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Excretory/secretory products from *in vitro*-cultured *Echinococcus granulosus* protoscoleces

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ABSTRACT

Cystic hydatid disease (CHD) is caused by infection with *Echinococcus granulosus* metacestodes and affects humans and livestock. Proteins secreted or excreted by protoscoleces, pre-adult worms found in the metacestode, are thought to play fundamental roles in the host–parasite relationship. In this work, we performed an LC–MS/MS proteomic analysis of the excretory–secretory products obtained from the first 48 h of an *in vitro* culture of the protoscoleces. We identified 32 proteins, including 18 that were never detected previously in metacestode proteomic studies. Among the novel identified excretory–secretory products are antigenic proteins, such as EG19 and P-29 and a calpain protease. We also identified other important protoscolex excretory–secretory products, such as thioredoxin peroxidase and 14-3-3 proteins, which are potentially involved in evasion mechanisms adopted by parasites to establish infection. Several intracellular proteins were found in the excretory–secretory products, revealing a set of identified proteins not previously thought to be exposed at the host–parasite interface. Additionally, immunological analyses established the antigenic profiles of the newly identified excretory–secretory products and revealed, for the first time, the *in vitro* secretion of the B antigen by protoscoleces. Considering that the excretory–secretory products obtained *in vitro* might reflect the products released and exposed to the host *in vivo*, our results provide valuable information on parasite survival strategies in adverse host environments and on the molecular mechanisms underpinning CHD immunopathology.

1. Introduction

Cystic hydatid disease (CHD) is a zoonosis of global distribution caused by the metacestode larval stage (hydatid cyst) of the tapeworm *Echinococcus granulosus* (Cestoda, Taeniidae) [1]. The hydatid cyst develops in the internal organs (mostly the liver and lungs) of a variety of intermediate hosts, including domestic ungulates and, *via* incidental exposure, in humans.

Anatomically, the hydatid cyst is a unilocular structure filled with hydatid cyst fluid (HCF). Its wall is composed of two layers: an inner, thin germinal layer, which is the most external parasite tissue, and an outer acellular laminated layer of variable thickness [2]. The germinal layer contains the tegument and several cell types, including proliferative undifferentiated cells responsible for forming brood capsules, which asexually develop into pre-adult worms called protoscoleces [3]. The laminated layer is a specialized extracellular matrix produced by the germinal layer, which is surrounded by a host-produced fibrous adventitial layer that results from the initial inflammatory reaction [2].

The capacity of helminth parasites to modulate the immune system underpins their longevity in the host body [4,5]; the modulation of the immune system is most likely to be effected through the release and/or secretion of molecules that ligate, degrade or otherwise interact with host immune cells [5]. Additionally, such excretory–secretory (ES) products may participate in a wide range of parasite functions, including penetration, establishment and

Abbreviations: AgB, antigen B; CHD, cystic hydatid disease; EgTeg, *Echinococcus granulosus* tegumental protein; EgTPx, *Echinococcus granulosus* thioredoxin peroxidase; ES, excretory/secretory; FABP, fatty acid-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCF, hydatid cyst fluid; LC–MS/MS, liquid chromatography–mass spectrometry; P-29, hydatid disease diagnostic antigen P-29.

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survival in host tissues and incorporation of metabolites from the host [6].

In *E. granulosus*, the successful establishment of an infection depends on the capacity of the cyst to subvert the immune defenses of the host [7]. This essentially depends on the molecules exposed by the parasite to the host, which in the *E. granulosus* metacestode are mainly represented by proteins exposed at the germinal layer surface and proteins excreted/secreted in the HCF by the germinal layer and the protoscoleces. ES products from the *E. granulosus* metacestode are known to modulate both the innate and adaptive host immune responses and appear to target both cellular and humoral responses [8,9]. Besides, ES products may interfere with other processes of parasite–host interaction, such as extracellular matrix degradation in pathology-related tissue remodeling, maintenance of the progressive growth capacity of the metacestode within host tissue and protection against oxidative damage. Therefore, the characterization of these ES products would aid in increasing the understanding of the mechanisms underpinning the strategies used by the parasite for increasing the efficiency and persistency of infection in the host.

The HCF is a reservoir of parasite ES products from both the germinal layer and protoscoleces, along with host proteins that permeate into the cyst. So far, HCF proteomic studies have been able to identify several *E. granulosus* ES products, including major antigens, such as antigen 5 (Ag5) and antigen B (AgB), and other components, such as thioredoxin peroxidase, metabolic enzymes and 14-3-3 proteins [10,11]. However, the presence of large amounts of host proteins in the HCF, such as albumin and immunoglobulins, significantly impairs the analysis and identification of *E. granulosus* ES products [10,12].

