Tropical Biomedicine 31(4): 579–591 (2014)

Identification of surface proteins of *Trichinella spiralis* muscle larvae using immunoproteomics

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Received 4 December 2013; received in revised form 5 January 2014; accepted 5 February 2014

Abstract. *Trichinella spiralis* surface proteins are directly exposed to the host's immune system, making them the main target antigens which induce the immune responses and may play an important role in the larval invasion and development process. The analysis and characterization of *T. spiralis* surface proteins could provide useful information to elucidate the host-parasite interaction, identify the early diagnostic antigens and the targets for vaccine. The purpose of this study was to identify the surface proteins of *T. spiralis* muscle larvae by two-dimensional gel electrophoresis (2-DE) Western-blot analysis and mass spectrometry. The 2-DE results showed that a total of approximately 33 proteins spots were detected with molecular weights varying from 10 to 66 kDa and isoelectric point (pI) from 4 to 7. Fourteen protein spots were recognized by sera of mice infected with *T. spiralis* at 42 dpi or at 18 dpi, and 12 spots were successfully identified by MALDI-TOF/TOF-MS, which represented 8 different proteins of *T. spiralis*. Out of the 8 *T. spiralis* proteins, 5 proteins (partial P49 antigen, deoxyribonuclease II family protein, two serine proteases, and serine proteinase) had catalytic and hydrolase activity, which might be the invasion-related proteins and the targets for vaccine. The 4 proteins (deoxyribonuclease II family protein, serine protease, 53 kDa ES antigen and hypothetical protein Tsp_08444) recognized by infection sera at 18 dpi might be the early diagnostic antigens for trichinellosis.

INTRODUCTION

Trichinella spiralis is a tissue-dwelling parasitic nematode that infects a wide variety of vertebrate hosts. Humans acquire the infection by the ingestion of raw or insufficiently cooked meat containing the *T. spiralis* infective larvae (Li *et al.*, 2012). The larvae at a muscle stage are an infective form of *T. spiralis*. In the host stomach, the infective larvae are released from the capsule in muscles with the aid of host gastric juice, and then invade, occupy and mobile in intestinal epithelium cells where they undergo four moults to emerge as sexually mature adults. The female begins to produce the second generation of larvae, which migrate through the whole body of the hosts. The establishment of *T. spiralis* in this habitat is the key step in which the larvae infect the hosts. With regard to the intestinal stage of infection, it has been suggested that proteases participate in intestinal invasion by *T. spiralis* (Ren *et al.*, 2013a; Wang *et al.*, 2013a; Zhang *et al.*, 2013). Although it has been known for many years that *T. spiralis* infective larvae invade the intestinal epithelium and the in vitro model of epithelial invasion by the larvae has been developed (Gagliardo *et al.*, 2002; Ren *et al.*, 2011), the mechanisms by which *T. spiralis* infective larvae recognize, invade, and migrate within the intestinal epithelium are unknown.

During their life cycle all nematodes undergo a series of moults in which they shed an external cuticle, consisting of an outermost membrane-like layer and a series of fibrillar layers similar to collagens. Because of this

structure, the cuticle has been viewed as an acellular exoskeleton with rather inert molecular components (Philipp *et al.*, 1980). It has showed that the cuticle surface of parasitic nematodes is recognized as antigenic in many infected hosts (Grencis *et al.*, 1986). In a number of experimental systems antibodies are produced against surface molecules and mediate antibody dependent cell mediated cytotoxic reactions (Butterworth, 1984). *T. spiralis* surface proteins include the cuticle proteins themselves and the excretory-secretory (ES) proteins which were incorporated on the cuticle (Ortega-Pierres *et al.*, 1984). They are directly exposed to the host's immune system and are the main target antigens which induce the immune responses, and may play an important role in the invasion and development process of *T. spiralis* larvae. Additionally, *T. spiralis* surface proteins are stage-specific and can induce the early serum antibody response to surface antigens of the homologous stage only (Philipp *et al.*, 1980; 1981). There has been special interest to study the *T. spiralis* surface proteins that interact at the interface between the parasite and the host to modify the parasite itself and its environment, either by modulating the host immune response for immune escape or even host cell gene expression, to ensure parasite invasion, development and survival (Bolas-Fernandez & Corral Bezara, 2006; Nagano *et al.*, 2009). Our previous study showed that the surface proteins could bind to the intestinal epithelial cells and might be related to invasion of intestinal epithelial cells by infective larvae (Wang *et al.*, 2011). The surface proteins may also be involved in the infective larvae-nurse cell complex formation and maintenance during the muscular stage of the infection for a longer period of time. Therefore, analysis and characterization of *T. spiralis* muscle larval surface proteins could provide useful information to elucidate the mechanism of parasite invasion, and possibly identify the early diagnostic antigens and the potential targets for vaccine.

Immunoproteomics could be defined as the combination of any proteomic technology with an immunological data presentation. Its development is vital in an age where it is increasingly becoming urgent to identify disease biomarkers and pathogenic target antigens for diagnosis and the development of new drugs and vaccines. Among the current proteomic techniques available (Steel *et al.*, 2005), 2-DE has often been chosen as the research tool in immuno-proteomic applications in combination with Western-blot (Klade, 2002). As effective tools for proteomics, the 2-DE Western-blot analysis combined with mass spectrometry (MS) has been used to characterize the differential expression profiles of different species of *Trichinella* spp. (Dea-Ayuela *et al.*, 2001; Bien *et al.*, 2012). Because the ES proteins can be easily prepared by the *in vitro* cultivation of *Trichinella* muscle larvae, the ES proteins were usually analyzed by utilizing 2-DE and immunoblotting techniques (Wang *et al.*, 2013c). To our knowledge, no surface proteins of *T. spiralis* muscle larvae have been analyzed and characterized by 2-DE Western-blot analysis and MS.

