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# The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke *Opisthorchis viverrini*

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## Abstract

Infection with the human liver fluke, *Opisthorchis viverrini*, is a serious public health problem in Thailand, Laos and nearby locations in Southeast Asia. Both experimental and epidemiological evidence strongly implicate liver fluke infection in the etiology of one of the liver cancer subtypes, cholangiocarcinoma (CCA). To identify parasite proteins critical for liver fluke survival and the etiology of CCA, OFFGEL electrophoresis and Multiple reaction monitoring were employed to characterize 300 parasite proteins from the *O. viverrini* excretory/secretory products (ES) and, utilizing selective labeling and sequential solubilization, from the host-exposed tegument. The ES included a complex mixture of proteins that have been associated with cancers, including proteases of different mechanistic classes and orthologues of mammalian growth factors and anti-apoptotic proteins. Also identified was a cysteine protease inhibitor which, in other helminth pathogens, induces nitric oxide production by macrophages, and, hence may contribute to malignant transformation of inflamed cells. Proteases in ES included cathepsins, calpain and a protein with homology to autoimmune prostatitis antigen 2. More than 160 tegumental proteins were identified using sequential solubilization of isolated teguments, and a subset of these was localized to the surface membrane of the tegument by labeling living flukes with biotin and confirming surface localization with fluorescence microscopy. These included annexins, which are potential immuno-modulators, and orthologues of the schistosomiasis vaccine antigens Sm29 and tetraspanin-2. Novel roles in pathogenesis were suggested for the tegument-host interface since more than ten biotinylated surface proteins had no homologues in the public databases. The *O. viverrini* proteins identified here provide an extensive catalogue of novel leads for research on the pathogenesis of opisthorchiasis and the development of novel interventions for this disease and CCA, as well as providing a scaffold for sequencing the genome of this fluke.

### Keywords

proteomics; Opisthorchis viverrini; tegument; biotinylation; cholangiocarcinoma

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## 1 Introduction

Infection with the human liver fluke *Opisthorchis viverrini* is a serious public health problem in many parts of Southeast Asia, including Thailand, Lao PDR, Vietnam and Cambodia. Infection is prevalent in areas where uncooked cyprinoid fish, the intermediate host of the parasite, are a staple of the diet. The parasites establish in the liver, extrahepatic bile ducts and gallbladder of the primary host, are long lived and can cause cholangitis, obstructive jaundice, hepatomegaly, fibrosis of the periportal system, periductal fibrosis, cholecystitis and cholelithiasis [1–3]. Both experimental and epidemiological evidence strongly implicate liver fluke infection in the etiology of one of the liver cancer subtypes - cholangiocarcinoma (CCA), or bile duct cancer [4,5]. In Thailand, in spite of the widespread implementation of community-based chemotherapy with the anthelmintic medication praziquantel, the prevalence of *O. viverrini* in some endemic areas approaches 70% [6]. Moreover, in some of these regions the rates of CCA are unprecedentedly high [7].

The onset of CCA occurs in a milieu of long-term hepatobiliary damage, inflammation, periductal fibrosis and cellular responses to *O. viverrini* antigens. These conditions may predispose to CCA, possibly through enhanced susceptibility of DNA to damage by carcinogens [4,8]. Long-term hepatobiliary damage is multi-factorial and arises from continued mechanical irritation of the epithelium by the suckers of the worm, secreted parasite metabolites [1] and immunopathological processes [9]. An increase in nitric acid synthase production by macrophages, mast cells and eosinophils in inflamed areas may contribute to a rise in *N*-nitroso compounds and, consistent with this, an increased level of endogenous nitrosation potential has been shown in humans infected with *O. viverrini* [10–12]. Local increases in concentration of N-nitroso compounds may increase the risk of CCA in inflamed tissue via alkylation and deamination of DNA [13–15]. In addition to endogenous sources, levels of N-nitroso compounds, and their precursors, may be supplemented during infection by exogenous compounds ingested with the traditional diet of fermented fish [13].

Apart from mechanical damage and inflammatory responses, factors secreted by *O. viverrini* also play an important role in the predisposition of cells to cancer. Metabolic products from *O. viverrini* appear to contribute to long-term hepatobiliary damage via epithelial and adenomatous hyperplasia [6]. *In vitro*, mouse fibroblasts co-cultured with adult worms but physically separated by a porous membrane induced proliferation of host cells [16], and we recently described the identification of a growth factor secreted by *O. viverrini* that is responsible for this mitogenic activity of excretory/secretory (ES) products [17]. In concert with mitogenic activity, ES products of *O. viverrini* may also down-regulate apoptosis of proliferating cells, an effect that has been demonstrated in the closely related liver fluke *Clonorchis sinensis* [18].

The ability of helminth parasites, such as *O. viverrini*, to ensure their reproduction, nutrition and continuing survival in a hostile environment is thought to depend mainly on the proteins exposed to the host—at the host-parasite interface. These proteins are thought to play key roles in parasite reproduction [19], host tissue penetration [20], modulation of host immune responses [21] and feeding [22]. In trematode parasites the host-parasite interface is comprised of proteins from two main sources; (1) the ES component, a complex mixture of proteins, carbohydrates and lipids secreted from the surface, oral openings or gut of the parasite; and (2) the tegument, the syncitial outermost surface of the parasite. The characteristics of the proteins from these compartments are expected to exemplify adaptive strategies of these parasitic worms at large, as well as to mediate infection-related pathogenesis, including cholangiocarcinogenesis.

We recently showed that OFFGEL electrophoresis (OGE) [23] was superior to the more traditional approaches, including one- and two-dimensional gel electrophoresis, followed by ingel digestion and MS/MS of eluted peptides, for characterization of the secretome of another helminth parasite, the hookworm *Ancylostoma caninum* [24]. Here we characterize both the ES products and the tegument of *O. viverrini* using OGE [23] of tryptic digests coupled with tandem mass spectrometry (MS/MS) and describe numerous proteins that are of interest due to their potential roles in pathogenesis, carcinogenesis and as potential vaccine antigens and drug targets.

## 2 Materials and Methods

### 2.1 Harvesting of adult worms and preparation of ES proteins

ES proteins were obtained following established methods [25]. In brief, *O. viverrini* metacercariae were obtained from naturally infected cyprinoid fish in Khon Kaen province, Thailand. The fish were digested with pepsin-HCl, washed and used to infect hamsters (*Mesocricetus auratus*) by stomach intubation. The hamsters were maintained at the animal research facility of the Khon Kaen University Faculty of Medicine, using protocols approved by the Khon Kaen University Animal Ethics Committee. Fresh adult flukes were recovered from bile ducts of euthanized hamsters infected for three months and washed several times in normal saline containing penicillin (200 U/ml) and streptomycin (200 U/ml). Dead or dying flukes were removed and ES products were prepared by incubating viable flukes in modified RPMI-1640 (Invitrogen) containing penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C/5% $CO_2$. Cultures were regularly examined for dead or dying flukes and these were removed immediately after detection. Supernatants containing the ES products were harvested twice over the course of a single day (Day 1 ES) or twice daily over seven days and pooled (Day 7 ES), concentrated 20-fold to 100–300 μg/ml using 3 kDa Jumbosep spin concentrators (Pall) and aliquoted for storage at −80°C.

