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Proteomics analysis of differentially expressed proteins in schistosomula and adult worms of *Schistosoma japonicum*

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ABSTRACT

Schistosoma japonicum has a complex lifecycle and exhibits dramatic changes in its biology and morphology at different developmental stages. The schistosomulum and adult worm are two stages of this complex lifecycle and differentially expressed proteins in these two stages should be important for survival, development, and reproduction of the parasites. In this study, soluble and hydrophobic proteins were extracted from eggs, cercariae, schistosomula (8 d and 19 d), and male and female adult worms (42 d) of *Schistosoma japonicum*, and separated by two-dimensional (2D) gel electrophoresis. A total of 1376 ± 52, 928 ± 61, 1465 ± 41, 1230 ± 30, 904 ± 34, and 1080 ± 26 soluble proteins and 1437 ± 44, 845 ± 53, 986 ± 22, 1145 ± 35, 1066 ± 39, and 1123 ± 45 hydrophobic proteins were separated from eggs, cercariae, schistosomula (8 d and 19 d), and male and female adult worms (42 d), respectively. There were 65 ± 14, 27 ± 7, 37 ± 17 and 48 ± 9 soluble protein spots only present in schistosomula (8 d and/or 19 d) and adult schistosomes (male and/or female). We successfully identified 22 spots from schistosomula and 11 spots from adult schistosomes by mass spectrometry. Quantitative real-time RT-PCR was used to examine six differentially expressed proteins at the transcription level. These proteins only found in schistosomula or adults stage by the proteomics analysis were highly expressed in the corresponding stage at mRNA level. Bioinformatics analysis showed that the differentially expressed proteins from schistosomula were mainly involved in cellular metabolic processes, stress response and developmental process. Differentially expressed proteins from adult schistosomes were involved with gene expression and protein metabolism processes. The results of this study might provide new insights to stimulate further exploration of the mechanism of growth and development in schistosomes and help identify candidate molecules for developing new vaccines or drugs.

1. Introduction

Schistosomiasis is caused by blood-dwelling flukes of the genus *Schistosoma* and it is one of the world's most prevalent tropical parasitic diseases, which affects over 200 million people in 74 developing countries and causes hundreds of thousands of deaths annually (Mathieson and Wilson, 2010; WHO, 2002). In China, comprehensive control approaches involving chemotherapy, snail elimination, sanitation improvement, and health education are used to control this disease. However, over 65 million people are at risk of infection and 500 000 infections were reported in 2007 (Wu et al., 2011). Thus, a prophylactic vaccine, alone or in combination with anthelmintic drugs may be more effective for long-term sustainable control of schistosomiasis (Bergquist et al., 2005). This goal demands the identification of promising vaccines or drug target molecules that are essential for growth, sex maturation, egg laying, evasion of the immune responses of the host, and other biological activities required by schistosomes to complete their complex life cycle.

Proteomic analysis is a powerful tool for high-throughput global protein expression analysis using gel or non-gel-based protein separation techniques, coupled with mass spectrometry and bioinformatics. This technique had been applied to identification of schistosome proteins from complex samples and the study of differentially expressed schistosome proteins (Cheng et al., 2005; Curwen et al., 2004; van Hellemond et al., 2007). For example, proteins expressed in different life cycle stages (Curwen et al., 2004), and genders (Cheng et al., 2005), and tegument and secretion proteins (Curwen et al., 2006; Knudsen et al., 2005; Perez-Sanchez et al., 2006) have been identified. The soluble proteins from cercariae, lung-schistosomula, adult worms and eggs of *Schistosoma mansoni* were analyzed by 2D gel electrophoresis. The 40 most abundant spots in each gel were selected for detailed investigation and the majority of dominant constituents were cytosolic in origin (Curwen et al., 2004). Important recent advances in research into

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the *Schistosoma japonicum* transcriptome, proteome, and genome have opened new opportunities for proteomic analysis of the parasite (Hu et al., 2003; Liu et al., 2008, 2006; Zhou et al., 2009). Protein expressions of various developmental cycle stages of *S. japonicum* life stages (cercariae, 14 d hepatic schistosomula, female and male adults, eggs, and miracidia) were analyzed using two-dimensional-nano-liquid chromatography (2D-nano-LC) and one-dimensional PAGE approach coupled with mass spectrometry. Moreover, the analysis of these proteins in different developmental stages of schistosomes was in tandem with nucleotide sequence analysis of the CDSs (Liu et al., 2006). The findings combined with comparative genomic, transcriptomic and proteomic analyses lead to a more profound understanding of schistosomes.

The schistosome has a complex life cycle, involving seven different developmental stages. The schistosomulum is the stage where the parasite migrates *via* the circulatory system of the host through the lungs to reach the hepatic portal system and it is critical for sexual maturation, coupling and the start of egg production. Successful development of the schistosome in the final host involves profound structural, biochemical, and physiological changes that are vital for adaptation to environmental variations (Hong et al., 2010). However, this developmental stage of the schistosome is susceptible to both vaccine and non-vaccine immune elimination (Loverde, 1998; Smithers and Terry, 1969). Therefore, proteins that are highly expressed in the schistosomulum stage might be good candidates for vaccines or new drugs targeted against schistosomiasis.

