTA, 8 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM iodoacetamide, and 2 mM leupeptine to inhibit proteases. Whole-worm extracts (*F. hepatica* and *S. mansoni*) were obtained by homogenizing 1 g of worms on ice in PBS using a Ten Broeck tissue grinder, followed by centrifugation (30,000 *g*, 4°C , 30 min). *Fasciola hepatica* and *S. mansoni* extracts were partially simplified by an ultrafiltration system using Amicon stirred cells (Millipore, Billerica, Massachusetts), in which proteins are passed through a high-flow membrane (YM-100) to exclude all proteins >100 kDa. Proteins that passed through membrane were stored at -20°C until use. Protein concentrations were determined using the bicinchoninic acid method (Stoscheck, 1990).

Serum samples

Serum samples obtained from 15 persons with chronic schistosomiasis were obtained from the serum library of the Institute of Tropical Medicine, Central University of Caracas, Venezuela. *Schistosoma mansoni* infection had been confirmed in all these patients by Kato-Katz techniques. Fifteen control serum samples were also obtained from the University of Puerto Rico serum library. Serum samples were serologically analyzed for schistosomiasis and fascioliasis by ELISA using *F. hepatica* and *S. mansoni* soluble extracts following a basic protocol with few modifications (Espino et al., 1987). Briefly, the protein concentration used to coat the polystyrene plate (Costar Corning, New York, New York) was 5 µg/ml for each extract; sera were diluted 1:100 in PBS containing 0.05% Tween-20 (PBST), and horseradish peroxidase-conjugate anti-human IgG was diluted 1:5,000 in blocking solution (3% bovine albumin in PBST). Substrate consisting of 10 mg orthophenylendiamine, 25 ml PBS, and 10 µl of 30% hydrogen peroxide was added to each well. The plates were incubated in the dark for 10 min at room temperature. Enzymatic reaction was stopped with 50 µl per well of 12.5% sulfuric acid, and absorbance values were read at 492 nm using a microplate reader (BioRad, Hercules, California). Serum samples with absorbance value 0.42 (cutoff value previously established), either in the *F. hepatica*-ELISA or in the *S. mansoni*-ELISA assay, were considered seropositive for schistosomiasis or fascioliasis.

Two-dimensional electrophoresis

Fasciola hepatica and *S. mansoni* samples containing 250 µg of total protein and rehydration buffer for pH 7–10 immobilized pH gradient (IPG) strips were used for rehydration (BioRad). After standing overnight at room temperature, the samples were mixed gently for 1 hr and then centrifuged at 18,000 *g* for 30 min. Separation in the first dimension (isoelectric focusing; IEF) was performed on IPG strips (passive in-gel rehydration 20 C, overnight) for a total 20,000 Vh on a BioRad protein IEF cell (maximum current 50 µA/strip). Afterward, the strips were reduced and alkylated in equilibration buffer (6 M urea, 2% SDS, 0.05 M Tris-HCl, pH 8.8, 50% glycerol, and 2% [w/v] dithiothreitol [DTT]). The second-dimension separation was performed in triplicate through a 4–20% gradient SDS-polyacrylamide gel (15 mA/gel for 15 min, then 30 mA/gel); 2-DE gels were stained with BioSafe Coomassie Blue G-250 (BioRad).

Image analysis of 2-DE gels using PDQuest software

BioSafe Coomassie Blue-stained gels were scanned using a VERSA-DOC Model 1000 imaging system (BioRad). Quantity One version 4.3.0 (BioRad) software and PDQuest version 7.4.0 (BioRad) software were used to detect common spots on *F. hepatica* and *S. mansoni* gels. The experimental set was created for pI 3–10 gels containing images of 6 gels (3 from *F. hepatica* and 3 from *S. mansoni*). After automatic detection of spots by the PDQuest software, the files were manually edited to correct artifacts. The software calculated individual spot “volumes” by density/area integration; then, to eliminate gel-to-gel variation, individual spot volumes for each gel were normalized relative to the total spot volume of that gel. Normalized spot volume data from each experimental set were exported to Microsoft Excel, where differences in expression of spots between the *F. hepatica* and *S. mansoni* groups were analyzed using Student’s *t*-test, with *P* < 0.05 as the criterion for statistical significance. Common protein spots between the 2 species showing 2-spot relative abundance were selected and marked for excision for further MS analysis. All experiments were performed in biological and technical triplicates.

