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Mass spectrometry analysis of the excretory-secretory (E-S) products of the model cestode *Hymenolepis diminuta* reveals their immunogenic properties and the presence of new E-S proteins in cestodes

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Abstract

Hymenolepis diminuta is an important model species in studies of therapeutics, biochemical processes, immune responses and other aspects of cestodiasis. The parasite produces numerous excretory-secretory (E-S) proteins and a glycocalyx covering its body. Our study focused on the mass spectrometry analysis of the E-S material with an objective to determine if E-S contains any new proteins, in particular those that can be identified as: antigens, vaccine candidates and drug targets. These proteins might engage directly in host-parasite interactions. Adult parasites collected from experimentally infected rats were cultured *in vitro* for 5 and 18h. Immunoblotting was used to verify which E-S protein bands separated in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) react with specific antibodies from sera of infected rats. We identified thirty-nine proteins by LC-MS/MS (liquid chromatography mass spectrometry). Results indicated the presence of proteins that have never been identified in cestode E-S material. Immunoblotting showed the immunogenicity of E-S products of *H. diminuta*, most probably associated with the presence of proteins known as antigens in other flatworm species. Among identified proteins are those engaged in immunomodulatory processes (eg. HSP), in response to oxidative stress (peroxidasin) or metabolism (eg. GAPDH). The predominant functions are associated with metabolism and catalytic activity. This is the first study identifying E-S-proteins in adult tapeworms, thus providing information for better understanding host-parasite interrelationships, and may point out potential targets for vaccines or drug discovery studies, as among the proteins observed in our study are those known to be antigens.

Keywords

Antigens, Cestoda, excretory-secretory products, proteins, proteomics, 1D immunoblotting

Introduction

Tapeworms comprise a large group of parasitic flatworms that may, both as in immature and in adult stages, seriously damage health or even cause death in animals and humans and thus have a great economic impact (Elias *et al.* 2007; Charlier *et al.* 2014). In spite of their great importance, cestodes, especially adult stages, are still rather neglected if compared to digenean trematodes and parasitic nematodes. Adult tapeworms are always intestinal parasites affecting hosts by competing with nutrient absorption and causing local inflammation of the intestinal epithelium. Their various immature stages (metacestodes) may invade different tissues (muscles, brain, liver tissue etc.) of humans and domestic animals, producing clinical changes in the invaded organs and causing disease in these intermediate hosts. In their mature adult stage, at least forty species of tapeworms cause intestinal cestodiasis in humans and many other species are known to be parasites of domes-

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tic animals (Cox 2002). The most common species include: *Taenia saginata*, *Taenia solium*, *Diphyllobothrium latum* and *Hymenolepis nana.* The last one is considered to be the most common cestode found in humans, affecting mainly children (Garcia and Bruckner 1997). Symptoms of intestinal cestodiasis include abdominal pain that may be relieved by eating and that may be accompanied by distention, flatulence, and nausea. Often, there are no symptoms, and the first notice of infection may occur only when proglottids (segments) of the worms are passed in the stools. Treatment may involve the use of antiparasitic drugs or surgery; however, new therapies and drug targets are urgently needed as numerous strains show drug resistance (Cox 2002; Tsai *et al.* 2013).

Although, our understanding of cestode biology has increased, nonetheless many aspects of interrelationships between cestodes and their hosts have remained mysterious. It is disquieting that work on parasitic models in areas such as developmental biology, cell death pathways, transport, metabolism, nutrition, host evasion strategies, etc. continues to be largely ignored in general review articles on these topics. One of the gaps in our understanding is related to the limited knowledge of antigenic proteins and the molecular basis of host-parasite interactions in cestodiasis. Therefore, continuous studies focused on the molecules engaged in host-parasite interaction are of high scientific interest. To address this gap, researchers need to find and use common model organisms. One of the most important model species in experimental cestodiasis is *Hymenolepis diminuta*, as we have greater understanding of its biology than of practically any other cestode, and probably any parasitic helminth in general (Arai 1980; Skrzycki *et al.* 2011; Bartikova *et al.* 2012; Graepel *et al.* 2013; Cadkova *et al.* 2014; Shostak 2014; Kosik-Bogacka *et al.* 2014; Woolsey *et al.* 2015; Reyes *et al.* 2015, Mansur *et al.* 2015). This is partly because the most medically important cestode, *T. solium*, can develop as an adult only in the human host and thus cannot be studied readily due to ethical constraints. Therefore *H. diminuta*, which develops as an adult readily in laboratory rats and as cysticercoid metacestodes in mealworms and adult tenebrionid beetles, seems to be the perfect model organism. Its proteomics and immunoproteomics should help to uncover mechanisms contributing to adaptation to environmental constraints and host-parasite interaction. These data provide crucial information to answer some of the existing questions about the molecular basis of the host-parasite relationship and potential usefulness of cestode antigens as immunomodulatory molecules.

The ultimate goal of the present study was to determine whether excretory-secretory (E-S) products of *H. diminuta* may contain proteins that are known as taking part in host-parasite interactions and/or are immunogenic (antigens). In addition, we searched for the presence of potential proteins already known as vaccine and drug target candidates. Our observations will be verified in future studies with the use of 2DE and 2D immunoblotting.

Materials and Methods

Experimental animals

Male Lewis rats aged about 3 months at the beginning of the experiment, to be used as experimental hosts, were kept in plastic cages in the animal house facilities of the Institute of Parasitology, PAS. They had continuous access to food and water and natural photoperiod conditions were provided. All experimental procedures used in the present study had been pre-approved by the 3rd Local Ethical Committee for Scientific Experiments on Animals in Warsaw, Poland (resolution nr 51/2012, 30th of May 2012).

Cultivation of *H. diminuta* and collection of E-S proteins

In the present study we used a *H. diminuta* strain kept at the Department of General Biology and Parasitology of the Medical University of Warsaw, known as Warsaw Medical School Line (strain WMS). Six-week-old cysticercoids reared in *Tribolium castaneum* beetles were fed in doses of 8–10 to 3-month-old rats (15 male rats). After six weeks, coproscopic examination of the rat faeces was performed to ascertain the presence of adult parasites. Serum was separated from blood samples collected every 2 weeks starting from the 2nd week of infection. Uninfected rats' sera were used as the controls. For the immunoblotting we prepared a pool of sera from different animals but from the same post-infection time-point. These sera were stored at –80°C until used. To collect adult parasites, rats were euthanized with Tiopental anaesthesia (Biochemie GmbH, Austria), administered in 100 mg/kg body weight (b.w.) intraperitoneally (i.p.). Collected tapeworms were divided into 2 groups: each of the groups containing 5 tapeworms/Petri dish and *in vitro* cultured at 37°C according to procedures described by Evans (Evans 1980) in Eagle's media with protease inhibitor cocktail added for the inhibition of serine, cysteine and metalloproteases (Roche Diagnostics GmbH, Germany). Excretory-secretory products were collected from both groups after 5 hours and 18 hours of cultivation. Viability of the parasites was assessed based on observing active body movements. Collected media was filtered through a 0.22μm syringe filter. E-S were dialysed through a Zellu-Trans cellulose membrane (Carl Roth GmbH + Co, Germany – pore size 10Å) and then concentrated by lyophilization. Samples were stored at −80 °C until used.