### 2.2 Preparation of O. viverrini tegument extracts

Sequential solubilization of *O. viverrini* tegument was performed as described [26]. Briefly, fresh adult flukes were retrieved from infected hamsters as described above. The flukes were examined to ensure their teguments were intact and only healthy flukes were retained. *O. viverrini* teguments were isolated using a freeze/thaw method [27] and then washed three times each with, in succession, (1) 40 mM Tris, pH 7.4 at 4°C; (2) 5M urea in 40 mM Tris, pH 7.4 at room temperature; (3) 0.1% SDS, 1% Triton X-100 in 40 mM Tris, pH 7.4 at room temperature. Each wash step included a 2 min vortex followed by incubation at the specified temperature for 20 min. The three washes from each solubilization step were combined and the proteins precipitated by addition of 9 volumes of methanol at −20°C. The three fractions and the final insoluble pellet were analyzed as described below.

### 2.3 Preparation of biotinylated tegument proteins by labeling live worms in culture

Live worms were labeled with biotin and biotinylated proteins were purified following published protocols [28]. In brief, adult liver flukes were harvested from infected hamsters and examined for indications of tegumental damage - only healthy flukes were retained. The flukes were then washed five times in Hanks Balanced Salt Solution (HBSS) and incubated for 30 min at 4°C in HBSS and 1 mM sulfosuccinimidyl-6-[biotinamido]hexanoate (EZ-LinkTM Sulfo-NHS-LC-Biotin; Pierce) to label free amines exposed at the surface of the fluke. Subsequently, the flukes were then washed three times in RPMI 1640 before immersion in liquid nitrogen with protease inhibitors. Frozen worms were thawed slowly on ice, washed briefly with TBS (10 mM Tris/HCl, 0.84% NaCl, pH 7.4), incubated for 5 min on ice in 10 mM Tris/HCl, pH 7.4 before vortexing five times for one second bursts. The supernatant was collected and teguments pelleted at 1000g for 30 min. Teguments were then

solubilized three times in 200 μl of 0.1% SDS, 1.0% Triton X-100 in 40 mM Tris, pH 7.4 (solubilizing solution) with pelleting at 15,000 g between each wash. These washes were combined and incubated with 120 μl of streptavidinagarose beads (Amersham Biosciences) for 2 h at room temperature with gentle end-over-end mixing. The streptavidin beads were pelleted, supernatants discarded, and the beads washed three times in solubilizing solution. To elute bound proteins, the beads were incubated at 90°C for 10 min in 2% SDS. The sample was then vortexed at maximum speed for 2 min and the supernatant removed after pelleting of the beads. The elution procedure was then repeated twice more. The supernatants were combined and proteins precipitated using 9 volumes of methanol at −20°C.

### 2.4 Fluorescence microscopy to observe surface biotinylation

For fluorescence microscopy, worms labeled with biotin were fixed in 4% paraformaldehyde in phosphate buffer for 30 min and embedded frozen in optimum cutting temperature embedding compound. Cryostat sections (5–7 μm) were mounted onto Superfrost slides and labelled with rabbit anti-biotin antiserum (Bethyl Laboratories) diluted 1:200 in 1% (w/v) bovine serum albumin (Sigma Fraction V) in PBS, followed by goat anti-rabbit serum conjugated to CY3 (Jackson Immunodiagnostics). Sections were mounted in Vectashield mounting medium with DAPI (Vector Labs). Sections were examined using a Leica DM IRB inverted fluorescence microscope. As controls, sections of worms that had been stripped of their tegument by freeze-thawing were prepared and labelled as described.

### 2.5 OFFGEL electrophoresis

Peptide digests from ES, sequentially solubilized tegument, except for the initial Tris wash, and biotinylated tegument preparations were analyzed using OGE. Proteins were reduced and alkylated before digestion with trypsin using established methods [24]. The 3100 OFFGEL Fractionator and OFFGEL Kit pH 3–10 (Agilent Technologies) with a 24-well setup were prepared as per the manufacturers' protocols. The tryptic digests were diluted in the peptide-focusing buffer, without the addition of ampholytes, to a final volume of 3.6 ml and 150 μl was loaded into each well. The samples were focused with a maximum current of 50 μA until 50 kVh were achieved. Peptide fractions were harvested and dried down using a vacuum centrifuge before mass spectral analysis. Proteins contained within the Tris wash were reduced and alkylated as described above but were not analyzed using OGE; instead, they were subjected to a slow LC gradient (below), prior to MS/MS.

### 2.6 Protein identification using LC-MS/MS-MS

OFFGEL fractions were chromatographically separated on a Dionex Ultimate 3000 HPLC using a Phenomenex C18 (2.1 mm × 25 cm) column using a linear gradient of 0–40% solvent B over 40min (or 80 min in the case of the Tris wash from the sequential solubilization) with a flow rate of 250 μl/min. The mobile phase consisted of solvent A (0.1% formic acid (aq)) and solvent B (90/10 acetonitrile/0.1% formic acid (aq)). Eluates from the RP-HPLC column were directly introduced into the TurboV ionisation source of a hybrid quadrupole/linear ion trap 4000 QTRAP MS/MS system (Applied Biosystems) operated in positive ion electrospray mode. All analyses were performed using Information Dependant Acquisition and the linear ion trap (LIT) acquisition modes. Analyst 1.4.1 software was used for data analysis. Briefly, the acquisition protocol consisted of the use of an Enhanced Mass Spectrum (EMS) scan as the survey scan. The two most abundant ions detected over the background threshold were subjected to examination using an Enhanced Resolution (ER) scan to confirm the charge state of the multiply charged ions. The ions with a charge state of +2 to +3 or with unknown charge were then subjected to collision-induced dissociation (CID) using a rolling collision energy dependent upon the *m/z* and the charge state of the ion. Enhanced Product Ion scans were acquired resulting in full product ion

spectra for each of the selected precursors which were then used in subsequent database searches. For the Day 7 ES preparation, LC-MS/MS analysis was performed using an Ultimate 3000 nanoLC system (Dionex) with a CAP-LC flow splitter coupled to a MicrOTOF-Q (Bruker) operated with a low flow electrospray needle. The solvents used were as described above except for solvent B, which was 80/20 acetonitrile/0.1% formic acid (aq). A Vydac monomeric C18 5 Å150 μm × 150 mm column operated at 1 μl/min was used with a linear gradient of 5–55% solvent B over 60 min, including an initial 10 min hold at 5% solvent B. The mass spectrometer was scanned between 50–3000 m/z and data were acquired for 50 min for each LC-MS/MS run. Data analysis was performed as above.

### 2.7 Multiple Reaction Monitoring

Multiple Reaction monitoring (MRM) experiments were performed on a hybrid quadrupole/linear ion trap 4000 QTRAP MS/MS system (Applied Biosystems). All analyses were performed using MRM-initiated IDA experiments in which a MRM survey scan triggered the acquisition of MS/MS spectra. Analyst 1.5 software was used for data analysis. The acquisition protocol to provide mass spectral data for both identification and characterization involved the following procedure of monitoring the HPLC eluant using MRM scans; ions over the background threshold of 200 counts per second were subjected to examination using the Enhanced Resolution scan (ER) to confirm charge states of the multiply charged molecular ions. The most and next most abundant ions in each of these scans with a charge state of +2 to +4 or with unknown charge were subjected to CID using a rolling collision energy dependant upon the m/z and the charge state of the ion. An Enhanced Product Ion (EPI) scan was then used to acquire the product ion spectrum. In the analysis of the ES for protease content, MRM transitions were generated from theoretical tryptic digest of target proteins using the MIDAS software program (Applied Biosystems).

