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Zygocotyle lunata: Proteomic analysis of the adult stage

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ABSTRACT

The somatic extract of *Zygocotyle lunata* (Trematoda: Paramphistomidae) adults collected from experimentally infected mice was investigated using a proteomic approach to separate and identify tryptic peptides from the somatic extract of *Z. lunata* adult worms. A shot-gun liquid chromatography/tandem mass spectrometry procedure was used. We used the MASCOT search engine (Matrix-Science) and ProteinPilot software v2.0 (Applied Biosystems) for the database search. A total of 36 proteins were accurately identified from the worms. The largest protein family consisted of metabolic enzymes. Structural, motor and receptor binding proteins and proteins related to oxygen transport were identified in the somatic extract of *Z. lunata*. This is the first study that attempts to identify the proteome of *Z. lunata*. However, more work is needed to improve our knowledge of trematodiasis in general and more specifically to have a better understanding about host–parasite relationships in infections with paramphistomes.

1. Introduction

The paramphistomids are cosmopolitan digenetic trematodes characterized by the absence of an oral sucker and with an acetabulum located near the posterior aspect of the body (Jones, 2005). Various species in different paramphistomid families cause diseases collectively referred to as paramphistomiasis (Sanabria and Romero, 2008). Paramphistomiasis is mainly a disease of domestic and wild animals and, given the high prevalence of these worms, it is likely that their importance is underestimated globally (Lofty et al., 2010). In ruminants, paramphistomes are responsible for a lower nutrient conversion resulting in a loss of weight and a decrease in milk production, causing great economic losses (Horak, 1971). Moreover, human paramphistomiasis caused by *Gastrodiscoides hominis*, *Watsonius watsoni*, and *Fischoederius elongatus* has also been reported (Toledo et al., 2006).

In spite of the economic importance of paramphistomes, only a few studies are available on the relationships of these host–parasite systems. In this context, proteomics may be a useful tool for studies on this topic. However, our current knowledge on the proteomics of paramphistomes is almost nil. For example, a search on NCBInr databases for each of the 10 families included in the Paramphistomoidea (Jones, 2005) only retrieves 7 proteins (5 of *Paramphistomum epiclitum* and 2 for *G. hominis*). This finding indicates a need for further research on this topic.

Zygocotyle lunata is a cosmopolitan digenean belonging to the family Zygocotylidae. It presents as an aquatic two host life cycle. Briefly, eggs are released with the faeces of the definitive host and the miracidia hatch in an aquatic environment and infect the snail intermediate host. A generation of sporocysts and two redial generations occur in the snail intermediate host. The cercariae leave the snail, encyst on substrata such as aquatic vegetation or the shells of snails and become metacercariae. The definitive host becomes infected following ingestion of the encysted metacercariae. Adult worms are located in the caecum of various species of birds and mammals in the wild (Fried et al., 2009). In the laboratory, *Z. lunata* larval stages can be grown in certain species of planorbid snails (*Helisoma* or *Biomphalaria*) and adults can be grown in various vertebrate hosts including domestic chickens, laboratory mice, rats and hamsters. Thus this parasite is a good model to study the host–parasite relationships of paramphistomes. The purpose of this study is to analyze the proteome of *Z. lunata* adult worms obtained from mice.

The present work is the first detailed study of the proteome of a paramphistomid. Identification of the components of *Z. lunata* proteome may be important for understanding host–parasite relationships in paramphistomiasis. The information reported herein provides baseline data that should be useful for future work which examines interactions of this group of digeneans with their hosts.

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2. Materials and methods

2.1. Parasites and experimental infections

Methods used to obtain and study this trematode have been previously described (LeSage and Fried, 2010). Encysted metacercariae of *Z. lunata* were collected after emergence from naturally infected *Helisoma trivolvis* snails and used to infect Balb/c mice (*Mus musculus*). Male mice weighing 25 g approximately were infected through a stomach tube with 20 metacercariae of *Z. lunata* each. The animals were maintained under standard conditions with food and water *ad libitum*. Worm egg release was investigated daily in each infected animal as described previously (Toledo et al., 2003).

2.2. Obtaining the somatic extract of Z. lunata

To prepare somatic (WWE) extract, adult worms were collected from the caecum of mice 6 weeks after infection, thoroughly washed with phosphate-buffered saline (PBS; pH 7.4), homogenized in culture medium of PBS containing 0.8 mM phenylmethyl-sulfonyl fluoride (Sigma, St. Louis, Missouri, USA), 100 U penicillin (Sigma), and 100 mg/ml streptomycin (Sigma), and centrifuged at low speed to remove larger debris The resulting supernatant was centrifuged at 15,000*g* for 30 min at 4 °C, measured (Bio-Rad protein assay) and adjusted to 1 mg/ml.