In the present study, the 2-DE Western-blot analysis and Matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF)/TOF-MS were used to characterize the surface proteins of *T. spiralis* muscle larvae. It will be valuable for further functional studies of surface proteins on the invasion, survival, and development of *T. spiralis* and their early diagnostic values for trichinellosis.

MATERIALS AND METHODS

Parasite and experimental animals

Trichinella spiralis isolate (ISS534) used in this study was obtained from a domestic pig in Nanyang city of Henan Province, China. The isolate was maintained by serial passages in Kunming mice in our laboratory. Six-week-old male Kunming mice were obtained from the Experimental Animal Center of Henan Province (Zhengzhou, China). The mice were maintained under specific pathogen-free conditions with sterilized food and water. All procedures of animal experiment of this study were

approved by the Life Science Ethics Committee of Zhengzhou University.

Collection of infection sera

BALB/c mice were orally infected with 300 muscle larvae/mouse and the serum samples from the infected mice were collected as described previously (Li *et al.*, 2010). About 100 µl of tail vein blood was collected daily from each mouse before infection and during 14-21 days post infection (dpi). When the forty infected mice were sacrificed at 42 dpi by deep ether anesthesia, their serum samples were also collected. Anti-*Trichinella* IgG antibodies in sera from infected mice at 14-21 dpi were assayed by ELISA and Western blot. By the above-mentioned two methods, the specific antibodies were firstly detected at 18 dpi and sustained to 42 dpi when the assay was ended. These infection sera were used to detect the following surface proteins.

Preparation of *T. spiralis* muscle larval surface proteins

The muscle larvae were recovered from the mice infected with 300 *T. spiralis* infective larvae at 42 days post infection (dpi) by artificial digestion of carcasses with 1% pepsin (1:3,000) and 1% hydrochloric acid as described previously (Gamble *et al.*, 2000; Li *et al.*, 2010). Muscle larval surface proteins were prepared as described previously with some modification (Pritchard *et al.*, 1985; Grencis *et al.*, 1986). Briefly, the live muscle larvae were cultured in phosphate-buffered saline (PBS; pH 7.4, 1/15 mol/L) containing 0.25% hexadecyl trimethyl ammonium Bromide (Sigma, USA) and 2% sodium deoxycholate (Sigma, USA) at 37ºC for 2.5 h. The supernatant was obtained by centrifugation at 4ºC, 11,000 ×g for 20 min, and dialyzed against deionized water at 4ºC for 2 days. The supernatant containing surface proteins was concentrated by a vacuum concentration and freeze drying (Heto Mxi-Dry-Lyo, Denmark). The protein concentration of surface proteins (4.62 mg/ml) was determined by the method described by Bradford (Bradford, 1976). The surface proteins were aliquoted and stored at -20ºC before use.

2-DE

The surface proteins were precipitated using trichloroacetic acid (TCA) and acetone as previously described method with some modifications (Ni *et al.*, 2010). Briefly, the sample was suspended in 10% TCA in acetone with 20mM DTT at -20ºC for 2 h. After centrifugation at 15,000 g at 4ºC for 15 min, the pellet was resuspended in cold acetone containing 20mM DTT and washed for three times. The final pellet was air-dried. The 2-DE was performed as previously described (Nareaho *et al.*, 2006). In brief, the pellet was suspended in rehydration buffer [7M urea, 2M thiourea, 4% CHAPS, 65 mM DTT, 0.2% IPG buffer (pH 3-10) and 0.001% bromophenol blue], containing 800 µg of the protein samples in a total volume of 500 µl and centrifuged at 12,000 ×g for 10 min at room temperature to remove the insoluble materials. The supernatant was loaded onto 24-cm pH 4-7 immobilized pH gradient (IPG) strips (Bio-Rad, USA) by over-night re-swelling and separated by isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad, USA). IEF was performed using a Protean IEF Cell at 20ºC as follows: S1: 250 V, 30 min; S2: 500 V, 30 min; S3: 1000 V, 1 h; S4: 10 000 V, 5 h; and S5: 10000V, 60 000 Vh (using a limit of 50 µA/strip). After IEF, the IPG strips were equilibrated sequentially, first in equilibration buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS and 20% glycerol) containing 2% dithiothreitol, then in equilibration buffer containing 2.5% iodoacetamide. The second dimension was performed on 12% SDS-PAGE using a Mini Protean cell (Bio-Rad, USA). Proteins were separated for 30 min at 16 mA and then at 24 mA until the dye front reached the bottom of the gel at 16ºC After 2D gel electrophoresis, proteins were stained with Coomassie blue R-250 for proteomic analysis as previously described (Wang *et al.*, 2011). The gel was scanned using ImageScanner (GE healthcare, USA). Spot detection and spot matching were performed by using Image Master 2D Platinum 6.0 (GE healthcare, USA). Three replicates were run for the sample. Only those protein spots that were clearly observed in three independent experiments were chosen for further analysis.