### 2.8 Mascot searching and databases

Searches were performed using version 2.2.02 of Mascot with a 20 ppm tolerance on the precursor, 0.5 amu tolerance on the product ions, allowing for methionine oxidation as a variable modification, carbamidomethylation as a fixed modification, two missed cleavages, charge states +2 and +3, trypsin as the enzyme and MudPIT scoring was used to derive protein scores. EST searches were conducted on a custom built database consisting of 4,194 *O. viverrini*, 2,970 *C. sinensis* and 205,892 *Schistosoma mansoni* DNA sequences (effective database size 1,278,336 sequences). Single peptide identifications were not retained. Identifications with shared peptides were retained if each contained at least one unique peptide above the significance threshold. For grouped proteins the highest scoring identification was reported. Additional searches were conducted against the NCBI non-redundant protein database (database size 8 483 808 sequences). Searches using this database were not subjected to any taxonomy limitations. Searches for host proteins were carried out against a database constructed using all Cricetinae (hamsters) nucleotide entries from the NCBI Entrez database. This database contained 18,806 sequences providing an effective database size of 112,836 sequences. False discovery rates were calculated for every search using the Mascot decoy database facility. In this case every time a database sequence is considered by the search engine a random sequence of the same length is also generated and considered. The number of significant decoy matches are then used to derive a false discovery rate (%) as a ratio of database and decoy database hits.

### 2.9 Electrophoresis and Western Transfer

Unbound and bound biotinylated tegument proteins from the streptavidin-agarose beads (described above) were incubated for 2 h at 37°C with an equal volume of Laemmli sample buffer. The samples were then applied to a 4% stacking/12.5% resolving gel (prepared using a Biorad PROTEAN 3 system) for SDS-PAGE according to Laemmli [29]. Electrophoresis

was carried out using a maximum of 40 mA/gel and 300 V. Proteins were stained using Deep Purple fluorescent protein stain (GE Healthcare). SDS-PAGE gels of the three soluble fractions from the sequential solubilization were prepared in a similar fashion but stained using Imperial™ Protein Stain (Thermo). Western blotting was used to visualize the biotinylation of the bound and unbound samples. Samples were electrophoresed on a SDS-PAGE gel as described above and transferred to Highbond-ECL nitrocellulose membrane (Amersham Biosciences) using a XCell II Blot Module (Invitrogen). The membrane was blocked using 5% skim milk, probed with streptavidin conjugated to horseradish peroxidase (streptavidin-hrp) (Sigma) and visualized using an ECL Western Blotting Analysis System (GE Healthcare).

### 2.10 Bioinformatics analysis

Transcript analysis was achieved by downloading all *O. viverrini* EST sequences from the NCBI nucleotide EST database. Sequences were cleaned and clustered with local copies of seq-clean and TGICL (http://compbio.dfci.harvard.edu/tgi/software/). Open reading frames (ORFs) were predicted using ESTScan [30] with the *Drosophila* smat file. All open reading frames were analysed for classical secretory signal sequence using a local version of SignalP [31] and transmembrane domains using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) [31]. A protein was deemed to be an integral membrane protein if it contained two or more predicted transmembrane domains. Descriptions of protein identifications arising from proteomic analysis of the EST database were assigned using BLASTX on the non-redundant protein databases from NCBI (bit score > 30) when the reading frame of the Mascot hit was the same as the blast hit. Gene ontology (GO) categories were assigned using a local copy of Interproscan (Version 17.0) [32] and sequence alignments were generated using ClustalW [33]. Predicted subcellular locations were assigned using a combination of the WoLF PSORT server (http://wolfpsort.org/) [34] and literature searches.

## 3 Results and Discussion

### 3.1 ESTs encoding secreted proteins in O. viverrini

To provide a context for the proteomic analysis of host-exposed proteins from *O. viverrini* ORFs were generated from available ESTs and subsequently analysed for secretory signal sequence and transmembrane domains. Cleaning and clustering of the 4,194 available EST sequences led to the identification of 897 potential ORFs, of which 78 were predicted to contain a secretory signal sequence and 42 were predicted to contain two or more transmembrane domains (Figure 6). Proteases were highly represented in the transcripts encoding secreted proteins with five different cathepsins, a legumain and a S1 type serine protease all predicted to contain a signal sequence. Egg proteins were also abundant as well as a large number of uncharacterized proteins, including 39 transcripts with no homology to any other protein in GenBank and four proteins that were homologous to proteins only found in the related fluke *S. japonicum*. Three transcripts encoded venom allergen-like (VAL) proteins, a group of proteins belonging to the sterol carrier protein family. A number of proteins from this family have been identified in *S. mansoni* [35], and are also abundantly present in hookworm ES products [24]. Predicted proteins containing two or more transmembrane domains represented a diverse range of functional categories (Figure 6), although transporters and structural proteins were highly abundant. Like the predicted secretory proteins, a large number of predicted transmembrane proteins were either unique to *O. viverrini*, with no homologous proteins in GenBank, or where homologous to uncharacterized proteins from closely related species. In general, the subcellular location of the putative transmembrane proteins was predicted to be the plasma membrane although a number of mitochondrial membrane and nuclear proteins were also predicted (Figure 6).

### 3.2 Proteomic analysis of O. viverrini ES using peptide OGE and multiple reaction monitoring

Approximately 100 μg of *O. viverrini* ES was obtained by culturing worms for a period of 7 days in RPMI. Proteomic characterization of this mixture resulted in 43 unique protein identifications (Figure 6). Using the spectral count as a guide to the relative abundance of proteins, the ten most abundant proteins included well characterized proteins such as glutathione S-transferase, enolase, myoglobin, and calpain. Three of the ten most abundant proteins were uncharacterized, including two proteins with no homologies to other proteins in GenBank and, the most abundant identification, a protein with homology to the poorly characterized experimental autoimmune prostatitis antigen 2.

Although worms are able to survive in culture for an extended period of time, after seven days it is possible that a subset of the protein identifications from Day 7 ES may have resulted from tegumental breakdown or processes resulting in the `leakage' of non-secreted proteins [36]. Accordingly, ES obtained from a single day of culture was also analysed using OGE and thirteen proteins were identified. All of these proteins had been identified in Day 7 ES and included the abundant Day 7 proteins glutathione-peroxidase (two isoforms), myoglobin and two of the three uncharacterized proteins. As in the Day 7 ES, the most abundant protein by spectral count was a protein with homologies to the experimental autoimmune prostatitis antigen. Thirty of the proteins identified in Day 7 ES were not identified in the OGE analysis of Day 1 ES (Figure 6).

Proteins from Day 7 ES, that were not identified in Day 1 ES, may have been present in Day 1 ES but in amounts too small for detection using the OGE methodology. To test this proposition MRM analysis was performed on Day 1 ES using peptide identifications from Day 7 ES. MRM is specifically designed to sensitively detect the presence of a target peptide using the precursor and product ion *m*/*z* values and the peptide identifications were confirmed by acquisition of the full product ion spectra. Fifty-four peptides, representing 26 proteins, suitable for MRM analysis were selected from Day 7 ES identifications (Supplementary Table 1). Fourteen of these peptides were subsequently identified in Day 1 ES and had significant ($p < 0.05$) scores when matched against their respective target proteins. The retention times of peptides identified in the MRM experiment and the Day 7 ES OGE analysis were compared by determining the difference between the observed retention times and, based on the gradients used in the two experiments, the calculated difference in retention times. In all cases the difference in retention time between Day 7 peptide identifications and Day 1 MRM transitions was less than or equal to one minute, with the exception of the identification for fatty acid binding protein (Supplementary Table 1). Thirteen proteins were represented by the identified peptides including a VAL protein, 14-3-3 epsilon and two uncharacterized proteins (Figure 6). It should be noted that a number of the MRM identifications, whilst obtaining significant scores were relatively weak in intensity and may represent proteins present in low abundance. Using the two approaches described here, 25 proteins, thirteen from MS/MS analysis of OGE and twelve MRM identifications, were positively identified as constituents of Day 1 ES, while the remaining 18 proteins identified in Day 7 ES are a likely consequence of the culturing process.