In-gel digestion of proteins, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and database searching

Spots of interest (identified as the same on 2-DE gels and confirmed later by MS/MS) were manually excised from Coomassie Blue-stained gels, destained using 40% methanol/10% acetic acid, reduced (with 10 mM DTT in 50 mM ammonium bicarbonate), and then alkylated (using 55 mM iodoacetamide in 50 mM ammonium bicarbonate). The resulting gel fragments were rinsed with 50 mM ammonium bicarbonate and acetonitrile and dried under a stream of nitrogen. Samples were digested with trypsin (Sigma, St. Louis, Missouri) and combined with an equal volume of saturated cyano-4-hydroxycinnamic acid in 50% acetonitrile/ 0.1% trifluoroacetic acid. Half of this mixture was applied to a MALDI target plate. MALDI TOF-MS was performed at the University of Texas Medical Branch, Proteomics Facility (Austin, Texas). Data were acquired with an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer (Foster City, California). Following MALDI MS analysis, MALDI MS/MS was performed on several (5–10) abundant ions from each sample spot. Applied Biosystems GPS Explorer™ (v. 3.6) software was used in conjunction with MASCOT to search NCBI nr databases to identify common proteins between the 2 species using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined by using expectation values and/or the MASCOT search engine (Matrix Science, London, U.K.).

Immunoblot analysis

Fasciola hepatica proteins separated by 2-DE were transferred to nitrocellulose membranes (BioRad) for 2 hr at 200 mA in transfer buffer (48 mM Tris/HCl, pH 8.3, 39 mM glycine, and 20% methanol) at 4 C. The membrane was blocked for 30 min with 5% skim milk in PBST at room temperature. After rinsing 3 times for 5 min with PBST, the membrane was incubated overnight at 25 C with a pool of schistosomiasis sera (diluted 1:100 in PBST) selected from those that were seropositive in the ELISA assay either for *S. mansoni* or *F. hepatica* extract. After incubation, the membrane was rinsed 3 times for 10 min and incubated with goat anti-human IgG secondary antibody conjugated to horseradish peroxidase (BioRad) at a dilution of 1:5,000 in PBST containing 5% skim milk for 1 hr at 37 C. After 3 more washes with PBST, the blots were developed using diaminobenzidine substrate.

RESULTS

2-DE analysis of *F. hepatica* and *S. mansoni* samples

Fasciola hepatica and *S. mansoni* extracts were first electro-focused using 3–10 linear immobilized pH gradient strips and then electrophoresed in 4–20% gradient polyacrylamide gels. Staining of these gels with Coomassie Blue permitted the detection of 361 spots for *F. hepatica* and 433 for *S. mansoni* located in a broad MW range of 6–85 kDa and a pH range of 3–10 (Fig. 1A). Scanned 2-DE gel images were imported into PDQuest, and the multichannel viewer function was applied for gel image overlay (Fig. 1B). The *F. hepatica* gel was used as the master to compare *F. hepatica* to *S. mansoni*. PDQuest software allowed us to overlap shared spots between the 2 species based on MWs and isoelectric points. In total, 333 protein spots that did not overlap any other spots were detected in *F. hepatica*, whereas 405 were detected in *S. mansoni*. Twenty-eight overlapping spots that were significant at $P < 0.05$ were detected on 2D gels and identified by peptide mass fingerprinting. Spots at the same position were assigned the same spot number by the software. A “3D” representation of the 2D gel sections containing 28 common spots from both parasites is shown in Figure 1A. Of the common proteins, 10 were expressed at lower levels in *F. hepatica* than in *S. mansoni* by 1.37- to 13.24-spot relative abundance, 9 were

expressed at 1.38- to 5.59-spot relative abundance in *F. hepatica* compared to *S. mansoni*, and 9 had similar relative abundance (Table I).