Western blotting

Proteins from 2-DE gels were transferred onto polyvinylidene difluoride (PVDF) membranes by semi-dry transfer cell (Bio-Rad, USA) for 1 h at 20 V (Wang *et al.*, 2013b). The membranes were blocked with 5% skimmed milk for 2 h at room temperature. Following three washes with TBST, the membranes were incubated overnight at 4°C with sera of infected mice (1:100 dilutions). After washing with TBST, blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, USA) (1: 5,000 dilutions) at 37°C for 1 h. Following three additional washes, the membranes were developed with the enhanced chemiluminescent (ECL) kit (CWBIO, China). Sera collected from mice before infection were used as negative controls. Immunoblot experiments were conducted in triplicate, with no variation in results observed. Images of immunoblots were captured using ImageScanner (GE healthcare, USA) and aligned with equivalent protein stained 2-DE gels using Image Master 2D Platinum 6.0 (GE healthcare, USA).

2-DE gel excision and tryptic digestion

2-DE protein spots recognized by mouse infection sera at 18 or 42 dpi were prepared for MALDITOF/TOF-MS analysis according to standard protocols (Li *et al.*, 1997). Fourteen spots were manually excised from the Coomassie blue-stained gels. The excised gel pieces carrying the spots were placed in a tube, destained for 20 min in 200 mmol/L NH_4HCO_3/30% acetonitrile and then lyophilized. All the lyophilized samples were digested overnight at 37°C with 12.5 ng/ml trypsin in 25 mmol/L NH_4HCO_3. The peptides were extracted three times with 60% ACN/0.1% trifluoroacetic acid (TFA). The extracts were pooled and dried completely by centrifugal lyophilization.

Protein identification by MALDI-TOF/TOF-MS

The resulting peptides from the above extraction were analyzed by MALDI-TOF/TOF-MS. The procedures were performed as described previously (Robinson *et al.*, 2007). Briefly, the purified tryptic peptide samples were spotted onto stainless steel sample target plates and mixed (1:1 ratio) with a matrix consisting of a saturated solution of a-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile-1% TFA. Peptide mass spectra were obtained on an Applied Biosystem Sciex 4800 MALDI-TOFD TOF mass spectrometer (Applied Biosystems, USA). Data were acquired using a CalMix5 standard to calibrate the instrument (ABI4700 Calibration Mixture). The MS spectra were recorded in reflector mode in a mass range from 800 to 4000 with a focus mass of 2000. For MS/MS spectra, up to 10 of the most abundant precursor ions per sample were selected as precursors for MS/MS acquisition, excluding the trypsin autolysis peaks and the matrix ion signals. In MS/MS positive ion mode, for one main MS spectrum, 50 subspectra with 50 shots per subspectrum were accumulated using a random search pattern. Collision energy was 2 kV, collision gas was air, and default calibration was set by using the Glu1-Fibrino-peptide B ([M+H] + 1, 570.6696) spotted onto Cal 7 positions of the MALDI target. Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed by using the MASCOT search engine 2.2 (Matrix Science, UK) and submitted to MASCOT Sequence Query server (http://www.matrixscience.com) for identification against nonredundant NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) with the following parameter settings: 100 ppm mass accuracy, trypsin cleavage (one missed cleavage allowed), carbamidomethylation set as fixed modification, oxidation of methionine was allowed as variable modification, and MS/MS fragment tolerance was set to 0.4 Da. The criteria for successfully identified proteins were as follows: ion score confidence index (CI) for peptide mass fingerprint and MS/MS data was $\geq$95%.

RESULTS

2-DE analysis of *T. spiralis* muscle larval surface proteins

The surface proteins of *T. spiralis* muscle larvae were separated on a 2-DE gel covering

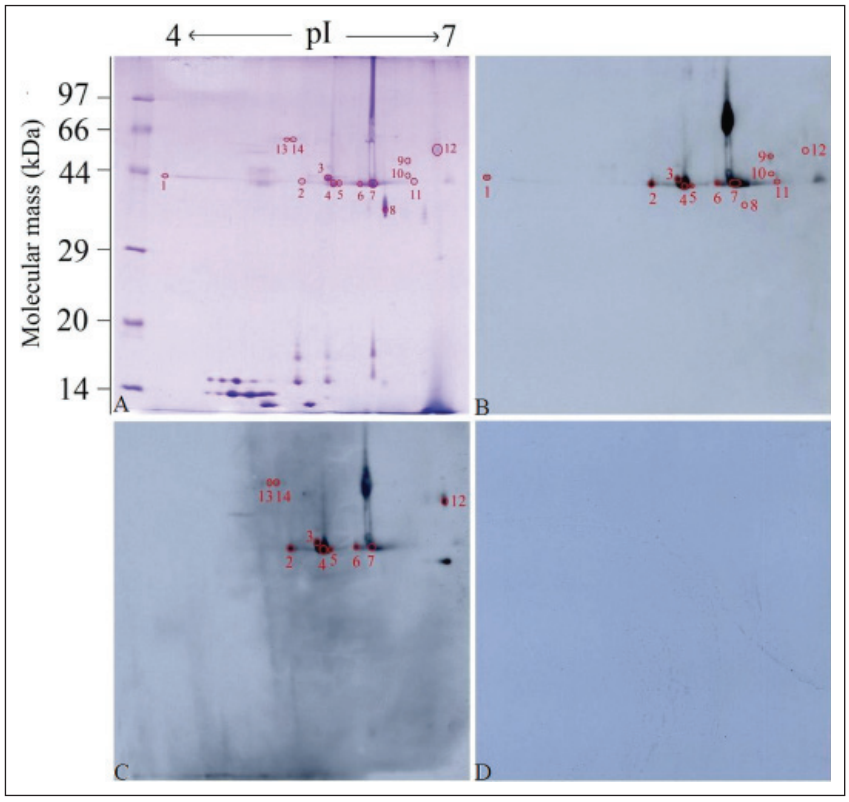

Figure 1. 2-DE and Western blot analysis of surface proteins of *T. spiralis* muscle larvae. (A) 2-DE gel of surface proteins separated in the first dimension in the pH range 4-7 and then in the second dimension on a 10% polyacrylamide gel. The gel was stained with Coomassie blue R-250, molecular weight standard is on the left, and pI values are indicated. Protein spots selected for analysis are numbered. (B) 2-DE Western blot of surface proteins probed by mouse infection sera at 42 days post infection (dpi), and the immunoreactive protein spots were detected by the enhanced chemiluminescent (ECL) kit. (C) 2-DE Western blotting analysis of surface proteins probed by mouse infection sera at 18 dpi. (D) Western blotting map of surface proteins probed by sera from mice before infection.