In the present study, traditional proteomics techniques were applied to separate soluble proteins and hydrophobic proteins extracted from eggs, cercariae, 8-d-old and 19-d-old schistosomula, and 42-d-old male and female adult worms of *S. japonicum*. Differentially expressed proteins that were present in particular developmental stages of the worms were identified by mass spectrometry (MS). The purpose of this study was to develop an improved understanding of the specific developmental mechanism of schistosomes at the molecular level by conducting a proteomic analysis that could provide valuable information for identifying new vaccine candidates or drug targets.

2. Materials and methods

2.1. Parasite maintenance

A Chinese strain (Anhui isolate) of *S. japonicum* was produced using New Zealand rabbits and *Oncomelania hapensis*. Rabbits were infected with cercariae, and worms were collected from the infected rabbits at 8, 19, and 42 d after the challenge, respectively. Male and female worms were detached manually after 42 d and all samples were stored in liquid nitrogen until use.

The eggs were collected as previously described (James and Colley, 1974; Lazdins et al., 1982). Briefly, the livers from rabbits infected with schistosome for 42 d were homogenized in phosphate buffered saline (PBS). The mixture was sequentially passed through 80, 120, 160, and 260 mesh screens to separate the eggs from the liver tissues. The solution collected was centrifuged at 5000 × *g* for 20 min and the pellet was washed three times with PBS. After the final wash, the pellet was resuspended in 100 mL PBS containing 7.5 U trypsin, 3 U collagenase II, 100 U ampicillin, and 100 U kanamycin. The mixture was then incubated at 37 °C for 7 h in a shaker to remove the remaining host proteins. The solution was centrifuged at 5000 × *g* for 15 min and the pellet was immediately rinsed three times with PBS. Finally, Percoll (Amersham Bioscience, USA) density separation was used to separate the eggs. The purified eggs were stored in liquid nitrogen until use.

2.2. Protein extraction

Frozen samples (100 mg eggs, cercariae, 8 d and 19 d schistosomula, 42 d male and female worms) were added to 2 mL Tris buffer (50 mM, pH 9.2) and homogenized using a Dounce homogenizer. The mixture was sonicated on ice for 12 s × 15 with an interval of 2 min, and was centrifuged at 10 000 × *g* for 10 min at 16 °C to remove tissue and cell debris. The supernatant was centrifuged at 40 000 × *g* for 1 h at 4 °C to obtain the soluble proteins. The pellets from the final step were washed twice with 50 mM Tris to remove the soluble proteins. Pellets were added to lysis buffer containing 7 M urea, 2 M thiourea, 40 mM Tris, 2% 3-(decyldimethylammonio) propanesulfonate inner salt, 30 mM DTT, 2% CHAPS, and 0.5% IPG buffer (pH 9.2). The mixture was treated as previously described and the surpernatant was retained as the hydrophobic proteins. Both soluble and hydrophobic proteins were treated using a 2-D Clean up Kit (Amersham Bioscience, USA), according to the manufacture's instructions, to remove contaminating materials. All samples were quantified using Bradford method and aliquots were stored at −80 °C until required for 2D electrophoresis.

2.3. Two-dimensional gel electrophoresis

2.3.1. Isoelectric focusing (IEF)

A 24 cm immobilized pH gradient (IPG) strip (pH 3–11; Amersham Bioscience, USA) was rehydrated overnight in IPG rehydration/sample buffer (8 M urea, 2% CHAPS, 15 mM DTT, 0.5% IPG buffer containing 800 μg of soluble protein in a total volume of 450 μL, or 7 M urea, 2 M thiourea, 2% CHAPS, 30 mM DTT, and 0.5% IPG buffer containing 800 μg of hydrophobic protein sample in a total volume of 450 μL). IEF was performed using an Ettan IPGphor II (Amersham Bioscience, USA) and focusing parameters were conducted by stepwise increase of the voltage as follows: 30 V for 14 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3500 V for 1 h, and 8000 V for 18 h.

2.3.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to SDS-PAGE, strips were equilibrated with gentle shaking in two steps for 15 min each in equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, 1% DTT, 50 mM Tris; pH 6.8) and equilibration buffer II (DTT was replaced with 2.5%) before strips were loaded onto a 12.5% (pH 8.8) acrylamide gel with a 1 cm 4% (pH 6.8) stacker gel. The strips were overlaid with 1% agarose in SDS running buffer containing 5 mg of bromophenol blue. Electrophoresis was performed in two steps: 15 mA gel^{-1} for 15 min and then 30 mA gel^{-1} at 15 °C until the bromophenol blue dye reached the bottom of the gels. All gels were stained by the silver-staining method (Lauber et al., 2001) and three replicates were run for each sample.

2.4. Image scanning, analysis, and in-gel digestion

Three 2-DE gels prepared for each sample were scanned into the computer with an Images Scanner (Amersham Bioscience, USA) in transmission mode and the images were transferred to ImageMaster 2D Platinum software (version 4.0, Amersham Bioscience) for direct analysis (Cheng et al., 2005). Protein spots were automatically detected after adjustments of smooth parameter, saliency value and minimum area value to remove high noise spots and intense artifact spots. A reference gel containing maximum spots was selected from one of the normal gels that set to compare with other gels together with 3 landmarks between all gels created manually for matching. Each paired spot was manually verified to ensure a high level of reproducibility between gels produced in triplicate data. Soluble proteins only existed in 8 d and/or 19 d schistosomula, and only in 42 d male and/or female worms were