Identification of common spots by MALDI-TOF MS analysis

The overlapping spots identified by their peptide mass fingerprinting correspond to 28 proteins, of which 10 are isoforms of 7 identified proteins (Table I). Quantitative analysis of the proteins that were common to both parasite species revealed substantial matching in the pattern of protein expression. All proteins listed in the table are the highest ranked candidates that were unambiguously identified in the MASCOT search. To confirm protein identification, a 2-DE spot from each parasite species was sequenced twice by MS. In 2 cases, spots were identified based on significant hits of proteins of organisms not related to *F. hepatica* or *S. mansoni*. These include spot 11 (related to *Archaeopotamobius sibiriensis* triose-phosphate isomerase, GenBank CAD29286) and spot 21 (related to *Marsupenaeus japonicus* heat shock protein, GenBank ABF8360). To confirm these 2 identifications, BLAST analysis was performed, and the amino-acid sequences were matched to the sequence alignment with the corresponding available *F. hepatica* and *S. mansoni* sequences. The sequences of *F. hepatica* Hsp-70 (GenBank ABS52704) and *S. mansoni* Hsp-70 (XP_002581472) were found to be 93% identical (score 5.3 e-88) to the *M. japonicus* sequence through 643 overlapping amino acids. The sequence of *S. mansoni* TPI (XP_002571861.1) was found to be 71.4% identical to *A. sibiriensis* TPI (score 7.4 e-86) over 244 overlapping amino acids. No records for TPI of *F. hepatica* were available in the database. The identified proteins together with changes in protein abundances are listed in Table I. The proteins identified were grouped into 4 categories of functionally related molecules (oxidative stress, energy metabolism, proteosomal proteins, and structural proteins).

F. hepatica/*S. mansoni* common antigenic proteins

To investigate the antigenicity of common *F. hepatica*/*S. mansoni* proteins, *F. hepatica* blots were incubated with a pool of negative sera and a pool of sera from patients with schistosomiasis that had been found doubly positive in the *S. mansoni*-ELISA and *F. hepatica*-ELISA assays. When *F. hepatica* blots were probed with negative control sera, no proteins were detected (data not shown). However, when probed with the pool of schistosomiasis sera, large numbers of immunoreactive spots were revealed. However, because our goal was to determine whether the common spots were antigenic, peptide mass fingerprinting was limited to those immunoreactive spots that matched with the common spots. The analysis revealed that the 28 common proteins identified herein were antigenic and, therefore, are cross-reactive proteins (Fig. 2).

DISCUSSION

In view of our aim to identify proteins that are common to *F. hepatica* and *S. mansoni*, in the present work we started by obtaining soluble extracts from whole adult worms. Although complex mixes of proteins make for difficult proteomic analysis, the examination of these crude extracts, instead of more simplified material, had the advantage of increasing the chance of detecting the most abundant proteins expressed by both parasites, and that could be relevant with respect to the aim of our study. As is well known, a simpler composition like, for instance, the excretory/ secretory products result in much lower protein yields, which are highly specific and useful for immunodiagnosis (Espino et al., 1987; Carnevale, Rodriguez, Guarnera et al., 2001; Carnevale, Rodriguez, Santillan et al., 2001; Salimi-Bejestani, Daniel, Felstead et al., 2005; Salimi-Bejestani, Daniel, McGarry et al., 2005; Arias et al., 2007).

To enable effective electrophoretic separation of the protein extracts, molecules of high MW (>100 kDa) were excluded, which facilitated conditions for protein sample preparation and solubilization for 2D-polyacrylamide gel electrophoresis (PAGE) that were easily optimized for each species (Jefferies et al., 2001; Gelhaus et al., 2005; Perez-Sanchez et al., 2006). The proteomic maps of *F. hepatica* and *S. mansoni* revealed considerable similarity with respect to the number of spots detected (361, 433) as the distribution of the spots across nearly the same pI ranges and MW. This similarity is not surprising since both extracts contain all of the proteins expressed by the adult worm, including those present on the exposed tegumental surfaces, somatic, and excretory-secretory proteins; both extracts are likely to share components, as was observed.

For adult *F. hepatica*, there are no equivalent proteomic maps with which to compare our results. However, there are maps for the soluble proteome of adult *S. mansoni* (Curwen et al., 2004) and *S. japonicum* (Cheng et al., 2005), containing between 847 and 1,288 spots. Differences in the number of spots obtained by us compared with previous studies could be due to differences in the extract preparation or in the sensitivity of staining used to reveal the protein spots. Our extracts were prepared by homogenization of adult worms and ultracentrifugation at 30,000 *g*, followed by a molecular sieving separation to exclude molecules >100 kDa. Extracts used in earlier studies were not partially purified (Curwen et al., 2004).