### 3.3 Proteases in O. viverrini ES

Despite their abundance in EST transcripts, proteolytic enzymes were under-represented in the ES of *O. viverrini*. This is highly unusual, particularly when *O. viverrini* ES is compared to the ES proteomes of other helminth parasites [24,37–39]. Indeed, the major ES constituents of the western liver fluke, *Fasciola hepatica*, are cathepsin L cysteine proteases [40,41]. Conversely, no characterized proteases were identified in Day 1 ES. At least two other proteases, cathepsin F [42] and an asparaginyl endopeptidase [43] have been detected

in Day 7 *O. viverrini* ES by immunoblot analysis using antibodies raised to recombinant proteins, but neither of these proteases was identified here, suggesting that very small quantities are produced and that they constitute a minor component of the ES products. To test this hypothesis, MRM analysis was again utilized to establish the protease content of *O. viverrini* ES. Three *O. viverrini* proteases were selected, cathepsins F, D and legumain, and their amino acid sequences were used to generate peptide targets for MRM analysis (Supplementary Table 2). Day 1 ES was then analysed resulting in the detection of three peptides from cathepsin D and one, low scoring, peptide from cathepsin F (Figure 6). Peptides from the legumain were not found. Although the presence of cathepsins D and F was demonstrated it is clear that these proteins are present in very low abundance and that *O. viverrini* differs significantly from *F. hepatica* in the composition and secretion of proteases in the ES. The absence, or low abundance, of these proteases may be a true reflection of low expression levels or may have been caused by limited regurgitation during culturing. It is noteworthy that these proteases have been immunolocalized to the gut of adult flukes, and at least some blood-feeding parasitic helminths, such as *Haemonchus contortus*, produce stable multi-protein complexes consisting predominantly of intestinal proteases that are anchored to the gut membrane and digest blood in the gut lumen of the worm [44]; a similar scenario might also occur in *O. viverrini* where gut proteases remain in the gastrodermis and are only secreted in small quantities. Alternatively, secretion of these enzymes might be regulated by the presence or absence of food, and the worms analyzed here were cultured in serum-free medium for collection of ES products.

### 3.4 ES products and cancer

Of particular interest in this study was the identification of secreted proteins that could contribute to carcinogenesis. One identification in Day 7 ES was a member of the granulin/epithelin protein family. The granulins are small proteins defined by a conserved domain containing 12 cysteines arranged into four cysteine pairs and flanked by two single cysteines at both the amino and carboxy termini [45]. In mammals, granulins are derived from a larger pro-form (pro-granulin) that contains multiple copies of the 12-Cys motif [46,47] and both the intact precursor and the single motif proteins are able to modulate cell growth [48–50]. *O. viverrini* granulin has a signal sequence and has been shown to be mitogenic at very low concentrations [17]. Granulin was identified by a single peptide in Day 7 ES. To confirm the presence of granulin in Day 1 ES, this peptide was used in a subsequent MRM analysis of the Day 1 ES. The peptide contained two Pro residues which gave rise to a product ion spectrum that was dominated by ions resulting from fragmentation at the amide bond preceding the Pro residues. Consequently, complete sequence ion coverage of the peptide was not attained, reducing the confidence of the identification. Accordingly, MRM experiments targeting this peptide were performed on Day 1 ES, and also on a tryptic digest of recombinant granulin. In both experiments a peptide matching the target peptide was identified with the same retention time and same product ion spectra [17]. This provided strong evidence that granulin is secreted by the parasite, and, its presence in the ES may contribute to the onset of CCA. Apart from granulin, proteomic analysis of Day 1 ES revealed two other proteins that may contribute to the carcinogenic potential at the site of infection. In particular, the identification of thioredoxin and cystatin are of interest. Thioredoxin has been shown to possess growth factor activity [51], is anti-apoptotic [52] and is over-expressed in many aggressive forms of cancer [53]. Cystatin, a cysteine protease inhibitor, stimulates interferon gamma-dependent nitric oxide production by macrophages [54], and hence may contribute to the carcinogenic potential at sites of inflammation via DNA damage and subsequent malignant transformation.

### 3.5 Comparison of the secreted transcriptome and proteome

Including cathepsin F, cathepsin D and granulin, 28 proteins were positively identified in the ES of *O. viverrini*. This can be contrasted with the putatively secreted proteins identified at the transcript level (Figure 6). Clearly, only a small subset of these proteins were present in the ES proteins identified in the proteomic analysis. Indeed, only six of the proteins predicted to be secreted at the transcript level were identified in the subsequent proteomic analysis of Day 1 ES, including the venom allergen protein 8, thioredoxin peroxidase, cystatin and one of the uncharacterized proteins. As was the case with granulin and cathepsins D and F, the absence of predicted secreted proteins in the proteomic analysis may be a result of very low abundance. Alternatively, secreted proteins may be targeted to compartments other than the ES, for example secreted egg proteins are likely to be retained within the egg shell rather than the ES component, such as appears to be the case in *F. hepatica* [41]. A number of non-secreted proteins were identified in Day 1 ES and many of these proteins have also been identified in the ES of closely related species (Supplementary Table 3). Although the identification of these proteins may be the result of tegumental shedding during the culturing process [55], morphological studies have suggested that the continuous *in vivo* shedding of the tegument may play an immuno-defensive role [41,56], thus providing a mechanism for the presence of these proteins in the ES. The *O. viverrini* ES characterized here is dominated by a single poorly characterized protein with homology to the experimental autoimmune antigen. No functional information could be found for this protein and comparison with homologus proteins suggest that the EST is N-terminally truncated and, thus, whether the protein contains a secretory signal sequence is also not known. Given the relatively small amount of sequence data available for *O. viverrini* it is also possible that the large spectral count for this protein stems from the existence of a number of different isoforms, as is also the case of cathepsin L in *F. hepatica*.

### 3.6 Sequential solubilization of the O. viverrini tegument

The tegument is a syncytium, that covers the entire parasite, and is connected to numerous cell bodies that lie beneath the musculature of the body wall [57]. Each cell body contain a nucleus, mitochondria and apparatus for the synthesis and packaging of proteins. The properties of membrane proteins, including their hydrophobicity and affinity for detergents, can be utilized to enrich samples for membrane associated proteins and, conversely, deplete water soluble cytosolic proteins. The use of sequential extraction in which samples are washed with buffers of increasing solubilizing strength has become widely used as a rapid and simple procedure to isolate membrane associated proteins without the use of specialized equipment [58,59]. Recently this technique was used to determine the host-exposed proteins of *S. mansoni* following removal of the tegument by freeze-thawing [26]. Accordingly, to determine proteins contained within and, in particular, proteins exposed at the surface of the *O. viverrini* tegument, we employed this technique for the sequential solubilization of the tegument and subsequent proteomic profiling of the solubilized fractions.