2.3. Liquid chromatography and tandem mass spectrometry (LC–MS/MS)

The proteins obtained were digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko et al., 1996). The digestion mixture was dried in a vacuum centrifuge, reconstituted with 20 μl of triethylamine (Sigma, St. Louis, MO, USA) 20 mM in water (pH 11.5), and injected to an UPLC Acquity system with a high pH stable RP column (Waters, Milford, MA, USA; C18; 2.1 mm × 15 cm) at a flow rate of 150 μl/min. The peptides were eluted with a mobile phase B of 5–45% linear gradient over 40 min (A, 20 mM TEA in water at pH 11.5; B, 20 mM TEA in acetonitrile). Ten fractions were collected (one every 4 min), evaporated under vacuum, reconstituted into 10 μl of 0.1% formic acid (pH 2). Five microliters of the resulting suspension were delivered to a trap column (LC Packings Amsterdam, C18 PepMap100, 5 μm, 300 μm × 5 mm) using capillary HPLC (Switchos, LC Packings) via an isocratic flow of mobile phase (0.1% TFA in water) at a rate of 30 μl/min for 3 min. The flow rate was then switched to 200 nl/min, and the peptides were flushed into the analytical column (LC Packings C18 PepMap100, 3 μ, 100 Å, 75 μm × 15 cm) and eluted via a mobile phase gradient: 15–50% B (A: 0.1% formic acid in water, B: 0.1% formic acid in 95% acetonitrile) over 120 min. The elution was directly applied to a nanospray source of a QSTAR XL instrument (Applied Biosystems, Framingham, MA, USA), and information-dependent acquisition analysis was carried out with IDA acquisition cycles in MS and MS/MS mode along all the chromatogram.

2.4. Database search

Database search on NCBInr databases was performed using MASCOT search engine (Matrix-Science) and ProteinPilot software v2.0.1 (Applied Biosystems). MASCOT searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications. A protein identification was considered accurate when overall MASCOT score was greater than 50 (Sotillo et al., 2008). ProteinPilot software uses the Paragon algorithm (Shilov et al., 2007), and then it is not necessary to fix mass tolerance or possible modifications because the algorithm explores all the possibilities.

3. Results and discussion

Z. lunata can be found globally in many natural definitive hosts including waterfowl and numerous species of ruminants. This trematode can be maintained easily in the laboratory because of its abbreviated two host lifecycle. In this context, *Z. lunata* is a good model to study the host–parasite relationships at a molecular level (Fried et al., 2009). As an approach to characterize the proteome of *Z. lunata*, we have analyzed the WWE of *Z. lunata* adult worms collected from Balb/c mice.

The fact that trematodes can not be cultured in vitro and their low number of sequences deposited in databases makes the analysis of spectrometric data and the study of the proteome of trematodes difficult. To overcome these difficulties, we have used two approximations following the methodology proposed by Sotillo et al. (2010a): (1) we have performed a shot-gun LC/MS-MS for the separation and identification of tryptic peptides from the complex mixture of proteins because 2D electrophoresis does not facilitate the identification of less abundant proteins; and (2) we have analyzed our spectrometric data using both MASCOT and ProteinPilot software to increase the detection of similarities with known proteins in NCBInr databases.

The number of peptides identified by LC/MS-MS was high (8528 peptides) although only 0.49% (42 peptides) showed significant homologies with known proteins. This could be related to the fact that very few paramphistomidae sequences are deposited in the databases. Moreover, no EST collection is available for *Z. lunata*, and no transcriptome projects have been done by any research groups, which complicate the identification of more proteins. In fact, all the proteins were identified because their homologies with proteins of other organisms.

A total of 36 proteins were accurately identified (15 using MASCOT and 21 using ProteinPilot). Using MASCOT, a total of 17 peptides (0.19%) showed similarities with known sequences, corresponding to 15 proteins (Table 1). Using ProteinPilot software, 25 peptides (0.29%) were similar to deposited sequences corresponding to 21 parasite proteins (Table 2). One protein was discarded for further analysis, as it seemed to be a host protein (Table 2).

From the total of identified proteins present in the WWE of *Z. lunata*, the most represented are the metabolic enzymes. This fact is consistent with other studies on trematodes (Bernal et al., 2006; Guillou et al., 2007; Sotillo et al., 2010a). From these proteins, four (aldolase, enolase, GAPDH, and 2-phospho-D-glycerate hydrolase) are glycolytic, three (phosphoglucomutase, phosphoenolpyruvate carboxykinase and malate dehydrogenase) are implicated in gluconeogenesis and the remainder are implicated in other mechanisms such as oxidative phosphorylation (NADH dehydrogenase) or other catabolic mechanisms (metallocarboxypeptidase, tyrosine-3-monooxygenase and adenylatecyclase). Their presence in WWE from *Z. lunata* should not be unexpected as they participate in the energetic processes needed by the parasite. Also, some of these proteins such as enolase, aldolase, phosphoenolpyruvate carboxykinase, phosphoglycerate kinase, triose phophate isomerase or GAPDH, have been described on the surface of different helminths such as *Echinostoma caproni*, *Echinostoma friedi*, *Schistosoma mansoni or Schistosoma japonicum* (Bernal et al., 2006; Perez-Sanchez et al., 2008; Marcilla et al., 2007; Liu et al., 2009;