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A proteomic approach to the identification of tegumental proteins of male and female *Schistosoma bovis* worms

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ABSTRACT

Schistosoma bovis, a parasite of ruminants, can live for years in the bloodstream in spite of the immune response of its host. The parasite tegument covers the entire surface of the worm and plays a key role in the host–parasite relationship. The parasite molecules involved in host immune response evasion mechanisms must be expressed on the tegument surface and are potential targets for immune or drug intervention. The purpose of the present work was to identify the tegumental proteomes of male and female *S. bovis* worms, in particular the proteins expressed on the outermost layers of the tegument structure. Adult worms of each sex were treated separately with trypsin in order to digest their tegumental proteins, after which the peptides released were analysed by LC–MS/MS for identification. This experimental approach afforded valuable information about the protein composition of the tegument of adult *S. bovis* worms. A range of tegumental proteins was identified, most of which had not been identified previously in this species. Although an absolute purification of the proteins expressed on the outermost layers of the tegument structure was not achieved, it is likely that present among the proteins identified are some of the molecules most closely associated with the tegument surface. Our study also suggests that there may be differences in the protein composition of the tegument of male and female schistosomes. Finally, the presence of actin and GAPDH on the surface of male and female worms and the presence of enolase exclusively on the surface of male worms were verified by confocal microscopy.

1. Introduction

Blood flukes of the genus *Schistosoma* cause human and domestic animal disease in tropical and subtropical areas. There are three major species that infect humans – *S. mansoni*, *S. japonicum* and *S. haematobium* – and at least 10 species that infect domestic ruminants. Among these latter species, *S. mattheei* and *S. bovis* are those most studied owing to their veterinary significance [1,2]. Studies on *S. bovis* are interesting from the perspectives of both veterinary and human medicine, since this species represents the genetic and immunological analogue of the human pathogen *S. haematobium* [3].

Like human schistosomes, adult *S. bovis* worms can survive for a long time in the vascular system of the immune-competent host despite the specific immunological response of the latter [4]. Such a long survival time is possible because schistosomes have evolved diverse mechanisms to evade host immune responses, many of which are dependent on the properties of the parasite tegument [5]. The tegument of adult schistosomes is a unique outer-surface structure and consists of a cytoplasmic syncytium attached to underlying cell bodies by narrow cytoplasmic connections. The nuclei, ribosomes, endoplasmic reticulum, mitochondria and Golgi apparatus are located in these cell bodies, and their vesicular products – the so-called discoid bodies and multilaminate vesicles – are transported to the tegument syncytium *via* the connections. The apical surface of the tegument is made of normal plasma membrane overlain by a membrane-like secretion, which has been termed membranocalyx, by analogy with the glycocalyx of eukaryotic cells [6,7].

This tegument covers the entire surface of the worm, constitutes a major interface between the parasite and its host, and is critically involved in the complex host–parasite relationship, which comprises nutrient uptake, excretion, osmoregulation,

Abbreviations: 2-D WB, two-dimensional Western blot; 2-DE, two-dimensional electrophoresis; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; GO, gene ontology database; IEF, isoelectric focusing; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; TG, tegument extract.

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sensory reception, signal transduction, and interaction with the host immune and haemostatic systems [8–10]. Thus, the identification and characterization of schistosome tegumental molecules is essential for a better understanding of the host–parasite relationship and for defining novel immunological, pharmacological and diagnostic targets [6,11].

Major advances in the identification of proteins from the schistosome tegument have been made in recent studies, in which tegument-enriched preparations of adult *S. mansoni* worms were subjected to proteomic analyses [12–15]. In the first of these studies, van Balkom et al. [12] detached the tegument from the underlying worm body. The proteins in the whole tegument and the body fractions were separated by electrophoresis and liquid chromatography and were identified by tandem mass spectrometry (LC–MS/MS). In this way, the authors were able to identify 740 proteins, among which 43 were tegument-specific, and many of them showed no homology with any non-schistosomal protein, demonstrating that the schistosomal outer-surface comprises specific, unique proteins.

In a later work aimed at determining the protein composition of the tegumental surface [13], the detached tegument was first enriched in surface membranes by sucrose gradient centrifugation and then subjected to a differential extraction procedure. All fractions were analysed by mass spectrometry, and many of their components were identified and classified in cytosolic, cytoskeletal, membrane, and secreted categories. However, this work did not verify the localization of individual components within the surface complex [6,14].