SDS-PAGE and immunoblot analysis

Collected E-S proteins of *H. diminuta* were purified by using PlusOne SDS-PAGE Clean-Up kit (GE Healthcare, USA) dedicated to samples of low protein concentration. Proteases are generally inactive in this solution, therefore we decided not to add protease inhibitors. SDS-PAGE separation of proteins was performed in Criterion AnyKD TGX precast gels (Bio-Rad, USA) using a pre-stained protein standard broad range marker

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(Bio-Rad, USA) for estimating the molecular weight of the proteins. Electrophoresis was carried out using a Midi-Protean Tetra Cell (Bio-Rad, USA) at 200 V constant voltage for 45 min. Proteins were either stained with silver according to the manufacturer's protocol (Krzysztof Kucharczyk Techniki Elektroforetyczne, Poland), or electrotransferred onto nitrocellulose membranes (NCM) using a Bio-Rad Trans-Blot Cell at 100 V constant voltage for one hour. After evaluating different quantities of proteins and dilutions of both primary and secondary antibodies, we established as optimal conditions: 10 µg of antigenic protein by lane for SDS-PAGE; 1/200 dilution of the anti-*H. diminuta* rat sera and 1/3000 dilution of the goat anti-rat IgG for immunoblot analysis. Afterwards, the transferred proteins were blocked with Pierce Protein-Free T20 blocking buffer (Thermo Scientific, USA) at room temperature with shaking for 60 min. NCM was washed (3 × 5 min) in PBS+ Tween 0.5% and incubated for 1–2 h with specific anti-*H. diminuta* rat sera diluted in the above-mentioned blocking buffer (1/200 dilution). Serum sample taken before the infection at day 0 was used as a negative control. To visualize cross-reacting antigens, the NCM was washed three times (5 min each) in the washing buffer composed of 20mM Tris, pH 8.0 and 0.9% NaCl and developed with goat anti-rat IgG (Whole Molecule) peroxidase conjugated (Sigma-Aldrich, USA) for 1 h in the dark at room temperature. NCM was washed in the Wash Buffer (3 × 5 min) and then immunoreactive signals were visualized with an Opti-4CN Substrate Kit (Bio-Rad, USA). Gels and membranes were digitalized and analyzed using a GS-800 Densitometer (Bio-Rad, USA) combined with 1-D Analysis Software Quantity 1 (Bio-Rad, USA). Gels were analyzed with use of the aforementioned software and optical density (OD) indicating the level of protein abundance was compared between two experimental groups. After imaging, the selected silver-stained bands containing the immunogenic proteins were excised manually (under magnification x 10) from the gels and proteins of interest were subjected to mass spectrometric identification.

Proteomic analyses – mass spectometry (MS) and protein identification

Selected immunoreactive protein bands were excised from the gel and analyzed by liquid chromatography coupled to the mass spectrometer (LC-MS/MS) in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Samples were subjected to standard procedure of trypsin digestion, during which proteins were reduced with 10 mM DTT for 30 min at 56°C and alkylated with iodoacetamide in darkness for 45 min at room temperature and digested overnight with 10 ng/ul trypsin. After blocking the trypsin reaction with TFA, the resulting tryptic peptides were recovered with the buffer, concentrated and desalted on a RP-C18 pre-column (Waters, 2G-V/M Trap 5 µm Symmetry C18 column, 180 µm i.d., 20 mm long). Further peptide separation was achieved on a nano-Ultra Performance Liquid Chromatography (UPLC) RP-C18 column (Waters, BEH130 C18 column, 75 µm i.d., 250 mm long) of a nanoACQUITY UPLC system, using a 45-min linear acetonitrile gradient. Column outlet was directly coupled to the electrospray ionization (ESI) ion source of the Orbitrap Velos type mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), working in the regime of data dependent MS to MS/MS switch with HCD type peptide fragmentation. An electrospray voltage of 1,5 kV was used. Raw data files were pre-processed with Mascot Distiller software (version 2.4.2.0, MatrixScience). The obtained peptide masses and fragmentation spectra were matched to the National Center Biotechnology Information (NCBI) non-redundant database (72430760 sequences/25686591311 residues), with a *Cestoda* filter (68709 sequences) using the Mascot search engine (Mascot Daemon v. 2.4.0, Mascot Server v. 2.4.1, MatrixScience). The following search parameters were applied: enzyme specificity was set to trypsin, peptide mass tolerance to ± 30 ppm and fragment mass tolerance to ± 0.1 Da. The protein mass was left as unrestricted, and mass values as monoisotopic with one missed cleavage being allowed. Alkylation of cysteine by carbamidomethylation as fixed, oxidation of methionine and carboxymethylation on lysine were set as a variable modification.

Protein identification was performed using the Mascot search engine (MatrixScience), with the probability based algorithm. The expected value threshold of 0.05 was used for analysis, which means that all peptide identifications had less than 1 in 20 chance of being a random match.

The proteins characterized by the highest Mascot-assigned protein score – Multi- dimensional Protein Identification Technology-type (MudPIT-type) and/or the highest number of peptide sequences, were selected. MudPIT score is a type of protein scoring, which eliminates the influence of low-scoring peptide matches.

Identified proteins were categorized by their molecular function, cellular component and biological processes according to gene ontology information obtained from UniProtKB (http://web.expasy.org/docs/swiss-prot_guideline.html) and QuickGO (http://www.ebi.ac.uk/ QuickGO/) databases.

Results

SDS-PAGE and optical density (OD) analysis

Cestodes removed from the small intestines of the experimentally infected rats were kept in media for 5 and 18 hours to study the potential differences in the protein expression estimated on the basis of OD (Figs. 1 and 2).

In each group, five tapeworms were kept in culture media, as we estimated that this number of parasites is optimal for the effective detection of E-S products by SDS-PAGE. The production of E-S proteins by the adult *H. diminuta* was followed by SDS-PAGE separation of the collected and pre-prepared

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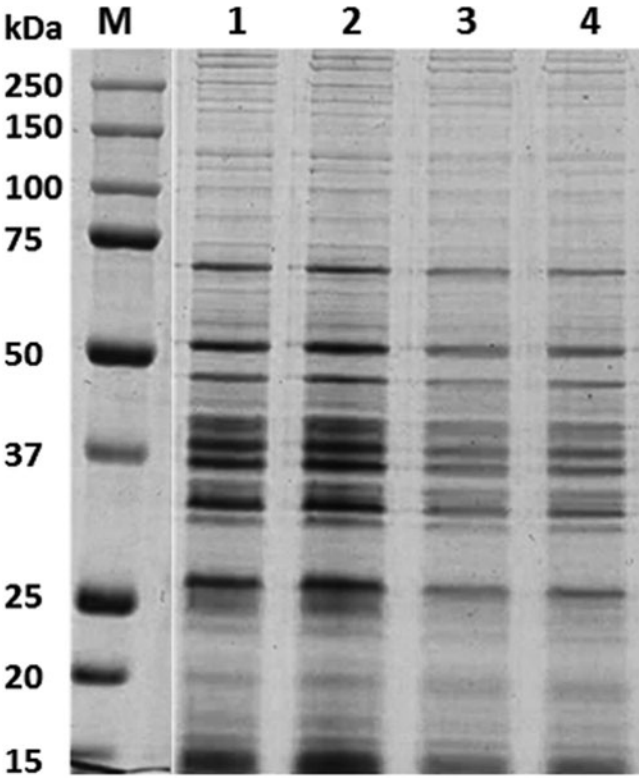