a pH 4-7 nonlinear, and the protein spots were visualized following Coomasie R-250 staining (Figure 1A). A total of approximately 33 spots were detected on the Coomassie bule stained 2-DE gels, with molecular weight (MW) varying from 10 to 66 kDa and isoelectric point (pI) from 4 to 7. Major protein spots were located in acidic range (pH 4-6) migrating at 30-60 kDa and 10-20 kDa. The 2-DE was repeated three times, and the patterns were highly reproducible.

Western blot analysis of surface proteins following 2-DE

As shown in Figure 1B, there were about 14 spots displaying reactivity to the infection sera at 42 dpi. Once photographed, the immunoblot and their homologous Coomassie blue-stained gel were aligned and then matched by Image Master 2D Platinum 6.0 software and artificial recognition. A total of 12 immunoreactive protein spots could be confidently matched to the corresponding protein spot in Coomassie blue-stained gels. These matched spots named spot 1 to 12 were selected to be further analyzed by MS. In comparison, when the immunoblot was performed with infection sera at 18 dpi, there were additional 2 positive spots named 13-14 except the above-mentioned 7 spots (2-7, 12) recognized by infection sera at 42 dpi, indicating that the 9 positive spots were

recognized by the early infection sera at 18 dpi (Figure 1C). However, the sera collected from mice before infection did not show detectable immunoreactivity with any of the protein spots (Figure 1D).

Identification of surface proteins by MALDI-TOF/TOF-MS

Eleven of 12 protein spots recognized by infection sera at 42 dpi were successfully identified as 7 different proteins of *T. spiralis* (Table 1). Additionally, 5 spots (2, 4, 5, 7 and 11) of the 12 immunoreactive spots were identified as the same *T. spiralis* proteins (deoxyribonuclease II family protein) by MS. Seven of 9 protein spots recognized by infection sera at 18 dpi spots were successfully identified as 4 different proteins of *T. spiralis* (Table 2).

Functional categorization of surface proteins by gene ontology

Gene Ontology (GO) signatures of 5 proteins [partial P49 antigen; deoxyribonuclease II family protein; serine protease (gi|168805931); serine protease (gi|168805933); serine proteinase] out of the 8 proteins identified were available. To further understand the functions of the proteins identified in this study, we queried against the InterPro databases and those resultant proteins were classified into molecular function and biological process according to GO hierarchy using WEGO (Fig. 2).

For the molecular function ontology, the classification results showed that all the above- mentioned five *Trichinella* genes were annotated with catalytic activity (GO:

Table 1. Identification of *T. spiralis* surface protein spots recognized by mouse infection sera at 42 dpi by using MALDI-TOF/TOF-MS

Spot No.	Protein name	Accession No.	Theoretical Mr/pI [a]	MOWSE score	Coverage (%)	No. Matched peptides	*p*-value
1	partial P49 antigen,	gi|162542	34.5/5.23	80	15	3	0.00032
2	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	97	10	2	6.8e-006
3	serine protease	gi|168805931	35.7/5.97	245	18	4	1.1e-020
4	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	382	22	6	2.1e-034
5	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	315	22	6	1.1e-027
6	Not identified	–	–	–	–	–	–
7	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	447	28	7	6.6e-041
8	ps73f07.y1 *T. spiralis* adult pAMP1 v1 *T. spiralis* cDNA 5', mRNA sequence	gi|21410467	22.3/4.83	371	39	9	2e-032
9	serine protease	gi|168805933	48.7/6.33	145	9	2	1.1e-010
10	serine proteinase	gi|13641204	48.7/6.33	116	12	5	8.4e-008
11	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	312	30	9	2.1e-027
12	53kDa excretory / secretory antigen	gi|805126	4.71/8.42	280	21	8	3.3e-024

[a] Theoretical molecular mass (kDa) and isoelectric point (pI)

Table 2. Identification of *T. spiralis* surface protein spots recognized by mouse infection sera at 18 dpi using MALDI-TOF/TOF-MS

Spot No.	Protein name	Accession No.	Theoretical Mr/pI [a]	MOWSE score	Coverage (%)	No. Matched peptides	*p*-value
2	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	97	10	2	6.8e-006
3	serine protease	gi|168805931	35.7/5.97	245	18	4	1.1e-020
4	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	382	22	6	2.1e-034
5	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	315	22	6	1.1e-027
6	Not identified	-	-	-	-	-	-
7	deoxyribonuclease II family protein	gi|339241449	38.1/5.95	447	28	7	6.6e-041
12	53kDa excretory/ secretory antigen	gi|805126	4.71/8.42	280	21	8	3.3e-024
13	Not identified	–	–	–	–	–	–
14	hypothetical protein Tsp_08444	gi|339247637	18.9/9.70	62	57	7	0.023

