International Journal for Parasitology: Parasites and Wildlife 3 (2014) 67–74

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Proteomic profile of *Ortleppascaris* sp.: A helminth parasite of *Rhinella marina* in the Amazonian region

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ARTICLE INFO

Article history:
Received 12 February 2014
Revised 20 March 2014
Accepted 20 March 2014

Keywords:
Ortleppascaris
Nematode
Amazonian amphibian
Somatic proteome
Mass spectrometry

ABSTRACT

Ortleppascaris sp. is a helminth that, in its larval stage, infects the liver parenchyma of the amphibian *Rhinella marina*, resulting in severe physiological and pathological changes. This study used a proteomic approach to determine the overall profile of proteins expressed in a somatic extract from the nematodes to investigate the relationship between the parasite and its host. A total of 60 abundant proteins were selected from the two-dimensional electrophoresis, identified by peptide mass fingerprinting, and grouped based on their Gene Ontology by the biological processes in which they are potentially involved. Important helminthic derivatives, such as the immunoreactive As37 antigen, guanylyl cyclases, proteolytic enzymes, and other proteins conserved among different parasites, were identified through homology. This study represents a new approach to helminth-related proteomic studies using an amphibian animal model. Furthermore, this study identified protein markers that are important to the host–parasite relationship and the viability, development, infectivity, and virulence of helminths.
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1. Introduction

Helminths are complex, often dangerous organisms that continue to be neglected, even in this era of health system globalisation (Hotez, 2009). However, with the emergence of "omics" tools, particularly proteomics, these organisms have been investigated at the molecular level (Loukas et al., 2011). Proteomics has provided ample information and promising results, identifying a repertoire of helminth derivatives that may reveal their biology, mode of infection, and the pathology associated with infection (Northern and Grove, 1987; Jenkins et al., 2005; Craig et al., 2006; Hewitson et al., 2008; Mutapi, 2012).

Molecular studies are needed for the design of future interventions, such as antihelminthic vaccines, which may directly influence the quality of life of approximately two billion infected people worldwide. In addition, the occurrence of infection in animals and plants, which are the organisms most affected by parasitic infections, represents an important public health problem and is of global economic significance (Nagaraj et al., 2008; Garg and Ranganathan, 2012).

As in the evolutionary dynamic proposed in the Red Queen Hypothesis by Valen (1973), many studies have indicated (Brindley et al., 2009; Jackson et al., 2009; Allen and Maizels, 2011) that helminths have co-evolved along with their hosts, resulting in a favourable environment for the survival and co-existence of both antagonistic parties in a complex adaptive mechanism. This host–parasite adaptation is closely related to the host immune system, which detects and attempts to inactivate and/or destroy the invader. The parasite, in turn, develops strategies for escape by mimicking and manipulating the host immune system, thereby adapting the host response to parasitism (Mckay, 2006).

Research has shown that by manipulating their immune systems, the hosts can become vulnerable to other infections or, conversely, suppress their immunopathological reactions. These processes also depend, in evolutionary terms, on the duration of the relationship between helminth parasites and their hosts (Mckay, 2006; Soblik et al., 2011). Johnston et al. (2009) suggest that bioactive molecules, mainly proteins, are responsible for modulating the host immune response in favour of the parasites.

These biomolecules are structurally and functionally diverse and include cytokines, chemokines, hormones, digestive enzymes, antibodies, extracellular proteinases, toxins, and antimicrobial peptides. Some of these proteins are involved in vital biological processes, such as oxidation–reduction processes, cell adhesion and migration, cell–cell communication, differentiation, proliferation, morphogenesis, defence, virulence factors, and immune responses (Maizels and Yazdanbakhsh, 2003; Bonin-Debs et al.,

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http://dx.doi.org/10.1016/j.ijppaw.2014.03.003
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2004; Geary et al., 2012). According to Craig et al. (2006), these substances are derived from the surface of the helminth or from specialised excretory/secretory glands and are often released at specific stages. Among the group of helminths that release immunomodulating substances, the phylum Nematoda is important because its members have been used in clinical trials for the treatment of immunological diseases and vaccine development (Mckay, 2006; Babayan et al., 2012; Pearson et al., 2012).

In the present study, helminths of the genus *Ortleppascaris* were used. As adults, these parasites are found in the gastrointestinal tract of Crocodilians (Goldberg et al., 1991), and in their larval form, they are found in the liver of amphibians (Moravec and Kaiser, 1995; González and Hamann, 2006, 2007), including *Rhinella marina* in Brazil, as previously described by Silva et al. (2013a).