Fig. 1. Representative overlay of silver-stained gel image of *H. diminuta* E-S proteins after separation by SDS–PAGE. Lanes 1-2– experimental group kept in the media for 18h; Lanes 3-4 – experimental group kept in the media for 5h

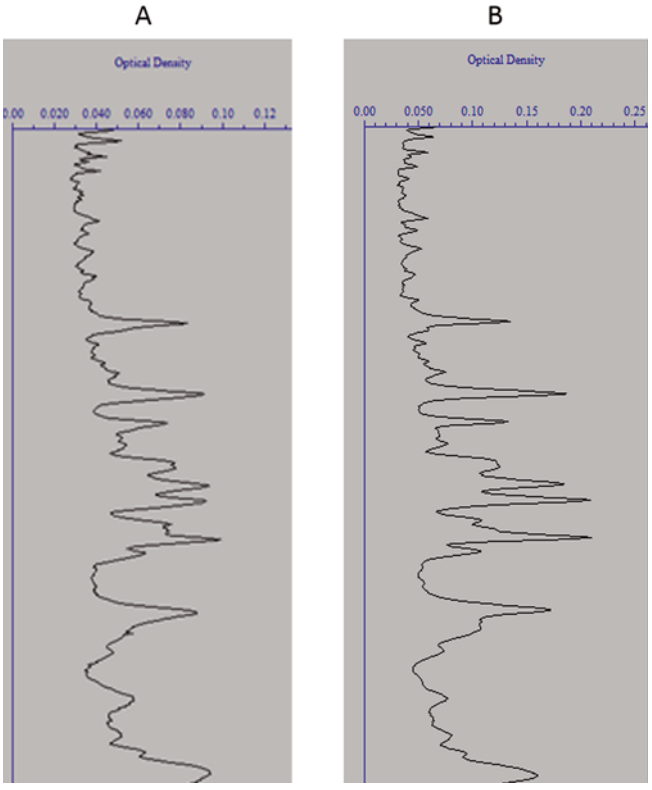

Fig. 2. The abundance of E-S proteins in the groups kept in the media for 5h (A) and 18h (B) expressed by the level of signal intensity distribution (OD)

media containing E-S proteins from two above-mentioned experimental groups. Despite the fact that the same amount of total proteins was analyzed in two experimental groups, the production of E-S proteins in the groups kept in the media for 18h was higher than in the groups where E-S products were collected after 5h (Figs. 1–3).

Taking into account the higher protein abundance measured by OD level, it was higher in the group of parasites with longer time spent in media (Fig. 3). The increase in the OD level was the highest in high-abundance bands with molecular masses ranging from 27 kDa to 70 kDa. However, the number of protein bands in both groups was the same and did not

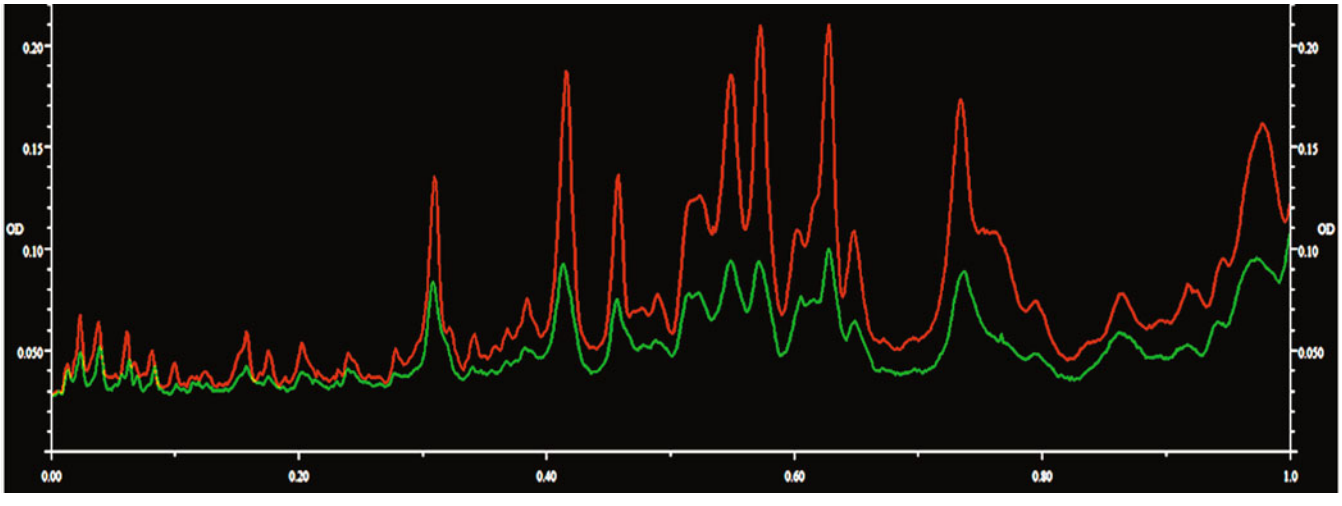

Fig. 3. Showing evident differences in the level of signal intensity distribution (OD) related to the expression (abundance) of proteins in two experimental groups

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vary with time (Fig. 1). Protein bands masses ranged from less than 15 kDa to over 250 kDa (Fig. 1). The highest abundance of proteins, related to OD level, was observed with bands having molecular masses between 25kDa to 70 kDa (Figs. 1–3). Accumulation of high-abundance protein bands was present in the gel at the spectrum from 25 kDa to 50 kDa (Figs. 1–3).

Immunoblot and selection of immunoreactive protein bands

In order to study the presence of proteins with antigenic properties in the E-S products of adult *H. diminuta*, the same samples that were separated by SDS-PAGE were immunoblotted using a pool of sera collected from infected rats (Fig. 4).

High reproducibility was achieved for both the SDS-PAGE gels and immunoblots (>99%, with ten repetitions). The level of reproducibility was analyzed by using Quantity One 1-D Analysis Software (Bio-Rad, USA). Numerous immunoreactive protein bands were observed in both E-S group products with no visible variation between them (Fig. 4). The high OD level observed with the immunoreactive bands was not always related to the high-abundant protein bands detected after SDS-PAGE (Figs 1 and 4). For instance some high-abundance protein bands separated on SDS-PAGE displayed no immunogenic reaction, and in contrast, selected bands of low reactivity to silver staining were highly immunoreactive. The molecular masses of the immunoreactive protein bands ranged from over 30 kDa to over 250 kDa (Fig. 4). For identification, ten protein bands containing proteins cross-reacting with the antisera were recovered from the silver-stained gels for mass spectrometry identification.