The identified common protein spots (28) between adult *F. hepatica* and *S. mansoni* were revealed by Coomassie Blue staining. It is not surprising that the number of immunoreactive spots detected by Western blot is higher than the number revealed by Coomassie Blue staining since Western blot is a much more sensitive technique. Therefore, this preliminary study doesn't rule out the existence of other shared spots, but emphasizes the most abundant proteins common between both species. The great amount of information available in the databases on the proteome, transcriptome, and genome of *S. mansoni* (Berriman et al., 2009; Criscione et al., 2009; Mathieson and Wilson, 2010; Dewalick et al., 2011; Farias et al., 2011) and *F. hepatica* (Cancela et al., 2010; Alasaad et al., 2011; Chemale et al., 2011) confirms this hypothesis.

Fasciola hepatica and *S. mansoni* have evolved in similar ways to avoid the immune responses of their hosts (McManus and Dalton, 2006). Therefore, it was not surprising that several of the common proteins identified herein have biological functions related to immune evasion mechanisms. Thioredoxin (TRX) is a low MW (~12 kDa) protein that is maintained in its active, reduced form by the flavoenzyme thioredoxin reductase. TRX has been found in many parasites, including *F. hepatica* (Salazar-Calderon et al., 2001) and *S. mansoni* (Alger et al., 2002). These findings and growing evidence that TRX acts as a redox-regulating molecule to maintain the cellular redox state suggest that this protein plays a role in protecting parasites from host immune responses (Alger et al., 2002). Thiol-specific antioxidant protein (TSA) protects cellular components from oxidative damage caused by reactive oxygen species (ROS) (O_2^- , H_2O_2 , and HO^-). Protection is achieved through the removal of hydrogen peroxide, preventing the formation of hydroxyl radicals. It has been demonstrated that in the very early stages of *F. hepatica* infection, the parasite secretes proteins, including TSA, that participate in the recruitment of an alternative activated macrophage population. These activated macrophages secrete large amounts of IL-4 and low quantities of IL-12, which are typical of a Th2 response (Donnelly et al., 2005). The development of a Th2 response is associated with infection susceptibility and favors chronic disease (O'Neill et al., 2000, 2001; Donnelly et al., 2005). Members of the TSA family also participate in egg granuloma formation during *S. mansoni* infection, which is mediated by $CD4^+$ T helper cells sensitized to egg antigens (Williams et al., 2001).

Glutathione-S-transferase (GST) is an enzyme found in all animals, and it plays a role in the detoxification and removal of harmful molecules. The 26-kDa GST from *F. hepatica* and *S. mansoni* is an antigenic protein excreted by the parasites and transiently expressed on tegumental surfaces (Abath and Werkhauser, 1996). For animal schistosomiasis, GSTs have been reported to provide a partial protection (Grzych et al., 1998), but also to be ineffective (De Bont et al., 2003). For animal fascioliasis, GSTs are known to be effective in inducing protection (Sexton et al., 1994; Paykari et al., 2002; Preyavichyapugdee et al., 2008). It has been recently demonstrated that GSTs can confer protection against *F. hepatica* and *S. mansoni* infection (Preyavichyapugdee et al., 2008).

Many parasites deploy proteinases such cathepsin-D and -L to accomplish some of the tasks necessitated by a parasitic life style, including tissue penetration, digestion of host tissue for nutrition, and evasion of host immune responses. Cathepsin-D is a protease that plays a role in the proteolysis of host-derived hemoglobin. This protease is expressed in the gastrodermis and the cecal lumen of adult schistosomes of both sexes and on the dorsal and lateral surfaces of the tegument of male *S. mansoni* and *S. japonicum* (Bogitsh et al., 2001; Verity, Loukas et al., 2001; Verity, McManus, and Brindley, 2001). Some reports indicate that after vaccination with this protein, mean total worm burdens were significantly reduced by 21–38% in mice challenged with *S. japonicum* cercariae (Verity, Loukas et al., 2001).