According to Silva et al. (2013b), the nematode infects the liver of *R. marina* in its larval parasitic form and develops inside fibrous cysts, which are apparently filled with amorphous material and cellular debris. Infection with these larvae induces a significant increase in the area occupied by melanomacrophages, which are phagocytic immune system cells in amphibians, and other pathological changes, such as the presence of inflammatory infiltrates distributed throughout the liver parenchyma. However, despite severe physiological changes, the defence system of the anuran is, in most cases, unable to curb the presence of the parasite because the larvae remain alive and develop in these organs from their elementary stages.

The proteomic analysis of biomolecules from *Ortleppascaris* sp. larval parasites of *R. marina* contributes to the understanding of the host–parasite relationship. Further, the screening of molecular markers generates information for the study of immunomodulatory products, which are targets of interest for the control of helminth infections in humans and animals.

2. Materials and methods

2.1. Parasites

Ten adult synanthropic specimens of *R. marina* (six females and four males) were caught in the urban area of the city of Belém, Pará, Brazil (01° 27′ 20″ S and 48° 30′ 15″ W). These hosts were naturally infected with *Ortleppascaris* larvae, which were collected from the interior of hepatic cysts. Approximately 200 parasites were subjected to three washes steps with PBS (pH 7.4) and then stored in extraction buffer (7 M urea, 2 M thiourea, 2% CHAPS) at −20 °C.

2.2. Preparation of a somatic extract of Ortleppascaris sp.

Somatic protein extracts were obtained by macerating approximately 200 larvae in extraction solution (7 M urea, 2 M thiourea, 2% CHAPS) whilst being cooled with liquid nitrogen and then centrifuging the extracts at 13,000×*g* for 15 min at 4 °C. The supernatant was directly used for protein analysis.

2.3. Two-dimensional electrophoresis

The total protein concentration was determined using the Bradford method (Bradford, 1976), and the samples were stored at −80 °C until use. Aliquots of protein extract containing 100 μg of sample were diluted to a final volume of 125 μL in Destreak solution (GE healthcare) and 2% IPG buffer (pH 3–10) (GE healthcare). Seven-centimetre strips (Immobiline, GE healthcare) with an immobilised pH gradient in the range of 3–10 were rehydrated with the protein extract for 17 h using IPGBox (GE Healthcare). Isoelectric focusing was initiated immediately after rehydration.

Isoelectric focusing was performed with an automated system (Ettan IPGphor III GE Healthcare) at 20 °C with a constant current of 50 μA/strip and a total of 5.0–6.5 kVh following a four-step programme: 300 V for 4 h; linear gradient to 1000 V for 30 min; linear gradient to 5000 V for 1:20 h; and 5000 V for 30 min.

After isoelectric focusing, the strips were reduced in equilibration buffer (6 M urea, 0.075 M Tris HCl (pH 8.8), 29% glycerol, 2% SDS, and 0.02% bromophenol blue) containing 2% dithiothreitol (DTT) for 30 min and then alkylated for 30 min in equilibration buffer containing 2.5% iodoacetamide.

For the second dimension, the strips were placed on a 12.5% polyacrylamide gel in a Mini Protean Cell system (Bio-Rad). Electrophoresis was performed at a constant 80 V for 2 h. The gels were stained with Coomassie Blue G-250 solution overnight with stirring and scanned with an ImageScanner III (GE Healthcare) using Labscan software (GE Healthcare).

2.4. In-gel tryptic digestion and mass spectrometry

Spots detected by ImageMaster 2D Platinum 7.0 software (GE Healthcare) and observed with the naked eye were manually excised, treated with washing solution (50% methanol, 5% acetic acid), and then dehydrated in 100% acetonitrile in a vacuum centrifuge at room temperature. The proteins were subsequently subjected to reduction (10 mM DTT) and alkylation (100 mM iodoacetamide).

The samples were digested at 37 °C overnight with proteomic-grade trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate (final concentration: 20 ng/μL). Tryptic peptides were extracted from the gel solution with 50% acetonitrile in 5% formic acid.

The extracted peptides were transferred to a sterile tube and treated with 100 mM ammonium bicarbonate, dried in a vacuum centrifuge, and resuspended in a solution of 50% acetonitrile, 0.05% formic acid, and 0.1% trifluoroacetic acid.

