Letters

Analysis of Protoscoleces-specific Antigens from *Echinococcus Granulosus* with Proteomics Combined with Western Blot*

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Abstract

Objective To establish and optimize the proteomic analysis of protoscoleces-specific antigens from *Echinococcus granulosus*. To provide a foundation for identifying specific antigens in the soluble proteins of *E. granulosus* protoscoleces for further research.

Methods Brood capsules were collected aseptically from fertile *E. granulosus* cysts from the livers of an infected patient. The fertile *E. granulosus* cysts were fractured, and protoscoleces were collected by centrifugation. The soluble proteins of protoscoleces were acquired using the 2D Quant kit according to the manufacturer's instructions. We employed two-dimensional electrophoresis (2-DE) combined with immunoblot assay (Western blot) to analyze the soluble components of *E. granulosus* protoscoleces antigens. The 2-DE and immunoblot maps obtained were analyzed with PDQuest 8.0 image analysis software.

Results About 233 soluble protein spots were identified with Coomassie-stained gels. Most of the proteins had a molecular weight of 16 000 Da to 117 000 Da, and an isoelectric point value of 3.0 to 10.0. 2-DE immunoblot was conducted and 57 specific antigen spots were observed, among which 23 spots were identified.

Conclusion 2-DE combined with Western blot is the key to successful proteomic analysis and presents a new possibility for searching the specific *E. granulosus* protoscoleces antigens.

Key words: *Echinococcus granulosus* protoscoleces; Proteomics; Specific antigen; Two-dimensional electrophoresis (2-DE); Western blot

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INTRODUCTION

Cystic echinococcosis is caused by the larval form of the cestode *Echinococcus granulosus*. The disease is one of the most serious emerging zoonotic parasitic diseases throughout the world with a considerable impact on both human and animal health, causing important socio-economic consequences in endemic areas[1]. The adult worm develops in the small intestine of definitive hosts, mainly dogs and other canids. The metacestode or hydatid cyst develops mostly in the

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liver and lungs of intermediate hosts, affecting humans and a wide range of livestock species[2]. The fully developed hydatid cyst of *E. granulosus* is unilocular and fluid (hydatid fluid, HF)-filled. The hydatid cyst consists of an inner germinal layer supported externally by a noncellular laminated layer, which is surrounded externally by host-produced fibrous adventitial tissue. Protoscoleces (PSCs), which are produced by the germ layer via asexual reproduction, develop in the adult worm when ingested by the definitive host. In past decades, despite great efforts to educate people at risk and the success of control programs in some countries, cystic echinococcosis continues to cause substantially morbidity and mortality in numerous parts of the world[3-4]. Finding vaccines for intermediate hosts is a highly effective and convenient approach to reduce the incidence and transmission of hydatid disease due to the definitive host not eating organs with PSCs. *E. granulosus* has numerous stages in its life cycle, including oncosphere, PSCs, adult worm, and egg. Single antigens that induce effective protective responses are limited. Thus, single antigen molecules can not completely protect against the different development stages of *E. granulosus*. Finding numerous new potential vaccine candidates or drug action targets for preventing infection or transmission is imperative. A cocktail vaccine, combined with several immunoprotective antigens, can be used for this purpose. Therefore, finding specific antigenic components of *E. granulosus* for diagnosis or drug target and vaccine study is important. Protein identification and characterization for *E. granulosu*s metacestode may help reveal new candidates for immunodiagnosis and vaccines. Proteomics offers a set of tools for investigating proteins from parasites, even in those without a genome project[5-6]. Thus, we applied proteomic technology to the field to investigate the proteomics of *E. granulosus* PSCs. Proteomics can find important functional proteins, which can be used for diagnosis or drug target and vaccine candidate molecules. Proteomics can also help uncover the host immune response and infection mechanism of *E. granulosus.*

MATERIALS AND METHODS

Collection of E. granulosus PSCs

PSCs were collected aseptically from fertile *E. granulosus* cysts removed from the liver of an infected patient, who was hospitalized in the Department of Liver Surgery, the affiliated hospital of Ningxia Medical University. The collected PSCs were washed in phosphate-buffered saline (PBS-1%) and Hanks' balanced salt solution (Sigma, St. Louis, USA) containing 100 U/mL penicillin G and 100 U/mL streptomycin sulfate. The PSCs were then stored in liquid nitrogen until use.