The teguments of approximately 300 adult *O. viverrini* were stripped by freeze thawing followed by sequential extraction with solutions of increasing solubilization strength. These extracts and the residual insoluble pellet were analysed using SDS-PAGE (Figure 6A) and digested with trypsin. Proteomic analysis of these fractions resulted in 139 unique protein identifications (Supplementary Table 3). As expected the majority of these proteins (64%) were of cytosolic, cytoskeletal or mitochondrial origin; three proteins originated from the endoplasmic reticulum or golgi apparatus and four miscellaneous proteins were identified from an indeterminate cellular location. Of the remainder, nine were known plasma membrane associated proteins and a large proportion (14 proteins) were uncharacterized proteins with no known homology. In general, the cellular location of an identified protein correlated to the fraction in which it was identified. Figure 6B groups the identified proteins

by their cellular location and shows the fractions in which they were identified. Nearly half (45%) of the identifications resolved to a single fraction, and when the fraction in which the highest scoring identification was taken into account, the majority of cytosolic and cytoskeletal proteins were identified in the first fraction, matrix mitochondrial proteins in the second fraction and membrane associated proteins of either mitochondrial or plasma membrane origin in the third and fourth fractions. Fourteen proteins had no known homology and accordingly could not be assigned a cellular location.

A number of known membrane associated proteins were identified (Figure 6C). These included three annexins (gi|114323473, gi|126255354 and gi|114322469), a protein containing a Ly6 domain (gi|126255127), and a SH3-domain containing protein (gi|146159637). Annexins, normally located at the cytoplasmic side of the plasma membrane, are host-exposed in *S. mansoni* and in other organisms [28,60,61]. In humans, non-cytoplasmic annexins play roles in the reduction of the inflammatory response via interactions with the annexin A-1 receptor on leukocytes [62] and may also act to protect lipid bilayers from the effects of the coagulation pathway [63]. Annexins on the surface of *O. viverrini* may serve similar functions in reducing the inflammatory response to the worm or inhibition of coagulation around the site of infection. Nine proteins were identified in fraction three that had no homologies in the NCBI non-redundant database, including three that possessed predicted transmembrane domains (Figure 6B). Although these proteins may be associated with membranes of the mitochondria, the relative ubiquity of processes occurring at these membranes makes it more likely that novel proteins, specific to *O. viverrini* biology, would be found at the plasma membrane. As such, these proteins represent a reservoir of novel activities and may play important roles in the survival and reproduction of *O. viverrini*.

### 3.7 Identification of biotinylated proteins after surface labeling of live worms

Biotinylation of whole cells or tissues is a technique that has been used to confirm exposure of integral membrane proteins to the environment as well as an enrichment mechanism for the characterization of exposed proteins [28,61,64]. The technique relies on the reagent reacting with exposed primary amines, for example those found in the side chain of lysine residues, and the subsequent purification of labeled proteins on immobilised streptavidin. As it contains a sulfo group, the reagent used in this study (Sulfo-NHS-LC-Biotin) is water soluble and hence is excluded by the lipid bilayer of the tegument and preferentially labels surface exposed proteins. This has also been confirmed experimentally where the reagent was used to label only surface exposed proteins on the tegument of *S. mansoni* [28]. As the reaction occurs in an amino-acid free, balanced-salt solution instead of normal culture medium, the reaction time was kept to a minimum (30 min) and performed at 4°C to minimize tegumental degradation.

Approximately 300 live and undamaged adult *O. viverrini* worms were incubated with biotin and the extent of labeling and the depth to which the biotin penetrated the membrane was assessed using fluorescence microscopy. Localization of biotin on the labeled worms using fluorescence microscopy showed a clear zone of labeling around the exterior of the worm (Figure 6A and B), but biotin did not appear to penetrate the tegument membrane, nor was it ingested during the labeling process because the gut membrane did not fluoresce. Worms that had been stripped of their teguments by freeze-thawing and then probed with Cy3-streptavidin did not fluoresce (Figure 6C). After stripping and solubilization of the tegument, biotinylated proteins were purified using streptavidin immobilized on agarose beads. SDS-PAGE gels stained with Deep Purple revealed, as expected, that the majority of protein remained in the unbound (unlabeled) fraction of the tegument. Immunoblot analysis of these same samples with streptavidinhrp showed that the unbound fraction, despite having a great deal more total protein content, yielded a considerably weaker signal with

streptavidin-hrp than did the proteins that bound to streptavidin-agarose (Supplementary Figure 1).

The bound sample was analyzed using a peptide OGE as described for ES products, resulting in the non-redundant identification of 25 proteins (Table 1). These proteins were then compared to proteins identified from the teguments of *S. mansoni* [26,28,65] and *S. bovis* [66,67] (Table 1). Only two cytosolic proteins were identified in the biotinylated tegument preparation - glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the tegumental calcium-binding EF-hand protein. GAPDH has been previously localized to the surface of larval schistosomes [68] and was strongly recognized by sera from hosts infected with *S. japonicum* [69] and *S. bovis* [66]. Likewise, the tegumental calcium-binding EF-hand protein is found in a number of helminths [70,71], and although classified as cytosolic, it possesses both calcium binding EF hand and dynein-like C-terminal domains, suggesting that it may play a role in the cytoskeleton and may be exposed, along with actin and dyneins, at the tegumental spines as was the case in *S. mansoni* [28]. The tegumental spines of *O. viverrini* are covered by the outer membrane, with their bases attached to the inner membrane [72], a position in which they may have been exposed to biotin. A subset of mitochondrial proteins was identified in the biotinylation experiment. A number of these proteins, including ATPase [73], pyruvate dehydrogenase [74] and the voltage-dependent anion channel 1 [28], have been localized to the plasma membrane surface in various models and their identification here suggests that they occupy a similar location in *O. viverrini*. An ultrastructural investigation showed that the *O. viverrini* tegument is rich in mitochondria that, in many cases, were proximate to the cytoplasmic side of the tegumental plasma membrane [72]. This can be contrasted with the schistosomes in which the mitochondria are generally located basally in the tegumental cytoplasm [75]. Whether this is a result of harvesting and culturing conditions or reflects the true nature of the *O. viverrini* tegument is not fully clear, but this may provide an explanation for biotin labeled mitochondrial proteins. Another possible explanation is endogenous biotinylation - at least one of the identified proteins, pyruvate carboxylase, is known to be endogenously biotinylated [76], although endogenous biotinylation is normally restricted to carboxylases, and 3-methylcrotonyl carboxylase and propionyl carboxylase, proteins also known to be endogenously biotinylated [77], were not identified.

### 3.8 Host-exposed membrane proteins of O. viverrini

A number of proteins were identified in both the sequentially solubilized tegument and the biotinylated tegument preparation. Annexins were identified with both approaches, confirming a role for these proteins at the surface of *O. viverrini*. A greater number of annexins were identified in the sequentially solubilized membrane fraction than in the biotinylated proteins, which may reflect different types of annexins that associate with the cytoplasmic or host-exposed sides of the apical membrane [63]. Proteins containing Ly6 and SH3 domains were also identified in both cases. The protein with an SH3 domain had homology to endophilin and is most likely involved in endocytosis at the tegument plasma membrane [78]. The protein with a Ly6 domain shared homology with the *S. mansoni* tegument surface protein Sm-29 [79], which also contains a Ly6 domain. The Ly6 (also known as Ly6/urokinase-type plasminogen activator receptor, uPAR) superfamily of molecules are cysteine-rich, generally glycosylphosphatidyl inositol (GPI) anchored, cell surface proteins [80]. Little is known about their functions, apart from the proteins CD59 and uPAR. CD59 is an inhibitor of the complement cascade [81] and uPAR plays an important role in the proteolysis of extracellular matrix proteins [82]. In *S. mansoni*, Sm29 has been shown to be effective as a vaccine antigen [83], suggesting that this protein is vital to the survival of the worm. By extension, a similar protein at the surface of *O. viverrini* is now an attractive vaccine target for opisthorchiasis.