Aliquots of 0.5 μL of each sample were applied to a steel plate at a 1:1 ratio with 2,5-dihydroxybenzoic acid matrix (Sigma). After crystallisation, the plate was inserted into the mass spectrometer for analysis. All MS spectra were obtained in the *m*/*z* range of 800–4000 kDa using a MALDI-TOF Ultraflex III spectrometer (Bruker Daltonics). The spectra were analysed using FlexAnalysis 3.3 software (Bruker Daltonics) for the determination of peaks.

The search for protein homology by peptide mass fingerprinting (PMF) was performed using the Blaxter Lab database (NEMBASE4) and Mascot Daemon software (Matrix Science). The search parameters were set as follows: up to two missed cleavage sites; 0.1 kDa error for the identification of peptides; carbamidomethylation of cysteines as a fixed modification and oxidation of methionine as a variable modification. PMF data analysis was supplemented by linking with Gene Ontology (GO) through the UniProt database to infer the biological processes in which the identified molecules are involved. The identified proteins were grouped into functional categories and subcategories according to their ontology.

3. Results

To characterise the protein profile of the nematode's somatic extract, the most abundant proteins detected with Image Master 2D Platinum software, shown in Fig. 1 and indicated as spots 1–60, were analysed by PMF using mass spectrometry.

The list of marked spots on the two-dimensional gel (Fig. 1) with their proteins and corresponding helminth according to the NEMBASE4 database are summarised in Table 1. Of these, 26 spots were identified, 24 spots were identified as hypothetical proteins,

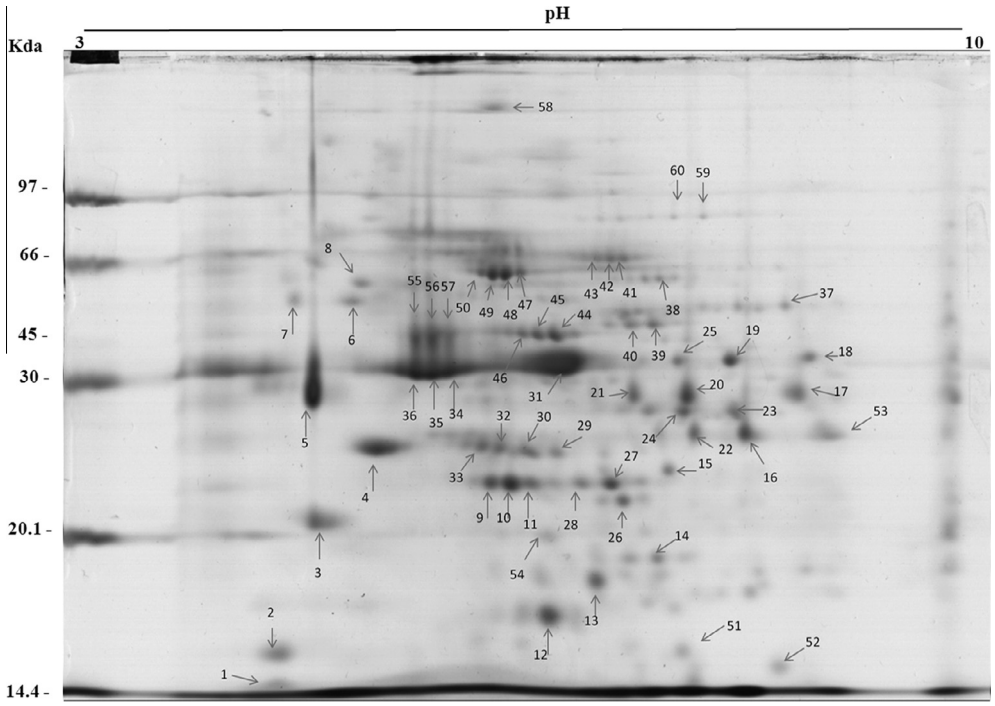

Fig. 1. 2D gel containing the somatic extract of *Ortleppascaris* sp. larvae. See Table 1 for details.

Table 1
Summary of somatic extract proteins of *Ortleppascaris* sp. identified by PMF using NEMBASE4 database. (See Supplemental Table 1 for details.)