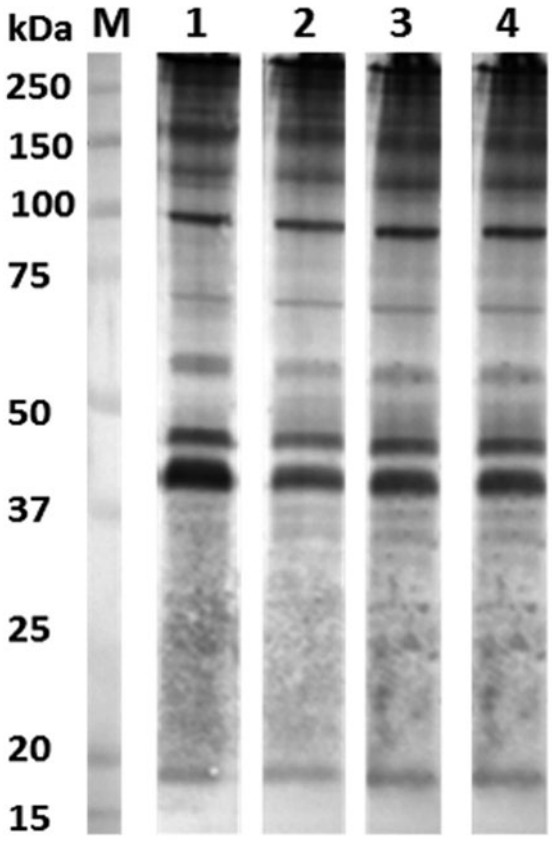

Fig. 4. Recognition pattern of *H. diminuta* antigens by *H. diminuta* infected rats antibodies. E-S antigens after electrophoresis were blotted onto a PVDF membrane. Higly immunoreactive bands were selected for LC-MS/MS analysis. Lanes 1-2– experimental group kept in the media for 18h; Lanes 3-4 – experimental group kept in the media for 5h

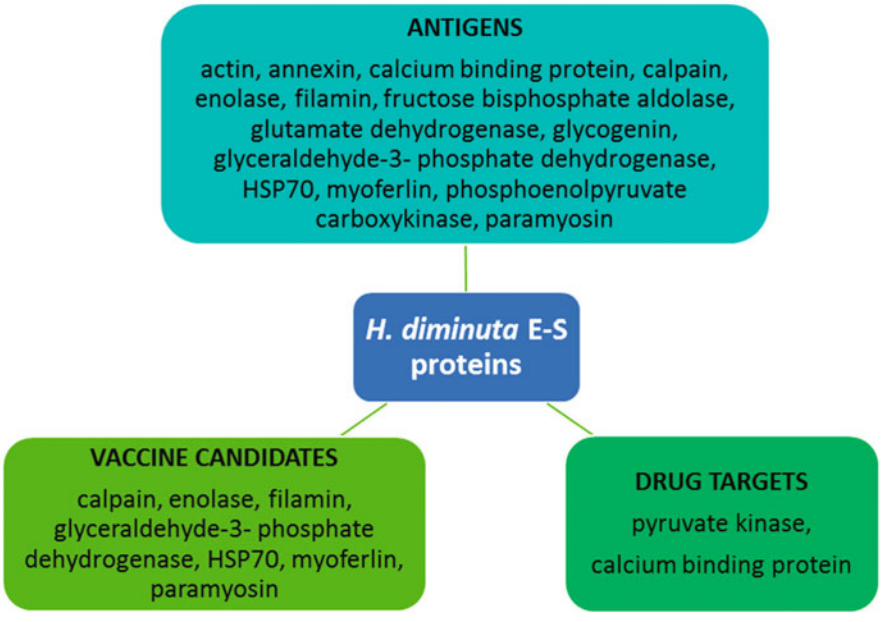

Fig. 5. Selected proteins found in *H. diminuta* E-S products subdivided into three subgroups: antigens, vaccine candidates, drug targets – according to available literature

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Table I. Results of the LC-MS/MS analysis of selected immunoreactive bands. Proteins identified for the first time in E-S products are shown in bold

Band	Database	Accession No	Score	Mass	N. matches	N. sequences	Description
1	NCBInr	gi|555936767	476	782807	11	8	**titin (*Hymenolepis microstoma*)**
	NCBInr	gi|556519647	408	348377	8	6	**neurogenic locus notch protein (*Echinococcus granulosus*)**
	NCBInr	gi|555936066	170	899497	5	5	**basement membrane specific heparan sulfate (*Hymenolepis microstoma*)**
	NCBInr	gi|556521040	156	868644	2	2	**basement membrane specific heparan sulfate (*Echinococcus granulosus*)**
2	NCBInr	gi|555936193	821	235262	23	19	**myoferlin (*Hymenolepis microstoma*)**
	NCBInr	gi|556513330	380	232251	10	10	**myoferlin (*Echinococcus granulosus*)**
	NCBInr	gi|555937336	549	308234	14	14	**filamin (*Hymenolepis microstoma*)**
	NCBInr	gi|576700013	296	308295	9	8	**filamin-A (*Echinococcus granulosus*)**
	NCBInr	gi|555931525	141	152617	4	4	**peroxidasin extracellular matrix-associated peroxidase (*Hymenolepis microstoma*)**
	NCBInr	gi|556521636	134	151182	3	3	**peroxidasin (*Echinococcus granulosus*)**
3	NCBInr	gi|556519535	137	246580	12	1	**receptor type tyrosine protein phosphatase (*Echinococcus granulosus*)**
	NCBInr	gi|555929800	134	117232	3	3	**anoctamin (*Hymenolepis microstoma*)**
	NCBInr	gi|556518785	131	118299	3	3	**anoctamin (*Echinococcus granulosus*)**
	NCBInr	gi|555933972	126	217498	5	4	**alpha 2 macroglobulin (*Hymenolepis microstoma*)**
4	NCBInr	gi|555938008	362	106047	11	10	alpha actinin, sarcomeric (*Hymenolepis microstoma*)
	NCBInr	gi|556513629	154	109048	5	5	alpha actinin sarcomeric (*Echinococcus granulosus*)
	NCBInr	gi|555933491	317	168588	5	4	**expressed protein (*Hymenolepis microstoma*)**
	NCBInr	gi|555931956	184	104838	5	4	**puromycin sensitive aminopeptidase (*Hymenolepis microstoma*)**
	NCBInr	gi|556522068	64	104922	2	2	**puromycin sensitive aminopeptidase (*Echinococcus granulosus*)**
	NCBInr	gi|555936912	158	99179	5	5	**paramyosin (*Hymenolepis microstoma*)**
	NCBInr	gi|42559495	121	98822	4	4	**paramyosin (*Taenia saginata*)**
	NCBInr	gi|555935186	102	129695	3	3	**phospholipid transporting ATPase IIB (*Hymenolepis microstoma*)**
	NCBInr	gi|556518758	99	120788	3	3	**phospholipid transporting ATPase IIB (*Echinococcus granulosus*)**
5	NCBInr	gi|555931973	5482	71158	139	24	phosphoenolpyruvate carboxykinase (*Hymenolepis microstoma*)
	NCBInr	gi|283466460	3972	55577	95	13	phosphoenolpyruvate carboxykinase, partial (*Echinococcus multilocularis*)
	NCBInr	gi|283466492	3511	55077	90	11	phosphoenolpyruvate carboxykinase, partial (*Taenia serialis*)
	NCBInr	gi|555932344	1288	70972	19	14	heat shock protein 70 (*Hymenolepis microstoma*)
	NCBInr	gi|556515519	1207	72909	18	13	heat shock 70 kDa protein 4 (*Echinococcus granulosus*)
	NCBInr	gi|556520512	1079	71324	16	12	heat shock 70 kDa protein 4 (*Echinococcus granulosus*)
	NCBInr	gi|124783175	758	21731	9	5	heat shock protein 70 (*Taenia asiatica*)
	NCBInr	gi|555932347	407	37695	9	7	**heat shock protein 71 kDa protein (*Hymenolepis microstoma*)**
	NCBInr	gi|556517487	327	76984	6	6	**Armadillo type fold (*Echinococcus granulosus*)**
	NCBInr	gi|555937827	228	84690	5	5	calpain (*Hymenolepis microstoma*)
	NCBInr	gi|555931467	210	62859	4	3	**alkaline phosphatase (*Hymenolepis microstoma*)**

