Parasitol Res (2017) 116:1687–1693
DOI 10.1007/s00436-017-5443-9

ORIGINAL PAPER

Identification of antigenic proteins in *Strongyloides stercoralis* by proteomic analysis

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Received: 8 February 2017 / Accepted: 12 April 2017 / Published online: 29 April 2017

Abstract *Strongyloides stercoralis* is an intestinal helminth that infects people worldwide. Hyperinfection or disseminated human strongyloidiasis can involve vital organs, leading to lethal outcomes. We analyzed immunoproteomics of antigenic spots, derived from *S. stercoralis* third-stage larvae and reacted with human strongyloidiasis sera, by two-dimensional gel electrophoresis and immunoblotting. Of 26 excised antigenic spots analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry, 20 proteins were identified. Most proteins were associated with enzymes involved in the metabolic process, energy generation, and oxidation–reduction. The proteins relate to promotion of worm development, cell division, cell signaling and transportation, and regulation of muscular contraction. Identification of antigenic proteins shows promise in helping to discover potential diagnostic protein markers or vaccine candidates for *S. stercoralis* infection.

Keywords *Strongyloides stercoralis* · Intestinal helminth · Antigenic proteins · Proteomic analysis · Mass spectrometry

Introduction

Strongyloides stercoralis, a soil-transmitted helminth, is an intestinal roundworm that infects people worldwide (Schar et al. 2013). The life cycle of *S. stercoralis* is complex, including direct, auto-infective, and free-living cycles (Schar et al. 2013). Infection, resulting in human strongyloidiasis, can occur through skin penetration of infective third-stage larvae (L3) (Schar et al. 2013). It occurs primarily in poor communities, travelers and former war veterans, immigrants, immunocompromised populations, and people exposed to soil (Beknazarova et al. 2016). Some patients present gastrointestinal symptoms and hyperinfection or disseminated strongyloidiasis, which can affect several organs, leading to fatal outcomes (Grove 1996). Presently, the immunology/immunopathology, protective immune response in humans, and biology of *S. stercoralis* are not fully understood. This study reported the biochemical properties of antigenic protein spots derived from *S. stercoralis* L3 using immunoproteomic and mass spectrometry techniques. Until now, there are few reports characterizing the *S. stercoralis* L3 antigenic proteins by proteomic analysis (Rodpai et al. 2016; Marcilla et al. 2010). The proteomic technique is a strategy for studying the patterns of protein expression in organisms and interpreting mass spectrometry data using cross-species databases. The results could augment our knowledge of the immune response in humans,

Electronic supplementary material The online version of this article (doi:10.1007/s00436-017-5443-9) contains supplementary material, which is available to authorized users.

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which may lead to the discovery of diagnostic protein markers or valuable vaccine candidates for helminth infection.

Materials and methods

Strongyloides stercoralis worms and protein extraction

Direct developed *S. stercoralis* third-stage larvae (L3) were collected from the fecal samples of infected asymptomatic patients using filter paper culture technique (Harada and Mori 1955). The L3 extract was prepared as has previously been described (Rodpai et al. 2016). The Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Inc., CA) was used to detect the protein concentration.

Two-dimensional gel electrophoresis (2DE)

The L3 extract was treated with the 2-D Clean-Up Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). One hundred fifty micrograms of the L3 extract was resuspended in rehydration solution and loaded onto a 7-cm Immobiline DryStrip gel (IPG) with a non-linear pH gradient of 3–11 (GE Healthcare). The sample was focused according to the manufacturer's instructions using the Ettan IPGphor 3 (GE Healthcare). Each of the focused IPG strips was then done in the second dimension on 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was then transferred to nitrocellulose membranes for immunodetection. Another gel was also stained with colloidal Coomassie blue, and the target spots were excised for protein identification by mass spectrometry. The experiments were conducted in triplicate.