Spot	Protein [helminth]
01	Hypothetical protein CAEBREN_23080 [*Caenorhabditis brenneri*]
02	CRE-ATH-1 protein [*Caenorhabditis remanei*]
03	Hypothetical protein CAEBREN_12726 [*Caenorhabditis brenneri*]
04	As37 [*Ascaris suum*]
05	Sulphotransferase domain containing protein [*Brugia malayi*]
06, 33, 34, 37–40	Cell division protein kinase 7 [*Ascaris suum*]
07	CBN-CDC-37 protein [*Caenorhabditis brenneri*]
08	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit [*Ascaris suum*]
09, 29, 48, 60	Hypothetical protein CAEBREN_00296 [*Caenorhabditis brenneri*]
10	Hypothetical protein CAEBREN_00853 [*Caenorhabditis brenneri*]
11, 16, 41, 42	LONg family member (lon-3) [*Caenorhabditis elegans*]
12	Hypothetical protein F46F3.3 [*Caenorhabditis elegans*]
13, 50	Guanylyl cyclase [*Heterodera glycines*]
14	CRE-NSY-4 protein [*Caenorhabditis remanei*]
15	Cleavage and polyadenylation specificity factor subunit 2 [*Trichinella spiralis*]
17, 19, 44, 49	Uncharacterised protein family UPF0005 containing protein [*Brugia malayi*]
18	Hypothetical protein LOAG_04901 [*Loa loa*]
21	Hypothetical protein LOAG_06031 [*Loa loa*]
22	GTP-binding protein SAR1 [*Brugia malayi*]
23	Hypothetical protein CRE_11672 [*Caenorhabditis remanei*]
25	Hypothetical protein CAEBREN_23609 [*Caenorhabditis brenneri*]
26	Hypothetical protein LOAG_09802 [*Loa loa*]
27	Hypothetical protein CRE_25790 [*Caenorhabditis remanei*]
28, 31, 35, 55, 56	Actin variant 1 [*Dictyocaulus viviparus*]
30, 59	[Pyruvate dehydrogenase acetyl-transferring-phosphatase 1 [*Ascaris suum*]
32	Formyl-CoA transferase [*Micromonospora aurantiaca* ATCC 27029]
36	Hypothetical protein LMOh7858_0493 [*Listeria monocytogenes* str. 4b H7858]
43	Hypothetical protein CBG09068 [*Caenorhabditis briggsae*]
46, 47, 52	Hypothetical protein AbauAB_10162 [*Acinetobacter baumannii* AB900]
51	rnf126-prov protein [*Brugia malayi*]
53	Nodal modulator 1 [*Ascaris suum*]
57	Hypothetical protein CBG_14574 [*Caenorhabditis briggsae* AF16]
58	Hypothetical protein LOAG_01253 [*Loa loa*]

and six spots were not characterised. Four spots did not produce spectra of sufficient quality to allow their identification because they had insufficient homology with any protein from the database.

Table 2 shows the list of identified proteins with their respective spot numbers. They are grouped into functional categories and subcategories according to their GO terms (see also Supplemental Table 1). Among the proteins identified, we found a number

Table 2
Summary of the identified proteins from the gel of the somatic extract and the category of biological process in GO terms. See Supplemental Table 1 for additional information.

GO term	Process/biological function	Spot	Protein
Molecules of metabolic processes and energy production			
GO: 0006535	Biosynthesis of cysteine from serine	01	Hypothetical protein CAEBREN_23080
GO: 0016787	Hydrolase	02	CRE-ATH-1 protein
GO: 0008152	Metabolic process	03	Hypothetical protein CAEBREN_12726
		32	Formyl-CoA transferase
GO: 0008146	Sulphotransferase	05	Sulphotransferase domain containing protein
GO: 0019901	Protein kinase ligand	07	CBN-CDC-37 protein
GO: 0016787/ GO: 0000104	Hydrolase/succinate dehydrogenase action	15	Cleavage and polyadenylation specificity factor subunit 2
GO: 0016787/ GO: 0006470	Hydrolase/protein dephosphorylation	30, 59	Pyruvate dehydrogenase acetyl-transferring-phosphatase 1
Structural molecules and organic morphogenesis			
GO: 0051301/ GO: 0004674	Cell division/protein phosphorylation	06, 33, 34, 37–40	Cell division protein kinase 7
GO: 0005856	Cytoskeleton	10	Hypothetical protein CAEBREN_00853
		57	Hypothetical protein CBG_14574
		28, 31, 35, 55, 56	Actin variant 1 [*Dictyocaulus viviparus*]
GO: 0010171/ GO: 0042329	Body morphogenesis/constituents of cuticle structure	11, 16, 41, 42	LONg family member (lon-3)
GO: 0016021	Membrane protein	14	CRE-NSY-4 protein
		17, 19, 44, 49	Uncharacterised protein family UPF0005 containing protein
Immunoreactive molecules and virulence factors			
GO: 0005515	Protein binding	04	As37
GO: 0006508	Proteolysis	43	Hypothetical protein CBG09068
Molecules of the biosynthetic/secretory pathway			
GO: 0004579/ GO: 0018279	Glycotransferase/N-terminal glycosylation in asparagine	08	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit
GO: 0016192	Transport mediated by vesicles	22	GTP-binding protein SAR1
Molecules of the chemosensory process			
GO: 0035556	Intracellular signal transduction	13, 50	Guanylyl cyclase