Using available *O. viverrini* EST sequences, 43 transcripts were identified as containing transmembrane domains, but only four of these transcripts were subsequently identified by proteomic means, either as putatively host-exposed or as a constituent of the mitochondrial membrane (Figure 6). Nonetheless, unlike ES in which the presence of a secretory signal sequence can be expected, the presence of transmembrane domains is not indicative of either a tegumental location, as proteins may be localised to a number of different cell types and membranes, or a prerequisite for a membrane association, as proteins may associate with membranes in a number of ways, including GPI anchoring and protein–protein interactions. Despite the lack of transmembrane domains, a number of the putatively surface exposed proteins identified in this study are also host-exposed in schistosomes. In biotinylation experiments on *S. mansoni* [28] and *S. japonicum* [61], proteins such as tetraspanins, annexins, calpain and Sm29 were identified at the surface of the parasites and these were also identified in the biotinylation or sequential solubilization experiments of *O. viverrini*. Notably, a number of proteins that are host-exposed in schistosomes were not identified here, including glucose transport proteins, alkaline phosphatase, sodium-potassium ATPase and aquaporin. However, of these proteins only aquaporin was represented in the *O. viverrini* EST sequences. Given the poor sequence coverage for *O. viverrini* it is possible that these proteins are present but unidentifiable because of the lack of sufficient sequence coverage, or that these proteins are not essential for the lifestyle of *O. viverrini*. Considering the different biological fluids in which each parasite resides — the blood for schistosomes and the bile ducts for *O. viverrini*—significant differences in the composition of host-exposed proteins could be expected.

### 3.9 False positive calculations, host protein and bacterial contamination

In addition to the searches described above a number of additional searches were performed: (1) to screen for contamination all datasets were searched against the NCBI nr protein database with no taxonomy restrictions; (2) a hamster EST database was constructed and was searched with all datasets to identify host proteins present in the samples; and (3) to estimate false positive identifications every search performed was coupled to a decoy database search using Mascots decoy search procedure. Searches of the NCBI nr protein database and the hamster database resulted in only a very small number of identifications of bacterial, fungal or host origin, confirming that *O. viverrini* samples had not been contaminated during processing (Supplementary Table 4). Decoy database searches of the *O. viverrini*/*C. sinensis*/*S. mansoni* database had an estimated false positive rate well below that used as the identification significance threshold (5%), with the exception of the biotinylated tegument search which had a false positive rate equal to the threshold (Supplementary Table 4). Searches of the NCBI non-redundant protein and hamster EST databases all had false positive rates above the significance threshold reflecting the low number of identifications made in these databases with the MS/MS datasets used.

### 3.10 Concluding Remarks

The aim of this investigation was to characterize the secreted and surface exposed proteins of *O. viverrini* and use this information to enhance our understanding of the molecular mechanisms by which *O. viverrini* establishes a chronic infection, avoids the host immune response, and ultimately contribute to the onset of cancer. Proteins that potentially play roles in all of these processes were identified, which should facilitate deeper, more focused investigations of the enigmatic host-parasite interactions of human infection with *O. viverrini* and its association with CCA. Some of these macromolecules will hopefully find utility as (1) biomarkers of disease progression, particularly towards tumorigenesis, (2) recombinant vaccines against chronic opisthorchiasis (and therefore as a prophylaxis for CCA), and (3) targets for new anthelmintic drugs. Finally, using de novo sequencing protocols and MS-BLAST it was established that a large number of proteins have yet to be

identified due to the meagre nucleotide sequence coverage of *O. viverrini* and other liver flukes. Accordingly, our complete datasets have been made available at the Helminth Proteomics Server (http://helminth.qimr.edu.au/proteomics) so that they can be of further utility as greater sequence coverage becomes available for *O. viverrini* and related parasites.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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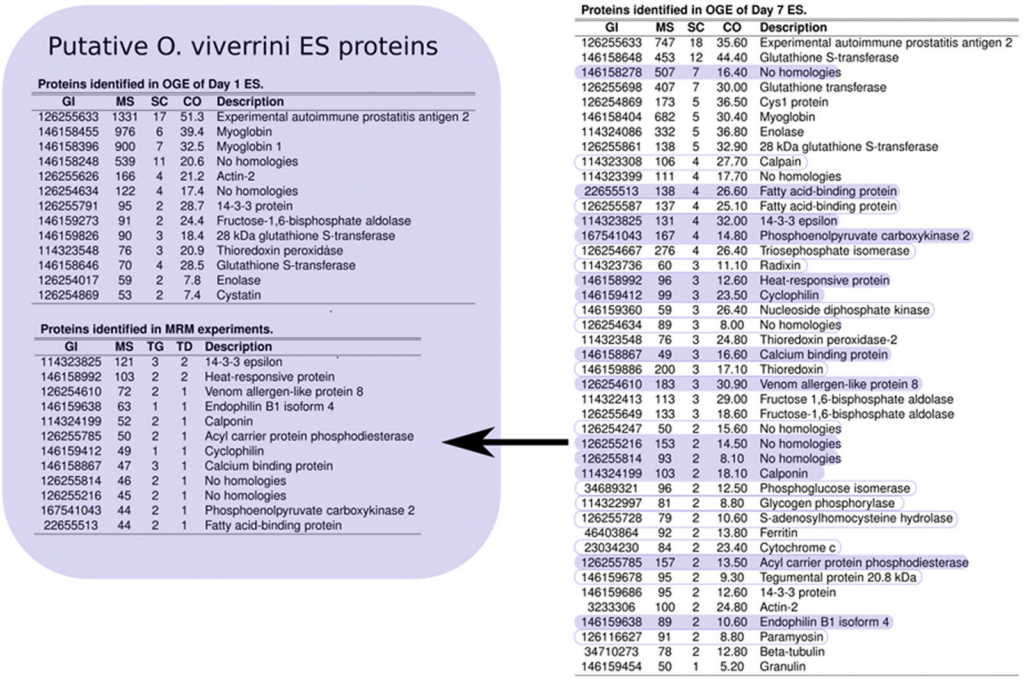
**Secretory proteins encoded by *Opisthorchis viverrini* ESTs.**

| Description | Species | ESTs | PI |
|---|---|---|---|
| Vitelline B precursor protein | *Opisthorchis viverrini* | 5 | - |
| Eggshell protein, chorion | *Schistosoma japonicum* | 1 | - |
| Eggshell precursor protein | *Schistosoma japonicum* | 1 | - |
| Cathepsin F-like cysteine protease | *Opisthorchis viverrini* | 4 | - |
| Cathepsin F-like cysteine protease | *Opisthorchis viverrini* | 3 | + |
| Cathepsin F-like cysteine protease | *Opisthorchis viverrini* | 1 | - |
| Cathepsin B2 | *Clonorchis sinensis* | 1 | - |
| Cathepsin D | *Opisthorchis viverrini* | 2 | + |
| S1 family serine protease | *Schistosoma mansoni* | 1 | - |
| Legumain | *Opisthorchis viverrini* | 3 | - |
| Venom allergen-like protein 1 | *Schistosoma mansoni* | 1 | - |
| Venom allergen-like protein 9 | *Schistosoma mansoni* | 1 | - |
| Venom allergen-like protein 8 | *Schistosoma mansoni* | 1 | + |
| Thioredoxin peroxidase | *Opisthorchis viverrini* | 1 | + |
| Saposin-like protein | *Clonorchis sinensis* | 1 | - |
| Tumor protein D52-like 2 | *Rattus norvegicus* | 1 | - |
| Mitogen-activated protein kinase 11 | *Bos taurus* | 1 | - |
| Shikimate kinase | *Xenopus tropicalis* | 1 | - |
| Cystatin | *Fasciola hepatica* | 1 | + |
| Histone H1 | *Schistosoma mansoni* | 2 | - |
| Granulin | *Opisthorchis viverrini* | 1 | + |
| SJCHGC02082 protein | *Schistosoma japonicum* | 1 | - |
| SJCHGC05602 protein | *Schistosoma japonicum* | 1 | - |
| SJCHGC08380 protein | *Schistosoma japonicum* | 1 | - |
| SJCHGC05639 protein | *Schistosoma japonicum* | 1 | - |
| SJCHGC02087 protein | *Schistosoma japonicum* | 1 | - |
| Unknown proteins (No homology) | | 39 | - |