Immunodetection

The experiment was independently conducted and visualized in triplicate as has previously been described (Rodpai et al. 2016). Each of the skim milk-blocked membranes was reacted with a 1:100 diluted pool of seven human serum samples from parasitologically proven strongyloidiasis patients or a 1:100 diluted pool of seven serum samples from healthy adult volunteers without intestinal parasitic infection in their stool from non-endemic areas and then probed with a 1:10,000 diluted goat anti-human IgG (H+L) antibody, horseradish peroxidase conjugate (Invitrogen Corporation, Camarillo, CA).

Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) and data analysis

Coomassie-stained protein spots that matched with the reacted spots against pooled human strongyloidiasis sera but not by healthy human sera were excised from the gel using an Ettan Spot Picker (GE Healthcare Bio-Sciences AB). Each protein in-gel spots was digested with trypsin (Promega, Madison, WI). The tryptic digested samples were analyzed with LC–ESI–MS/MS. NCBI's protein database (Metazoa) searching via the MASCOT MS/MS Ion Search program (www.matrixscience.com) was used to identify proteins. The instrument was provided by Khon Kaen University Research Instrument Center, Thailand.

The identified peptide sequences from LC–ESI–MS/MS and the MASCOT search (which generates sequences for the individual peptides) were assigned to the UniProtKB database (peptide search) (http://www.uniprot.org/) and described in gene ontology (GO) terms based on the biological process, molecular function, and cellular components. Since there was limited information regarding *S. stercoralis* gene sequences in the Mascot Server, peptide sequences from LC–ESI–MS/MS were subjected to the UniProtKB and WormBase ParaSite databases, in which the genome sequence of *S. stercoralis* is available (Hunt et al. 2016).

Results

Twenty-six antigenic protein spots related to protein spots in the Coomassie-stained gel, which were recognized by human strongyloidiasis sera, are shown in Fig. 1. The target protein spots were excised from the gels and digested. The resulting peptides were then identified using mass spectrometry (Table S1) based on matches to homologous proteins from related round worms (*Strongyloides ratti*, *Caenorhabditis remanei*, *Caenorhabditis elegans*, and *Haemonchus contortus*). Twenty different proteins were identified based on matches to *S. stercoralis* gene sequences in the UniProtKB and WormBase ParaSite databases (Table 1 and Table S2).

Out of a total of 20 proteins, 17 were annotated with gene ontology (GO) terms based on InterPro and QuickGO term annotations (Fig. 2 and Table S2), while three were not annotated. They were grouped according to the biological process (Fig. 2a), molecular function (Fig. 2b), and cellular component (Fig. 2c). The proteins were related to nine biological processes, 26 molecular functions, and five cellular components.

Discussion

There has already been a proteomic study of *S. stercoralis* antigenic protein recognized with human strongyloidiasis sera (Rodpai et al. 2016). Characterizations of the diagnostic 26- and 29-kDa polypeptide bands of *S. stercoralis* L3 have been reported (Rodpai et al. 2016). Here, we identified additional antigenic proteins of *S. stercoralis* recognized by human