of molecules involved in the following processes: metabolic processes and energy production molecules (spots 01, 02, 03, 05, 07, 15, 30, 32 and 59); structural molecules and organic morphogenesis (spots 06, 10,11, 14, 16, 17, 19, 28, 31, 33, 34, 35, 37, 38, 39, 40, 41, 42, 44, 49, 57, 55 and 56); immunoreactive molecules and virulence factors (spots 04 and 43); biosynthetic/secretory pathway molecules (spots 08 and 22); and chemosensory process molecules (spots 13 and 50).

The bioproducts detected in the somatic extract included enzymes involved in metabolic processes and energy production (spots 1, 2, 3, 15, 30 and 59), such as hydrolases of the protein phosphatase 2C family (PP2C), sulphotransferases (spot 5) and transferases from CoA transferase family III (spot 32), which are evolutionarily conserved and related to energy production in helminths and protozoa.

Some of the abundant proteins were associated with body morphogenesis (spots 11, 16, 41 and 42), which corresponds to the collagen present in the cuticle of helminths. Similarly, other structural components, such as cytoskeletal proteins, were indicated among the hypothetical proteins (spots 10 and 57) or identified as proteins from the actin family (spots 28, 31, 35, 55 and 56).

Seven proteins (spots 06, 33, 34, 37, 38, 39 and 40) related to cell division and protein phosphorylation processes, which are important to the developmental process of the larvae, were found.

Endoplasmic reticulum (ER) membrane proteins related to the biosynthetic/secretory pathway are also included in this somatic proteome; these molecules are involved in the process of asparagine glycosylation (spot 8) and vesicular trafficking between the ER and Golgi, such as the SAR1 protein (spot 22).

Guanylyl cyclases (spots 13 and 50), molecules of great importance to the biology of chemosensory processes in helminths, were identified. They are involved in cell signalling, neural circuits, host recognition, and host–parasite relationships.

Surprisingly, some other relevant proteins were identified, such as As37 (spot 4), which is a member of the immunoglobulin family and a highly reactive antigen first recorded in *Ascaris suum*. Also in this set, proteolytic enzymes (spot 43), which are virulence factors for the establishment and maintenance of helminthic infection, were identified.

4. Discussion

Problems with the identification of peptide sequences in the proteomic analysis are related to peaks which do not achieve significant scores due to many of these databases are incomplete or not well established (Medina et al., 2005). According to Antunes et al. (2008), these data should be considered even when they present low scores due to the lack of a complete and specific database for the organism in question. The scarcity of database entries for each sequence is responsible for the low scores. However, detecting these molecules is expected to be a strong indication of their presence in the studied systems. Unfortunately, there is no database specific for *Ortleppascaris* and for many other helminths of veterinary interest, particularly those associated with wildlife. Therefore, using NEMBASE4, we integrated transcriptomics data from helminths that are taxonomically related into a proteomics analysis of *Ortleppascaris* as a strategy to predict proteins in our study.

The molecules present in the proteomic profile in question give an alternate view of a parasitism that is well established through co-evolution guided to adaption for both organisms. This infection may represent an example of common strategies developed by larval helminths through exploitation of the host tissue.

These larvae obviously express a group of molecules that are needful to maintain the larva's own development without external complications. These products mainly comprise molecules involved in basal metabolism; energy production; the development of body structure; virulence factors, which are responsible for maintaining the infection; and finally, chemosensory proteins, which give these larvae the special "skills" needed to map the environment in which they are living.

4.1. Metabolic process and energy production molecules

The somatic extract of *Ortleppascaris* includes molecules that are related to basic metabolism. This group of molecules includes a large group of enzymes, such as hydrolases. Ishii et al. (1998) showed that these proteins catalyse the breakage of several covalent bonds that are important for fighting aging in nematodes. These molecules in the proteome characterised herein may facilitate natural development and maturation during larval phases.

In proteomic studies of *Brugia malayi*, Hewitson et al. (2008) suggested that some of these hydrolases are essential for resisting the oxidative stress caused by reactive oxygen species and nitrogen produced by immune cells. These molecules may be involved in evasion of the host immune system and are perhaps being expressed by *Ortleppascaris* in an attempt to escape the immune system of the *R. marina*.