[a] Theoretical molecular mass (kDa) and isoelectric point (pI)

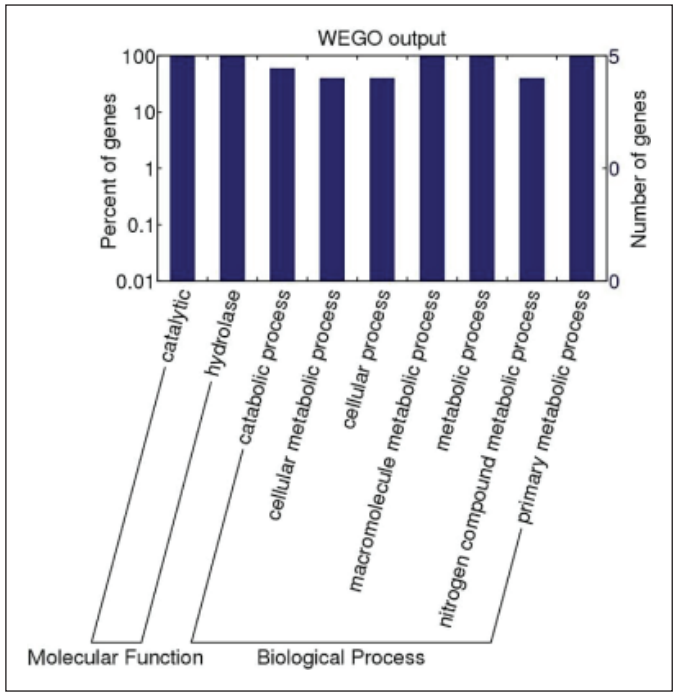

Figure 2. GO categories of surface proteins of *T. spiralis* muscle larvae. The proteins were classified into molecular function and biological process by WEGO according to their GO signatures. The number of genes denotes that of proteins with GO annotations.

0003824). Catalytic activity specifically refers to hydrolase activity (GO: 0016787).

In the biological process category, five proteins of *T. spiralis* were related to metabolic process (GO: 0008152, 5 proteins, 100% of 5 annotated peptides) and cellular process (GO: 0009987, 2, 40%). Most of the assigned metabolic process could be assigned to nitrogen compound metabolic process (GO: 0006807), catabolic process (GO:0009056), macromolecule metabolic process (GO:0043170), cellular metabolic process (GO: 0044237), primary metabolic process (GO:0044238). The proteins in the cellular process group are related to cellular metabolic process (GO: 0044237). Most of the cellular and metabolic processes were related to synthesis and degradation of macromolecules, particularly carbohydrates, nucleotides and proteins, which might be associated with the invasion and development of *T. spiralis* infective larvae.

DISCUSSION

The cuticle surface of *T. spiralis* muscle larvae is directly exposed to the host's immune system, the primary site for immune recognition and present key target antigens that induce the host immune responses. Clearly, the essential host-parasite interface is at the parasite surface, and it is the immune recognition at this site that will initiate parasite rejection (Bolas-Fernandez & Corral Bezara, 2006). Hence, the surface proteins may play an important role in the invasion, expulsion or immune evasion of the larvae. The surface protein is a group of proteins that can signal various biological processes, including immune reactions, adhesion molecules and enzymes. The molecular identification of the *T. spiralis* surface proteins is very important in elucidating host-parasite interaction. The recent development of immunoproteomics provides a powerful new approach to the analysis of the surface proteins. Such technique also allows the identification and characterization of surface proteins, which can subsequently be tested as the potential targets for vaccines and the possible early diagnostic antigens.

Previous studies demonstrated that after IEF in pH 3-10 nonlinear IPG strips and separation by 12% SDS-PAGE, the 2-DE gel was stained with Coomasie blue R-250, and most of the *T. spiralis* protein spots were located between pH 4 and 7 (Wang *et al.*, 2013c). With the purpose of improving the resolution of the spots, IEF was performed in narrow pH strips (pH 4-7) in this study. Our results showed that a total of 14 protein spots in surface proteins were recognized by sera at 42 dpi or at 18 dpi, and 12 spots were successfully identified by MALDI-TOF/TOF-MS, which represented 8 different proteins of *T. spiralis*. Out of the 12 successfully identified protein spots, five spots (2, 4, 5, 7, and 11) were identified as the same deoxyribonuclease II family protein, and they have the same MW and pI. Two spots (3 and 9) were identified as the same serine protease, but they have the different MW and pI. Several proteases (such as serine and cysteine) have been identified by substrate gel electrophoresis and characterized according to their pH optima, substrate specificities and inhibitor sensitivities (Todorova, 2000; Robinson *et al.*, 2007). Moreover, protease can serve as an immunodominant antigen, stimulating a protective immune response (Nagano *et al.*, 2003; Romaris *et al.*, 2002b). A previous study has also demonstrated that *T. spiralis* may express more than one isoforms of the protein and that a common precursor protein could undergo variable post-translational processing (Robinson *et al.*, 2007; Bien *et al.*, 2012). These modifications could be related to phosphorylation or acetylation of the proteins after translation, and they could be vital for the protein's biological functions, such as parasite survival, immune escape and immunopathogenesis. There is also a possibility that these proteins are members of the same protein family which share functional domains. Two protein spots (6 and 13) failed to match the proteins of *T. spiralis* ESTs or any sequence of other species of the genus *Trichinella*, which may be due to the low concentrations of the proteins and

therefore failed to produce high quality mass spectrometric data. It is also possibly because the proteins of the two spots were not included in the databases and these proteins have not yet been described.