Collection of Pooled Antisera

Blood samples from 40 patients diagnosed with echinococcosis were collected and centrifuged at 4000 g to acquire the antisera. The antisera were pooled together and frozen at -20 °C to use for subsequent experiments.

2D Sample Preparation and Electrophoresis

Frozen samples were pulverized in a liquid nitrogen-cooled percussion mortar and solubilized directly into 200 mL sample buffer consisting of 8 mol/L urea, 0.5% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 20 mmol/L dithiothreitol, and 0.5% (v/v) immobilized pH gradient buffer with a pH of 3 to 10. The samples were allowed to mix gently overnight at 4 °C and then centrifuged at 8000 g for 30 min. The supernatant was kept and assayed for protein content using the 2D Quant kit (Amersham Biosciences). Proteins were aliquoted into single-use samples and stored at -80 °C. In the first dimension, samples were run respectively on two 18 cm Immobiline Dry Strips (Bio-Rad) on an IPGphor isoelectric focusing system as recommended by the manufacturer for samples run on pH 3 to 10 strips. Two strips were equilibrated in equilibration buffer [50 mmol/L Tris-Cl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), trace of bromphenol blue] containing 10 mg/mL dithiothreitol for 15 min. The strips were further equilibrated in equilibration buffer containing 25 mg/mL iodoacetamide for 15 min and sealed to 12% acrylamide gels using 0.5% agarose in standard Tris-glycine electrophoresis buffer. Second dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run in a Bio-Rad apparatus at 40 mA/gel and 15 °C until the tracking dye ran off the gel.

Gel Staining, Imaging, and Image Analysis

One of the two gels used was Coomassie blue stain. The images were scanned using Bio-Rad GM800 scanner and saved in the Tagged Image File Format (.tif) via UVPs Grap-It. Spot numbers were

measured and analyzed using PDQuest software, version 6.2.1 (Bio-Rad).

Mass Spectrometry

Coomassie blue-stained 2-D gel was used to analyze proteins combined with mass spectrometry. Gel plugs containing the proteins of interest were excised by hand (confirmed by rescanning the gel) and for peptide mass fingerprinting. Gel plugs were placed in 96-well plates and washed with water. Tryptic digestions were performed on a MassPrep liquid handling robot (MicroMass) according to the manufacturer's specifications using sequencing- grade modified trypsin (Promega). After gel extraction into 50% acetonitrile, peptides were lyophilized in a speed vacuum and resuspended in 3 mL of 0.1% trifluoroacetic acid solution. The peptide sample solution was then combined with an equal volume of matrix and spotted onto a matrix-assisted laser desorption/ionization (MALDI) sample plate. The sample/matrix solution was allowed to air dry at room temperature and was then washed thrice with 2 ml of 0.1% trifluoroacetic acid. Mass spectra were acquired on a MALDI-time-of-flight (MALDI-TOF) mass spectrometer (waters) operating in the positive ion reflector delayed extraction mode. Protein identifications were performed using MASCOT (Matrix Science) and by searching for matching peptide mass fingerprints in a protein database. The search criteria used were the complete carboxamidomethylation of cysteine, partial methionine oxidation, and mass deviation smaller than 60 ppm. A score greater than 51 was considered significant (P<0.05). We also required at least six matched peaks per protein and considered the experimental accuracy to theoretical isoelectric point (pI) and molecular weight.

Western Blot

One of the 2-D gels was used to screen specific immunity antigen. The 2-D gels were blotted to nitrocellulose membranes. The blot was blocked for 1 h in 3% bovine serum albumin in PBS. Pooled antisera were diluted 1:2000 in blocking solution and incubated for 1 h with the blot. The blot was washed for 3 min to 5 min in PBS containing 0.02% Tween 20 and incubated with horseradish peroxidase-conjugated sheep-anti mouse IgG (Amersham Biosciences) diluted to 1:1000 in blocking solution. The blot was washed, incubated with ECL Plus chemiluminescent substrate (Amersham Bio-sciences), and exposed to X-ray film.