Succinate dehydrogenase was also identified in some spots, and according to Schilling et al. (2006) and Huang and Lemire (2009), they are involved in the mitochondrial electron transport chain, cell signalling, and apoptosis. They play a role in the typical process of tissue growth and differentiation and are present in the moulting of helminths.

The presence of the CoA-transferase family of enzymes in these nematode larvae may be related to hypoxic environments or anaerobic conditions in which the parasite is inserted. According to Tielens et al. (2010), these enzymes are widely observed in helminths parasitising their hosts in these conditions; furthermore, they are responsible for acetate production from acetyl-CoA, which is directly associated with the production of metabolic energy in various helminths and protozoa.

Phosphatases from the PP2C family, one of the largest families of serine/threonine phosphatases, are also involved in the metabolism of *Ortleppascaris* sp. According to Wenk et al. (1992), these enzymes have a broad spectrum of specific substrates, including enzymes that regulate many metabolic pathways and energy production and are likely involved in the metabolic processes of the helminths in the present study.

Homologous proteins of this PP2C family have been found in *Caenorhabditis elegans* and are encoded by the FEM-2, F42G9.1, and T23F11.1 genes, which may be related to gender determination in helminths. Although no effective sexual differentiation has been observed in these larvae, this may be an indication of the worm's attempts to differentiate into an adult parasite. According to Stothard et al. (2002) and Kuwabara (2007), the FEM-2 gene encodes a protein with phosphatase activity that leads to somatic male development and sperm production. Because protein phosphorylation is a major mechanism of cellular regulation and these proteins are highly evolutionarily conserved (Rodriguez, 1998), we believe that these molecules may be involved in the metabolic processes of *Ortleppascaris* sp. and must have some function in the sexual differentiation of the larvae in an effort to reach an L4 larval stage in the interior of the liver.

4.2. Structural molecules and organic morphogenesis

Similar to proteomic studies performed with other parasitic nematodes, many of the abundant proteins from the somatic extract of *Ortleppascaris* sp. are associated with cell division, the cytoskeleton, or even the structural constitution of the helminth; these proteins may be useful to larvae because of the intense morphological changes that occur during the larval stage. Evidence of high larval mitotic activity was observed based on the identification of seven proteins (spots 6, 33, 34, 37, 38, 39 and 40) that are related to cell division and protein phosphorylation, which are processes required for larval viability during fully active development.

Structural molecules that were identified in *C. elegans* are also present in the set of proteins studied in *Ortleppascaris*. According Nyström et al. (2002) and Suzuki et al. (2002), these molecules are cuticular collagen, which is encoded by the gene *lon-3* and required in hypodermal cells during larval development; this gene specifies and controls body length.

This expression occurs on the hypodermal cords of helminths and plays an important role in the formation of the cuticle. Because the surface of the parasite is the main contact surface and interacts directly with the hepatic fluids and tissues of *R. marina*, in *Ortleppascaris* biology, it may be thought of as a mechanism of antigenic variation on the surface of the nematode to facilitate evading the immune response and enhance their chances of survival.

Cox et al. (1981a,b) state that cuticular collagens are synthesised and secreted by hypodermal cells and polymerise on the apical surface of the epithelium, forming a complex structure with six layers. Furthermore according to Johnstone and Barry (1996), different genes encoding collagen are expressed in discrete time periods during nematode larval stages. In *Ortleppascaris*, this finding may reaffirm the attempt by the larvae to progress to an adult phase.

The identification of these homologs in the somatic extract from *Ortleppascaris* sp. suggests that the helminth undergoes a developmental process in the host, as shown histologically by Silva et al. (2013b).

Moreover, Van der Eycken et al. (1994) showed that genes encoding collagen appear to be highly evolutionarily conserved, even among free-living nematodes and nematode parasites, such as *C. elegans*, *A. suum*, *Haemonchus contortus*, and *Meloidogyne incognita*. These findings suggest that *Ortleppascaris* sp. and other nematodes may be used as an experimental model to investigate the processes regulating body size and shape and other aspects of development because many nematode processes, as indicated by Conlon and Raff (1999) and Suzuki et al. (2002), remain poorly understood.

Spots 28, 31, 35, 55 and 56 were identified as actin variant 1, which has also been characterised in *Dictyocaulus viviparous*. Actin is a major component of the cytoskeleton that is important for cell shape maintenance, endocytosis, organelle movement, and cell division. These proteins interact with myosin, and in invertebrates, they are structural constituents of the contractile machinery.