Cathepsin-L is a lysosomal cysteine proteinase of the papain superfamily involved in the catabolism of mammalian cell proteins. It has been demonstrated that cathepsin-L can disrupt immune defense mechanisms directed against parasites by facilitating both migration of parasites through the host tissues and acquisition of nutrients from the host (Dalton et al., 1996). *Fasciola hepatica* cathepsin-L is probably the most studied trematode peptidase, with well-characterized critical functions. Due to the importance of peptidases in host–parasite interactions, they are considered to be promising targets for the development of novel chemotherapeutic drugs, vaccines, and also as selective diagnostic markers of disease (Perez-Sanchez et al., 2006).

The Kunitz-type (KT) molecule of *F. hepatica* is a single polypeptide of 58 amino acids (6.75 kDa) that was identified by its significant similarity to the Kunitz-type (BPTI) family of proteinase inhibitors of plants (Bozas et al., 1995). KT proteins typically inhibit serine proteinases, but the family also includes inhibitors of cysteine and aspartyl proteinases (Rawlings et al., 2004). Proteinases are involved in key areas of the host antiparasite immune response, including antigen presentation, effectors function, and tissue dissolution and remodeling. There is substantial evidence that parasites utilize protease inhibitors to protect themselves against degradation caused by host proteases (McKean and Pritchard, 1989), to facilitate feeding (Harrison et al., 2002), and to manipulate the host response to the parasite (Pfaff et al., 2002). Because KT and other types of inhibitors have been found either on, or within, the tegument of *F. hepatica* (Bozas et al., 1995) and *S. mansoni* (Blanton et al., 1994), a possible function could be to protect the tegument from attack by the host.

Stefin-1 is a poorly studied protein that has been classified as a type 1 cystatin, a cysteine protease inhibitor recently identified as a major released antigen of *Fasciola gigantica* (Tarasuk et al., 2009). This protein was found to be abundant in the parasite tegument, where it was found partially complexed with cathepsin-L. Stefin-1 is able to react with sera from rabbits experimentally infected with *F. gigantica*, and studies suggest protective functions, regulating intracellular cysteine activity, and possibly providing protection against extracellular proteolytic damage to the parasite's intestinal and tegumental surface proteins (Tarasuk et al., 2009).

Fatty acid-binding proteins (FABPs) are involved in the acquisition of fatty acids from mammalian host blood. It has also been suggested that FABPs play a role in the protection

of cell membranes via buffering against the effects of high fatty acid concentrations (Das et al., 1991). Several isoforms of the *F. hepatica*-FABP with pI values from 5.11 to 7.82 have been reported (Espino, Dumenigo, and Hillyer, 2001). The FABPs from *F. hepatica* (Fh15) and *S. mansoni* (Sm14) are the only pair of common proteins that to date have been targeted for dual vaccines against both parasites (Vilar et al., 2003).

An interesting common protein identified in the present study is enolase. This protein, also known as phosphopyruvate dehydratase, is a metalloenzyme that participates in the glycolytic pathway. It has also been identified on the surface of different pathogens such as bacteria, fungi, and protozoa (Pancholi, 2001) and, more recently, on helminths (Jolodar et al., 2003). This enzyme has also been implicated in autoimmune diseases, and its utilization by pathogens while invading host tissue is well documented (Pancholi, 2001). Enolase is a major protein in the excretory/secretory products of *F. hepatica* (Bernal et al., 2004) and, therefore, is a key player in understanding the host-parasite interaction, and it offers a target for chemo- and immunotherapy (Morphew et al., 2007). Enolase has been identified as one of the proteins used by *S. mansoni* to interfere with the innate immune system of the snail *Biomphalaria glabrata* (Guillou et al., 2007). The immunogenicity of enolase and its potential as vaccine have been demonstrated against *Streptococcus suis* (Feng et al., 2009; Zhang et al., 2009) and *Echinococcus granulosus* (Gan et al., 2010).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an important enzyme of glycolysis and gluconeogenesis. *Fasciola hepatica* adults are thought to continually shed their teguments to evade the host immune system (Morphew et al., 2007). Like many other proteins, GAPDH is located at the surface or just below the surface of the fluke, and it may be released into the surrounding medium as the tegument is sloughed. The presence of GAPDH on the surface of schistosomes may help to protect the parasite from oxygen-mediated attack by phagocytes; indeed, the presences of IgG antibodies to this enzyme are related to resistance to infection (Waite et al., 1993).