**Membrane-spanning proteins encoded by *Opisthorchis viverrini* ESTs.**

| Description | ESTs | TM | SL | PI |
|---|---|---|---|---|
| **Transporters/Channels** | | | | |
| Aquaporin-9 | 1 | 5 | PL | - |
| ATP synthase lipid-binding protein | 1 | 2 | MI | - |
| ATP:ADP antiporter | 1 | 4 | PL | + |
| Cation-transporting ATPase | 1 | 6 | PL | - |
| Oxalate:formate antiporter | 1 | 4 | PL | - |
| Sodium-bile acid cotransporter | 1 | 4 | PL | - |
| Vacuolar ATP synthase | 1 | 4 | PL | - |
| Bestrophin-related | 1 | 2 | PL | - |
| **Proteases/Hydrolytic** | | | | |
| Chitinase 18-18 | 1 | 2 | EC | - |
| Signal peptide protease | 1 | 4 | ER | - |
| **Structural/Membrane Organisation** | | | | |
| Glycine/tyrosine-rich eggshell protein | 1 | 2 | Egg | - |
| Innexin | 3 | 3 | PL | - |
| Neutral sphingomyelinase | 1 | 3 | PL | - |
| Sm-TSP-2 | 1 | 3 | PL | + |
| Tetraspanin-1 | 1 | 3 | PL | - |
| **Miscellaneous** | | | | |
| Homeobox protein | 1 | 2 | N | - |
| Collagen-binding surface protein | 1 | 5 | PL | - |
| Polycystic kidney and hepatic disease 1 | 1 | 3 | MI | - |
| Keratinocyte associated protein | 2 | 3 | PL | - |
| Lamin B receptor | 1 | 3 | N | - |
| Mannose-p-dolichol utilization defect 1 | 1 | 6 | PL | - |
| RING finger protein 121 | 1 | 4 | PL | - |
| RP140-upstream | 1 | 4 | PL | - |
| Succinate dehydrogenase | 1 | 2 | MI | + |
| Succinate dehydrogenase complex, subunit C | 1 | 3 | MI | + |
| Surfeit locus protein 4 | 4 | 7 | PL | - |
| Tumor suppressor candidate 3 | 1 | 3 | PL | - |
| TLC domain-containing protein 1 precursor | 1 | 3 | PL | - |
| **Unknown** | | | | |
| Hypothetical protein [Schistosoma mansoni] | 1 | 2 | PL | - |
| Hypothetical protein [Schistosoma mansoni] | 1 | 2 | ER | - |
| Hypothetical protein [Schistosoma mansoni] | 1 | 3 | PL | - |
| No homologies | 1 | 2 | PL | - |
| No homologies | 1 | 3 | PL | - |
| No homologies | 1 | 4 | PL | - |
| No homologies | 1 | 2 | PL | - |
| No homologies | 1 | 2 | PL | - |

**Figure 1.**



## Putative O. viverrini ES proteins

**Proteins identified in OGE of Day 1 ES.**

| GI | MS | SC | CO | Description |
|---|---|---|---|---|
| 126255633 | 1331 | 17 | 51.3 | Experimental autoimmune prostatitis antigen 2 |
| 146158455 | 976 | 6 | 39.4 | Myoglobin |
| 146158396 | 900 | 7 | 32.5 | Myoglobin 1 |
| 146158248 | 539 | 11 | 20.6 | No homologies |
| 126255626 | 166 | 4 | 21.2 | Actin-2 |
| 126254634 | 122 | 4 | 17.4 | No homologies |
| 126255791 | 95 | 2 | 28.7 | 14-3-3 protein |
| 146159273 | 91 | 2 | 24.4 | Fructose-1,6-bisphosphate aldolase |
| 146159826 | 90 | 3 | 18.4 | 28 kDa glutathione S-transferase |
| 114323548 | 76 | 3 | 20.9 | Thioredoxin peroxidase |
| 146158646 | 70 | 4 | 28.5 | Glutathione S-transferase |
| 126254017 | 59 | 2 | 7.8 | Enolase |
| 126254869 | 53 | 2 | 7.4 | Cystatin |

**Proteins identified in MRM experiments.**

| GI | MS | TG | TD | Description |
|---|---|---|---|---|
| 114323825 | 121 | 3 | 2 | 14-3-3 epsilon |
| 146158992 | 103 | 2 | 2 | Heat-responsive protein |
| 126254610 | 72 | 2 | 1 | Venom allergen-like protein 8 |
| 146159638 | 63 | 1 | 1 | Endophilin B1 isoform 4 |
| 114324199 | 52 | 2 | 1 | Calponin |
| 126255785 | 50 | 2 | 1 | Acyl carrier protein phosphodiesterase |
| 146159412 | 49 | 1 | 1 | Cyclophilin |
| 146158867 | 47 | 3 | 1 | Calcium binding protein |
| 126255814 | 46 | 2 | 1 | No homologies |
| 126255216 | 45 | 2 | 1 | No homologies |
| 167541043 | 44 | 2 | 1 | Phosphoenolpyruvate carboxykinase 2 |
| 22655513 | 44 | 2 | 1 | Fatty acid-binding protein |

**Proteins identified in OGE of Day 7 ES.**

| GI | MS | SC | CO | Description |
|---|---|---|---|---|
| 126255633 | 747 | 18 | 35.60 | Experimental autoimmune prostatitis antigen 2 |
| 146158648 | 453 | 12 | 44.40 | Glutathione S-transferase |
| 146158278 | 507 | 7 | 16.40 | No homologies |
| 126255698 | 407 | 7 | 30.00 | Glutathione transferase |
| 126254869 | 173 | 5 | 36.50 | Cys1 protein |
| 146158404 | 682 | 5 | 30.40 | Myoglobin |
| 114324086 | 332 | 5 | 36.80 | Enolase |
| 126255861 | 138 | 5 | 32.90 | 28 kDa glutathione S-transferase |
| 114323308 | 106 | 4 | 27.70 | Calpain |
| 114323399 | 111 | 4 | 17.70 | No homologies |
| 22655513 | 138 | 4 | 26.60 | Fatty acid-binding protein |
| 126255587 | 137 | 4 | 25.10 | Fatty acid-binding protein |
| 114323825 | 131 | 4 | 32.00 | 14-3-3 epsilon |
| 167541043 | 167 | 4 | 14.80 | Phosphoenolpyruvate carboxykinase 2 |
| 126254667 | 276 | 4 | 26.40 | Triosephosphate isomerase |
| 114323736 | 60 | 3 | 11.10 | Radixin |
| 146158992 | 96 | 3 | 12.60 | Heat-responsive protein |
| 146159412 | 99 | 3 | 23.50 | Cyclophilin |
| 146159360 | 59 | 3 | 26.40 | Nucleoside diphosphate kinase |
| 126254634 | 89 | 3 | 8.00 | No homologies |
| 114323548 | 76 | 3 | 24.80 | Thioredoxin peroxidase-2 |
| 146158867 | 49 | 3 | 16.60 | Calcium binding protein |
| 146159886 | 200 | 3 | 17.10 | Thioredoxin |
| 126254610 | 183 | 3 | 30.90 | Venom allergen-like protein 8 |
| 114322413 | 113 | 3 | 29.00 | Fructose 1,6-bisphosphate aldolase |
| 126255649 | 133 | 3 | 18.60 | Fructose-1,6-bisphosphate aldolase |
| 126254247 | 50 | 2 | 15.60 | No homologies |
| 126255216 | 153 | 2 | 14.50 | No homologies |
| 126255814 | 93 | 2 | 8.10 | No homologies |
| 114324199 | 103 | 2 | 18.10 | Calponin |
| 34689321 | 96 | 2 | 12.50 | Phosphoglucose isomerase |
| 114322997 | 81 | 2 | 8.80 | Glycogen phosphorylase |
| 126255728 | 79 | 2 | 10.60 | S-adenosylhomocysteine hydrolase |
| 46403864 | 92 | 2 | 13.80 | Ferritin |
| 23034230 | 84 | 2 | 23.40 | Cytochrome c |
| 126255785 | 157 | 2 | 13.50 | Acyl carrier protein phosphodiesterase |
| 146159678 | 95 | 2 | 9.30 | Tegumental protein 20.8 kDa |
| 146159686 | 95 | 2 | 12.60 | 14-3-3 protein |
| 3233306 | 100 | 2 | 24.80 | Actin-2 |
| 146159638 | 89 | 2 | 10.60 | Endophilin B1 isoform 4 |
| 126116627 | 91 | 2 | 8.80 | Paramyosin |
| 34710273 | 78 | 2 | 12.80 | Beta-tubulin |
| 146159454 | 50 | 1 | 5.20 | Granulin |