4.3. Immunoreactive molecules and virulence factors

A database search indicated the presence of As37, a highly immunoreactive 37 kDa antigen belonging to the immunoglobulin superfamily. As37 was previously identified in the larval stage of *A. suum* by Tsuji et al. (2002), who also suggested its presence in other nematodes, such as *Ascaris lumbricoides* and *Toxocara canis*. In addition, this antigen was recently identified in *Angiostrongylus costaricensis* by Rebello et al. (2011) and in *Angiostrongylus cantonensis* by Morassutti et al. (2012). In the latter study, the authors also described peptide molecules with high homology to As37 in *Baylisascaris schroederi* and *B. malayi*.

This protein was identified among the molecules expressed by *Ortleppascaris* sp. and is a possible candidate target for an anti-helminth vaccine. The existence of As37 homologs in other nematodes suggests that As37 is specific to nematodes. The presence of this antigen in *Ortleppascaris* may be explained by the taxonomic proximity to other cited nematodes. The helminth used in this study also belongs to the order Ascaridida, which is the order that includes *A. suum* and *A. lumbricoides*.

In addition to identification of immunoreactive antigens, homologs of proteolytic enzymes, such as the hypothetical protein CBG09068, are present in the somatic extract of *Ortleppascaris* sp. This group of proteins was considered by Dalton et al. (2003) to be a potential candidate for anti-parasitic interventions. They may be expressed in the intestines of parasites; alternatively, they may be constituents of excretions and secretions.

Xu and Dresden (1986), Hong et al. (1993), Chung et al. (1995) and Rhoads et al. (1998) also indicate that these enzyme homologs are involved in many of the developmental processes of helminths, including moulting, hatching, and encystment, which is consistent with the encystment and larval-stage development of *Ortleppascaris*.

In addition according to McKerrow et al. (2006) and Rebello et al. (2012), these are key molecules for invasion of the host by the helminth during the infective larval stage; they are considered major virulence factors for the establishment and maintenance of an infection. The proteolytic enzymes assist in the evasion of the host immune system by degrading the mucus barrier of the gastrointestinal tract, preventing blood coagulation, facilitating the feeding process, and aiding in the invasion of connective tissue (McKerrow, 1989; Dzik, 2006; Hasnain et al., 2012). Silva et al. (2013b) observed severe alterations in the hepatic parenchyma due to invasion into the connective tissue in *R. marina* parasitised by *Ortleppascaris* sp., where induction of the connective tissue to reach encystment without destruction of the hepatic tissue or vessels was shown.

Another important function of these proteolytic enzymes is the degradation of host haemoglobin, considered by Williamson et al. (2003) to be the main substrate of haematophagous nematodes. Haemoglobin degradation was also observed by Silva et al. (2013b) during parasitism in this study, where *Ortleppascaris* infestation caused an exacerbation of melanomacrophage centres in the parenchyma. Melanomacrophages are host defence cells that have a characteristic pigment formed by degradation of haemoglobin in the cytoplasmic compartment.

4.4. Molecules of the secretory biosynthetic pathway

Constituents of the vesicular formation and recruitment machinery are present among the abundant proteins of the somatic extract. In particular, there are several proteins involved in glycosylation and the export of proteins and lipids from the ER.

According to Varki (1993) and Berninsone (2006), complex carbohydrates are involved in multiple biological processes, including the immune response and parasite–host interactions. A membrane glycotransferase of the ER, which is responsible for the production of glycans with N-terminal oligosaccharides linked to asparagine, is present in *A. suum* and was also detected in *Ortleppascaris* sp.

Kelleher and Gilmore (2006) reported that glycosylation is one of the most common protein modifications in eukaryotes. The biosynthetic pathway of these glycans is highly conserved in most eukaryotes, such as *C. elegans* (Haslam and Dell, 2003; Berninsone, 2006), and is essential for the synthesis and transport of the glycolipid by ER vesicles.

Traffic along the biosynthetic secretory pathway occurs through packaging in vesicles that bud from the ER (Gillon et al., 2012). COPII vesicles are key components of this pathway, and the formation of such vesicles is initiated by the GTPase SAR1, which was found in the present study. SAR1 is responsible for recruiting additional proteins and coating the vesicles and may facilitate the trafficking of a variety of membrane glycoproteins, mainly on the cell surface and in the extracellular matrix, which act as surface antigens and excretory or secretory products of helminths.

According to Thomas and Harn (2004), glycoproteins can direct the host immune system toward a typical Th2 response or modulate the system for the maintenance of infection through dendritic cells, which may recognise these molecules as pathogen-associated molecular patterns.