Actins are highly conserved proteins that, associated with myosins, are responsible for many types of cell movements. Myosins are the prototype of a molecule-motor, a protein that converts chemical energy in the form of ATP to mechanical energy, thus generating force and movement. *Fasciola hepatica* and *S. mansoni* rely on a well-developed muscular system, not only for attachment, but for many aspects of their biology. Actin and myosin form part of the spines on the surface of the schistosomes (Jones et al., 2004). In *F. hepatica*, actin and myosin are located in the tegument and in the muscle surrounding the ventral sucker (Kumar et al., 2003; Tansatit et al., 2006). Previous studies identified actin as the receptor on the parasite surface that binds praziquantel, a drug widely used against schistosomiasis, facilitating its distribution throughout the worm (Tallima and El Ridi, 2007). Moreover, earlier studies have shown that a fragment of recombinant *S. mansoni* myosin induces high levels of protection in experimentally infected animals (Soisson and Strand, 1993). Based on these antecedents, we could anticipate that actin and myosin have potential as vaccine candidates or targets for drugs against both parasites.

Calcium-binding protein (CaBP) plays an important physiological role in the cell; it participates in calcium signaling pathways that contribute to all aspects of cell function. CaBPs have been found in all eukaryotic organisms, including *F. hepatica* (Russell et al., 2007) and *S. mansoni* (Mohamed et al., 1998). In *S. mansoni*, CaBP is associated with organ tissue remodeling via the modulation of key proteasome activity during transformation of cercariae to schistosomula (Ram et al., 2003). In *F. hepatica*, CaBP has been identified in the tegument in close association with myosin-light chain muscle contraction and is considered to be a good target for the development of new drugs (Russell et al., 2007).

Heat shock proteins (Hsp70) are the most conserved and ubiquitous proteins known to date (Gupta and Golding, 1993), and they are likely to play important roles in humoral responses against infection. Sm Hsp70 induces a dominant antibody response in humans and animals with both acute and chronic *S. mansoni* infection, and also in mice vaccinated with irradiated cercariae (Moser et al., 1990). This protein is strongly expressed in baboons infected with *S. mansoni* (Kanamura et al., 2002). Recently, the Hsp70 homologues of *F. hepatica* and *F. gigantica* were cloned and characterized (Smith et al., 2008); they are highly expressed in both species.

Other identified molecules include phosphatase 2A inhibitor and hemoglobin (Hb). The phosphatase 2A inhibitor is a multitasking protein involved in apoptosis, transcription, nucleosome assembly, and histone binding. A 28-kDa protein for *S. japonicum* was recently identified (Berriman et al., 2009), but its role in the biology of trematodes remains unknown. The physiological roles of trematode Hbs are a matter of debate. Most adult parasitic trematodes live mainly in a semi-anaerobic environment. Their Hbs displays such high oxygen affinities that it cannot simply serve in O₂ transport to the tissues. Therefore, other functions for these Hbs, such as oxygen scavenging, heme reserve for egg production, and NO dioxygenase, have been proposed (de Guzman et al., 2007). In addition, they may be involved in host–parasite interactions. Indeed, parasitic trematodes are known to secrete/excrete a set of proteins (ES proteins) into the host (Berasain et al., 2000). Among these, hemoglobin-like proteins have been identified (Dalton et al., 1996). The ES proteins, including Hbs, are potent antigens that are potentially useful in eliciting host immunological resistance against the parasitic infection through vaccination (Dewilde et al., 2008). A vaccine trial in cattle with Hb resulted in a 43.8% protection level against fluke infection (Dalton et al., 1996). Therefore, trematode Hbs appear to be important, yet to date scarcely characterized targets for new vaccination strategies.