In order to understand the biological activity of *T. spiralis* surface proteins, the surface proteins identified were functionally categorized based on the GO annotation of biological process and molecular functions. Accordingly, the proteins identified by MALDI-TOF MS might demonstrate their importance and contribution in the process of invasion and immune evasion of the larvae. The classification results of the 8 *Trichinella* genes showed that five genes were annotated with putative molecular functions. All of the five *T. spiralis* proteins (partial P49 antigen, deoxyribonuclease II family protein, two serine proteases, and serine proteinase) encoded by these genes have hydrolase activity. The results suggested that the larval invasion of intestinal epithelial cells was possibly mediated by these hydrolase in the larval surface proteins (ManWarren *et al.*, 1997, Ren *et al.*, 2013b). Out of the 8 different *T. spiralis* proteins identified in this study, 3 proteins had no assigned GO terms in the GO database. This is partially due to the limitation of the coverage of the current GO annotation system, and also due to some novel proteins previously described only as putative open reading frames (ORFs).

The partial P49 antigen of *T. spiralis* has been characterized and expressed in *Escherichia coli* by recombinant DNA methods (Su *et al.*, 1991). The recombinant P49 is a potentially valuable antigen both for vaccine development and immunodiagnosis. The deoxyribonuclease II family protein is known to be a lysosomal enzyme, capable of introducing single and double-stranded breaks into supercoiled plasmids in the presence of EDTA and mediating internucleosomal DNA digestion characteristic of apoptosis following intracellular acidification (Barry & Eastman, 1993; Shiokawa *et al.*, 1994). Serine proteases are important in a wide variety of biological processes, including digestion, blood coagulation and fibrinolysis. They are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the enzyme's active site (Hedstrom, 2002). In parasites, serine proteases are known to be involved in the invasion of host tissues and cells (Dzik, 2006), and in nematodes, they are likely to be important in moulting. Several secreted serine proteases have been identified in *T. spiralis* ES proteins, including the trypsin-like 45 kDa antigen and the serine protease TspSP-1(Romaris *et al.*, 2002b; Robinson & Connolly, 2005). Our previous studies showed that when *T. spiralis* muscle larvae were activated by bile and co-cultured with intestinal epithelial cells, the transcription and/or expression level of serine protease gene was obviously up-regulated, compared with the untreated normal muscle larvae (Liu *et al.*, 2013; Ren *et al.*, 2013a; Wang *et al.*, 2013b). The results of the present study further suggested that the serine proteases might be related to the larval invasion of intestinal epithelial cells, which require further investigation.

The 53kDa ES antigen is a glycoprotein derived from the β-granules of the larval stichosome and expressed in five *Trichinella* species (*T. spiralis*, *T. britovi*, *T. nativa*, *T. pseudospiralis*, and *T. papuae*), indicating that the 53 kDa protein is conserved among the five *Trichinella* species. The recombinant 53 kDa proteins of *T. spiralis* and *T. pseudospiralis* reacted with sera from mice infected with *T. spiralis* and *T. pseudospiralis* at 8 dpi, respectively. An antibody against the recombinant 53 kDa protein of *T. spiralis* recognized the 53 kDa protein in the crude extracts from adult worms, muscle larvae at 30 dpi and ES products from muscle larvae of *T. spiralis*, but did not recognize any proteins from *T. pseudospiralis*. The sera from the mice infected with *T. spiralis* strongly reacted with the 53 kDa recombinant protein of *T. spiralis* but did not react with the 53 kDa recombinant proteins of *T. britovi*, *T. nativa*, *T. pseudospiralis*, and *T. Papuae* (Romaris *et al.*, 2002a; Nagano *et al.*, 2008). These results showed that the 53-kDa ES antigen is a species-specific early diagnostic antigen

and could be used for the early diagnosis and species differential diagnosis of trichinellosis. Additionally, the 53 kDa ES antigen plays some roles in the continuation of the larval parasitism and modulation of host immune response (Nagano *et al.*, 2009).

The ES proteins of *T. spiralis* muscle larvae is the most commonly used diagnostic antigens for trichinellosis (Dupouy-Camet *et al.*, 2002), but their main disadvantages are the false negative results during the early stage of infection. Several studies have shown that the maximum detection rate of 100% of anti-*Trichinella* antibody IgG was not reached until at least 1-3 months after human infection with the parasite (Bruschi *et al.*, 1990; Gamble *et al.*, 2004). Our study showed the 4 proteins identified in *T. spiralis* surface proteins recognized by sera from early infection, indicating that they may be the antigens for early diagnosis of trichinellosis. However, this requires validation and confirmation in further experiments.

In conclusion, this study indicated that a total of 14 protein spots in surface proteins of *T. spiralis* muscle larvae were recognized by infection sera at 42 dpi or at 18 dpi, and 12 spots were successfully identified by MALDI-TOF/TOF-MS, which represented 8 different proteins of *T. spiralis*. Out of the 8 identified *T. spiralis* proteins, 5 proteins (partial P49 antigen, deoxyribonuclease II family protein, two serine proteases and serine proteinase) had catalytic and hydrolase activities. The identified *T. spiralis* surface proteins might be the invasion-related proteins and the targets for vaccine. The 4 proteins (deoxyribonuclease II family protein, serine protease, 53kDa ES antigen and hypothetical protein Tsp_08444) recognized by infection sera at 18 dpi may be the early diagnostic antigens for trichinellosis.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (No. 81271860, 81141084 and 81371843).

REFERENCES

Barry, M.A. & Eastman, A. (1993). Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Archives of Biochemistry and Biophysics* **300**: 440-450.