6	NCBInr	gi|556518187	1493	71003	27	5	**NADP dependent malic enzyme (*Echinococcus granulosus*)**
	NCBInr	gi|555933393	220	33456	9	4	**NADP dependent malic enzyme (*Hymenolepis microstoma*)**
	NCBInr	gi|555930128	1200	114130	24	15	**pyruvate kinase *(Hymenolepis microstoma*)**
	NCBInr	gi|556515455	939	62872	21	14	**pyruvate kinase (*Echinococcus granulosus*)**
	NCBInr	gi|555938520	309	88988	9	5	calpain A (*Hymenolepis microstoma*)
	NCBInr	gi|555929491	216	55360	6	6	glutamate dehydrogenase, mitochondrial (*Hymenolepis microstoma*)
	NCBInr	gi|556515747	88	47172	3	3	**glutamate dehydrogenase mitochondrial (*Echinococcus granulosus*)**
7	NCBInr	gi|555934171	8155	48584	137	22	**enolase (*Hymenolepis microstoma*)**
	NCBInr	gi|1839200	7618	17916	104	11	enolase, partial (*Hymenolepis diminuta*)
	NCBInr	gi|563425937	7592	46813	99	13	enolase (*Taenia solium*)
	NCBInr	gi|556518822	117	47250	5	3	enolase (*Echinococcus granulosus*)
	NCBInr	gi|6467323	311	29776	8	7	elongation factor 1-a (*Hymenolepis diminuta*)
8	NCBInr	gi|555935514	674	39225	26	4	**deoxyhypusine hydroxylase:monooxygenase (*Hymenolepis microstoma*)**
	NCBInr	gi|556516051	254	38406	15	3	**deoxyhypusine hydroxylase:monooxygenase (*Echinococcus granulosus*)**
	NCBInr	gi|467215	598	41700	9	8	actin, partial *(Diphyllobothrium dendriticum*)
	NCBInr	gi|555930350	501	114751	10	8	actin, cytoplasmic type 5 (*Hymenolepis microstoma*)
	NCBInr	gi|555930765	519	45609	20	10	**sarcoplasmic calcium binding protein (*Hymenolepis microstoma*)**
	NCBInr	gi|556518860	461	45528	17	6	**sarcoplasmic calcium binding protein (*Echinococcus granulosus*)**
9	NCBInr	gi|555930614	4481	39942	89	16	fructose 1,6 bisphosphate aldolase (*Hymenolepis microstoma*)
	NCBInr	gi|29336561	780	40159	11	5	fructose- bisphosphate aldolase (*Echinococcus multilocularis*)
	NCBInr	gi|556521309	733	39132	12	3	annexin (*Echinococcus granulosus*)
	NCBInr	gi|556513882	175	42226	11	1	**gelsolin (*Echinococcus granulosus*)**
10	NCBInr	gi|556517778	372	38114	6	5	**glyceraldehyde 3 phosphate dehydrogenase (*Echinococcus granulosus*)**
	NCBInr	gi|149364041	241	36523	5	4	**glyceraldehyde-3-phosphate dehydrogenase (*Taenia solium*)**
	NCBInr	gi|555934884	225	38330	5	4	**glycogenin 1 (*Hymenolepis microstoma*)**
	NCBInr	gi|555931050	187	36385	14	6	**lactate dehydrogenase (*Hymenolepis microstoma*)**