RESULTS

After 2-DE and Western blot, the resulting images were analyzed using PDQuest software, edited, and formed into two images. The first image (Figure 1A) generated from the gels spanning pH 3-10 contained 233 spots. In the Western blot image (Figure 1B), 57 protein spots were recognized, among which, 23 protein spots were identified. Determining the number of spots on the pH 3-10 gel was challenging due to significant streaking, a problem commonly encountered with IPG strips in the basic range. Spots of varying intensity were excised and digested with trypsin. The resulting peptides were subjected to MALDI-TOF analysis. Of the 57 good quality spectra acquired, 23 identifications were obtained (Table 1). A correlation was found between spot intensity and spectrum quality, although several intense spots produced few peaks by MALDI-TOF. Most proteins had a molecular weight of 16 000 Da to 117 000 Da (Table 2) and a pI value of 3.0 to 10.0. Peptide mass fingerprinting required at least six matched peaks per protein (Figures 2A and 2B). The identified proteins can be divided into four categories: 1) cell skeleton proteins, including paramyosin, myosin, tropomyosin, dynactin subunit 2, and paramyosin; 2) metabolic enzyme, including phospholipase A2, putative peroxiredoxin, glycogen synthase kinase-3, and transketolase; 3) signal transduction protein, including calcium-binding protein, serine/threonine-protein kinase, and protein tyrosine kinase; and 4) material transmission protein, including fatty acid-binding, cytochrome c, and ADP-ATP carrier protein 2.

DISCUSSION

The completion of several parasite genomes ushered in a new phase of parasite-related research and the potential for improved parasite interventions, drugs, and vaccines[7-9]. Although genome sequences are now available for numerous important medical and veterinary parasites, the whole genome sequence for *E. granulosus* is not yet available. Large-scale technologies such as proteomics combined with sophisticated computational algorithms and databases have already given researchers a clearer understanding of this complex human parasite. However, how proteomics, combined with other genomic strategies, will aid in identifying new drug and vaccine targets is still unclear. As limitations of proteomics continue to

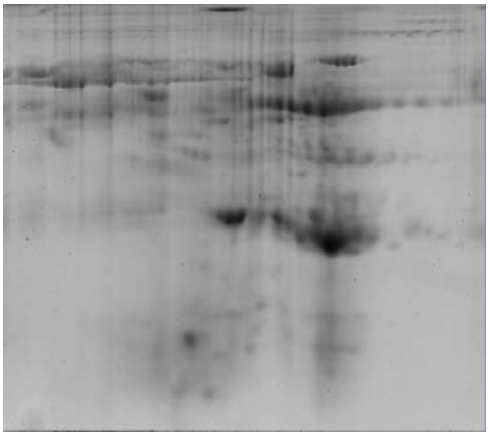

Figure 1A. 2-DE image mapping of Protoscoleces proteins from *Echinococcus Granulosus* 233 protein spot were revealed by PDQuest 8.0.

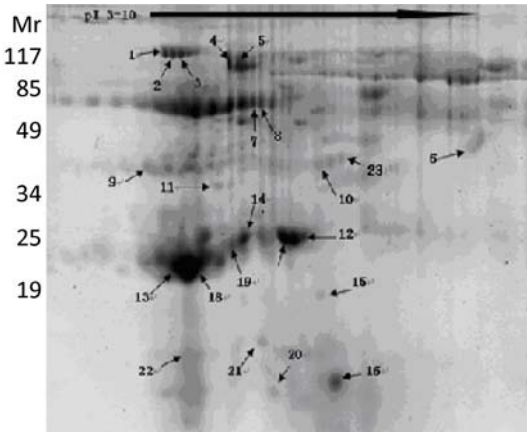

Figure 1B. Western-blotting shows proteins which been recognized by pooled antiserum and identified proteins spot (markedd with number and arrow).