The presence of proteins involved in production machinery and glycoprotein trafficking may contribute to the increased cellular immune response described by Silva et al. (2013b). In addition, glycoproteins may contribute to the export of sugars and lipoproteins to the larvae cell surface during its full development because many proteins have been identified as being part of the surface/cuticle composition of the nematode. For example, collagen trafficking is mediated by "mega vesicles" formed by COPII (Gillon et al., 2012) and may cause a strong immune response against the nematode.

4.5. Molecules of the chemosensory process

In summary, the sensory abilities of metazoans are due to second messengers, such as GMPc. Therefore, much has been speculated about helminths and their ability to sense odours or flavours, memorise temperature, and detect and respond to different oxygen levels. To maintain vital functions, the nematode must be able to recognise the "demand" for nutrients and initiate chemo- and thermotaxic processes to respond to environmental changes and host tropism. Guanylyl cyclase enzymes are present in the somatic protein group of *Ortleppascaris*.

In *C. elegans*, studies have identified the molecular basis of the genetic and behavioural plasticity of signalling proteins, including the guanylyl cyclase enzymes (Baude et al., 1997; Mori, 1999; Rankin, 2005; Hallem et al., 2011).

Guanylyl cyclases are ubiquitous enzymes found in organisms ranging from bacteria to humans. They catalyse the conversion of GTP to cGMP and are involved in cell signalling and other physiological processes, including smelling, muscle contractility, electrolyte homeostasis, and retinal phosphotransduction (Yan and Davis, 2002; Potter, 2011). Studies have shown that the genes encoding guanylyl cyclases are coincidentally expressed in sensory regions of the helminth body, such as in the nerve ring, amphid, and tail of the *Heterodera glycines* nematode (Yan and Davis, 2002) and in the gustatory neurons of *C. elegans* (Ortiz et al., 2009). Holden-Dye and Walker (2011) believe that such molecules have a significant role in the neural circuits involved in host recognition by chemotaxis and thermotaxis.

In helminths, it is hypothesised that guanylyl cyclases increase their expression at low oxygen concentrations (Gray et al., 2004). Enzyme upregulation likely occurs at the sites of *Ortleppascaris* sp. infection, which correspond to areas with low amounts of free oxygen. Moreover according to Hallem et al. (2011), enzyme expression is closely related to CO_2 concentration, which in turn appears to be an important chemotactic component for attracting nematodes. It is believed that the detection of CO_2 by *C. elegans* is mediated by evolutionarily conserved receptor-like guanylyl cyclases. This receptor, GCY-9, is well known in *C. elegans* and has orthologs in other nematodes, such as *H. glycines*, *M. incognita*, and the human nematode *B. malayi*, indicating that CO_2 is a determinant of the affinity of helminths for their respective hosts.

Therefore, *Ortleppascaris* sp. may be attracted to amphibian liver because it provides a high concentration of CO_2. This organ may thus be a potential chemotactic site for these larvae.

In conclusion, proteins identified in *Ortleppascaris* sp., such as actin; proteins related to *lon-3*; and PP2C, are highly evolutionary conserved, indicating that this organism has the potential for use in structural studies of the development and sexual differentiation of nematodes.

Other key molecules of helminth biology were found in the present study, such as immunoreactive antigens specific to nematodes and virulence factors that are considered potential anti-helminthic vaccine candidates and protein markers for helminth infection. Furthermore, guanylyl cyclases that are involved in the chemosensory and neurobiological processes of parasitic nematodes were found in the present study.

The set of molecules identified shows the diversity of processes that occur in these nematodes, including development, redox metabolism, infectivity, and virulence, and is a further indicator of the physiological complexity of these organisms.

The proteomic approach used here provides clues about the host–parasite relationship at the molecular level, which is relevant to co-evolutionary studies, the choice of antigen for vaccine development, and the duality of the physiological relationship between these two organisms.

Acknowledgements

This research was supported by PROCAD-NF 2009 [Programa Nacional de Cooperação Acadêmica – Ação Novas Fronteiras (National Program of Academic Cooperation – New Frontiers)], a special program of CAPES [Coordenação de Aperfeçoamento de Pessoal de Nível Superior (Coordination for the Improvement of Higher Education Personnel)] and PROPESP [Pró-Reitoria de Pesquisa e Pós-Graduação (Dean of Research and Postgraduate Studies)].

The authors thank NuBioMol (Núcleo de análises de Biomoléculas [Centre of Biomolecular Analysis]) for their equipment and technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijppaw.2014.03.003.

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