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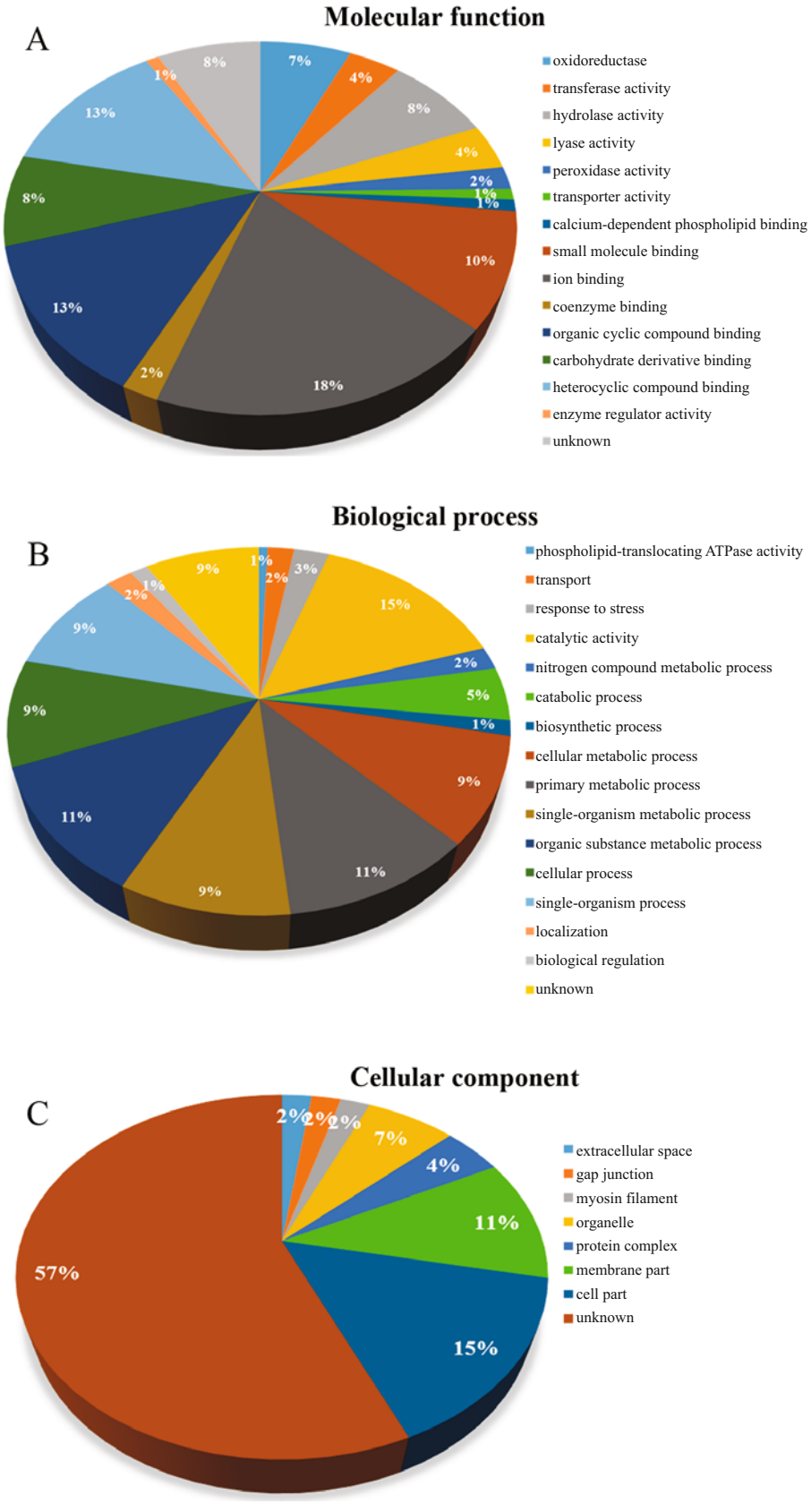

Fig. 6. Identified proteins categorized by their molecular function (A), biological processes (B) and cellular component (C) according to gene ontology (GO) information obtained from UniProtKB and QuickGO databases

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Protein identification

LC-MS/MS proteomic approach was used to analyze the immunogenic proteins from selected protein bands. All the proteins were identified by their homologies with proteins of other cestode species as there is no genome and/or proteome of *H. diminuta* available. Mass spectrometric analysis allowed identification of thirty-nine parasite proteins present in the immunoreactive bands (Table I).

Several proteins were identified from multiple bands (data not shown), they probably corresponded to protein isoforms or post-translational modifications. Only the proteins with the highest identification protein scores were selected for further analysis and discussion, and these are listed in Table I. These proteins were grouped according to their scientific importance into three categories: antigens, vaccine candidates and drug targets (Fig. 5).

The most promising new antigen candidates were identified from the immunoreactive protein bands 1, 2, 3 and 10 (Table I), containing proteins which have been identified for the first time in the E-S products of cestodes. None of these proteins has ever been observed in the E-S products of adult tapeworms (Table I).

Functional categories of proteins by gene ontology (GO)

Identified proteins were categorized by their molecular function, cellular component, and biological processes according to GO (Gene Ontology) (Fig. 6 A-C). The GO-based data illuminate the different functions and processes in which the identified E-S proteins are likely to be involved.

We were able to establish molecular function (GO:0003674) for thirty-one of the thirty-nine identified proteins (Fig. 6). Fourteen subcategories were assigned to molecular function ontology, including: oxidoreductase activity (GO:0016491, 7), transferase activity (GO:0016740, 4), hydrolase activity (GO:0016787, 8), lyase activity (GO:0016829, 4), peroxidase activity (GO:0004601, 2), transporter activity (GO:0005215, 1), calcium-dependent phospholipid binding (GO:0005544, 1), small molecule binding (GO:0036094, 10), ion binding (GO:0043167, 19), coenzyme binding (GO:0050662, 2), organic cyclic compound binding (GO:0097159, 13), carbohydrate derivative binding (GO:0097367, 8), heterocyclic compound binding (GO:1901363, 13), enzyme regulator activity (GO:0030234, 1). Among these functions, proteins of ion binding, heterocyclic compound and organic cyclic compound-binding properties were predominant (Fig. 6A). Only one protein was associated with the following functions: transporter activity (phospholipid transporting ATPase IIB), calcium-dependent phospholipid binding (annexin), and enzyme regulator activity (alpha 2 macroglobulin). Eight proteins had no molecular function associated with them and therefore remained "unclassified".

According to GO, we classified twenty-six proteins according to their biological process (GO:0008150) (Fig. 6B). These proteins were associated with fifteen subcategories: phospholipid-translocating ATPase activity (GO:0004012, 1), transport (GO:0006810, 3), response to stress (GO:0006950, 4), catalytic activity (GO:0003824, 22), nitrogen compound metabolic process (GO:0006807, 3), catabolic process (GO:0009056, 7), biosynthetic process (GO:0009058, 2), cellular metabolic process (GO:0044237, 14), primary metabolic process (GO:0044238, 16), single-organism metabolic process (GO:0044710, 14), organic substance metabolic process (GO:0071704, 17), cellular process (GO:0009987, 14), single-organism process (GO:0044699, 14), localization (GO:0051179, 3), biological regulation (GO:0065007, 2). A significant portion of proteins were classified to catalytic activity (22 proteins) and organic substance metabolic process (17 proteins), and only one protein (phospholipid transporting ATPase IIB) was associated with phospholipid-translocating ATPase activity (Fig. 6B). Among thirty-nine identified proteins, thirteen had no biological process established for them.

Regarding the association of E-S proteins of the adult *H. diminuta* with cellular components (GO:0005575), thirteen of the total thirty-nine proteins were found to belong to this GO category (Fig. 6C). The classification results show that these proteins were classified into seven subcategories: extracellular space (GO:0005615, 1), gap junction (GO:0005921, 1), myosin filament (GO:0032982, 1), organelle (GO:0043226, 3), protein complex (GO:0043234, 2), membrane part (GO:0044425, 5), cell part (GO:0044464, 7). Within this subcategory of GO, the most numerous were proteins associated with the last two subcategories, namely, membranes and cell parts (Fig. 6C).

Discussion

Modern proteomics tools can separate and identify individual proteins from a protein mixture (Pandey and Mann 2000). This approach provides a powerful tool in both basic and applied parasitology. Reproducible SDS-PAGE, two-dimensional electrophoresis (2DE), immunoblotting, mass spectrometry methods combined with bioinformatics has become commonplace in modern helminth parasitology research (Morphew *et al.* 2006). Proteomics has the potential to identify proteins differentially expressed in different parasite stages or in response to drugs, and excretory-secretory products (Jefferies 2001). Identification and characterization of parasite proteins might help to find new candidates for the immunodiagnostics, new therapies and vaccines. There are important benefits of using 1DE immunoblotting when compared to 2DE immunoblotting. In SDS-PAGE we can practically analyze all proteins from complex samples due to the presence of SDS, whereas in 2DE the problem is that several highly alkaline and/or hydrophobic proteins remain unanalyzed since SDS cannot be used for protein solubilization due to its charged character. For the aforementioned reasons, 1DE immunoblotting was the method of choice to achieve the goals of the present study.