In conclusion, we report through this study a novel and reproducible protocol for the analysis of the proteome of the 2 parasites in the adult stage, *F. hepatica* and *S. mansoni*, using 2-dimensional gel electrophoresis (2-DE) and mass spectrometry (matrix-assisted laser desorption ionization time-of-flight spectrometry [MALDI-TOF]). We were also able to determine the level of proteomic 2-DE spot matching between these 2 parasites, as well as conduct quantitative analysis of the proteins that were common to both parasites. Twenty-eight proteins common to *F. hepatica* and *S. mansoni* were identified as antigenic. This set will form the basis of further studies aimed at understanding the ways in which these proteins interact with their hosts. This study also improved our understanding of protein composition of *F. hepatica* and *S. mansoni*, such as actin, myosin, Kunitz-type, and Stefin, which can be candidates for investigating the underlying mechanisms and biological significance of their tegumental function. For functional analysis of the candidate proteins, RNA interference (RNAi) can be performed in future studies, since RNAi has been proven to be an indispensable method for achieving targeted knockdown of proteins. This was the case for *F. hepatica* cathepsin, for which the effect on larvae penetration through the intestine wall was found to be seriously hampered (McGonigle et al., 2008). A similar study on the suppression of this gene in *S. mansoni* could be explored in the future and could serve to provide bases for new strategies for developing drug targets or vaccines.

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Figure 1.

(A) Overview of Coomassie Blue-stained 2-DE gels showing numerous spots from *F. hepatica* and *S. mansoni* whole-worm extracts (*F. hepatica* [F.h.] and *S. mansoni* [S.m.]). Two-DE analyses were performed on an 11-cm, pH 3–10 strip in the first dimension and 4–20% continuous polyacrylamide gradient in the second dimension. A 3-dimensional (3D) rendering of matching spots between *F. hepatica* and *S. mansoni* whole-worm extracts is shown in the middle of figure. Numbers on 2-DE represent the standard spot number (SSP) designated for each matching spot, which were selected for spot picking and MS analysis. (B) Overlaid images of *S. mansoni* and *F. hepatica* proteomes with computer-generated color-coded intensity variations on the x- and y-axes.

Figure 2.

Immunoblot analysis of *Fasciola hepatica* whole-worm extract (*F.h.*) tested with a pool of sera from patients with chronic *Schistosoma mansoni* infection. Common immunoreactive spots are numbered as described in Figure 1 and Table I.

F. hepatica/*S. mansoni* common proteins identified by MS/MS and Mascot search engine (Matrix Science) database searching.

Table I

Spot no.	Protein categories	Protein ID*	Exp./Theor. [†]		NSV [‡] (±SD)			Score [§]	Matched peptide	Expected score	R _A
			Mass (kDa)	pI	<i>F. hepatica</i>	<i>S. mansoni</i>					
Oxidative stress-related proteins											
1	Thioredoxin	CAB65014	18.9/11.8	5.4/5.8	3.30 ± 0.26	4.13 ± 0.07	568	8	1.58 E-51
2	Thiol-specific antioxidant	CAA06158	19.2/21.9	7.3/6.3	8.43 ± 0.40	4.80 ± 0.25	105	1	3.15E-05
3	Glutathion S-transferase	ABI79450	25.2/24.6	8.7/8.8	13.3 ± 0.31	9.60 ± 0.26	396	10	2.5 E-34
4	GST class mu (GST51)	P30112	25.2/25.5	5.7/5.8	20.1 ± 0.76	20.53 ± 0.50	478	11	1.58 E-42
Energy metabolism-related proteins											
5	GAPDH	AAG23287	21.0/23.3	6.8/7.0	3.20 ± 0.20	4.13 ± 0.07	368	10	1.58 E-31
6	GAPDH	AAG23287	23.1/23.0	7.8/7.0	5.77 ± 0.25	7.10 ± 0.35	392	8	6.29 E-34
7	Fatty acid-binding protein-type 3	Q9U1G6	15.0/14.6	6.0/9.0	13.7 ± 0.52	13.9 ± 0.33	671	9	7.92 E-62
8	Fatty acid-binding protein-Fh15	Q7M4G0	15.5/14.7	5.4/5.9	22.4 ± 0.21	15.9 ± 0.45	187	5	1.99 E-13
9	Fatty acid-binding protein Fh15	Q7M4G0	11.55/14.8	5.54/5.91	175 ± 8.9	61.9 ± 5.6	121	6	1.25 E-06