Since the accessibility of a protein in the tegument is an important issue when selecting target molecules for vaccines and drugs, it is critical to bear in mind that not all proteins inside the tegument are exposed to host tissues [16]. To ascertain the identification and localization of the proteins exposed on the surface of the *S. mansoni* tegument, Braschi and Wilson [14] carried out a proteomic study in which they labelled the most exposed surface proteins of live adult schistosome worms by using two impermeant biotinylation reagents of different sizes. The labelled proteins were recovered by streptavidin affinity and were identified by tandem mass spectrometry. The study revealed 28 proteins; 13 of them labelled by the long-form reagent (four derived from the host), and the same 13 plus a further 15 labelled by the short-form reagent. This study demonstrated the relative accessibility of tegument components, those tagged exclusively by the short-form reagent having a more concealed localization within the membrane complex.

With the same aim, another two experimental approaches have been developed to enrich the membranocalyx from the plasma membrane and underlying tegument syncytium (in [15]). In the first approach, cationised ferritin was used to induce a rapid sloughing of the membranocalyx, and in the second one the surface tegumental proteins were labelled with rat anti-mouse erythrocyte ghost antibodies and then captured with goat anti-rat antibody-coated beads. In both approaches, the protein contents of the samples were analysed by LC–MS/MS. Although absolute purification of the membranocalyx was not achieved, both approaches served to enrich the peripheral-most proteins of the tegument, and the identities obtained probably represent the molecules most closely associated with the membranocalyx [15].

The foregoing studies have thus provided a wealth of data on the protein constituents of the *S. mansoni* tegument and their relative localization within this surface complex.

Regarding *S. bovis*, the information available concerning its tegumental proteins is much sparser than for *S. mansoni* and was obtained in two studies performed on a tegument extract prepared by solubilisation with Triton X-100. Proteomic analysis of this extract identified 21 tegumental proteins, although no studies were made to assess their relative localization in the tegument structure [10,17].

The aim of the present work was the identification of the tegumental proteomes of male and female *S. bovis* worms, in particular of the proteins expressed on the outermost layers of the tegument structure. To achieve this goal, whole adult worms of each sex were treated separately with trypsin in order to digest their tegumental proteins, after which the peptides released were analysed by LC–MS/MS for identification. A number of proteins from the tegument of the male and/or female worms are reported. In addition, the tegumental expression of some of the proteins identified was verified by confocal microscopy.

2. Materials and methods

2.1. Parasite material

A strain of *S. bovis* from Salamanca (Spain) was maintained in the laboratory in its natural hosts: *Planorbarius metidjensis* snails and sheep. In order to obtain cercariae, each snail was infected with five miracidia from eggs obtained from experimentally infected sheep faeces. The sheep were infected percutaneously with 2000 *S. bovis* cercariae by submerging a fore-limb for 30 min in a suspension of these cercariae. At 4 months post-infection, the sheep were sedated with ketamine (10 mg/kg) and then sacrificed by bleeding through the jugular vein. Adult *S. bovis* worms were recovered by dissection of the mesenteric vessels from the entire gut.

Schistosomes were rinsed in warm phosphate buffered saline (PBS) and their integrity was examined microscopically. Only intact parasites were used for the subsequent analyses; namely, trypsin digestion, immunofluorescence analysis, and the preparation of tegument protein extracts (see below).

2.2. Trypsin digestion of whole male and female worms

Freshly obtained adult *S. bovis* worms were incubated in PBS at 37 °C for 30 min with gentle shaking. In this way the couples separate spontaneously, allowing selective collection of individuals of each sex with minimal manipulation and risk of damage. Following this, batches of male and female adult worms were fixed in 70% methanol and processed separately.

Three similar batches of male worms (10 individuals per batch) were analysed in different assays. The trypsin digestion protocol used in each assay differed in the digestion time (4 h or 30 min), and/or in the time point at which the reduction and alkylation of cysteine residues – to disrupt and prevent the restoration of disulfide bonds – were carried out, which were done either on the intact worms, before trypsin digestion, or on the peptides released after digestion. From the harshest to the softest digestion protocols, the different assays were carried out as follows.

Assay 1 (reduction/alkylation and 4 h of digestion). Male worms were washed three times, 1 min per wash, with 50 mM NH_4HCO_3. The worms were reduced with 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 in a microwave oven for 3 min at 560 W, and alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min at room temperature in the dark. Following this, the worms were incubated with 5 ng/μl of sequencing grade trypsin (Promega) in NH_4HCO_3 at 37 °C for 4 h. The reaction was stopped with 5 μl of 10% trifluoroacetic acid (TFA) and the supernatant containing the released peptides was recovered and preserved at −20 °C.

Assay 2 (4 h of digestion and reduction/alkylation). Male worms were washed three times, 1 min per wash, with 50 mM NH_4HCO_3