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Present results demonstrate several proteins of *H. diminuta* that were previously characterized in other parasitic helminths as antigens or as being involved in mechanisms of host immune evasion and/or immune modulation (Chemale *et al.* 2003; Yatsuda *et al*. 2003; Bernal *et al.* 2004; Curwen *et al.* 2004; Knudsen *et al.* 2005; Nareaho *et al.* 2006; Wang *et al*. 2009; Kouguchi *et al*. 2010; Monteneiro *et al.* 2010; Nguyen *et al*. 2010; Santivanez *et al*. 2010; Aziz *et al*. 2011; De la Torre *et al.* 2011; Hewitson *et al.* 2011; Bien *et al.* 2012; Virginio *et al*. 2012; Cui *et al.*; 2013; Ludolf *et. al*. 2014; Dea-Ayuela *et al.* 2015). We also identified proteins known to be important vaccine candidates and/or drug targets. These proteins were grouped according to their scientific importance into three categories: antigens, vaccine candidates, and drug targets (Fig. 5). These findings prove that adult stage of *H. diminuta* secretes proteins, which may play an important role in host-parasite interactions.

Our results fit well within current research trends, as helminth proteomics has revealed new aspects of the host-parasite relationship, with results from experimental studies published on parasitic nematodes (Yatsuda *et al.* 2003; Nareaho *et al.* 2006; Rebello *et al*. 2011; Hewitson *et al.* 2011; Bien *et al*. 2012; Dea-Ayuela *et al*. 2015), digenean trematodes (Bernal *et al.* 2004; Curwen *et al.* 2004; Knudsen *et al.* 2005; Liu *et al.* 2009; De la Torre *et al.* 2011), and what is the most interesting for the results presented in this paper on cestodes. The latter were focused mainly on medically/veterinary important taeniid cestodes such as: *Echinococcus granulosus* (Chemale *et al.* 2003; Monteneiro *et al.* 2010; Aziz *et al*. 2011; Virginio *et al*. 2012; Cui *et al*; 2013), *Echinococcus multilocularis* (Wang *et al*. 2009; Kouguchi *et al*. 2010), and *Taenia solium* (Nguyen *et al*. 2010; Santivanez *et al*. 2010). Among non-taeniid cestodes, proteomic studies have been performed for the diphyllobothriid *Spirometra erinacei* (Kim *et al.* 2009) and mesocestoidid *Mesocestoides corti* (Laschuk *et al.* 2011). Most of the aforementioned authors described proteomic analysis of taeniid cestodes. Exceptions are two papers focused on proteomic survey of the cestode *M. corti* during the first 24 hour of strobilar development *in vitro* (Laschuk *et al.* 2011) and differential protein expression in *S. erinacei* according to its development in the final host (Kim *et al.* 2009).

According to our knowledge, antigenic proteins that are likely vaccine and drug target candidates have never been detected previously in E-S material of adult cestodes. Antigens of particular scientific interests include proteins such as: actin, annexin, calcium-binding protein, calpain, enolase, filamin, fructose bisphosphate aldolase (FBA), glutamate dehydrogenase (GDH), glycogenin, glyceraldehyde-3- phosphate dehydrogenase, Heat Shock Protein 70 kDa (HSP70), myoferlin, phosphoenolpyruvate carboxykinase and paramyosin (Fig. 5). Here, the identified proteins included some known vaccine candidates such as: myoferlin, filamin, enolase, glyceraldehyde-3-phosphate dehydrogenase, calpain, HSP70 and paramyosin (Fig. 5). Among proteins known as drug targets, we showed the presence of pyruvate kinase (PyK) and calcium-binding protein in the E-S products of adult *H. diminuta* (Fig. 5).

The proteomic study by Chemale *et al.* (2003) on metacestodes (hydatid cysts) of *E. granulosus* have provided interesting information about usefulness of gel-based proteomics and mass spectrometry in identification of proteins from the hydatid cysts. In spite of the absence of *E. granulosus* genome sequencing, available only from 2013 (Tsai *et al.* 2013), the aforementioned authors succeeded in identification of a number of antigenic proteins; among them are also those we have found in our studies (Hsp70, paramyosin, actin). As we observed similar proteins, this may indicate that some proteins with antigenic properties are common not only throughout the cestode life cycle, but in cestodes in general. Recent data (Monteneiro *et al.* 2010) indicate that helminth paramyosins are multifunctional modulators of the host immune response, binding complement components, immunoglobulins and secreted components of the cellular immune response. The presence of paramyosin in E-S products of *H. diminuta* may therefore play an important role in host immune system evasion and be crucial for cestode survival in the host environment.

In 2009, Wang *et al.* (2009) detected on immunoblots of *E. multilocularis* protoscoleces over fifty protein spots as antigens, and defined fifteen of them. These were classified as actins, trypomyosin, heat shock proteins, *etc*. Results of these studies showed that cytoskeletal and Hsp proteins are immunodominant antigens in alveolar echinococcosis. Taking into account the aforementioned data from immature stages, we can conclude that our results suggests that cytoskeletal and Hsp proteins may play crucial roles in host-parasite interaction and host immune system evasion. We have observed the higher protein abundance measured by OD level between the studied groups. Evident disproportionality was present and protein abundance was clearly higher in the group of parasites with longer time spent in media. These differences could be explained by the decrease in the concentration of nutritive substances in the culture media and simultaneous increase in the production of proteins engaged in nutrient uptake, and could be associated with shedding of the glycocalyx, which may result in the increase in concentration of proteins associated with the tegument into the culture media.

However, the immature stages of highly pathogenic taeniids are not the only cestodes able to interact with their hosts and to produce a number of mechanisms allowing them to adapt to the surrounding environment of the host. Adult parasites also need to evolve mechanisms and adaptations helping them to survive in an extremely hostile environment that prevails in the digestive system of their hosts. These must include production of proteins, some of them with antigenic properties, and is likely to have led to continuous evolution of host-parasite interrelationship.

Proteomic analyses of hydatid cysts and adults of *E. granulosus* were performed recently by Cui *et al.* (2013). This detailed paper revealed some novel insights into host-parasite interrelationships. According to these analyses, paramyosin,

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actin, phosphoenolpyruvate carboxykinase (PEPCK) and an antigen B (AgB) were the dominant adult proteins and were the most abundant adult proteins as determined by spectra count. PEPCK proteins are directly involved in many pathways including excretory pathway, endocrine pathways and carbohydrate metabolism pathways (Cui *et al.* 2013). Similar proteins were detected in our study and are considered to be antigenic. In addition, we noted the presence of some of the known vaccine candidates (Fig. 5). Some of them were also observed by Cui *et al.* (2013) to be expressed in the adult stage of *E. granulosus*. These include tegumental membrane vaccine protein enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Numerous proteins identified in E-S products of adult *H. diminuta* in the present study have never been detected among E-S proteins of any other cestode species including metacestode stages (shown as bold in Table I). As they were present in the immunoreactive bands, their immunomodulatory properties are possible; some of these proteins are known as antigens from other helminths (Fig. 5), therefore their possible role as antigenic factors in cestodiasis will be the subject of our future careful research based on 2DE and 2D immunoblotting. The most promising candidates were identified from the immunoreactive protein bands 1, 2, 3 and 10 (Table I). None of these proteins has ever been observed in the E-S products of adult tapeworms.