Table 1. Proteins Have been Identified by Mass Spectrometry

Number Of Spot	Name of Protein	Weight of Molecular	PI	Score
No1	Paramyosin	98682	5.21	78
No.16	Calcium-binding protein	15103	5.47	54
No22	Fatty acid-binding protein	18276.	5.21	56
No.14	Serine/threonine-protein kinase	37400	5.51	74
No.9	Myosin	46067	4.68	60
No.6	Cytochrome C	57779	8.71	58
No.13	Phospholipase A2	27346	5.29	95
No.19	Actin	37800	5.36	62
No.17	Protein tyrosine kinase	27342	6.45	72
No.10	Tropomyosin	41325	6.86	58
No.11	Dynactin subunit2	44359	5.64	52
No.21	ADP-ATP carrier protein2	24145	6.44	55
No4	Glycogen synthase kinse-3	83852	6.39	64
No7	Transketolase	72575	6.53	72
No.12	Protein tyrosine kinse	27342	6.45	73
No.2	Paramyosin	98682	5.21	70
No3	paramyosin	98682	5.21	70
No5	Glycogen synthase kinase-3	83852	6.53	68
No.18	Phospholipase A2	27346	6.39	58
No.8	Transketolase	72575	6.53	68
No.23	Tropomyosin	41325	6.86	73
No20	Calcium-binding protein	15103	5.47	52
No.15	Putative peroxire doxin	22176	6.75	52

Table 2. Value Scale of Identified Proteins Biochemical Character

Scale of Molecular Weight (KD)	Number of Protein Spot	IP Value	Number of Protein
10-30	25	4-5	16
30-50	56	5-6	23
50-70	82	6-7	61
70-90	39	7-8	68
90-110	25	8-9	55
110 above	13	9 above	17

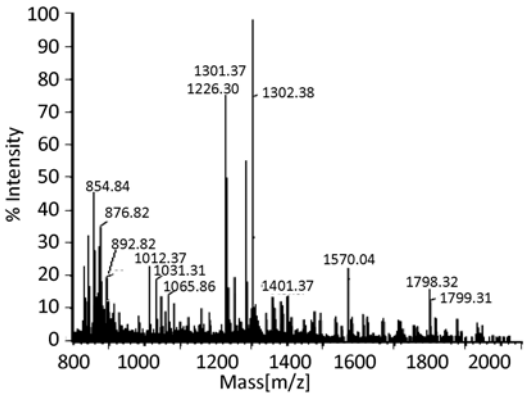

Figure 2A. Peptide mass fingerprinting of Protein spot No.7.

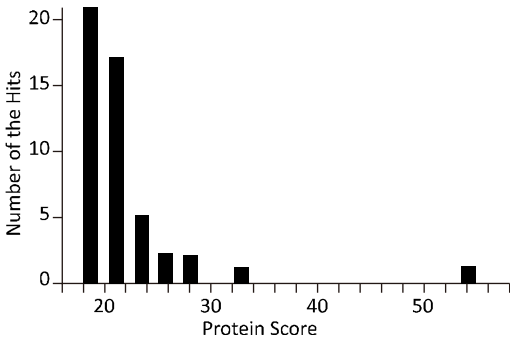

Figure 2B. Mascot identification result of protein spot No.7.

diminish with advanced technologies, creative approaches to investigating new drug targets are critical in maximizing available resources. These efforts offer hope that rational new drugs and vaccines are closer to reality.

Recent advances in 2-DE, mass spectrometry, and bioinformatics have significantly improved the possibilities for the mapping and characterization of protein populations. In the present study, we used a proteomics approach to analyze proteins expressed in E. granulosus protoscoleces. Over 200 to 240 protein spots were reproducibly separated by high-resolution 2-DE using the nonlinear pH range 3 to 10 and 12% SDS-polyacrylamide gel. The duplicate profiles from the same sample gave 88%-90% matching. No special treatment was required for sample preparation. Samples treated with Plus One 2-D Clean-Up kit did not give a superior number of spots or an improved profile. This observation indicates the proper protocol for sample solubilization in the present study. The overall distribution of protein profiles obtained for protoscoleces is similar to those obtained from other worms, e.g., *Caenorhabditis elegans*[10] and *Fasiola hepatica*[11]. 2-DE is one of the most widely accepted methods for separating and comparing complex protein mixtures. In addition, it has become the primary basis for proteomic studies. 2-DE has been used to identify protein expression changes and to map large numbers of proteins from different organisms and tissues. In the present study, we used 2-DE combined with Western blot to screen for specific immunity antigens. These results provide a framework for further proteomic studies of protoscoleces, and the tools used are valuable for the essential study of potential antigens associated with pathogenesis and/or carcinogenesis, drug targets, or vaccine development in the future.

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