The study of *in vitro* culture supernatants is an interesting alternative for the analysis of parasite ES products free of host contaminants. The analysis of ES products from *in vitro* cultures has been largely used for other helminth parasites [13–16], and such an approach is expected to allow the identification of some proteins that are undetectable in HCF sample analyses due to their underrepresentation in comparison to major parasite and host HCF components.

In this work, we investigated *E. granulosus* ES proteins produced by protoscoleces within their first 48 h of culture *in vitro*. The proteomic analysis of ES products from protoscoleces cultured *in vitro* allowed the identification of more than 30 proteins, including 18 that were never previously detected in proteomic studies of *E. granulosus* HCF. Additionally, an immunological analysis established the antigenic profile of the newly excreted or secreted proteins. Overall, our results established a methodology for the analysis and identification of newly produced ES products by *E. granulosus* protoscoleces, allowing the tracking of the dynamics of protoscoleces protein excretion/secretion over time.

2. Materials and methods

2.1. Protoscoleces

Protoscoleces were obtained by aseptic puncture from fertile liver and lung hydatid cysts of bovine origin collected from an abattoir in São Leopoldo in the state of Rio Grande do Sul (Southern Brazil). Protoscoleces were decanted by gravity, washed several times in phosphate-buffered saline (PBS) and cultured immediately after their extraction from the cyst. Viability was determined by trypan blue exclusion analysis [17], and only protoscolex samples with viability higher than 90% were selected for the assays. Protoscolex samples from 6 cysts (5 from lungs and 1 from liver) were individually analyzed.

2.2. Protoscolex cultures and ES products collection

Protoscoleces were cultured as previously described [18], with some modifications. Briefly, protoscoleces were cultured in 10 ml of sterile PBS supplemented with 10% glucose and 100 μg/ml of gentamicin at 37 °C in 5% CO_2. The culture supernatants containing the parasite ES products were collected at different and non-cumulative time intervals, from 13 h to 48 h. At the end of each time interval, the entire 10 ml of culture medium (culture supernatants) was removed and replaced with the same volume of fresh medium. The protoscoleces were then incubated under the same conditions. With each change of culture medium, the viability of the protoscoleces was determined as described above. The culture supernatants were concentrated 100-fold using Amicon Ultra-15 centrifugal filters with a 5000 MWCO membrane (Millipore, Billerica, MA, USA). From each concentrated sample, 100 μl was then used for electrophoretic and immunoblotting analyses. The remaining concentrated supernatant samples from all time intervals and from all hydatid cysts were then pooled for liquid chromatography–mass spectrometry (LC–MS/MS) protein identification.

2.3. SDS-PAGE and immunoblot analysis of protoscolex ES products from E. granulosus

Proteins contained in the ES products were separated by SDS-PAGE (12% or 15%) under reducing conditions [19] and were visualized by silver staining [20]. For immunoblot analysis, the proteins were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) [21]. After blocking in Blotto (PBS containing 5% milk powder), the membranes were incubated with primary antibodies for 1.5 h at room temperature. Membranes were probed with a pool of five sera from surgery-confirmed CHD patients or with a pool of rabbit polyclonal monospecific sera against AgB recombinant subunits AgB8/1, AgB8/2 and AgB8/3, diluted 1:500 and 1:30,000 in Blotto, respectively. Peroxidase-conjugated goat anti-human IgG (Sigma–Aldrich, St. Louis, MO, USA) or rabbit anti-IgG (GE Healthcare) were used at 1:1000 and 1:2000 dilutions, respectively, in Blotto for 1 h at room temperature. Antigen–antibody reactions were developed with ECL detection reagent (GE Healthcare). Due to ES protein limitations, negative control sera from clinically healthy individuals or rabbit pre-immune sera were assayed in parallel by immunodot analysis [22].

2.4. Mass spectrometry analysis of protoscolex ES products from E. granulosus

Samples of all culture supernatants were pooled to obtain sufficient protein amounts for mass spectrometry (MS) analysis and three technical replicates. Proteins were diluted in denaturing buffer (25 mM NH_4HCO_3/8 M urea, pH 8.9), reduced by adding DTT (0.02 μg/μg protein), and alkylated with iodoacetamide (0.01 μg/μg protein). Samples were then diluted to 1 M urea with 25 mM NH_4HCO_3 (pH 8.9) and digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA) at a ratio of 0.01 μg/μg protein. After digestion for 4 h at 37 °C, a new aliquot of enzyme was added, and samples were further incubated for 16–20 h at 37 °C. The resulting peptides were desalted using an OASIS®HLB Cartridge column (Waters, Milford, MA, USA) and analyzed by LC–MS/MS as previously described [10]. MS analyses were performed using an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) Ultima API mass spectrometer (Micromass, Manchester, UK) coupled to a capillary liquid chromatography system (CapLC, Waters). The peptides were separated in a Nanoease C18 (75 μm ID) capillary column by elution with a