**Figure 2.**

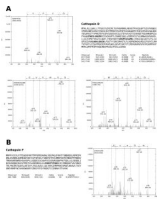


**Figure 3.**

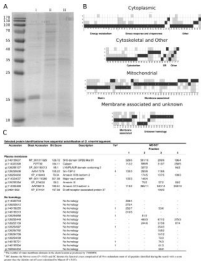



**Figure 4.**

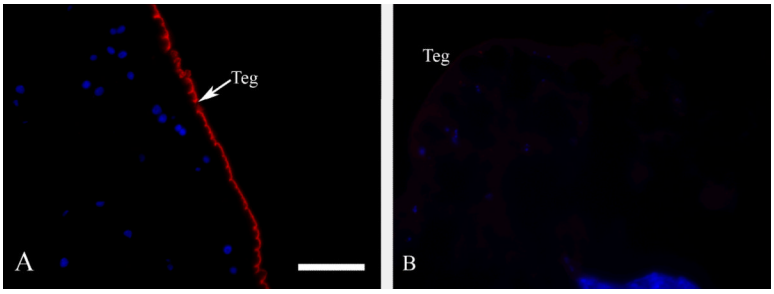



**Figure 5.**

*Proteomics*. Author manuscript; available in PMC 2011 March 1.

**Table 1**

Proteins identified from *Opisthorchis viverrini* biotinylated tegument preparation[a].

| GI | MS | SC | CO | Description | SS | Trematoda | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Sm1 | Sm2 | Sm3 | Sm4 | Sb1 | Sb2 |
| **Cytosol energy metabolism** | | | | | | | | | | | |
| 146159601 | 114 | 4 | 12.9 | GAPDH | 1 | − | − | + | − | + | + |
| **Cytosol/other** | | | | | | | | | | | |
| 126255683 | 111 | 6 | 21.7 | Calcium-binding EF-hand protein | 1 | − | − | − | − | + | + |
| **Cytoskeleton** | | | | | | | | | | | |
| 126255581 | 99 | 4 | 8.9 | Actin 2 | 1 | − | − | − | SC | + | + |
| 166249691 | 80 | 2 | 31.7 | Actin 3 | 1 | − | − | − | SC | + | + |
| **Other** | | | | | | | | | | | |
| 114322940 | 310 | 3 | 12.0 | Putative peroxidase | 3 | − | − | − | − | − | − |
| 126255633 | 56 | 6 | 16.0 | FAM139A | 1 | − | − | − | − | − | − |
| **Mitochondrial matrix** | | | | | | | | | | | |
| 126254590 | 391 | 5 | 15.0 | Succinate dehydrogenase subunit A | 2 | − | − | − | − | − | − |
| 114323037 | 250 | 4 | 22.4 | Pyruvate carboxylase | 2 | − | − | − | − | − | − |
| 114323615 | 186 | 5 | 18.4 | Succinate dehydrogenase | 2 | − | − | − | − | − | − |
| 166324032 | 73 | 3 | 63.2 | Succinate dehydrogenase | 2 | − | − | − | − | − | − |
| 146159582 | 64 | 3 | 13.5 | Succinate dehydrogenase cytochrome B | 2 | − | − | − | − | − | − |
| 34625702 | 52 | 2 | 13.2 | Pyruvate dehydrogenase | 2 | − | − | − | − | − | − |
| **Mitochondrial membrane** | | | | | | | | | | | |
| 114324249 | 414 | 6 | 29.1 | ADP,ATP carrier protein 1 | 3 | − | − | − | − | + | − |
| 114322316 | 187 | 2 | 13.5 | Voltage-dependent anion channel 1 | 3 | − | − | − | SC | − | − |
| 114322861 | 145 | 8 | 44.2 | Glutamate dehydrogenase | 4 | − | − | − | − | − | − |
| 114322605 | 129 | 5 | 25.1 | Glutamate dehydrogenase | 4 | − | − | − | − | − | − |
| 114322752 | 87 | 2 | 14.7 | Glycerol kinase | 4 | − | − | − | − | − | − |
| 166304141 | 79 | 2 | 19.1 | Aldehyde dehydrogenase 4A1 | 4 | − | − | − | − | + | − |
| 34671317 | 77 | 3 | 16.5 | ATPase alpha subunit | 3 | − | − | − | SC | + | − |
| 126255408 | 59 | 2 | 15.4 | NADH dehydrogenase isoform 2 | 3 | − | − | − | − | − | − |
| **Plasma membrane** | | | | | | | | | | | |
| 114323473 | 131 | 5 | 24.2 | Annexin A13 | 3 | + | + | + | LC | − | − |

| GI | MS | SC | CO | Description | SS | Trematoda | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Sm1 | Sm2 | Sm3 | Sm4 | Sb1 | Sb2 |
| 126255127 | 85 | 1 | 10.9 | LY6/PLAUR domain containing 2 | 2 | + | + | + | LC | − | − |
| 126255767 | 70 | 2 | 11.2 | SH3-domain GRB2-like B1 | 2 | − | − | − | − | − | − |
| 126254202 | 51 | 3 | 8.4 | Annexin XIIIb isoform 2 | 2 | + | + | + | LC | − | − |
| **No homology** | | | | | | | | | | | |
| 126254997 | 91 | 1 | 17.7 | No homology | 3 | − | − | − | − | − | − |

[a]Abbreviations used are as follows: GI — gene accession; MS — Mowse score; SC — Spectral count; CO — Percent cover; SS — Corresponding fraction from the sequential solubilisation experiment; Sm1, Sm2, Sm3 and Sm4 — *Schistosoma mansoni* (refs [26,28,65]); Sb1 and Sb2 — *Schistosoma bovis* (refs [66,67]). The presence of a protein in the relevant study is denoted with a `+' and the absence with a `−'. In the case of Sm4 `LC' denotes detection by long-chain form of biotin and `SC' denotes detection by the short-chain form.