Spot no.	Protein categories	Protein ID *	Exp./Theor. [†]			NSV [‡] (±SD)			Score [§]	Matched peptide	Expected score	RA
			Mass (kDa)	pI		<i>F. hepatica</i>	<i>S. mansoni</i>					
10	Enolase	Q27655	45.8/46.2	6.9/6.3		7.37 ± 0.32	3.00 ± 0.17		1020	19	9.97 E-97
11	Triose-phosphate isomerase	CAD29196	21.6/24.7	5.8/5.3		4.87 ± 0.15	12.8 ± 0.12		131	5	7.98 E-8
12	Chain A, hemoglobin	B5KY09	12.33/16.5	6.86/7.23		13.5 ± 1.8	34.5 ± 0.6		518	6	2.51 E-46
13	Chain A, hemoglobin	B5KY09	12.66/16.5	7.83/7.23		8.1 ± 0.2	107.3 ± 2.5		531	6	1.26 E-47
Proteosomal proteins												
14	Secreted cathepsin-L	AAB41670	37.1/37.1	5.0/6.2		7.46 ± 0.42	29.7 ± 0.13		280	7	9.97 E-23
15	Secreted cathepsin-L	AAB41670	12.32/37.5	3.87/5.43		72.7 ± 0.4	13 ± 2.0		227	7	3.15 E-17
16	Cathepsin-L1	AAR99519	26.5/26.5	6.2/5.6		3.43 ± 0.4	21.5 ± 0.45		428	8	1.58 E-37
17	Cathepsin-D	XP_002572773	50.2/49.4	5.7/5.5		1.50 ± 0.44	6.07 ± 0.16		413	7	7.92 E-49
18	Kunitz-type (Fh-KTM)	AAB46830	9.3/6.9	3.9/6.3		8.0 ± 0.20	6.00 ± 0.20		70	2	0.09976
19	Kunitz-type (Fh-KTM)	AAB46830	7.9/6.9	3.8/6.3		10.6 ± 0.53	6.23 ± 0.20		72	2	0.06294

Spot no.	Protein categories	Protein ID *	Exp./Theor. [†]			NSV [‡] (±SD)			Score [§]	Matched peptide	Expected score	RA
			Mass (kDa)	pI		<i>F. hepatica</i>	<i>S. mansoni</i>					
20	Kunitz-type (Fh-KTM)	AAB46830	8.90/69.27	7.96/6.34		9.7 ± 0.7	102.1 ± 5.3		101	4	5.94 E-7
21	Heat shock protein-70	ABF8360	59.0/71.8	6.2/5.3		3.06 ± 0.12	4.20 ± 0.25		431	11	7.92 E-38
22	Phosphatase 2A inhibitor (protein SET inhibitor)	Q5DA15-1	12.76/28.838	4.79/4.87		82.1 ± 0.8	32.3 ± 1.5		102	3	9.97 E-05
23	Stefin-1	C6GC97	9.24/11.3	6.5/7.68		32.6 ± 1.6	125.6 ± 0.7		235	4	5 E-05
Structural proteins												
24	Myosin light chain	AAD41591	13.4/18.3	6.3/4.5		6.20 ± 0.20	5.97 ± 0.02		169	7	6.29 E-11
25	Actin-2	C4QH21	44.56/42.05	4.89/5.3		12.3 ± 0.20	136.5 ± 0.02		301	10	9.98 E-27
26	Ca+ binding protein	AAP06154	19.1/17.9	4.4/4.3		3.23 ± 0.25	3.13 ± 0.15		269	4	1.25 E-21
27	Ca+ binding protein	AAP06154	26.5/17.9	3.19/4.34		25.1 ± 0.7	16.5 ± 0.2		108	3	2.5 E-05
28	Unknown (<i>S. japonicum</i>)	AAW25255	33.3/21.8	7.8/5.6		4.03 ± 0.15	3.90 ± 0.06		88	5	0.00158

* NCBI protein accession number.

[†]Theoretical molecular mass and isoelectric point estimated by Swissprot database at <http://ca.expasy.org/sprot/>.

[‡]NSV: Normalized spot volume.

[§]Scores > 61 were significant ($p < 0.05$).

//RA: Relative abundance, refers to spots with intensity ratio more than two. Gray bar (left) corresponds to *Fasciola hepatica*, and black bar (right) corresponds to *Schistosoma mansoni*.