Bien, J., Nareaho, A., Varmanen, P., Gozdzik, K., Moskwa, B., Cabaj, W., Nyman, T.A. & Savijoki, K. (2012). Comparative analysis of excretory-secretory antigens of *Trichinella spiralis* and *Trichinella britovi* muscle larvae by two-dimensional difference gel electrophoresis and immunoblotting. *Proteome Science* **10**: 10.

Bolas-Fernandez, F. & Corral Bezara, L.D. (2006). TSL-1 antigens of *Trichinella*: an overview of their potential role in parasite invasion, survival and serodiagnosis of trichinellosis. *Research in Veterinary Science* **81**: 297-303.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Bruschi, F., Tassi, C. & Pozio, E. (1990). Parasite-specific antibody response in *Trichinella* sp. 3 human infection: a one year follow-up. *The American Journal of Tropical Medicine and Hygiene* **43**: 186-193.

Butterworth, A.E. (1984). Cell-mediated damage to helminths. *Advances in Parasitology* **23**: 143-235.

Dea-Ayuela, M.A., Ubeira, F.M., Pitarch, A., Gil, C., Martinez-Fernandez, A.R. & Bolas, F. (2001). A comparison of antigenic peptides in muscle larvae of several *Trichinella* species by two-dimensional western-blot analysis with monoclonal antibodies. *Parasite* **8**: S117-119.

Dupouy-Camet, J., Kociecka, W., Bruschi, F., Bolas-Fernandez, F. & Pozio, E. (2002). Opinion on the diagnosis and treatment of human trichinellosis. *Expert Opinion on Parmacotherapy* **3**: 1117-1130.

Dzik, J.M. (2006). Molecules released by helminth parasites involved in host colonization. *Acta Biochimica Polonica* **53**: 33-64.

Gagliardo, L.F., McVay, C.S. & Appleton, J.A. (2002). Molting, ecdysis, and reproduction of *Trichinella spiralis* are supported in vitro by intestinal epithelial cells. *Infection and Immunity* **70**: 1853-1859.

Gamble, H.R., Bessonov, A.S., Cuperlovic, K., Gajadhar, A.A., van Knapen, F., Noeckler, K., Schenone, H. & Zhu, X. (2000). International Commission on Trichinellosis: recommendations on methods for the control of *Trichinella* in domestic and wild animals intended for human consumption. *Veterinary Parasitology* **93**: 393-408.

Gamble, H.R., Pozio, E., Bruschi, F., Nockler, K., Kapel, C.M. & Gajadhar, A.A. (2004). International Commission on Trichinellosis: recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man. *Parasite* **11**: 3-13.

Grencis, R.K., Crawford, C., Pritchard, D.I., Behnke, J.M. & Wakelin, D. (1986). Immunization of mice with surface antigens from the muscle larvae of *Trichinella spiralis*. *Parasite Immunology* **8**: 587-596.

Hedstrom, L. (2002). Serine protease mechanism and specificity. *Chem Rev* **102**: 4501-4524.

Klade, C.S. (2002). Proteomics approaches towards antigen discovery and vaccine development. *Current Opinion in Molecular Therapeutics* **4**: 216-223.

Li, F., Cui, J., Wang, Z.Q. & Jiang, P. (2010). Sensitivity and optimization of artificial digestion in the inspection of meat for *Trichinella spiralis*. *Foodborne Pathogens and Disease* **7**: 879-885.

Li, G., Waltham, M., Anderson, N.L., Unsworth, E., Treston, A. & Weinstein, J.N. (1997). Rapid mass spectrometric identification of proteins from two-dimensional polyacrylamide gels after in gel proteolytic digestion. *Electrophoresis* **18**: 391-402.

Li, X., Liu, W., Wang, J., Zou, D., Wang, X., Yang, Z., Yin, Z., Cui, Q., Shang, W., Li, H., Wei, X., Cui, J., Wang, Z., Huang, L. & Yuan, J. (2012). Rapid detection of *Trichinella spiralis* larvae in muscles by loop-mediated isothermal amplification. *International Journal for Parasitology* **42**: 1119-1126.

Liu, R.D., Wang, Z.Q., Wang, L., Long, S.R., Ren, H.J. & Cui, J. (2013). Analysis of differentially expressed genes of *Trichinella spiralis* larvae activated by bile and cultured with intestinal epithelial cells using real-time PCR. *Parasitology Research* **112**: 4113-4120.

ManWarren, T., Gagliardo, L., Geyer, J., McVay, C., Pearce-Kelling, S. & Appleton, J. (1997). Invasion of intestinal epithelia in vitro by the parasitic nematode *Trichinella spiralis*. *Infection and Immunity* **65**: 4806-4812.

Nagano, I., Wu, Z., Nakada, T., Boonmars, T. & Takahashi, Y. (2003). Molecular cloning and characterization of a serine proteinase gene of *Trichinella spiralis*. *The Journal of Parasitology* **89**: 92-98.

Nagano, I., Wu, Z. & Takahashi, Y. (2008). Species-specific antibody responses to the recombinant 53-kilodalton excretory and secretory proteins in mice infected with *Trichinella* spp. *Clinical and Vaccine Immunology* **15**: 468-473.

Nagano, I., Wu, Z. & Takahashi, Y. (2009). Functional genes and proteins of *Trichinella* spp. *Parasitology Research* **104**: 197-207.

Nareaho, A., Ravanko, K., Holtta, E. & Sukura, A. (2006). Comparative analysis of *Trichinella spiralis* and *Trichinella nativa* proteins by two-dimensional gel electrophoresis. *Parasitology Research* **98**: 349-354.