Using LC-MS/MS we identified a number of proteins that have never been noted in E-S products of any other cestode species. Similar to the research done by Liu *et al.* (2009) who studied secretome of schistosomes, structural proteins including cytoskeleton and muscle proteins were observed also in E-S material of *H. diminuta*. These structural proteins, which were noted in cestode E-S material for the first time, included titin, filamin, myoferlin, paramyosin, gelsolin, and neurogenic locus notch protein. Studies by Sotillo *et al*. (2008) indicated that structural proteins such as actins may induce early IgM response and help the worm to survive. Besides the presence of HSP 70kDa, which is commonly observed in the secretomes of metacestodes, we report here the high abundance of HSP 71kDa protein. We suppose that similarly to other studies, this HSP protein may play a role in immunostimulatory and immunosuppresive processes (Asea 2008, Pockley *et al.*2008).

We suspect that some of the proteins that are observed for the first time are involved directly in the complex host-parasite interaction and parasite survival inside its host. Our results indicate the presence of detoxification proteins such as peroxidasin, which is known to play a role in the response to oxidative stress (Tsai *et al.* 2013). Other proteins associated with detoxification are: receptor type tyrosine protein phosphatase, glutamate dehydrogenase and puromycin sensitive aminopeptidase. The first one, among the others, is believed to be associated with T-cell activation.

Some of the newly observed proteins were associated with transport (basement membrane specific heparan sulfate, phospholipid transporting atepase, armadillo type fold), ion-binding and/or ion channels (anoctamin, sarcoplasmic calcium binding protein), whereas alpha 2 macroglobulin is a protein engaged in cell communication. The presence of proteins belonging to ion channels, transport proteins and receptors are important from the practical point of view as they are considered as drug targets (Tsai *et al.* 2013).

Numerous proteins are involved in metabolic processes: pyruvate kinase, deoxyhypusine hydroxylase, glyceraldehyde 3 phosphate dehydrogenase, glycogenin, lactate dehydrolase. They play an important role in key metabolic processes, for instance malate metabolism (NADP dependent malic enzyme) and glycolysis (pyruvate kinase), or as enzymes of catalytic activity (glycogenin, lactate dehydrolase, glyceraldehyde 3 phosphate dehydrogenase, deoxyhypusine hydroxylase). The presence of cytosolic enzymes in the E-S material of the adult parasitic flatworms was previously observed by Liu *et al.* (2009). Pyruvate kinase is one of the immunodominant proteins of *Neospora caninum* and was recognized by sera from the infected animals. This suggests that it might be exposed to the host immune system. Some of the metabolic enzymes may be derived from the tegument. For instance, glyceraldehyde 3 phosphate dehydrogenase, a multifunctional protein bound to the cell membrane (Sirover 1999), was identified by Perez-Sanchez *et al.* (2008) as a tegumental protein of *Schistosoma bovis*. Monteneiro *et al.* (2010) point out that the intracellular and membrane proteins identified in *E. granulosus* hydatid fluid may be engaged in the process of immune evasion. These proteins may represent products of parasite tegument shedding. Our study shows that intracellular and membrane proteins are present in the E-S material of the adult tapeworm; this may confirm the role of tegumental proteins in host immune system evasion.

One of the important aspects of parasitic helminth antigens is their potential use as immunomodulatory and anti-inflammatory molecules. The concept of helminth-derived molecules as a source of immunomodulatory agents is fully established (Harnett 2014), and the literature is flush with examples of how crude extracts and partially characterized molecules from many species of parasitic helminth can affect virtually all aspects of innate and adaptive immune responses (Harnett 2014; Johnston *et al.* 2009, 2010; Shi *et al.* 2011; Hernandez *et al.* 2013; Reyes *et al.* 2015). Helminth parasites provoke multicellular immune responses in their hosts that can suppress concomitant disease (Reyes *et al.* 2015). Despite the progress in research on potential use of parasite antigens as new drugs in therapies of inflammations of different kinds to date, this wealth of data has not been converted into new drugs. As was suggested by Johnston *et al.* (2009) the promise of parasitic helminths as a pharmacopeia has yet to be fulfilled, given the advent of technology for the purification and characterization of molecules from small amounts of tissues and the synthesis of agents for testing in high throughput immunological assays. It has been demonstrated that the low-pathogenic cestode *H. diminuta* may regulate inflammation and inflammatory diseases (Johnston *et al.*

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2009, 2010, Hernandez *et al.* 2013; Reyes *et al.* 2015). In the use of *H. diminuta* as a potential organism in helminth therapy, its low pathogenicity might be important. Unlike other parasites assessed for helminth therapy, it causes no host tissue damage, while potently suppressing murine colitis (Reyes *et al.* 2015). This is why *H. diminuta* is used as a model organism with immunomodulatory potential (Johnston *et al.* 2009, 2010; Melon *et al.* 2010; Shi *et al.* 2011; Graepel *et al*. 2013; Hernandez *et al.* 2013; Reyes *et al.* 2015). However, to date little is known about which parasite proteins take part in the process of regulation and suppression. Our data indicate, for the first time, which proteins of *H. diminuta* may be associated with the process of immunomodulation. In this respect our data fill the gap and indicate which proteins of *H. diminuta* may be of particular importance when considered as immunomodulators. We have found a number of proteins previously known as antigenic, as well as new antigenic candidates (Fig. 5 and Table I); some of them we observed for the first time in the E-S products of any cestode species, including larval and metacestode stages. In addition, our results point out potential candidates. These potential antigens will be analyzed in our future research with use of 2D immunoblotting.

Despite the increasing knowledge of helminth genomics and proteomics, many aspects of host-parasite interactions, especially interrelation between the adult tapeworms and their definitive hosts, still remain to be elucidated. Therefore extensive research dedicated to this low-pathogenic and neglected developmental stage is needed for better understanding of tapeworm evolutionary success and their influence on the host organism.

Our studies show that E-S proteins of *H. diminuta* play an important role in host-parasite interactions at the molecular level. Among them are proteins known as antigens and vaccine candidates, whereas the others might represent drug targets for new therapies. Some of the E-S-proteins identified here have never been detected in the E-S-products of any adult tapeworm before and, as they are present in the immunoreactive bands, they may represent new potential antigens. Therefore further studies with the use of 2DE and 2D immunoblotting are planned to test their immunogenic properties.

Competing interests. The authors declare that they have no competing interests.

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