Ni, X.D., Wang, N., Liu, Y.J. & Lu, C.P. (2010). Immunoproteomics of extracellular proteins of the *Aeromonas hydrophila* China vaccine strain J-1 reveal a highly immunoreactive outer membrane protein. *FEMS Immunology and Medical Microbiology* **58**: 363-373.

Ortega-Pierres, G., Chayen, A., Clark, N.W. & Parkhouse, R.M. (1984). The occurrence of antibodies to hidden and exposed determinants of surface antigens of *Trichinella spiralis*. *Parasitology* **88 (Pt 2)**: 359-369.

Philipp, M., Parkhouse, R.M., Ogilvie, B.M. (1980). Changing proteins on the surface of a parasitic nematode. *Nature* **287**: 538-400.

Philipp, M., Taylor, P.M., Parkhouse, R.M., Ogilvie, B.M., (1981). Immune response to stage-specific surface antigens of the parasitic nematode *Trichinella spiralis*. *The Journal of Experimental Medicine* **154**: 210-215.

Pritchard, D.I., Crawford, C.R., Duce, I.R. & Behnke, J.M. (1985). Antigen stripping from the nematode epicuticle using the cationic detergent cetyltrimethylammonium bromide (CTAB). *Parasite Immunology* **7**: 575-585.

Ren, H.J., Cui, J., Wang, Z.Q. & Liu, R.D. (2011). Normal mouse intestinal epithelial cells as a model for the in vitro invasion of *Trichinella spiralis* infective larvae. *PloS One* **6**: e27010.

Ren, H.J., Cui, J., Yang, W., Liu, R.D. & Wang, Z.Q. (2013a). Identification of differentially expressed genes of *Trichinella spiralis* larvae after exposure to host intestine milieu. *PloS One* **8**: e67570.

Ren, H.J., Liu, R.D., Wang, Z.Q. & Cui, J. (2013b). Construction and use of a *Trichinella spiralis* phage display library to identify the interactions between parasite and host enterocytes. *Parasitology Research* **112**: 1857-1863.

Robinson, M.W. & Connolly, B. (2005). Proteomic analysis of the excretory-secretory proteins of the *Trichinella spiralis* L1 larva, a nematode parasite of skeletal muscle. *Proteomics* **5**: 4525-4532.

Robinson, M.W., Greig, R., Beattie, K.A., Lamont, D.J. & Connolly, B. (2007). Comparative analysis of the excretory-secretory proteome of the muscle larva of *Trichinella pseudospiralis* and *Trichinella spiralis*. *International Journal for Parasitology* **37**: 139-148.

Romaris, F., Escalante, M., Lorenzo, S., Bonay, P., Garate, T., Leiro, J. & Ubeira, F.M. (2002a). Monoclonal antibodies raised in Btk(xid) mice reveal new antigenic relationships and molecular interactions among gp53 and other *Trichinella* glycoproteins. *Molecular and Biochemical Parasitology* **125**: 173-183.

Romaris, F., North, S.J., Gagliardo, L.F., Butcher, B.A., Ghosh, K., Beiting, D.P., Panico, M., Arasu, P., Dell, A., Morris, H.R. & Appleton, J.A. (2002b). A putative serine protease among the excretory-secretory glycoproteins of L1 *Trichinella spiralis*. *Molecular and Biochemical Parasitology* **122**: 149-160.

Shiokawa, D., Ohyama, H., Yamada, T., Takahashi, K. & Tanuma, S. (1994). Identification of an endonuclease responsible for apoptosis in rat thymocytes. *European Journal of Biochemistry / FEBS* **226**: 23-30.

Steel, L.F., Haab, B.B. & Hanash, S.M. (2005). Methods of comparative proteomic profiling for disease diagnostics. *Analytical Technologies in the Biomedical and Life Sciences* **815**: 275-284.

Su, X.Z., Prestwood, A.K. & McGraw, R.A. (1991). Cloning and expression of complementary DNA encoding an antigen of *Trichinella spiralis*. *Molecular and Biochemical Parasitology* **45**: 331-336.

Todorova, V.K. (2000). Proteolytic enzymes secreted by larval stage of the parasitic nematode *Trichinella spiralis*. *Folia Parasitologica* **47**: 141-145.

Wang, B., Wang, Z.Q., Jin, J., Ren, H.J., Liu, L.N. & Cui, J. (2013a). Cloning, expression and characterization of a *Trichinella spiralis* serine protease gene encoding a 35.5 kDa protein. *Experimental Parasitology* **134**: 148-154.

Wang, L., Wang, Z.Q. & Cui, J. (2013b). Protein changes in *Trichinella spiralis* muscle larvae *in vitro* induced by bovine bile. *Veterinary Parasitology* **194**: 164-167.

Wang, L., Wang, Z.Q., Hu, D.D. & Cui, J. (2013c). Proteomic analysis of *Trichinella spiralis* muscle larval excretory-secretory proteins recognized by early infection sera. *BioMed Research International* **2013**: 139745.

Wang, S.W., Wang, Z.Q. & Cui, J. (2011). Protein change of intestinal epithelial cells induced *in vitro* by *Trichinella spiralis* infective larvae. *Parasitology Research* **108**: 593-599.

Zhang, Y.L., Wang, Z.Q., Li, L.G. & Cui, J. (2013). Molecular characterization of *Trichinella spiralis* aminopeptidase and its potential as a novel vaccine candidate antigen against trichinellosis in BALB/c mice. *Parasites & Vectors* **6**: 246.