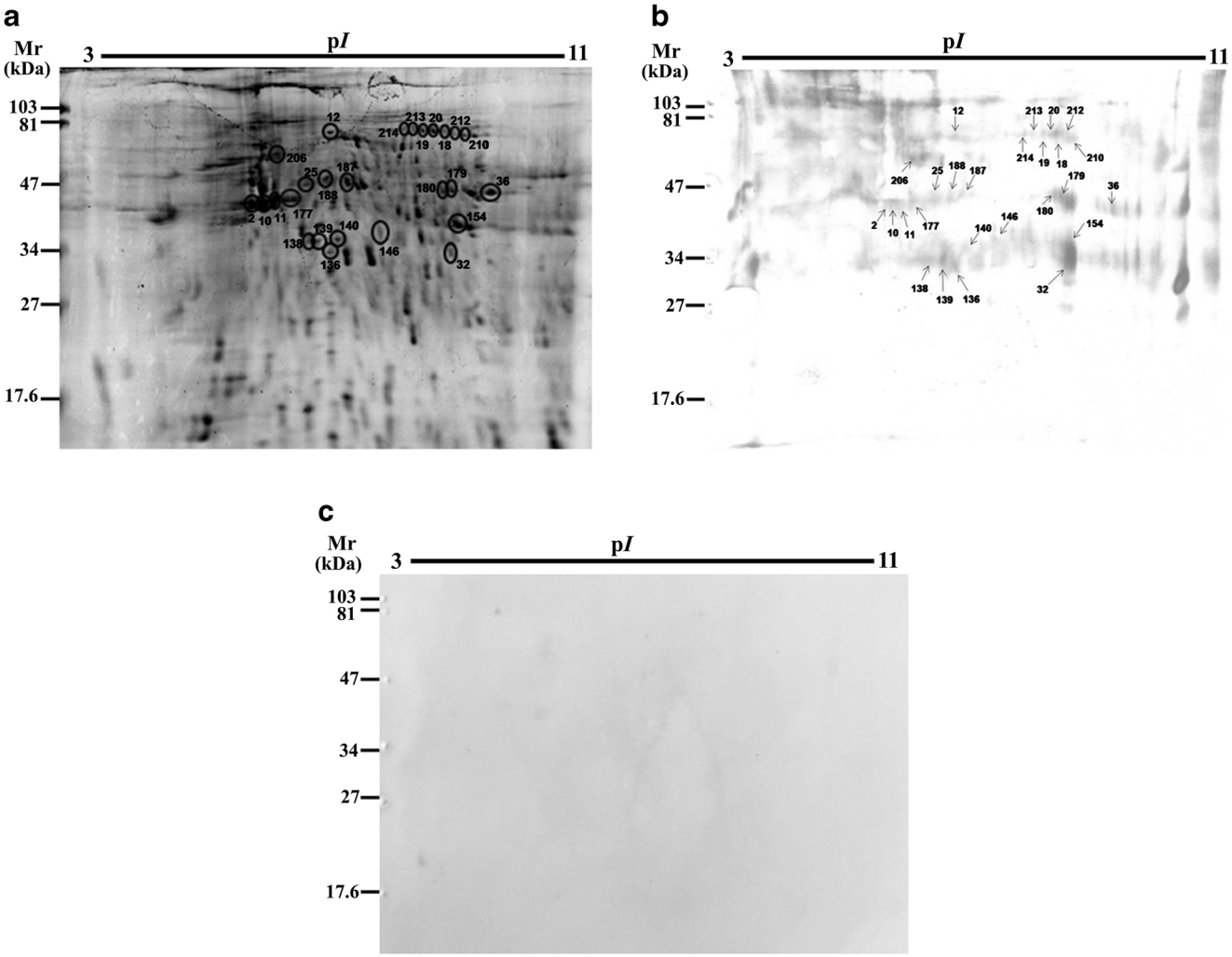

Fig. 1 Showing representative of Coomassie-stained 2DE gel and immunoblotting reaction patterns. The *Strongyloides stercoralis* L3 protein extract (150 μg) was separated using two-dimensional gel electrophoresis. Each colloidal Coomassie blue-stained gel (**a**) and the other were transferred onto a nitrocellulose membrane and then probed with human strongyloidiasis sera (**b**) and healthy sera (**c**) using immunoblotting. Coomassie-stained protein spots that matched with the reacted spots against pooled human strongyloidiasis sera were excised and subjected to LC–ESI–MS/MS. Molecular weight markers are indicated at the *left* of each image. *Numbers* and *circles* or *arrows* on images indicated the spot identity used in Table 1, Table S1, and Table S2, in which details of their identification are given

strongyloidiasis in patient sera using an immunoproteomic analysis.

Base on GO database comparisons, each immune-reactive protein was identified either as a cellular component, as having a molecular function, or as participating in at least one biological process. Metabolic process and energy generation, oxidation–reduction, promotion of worm development activation, cell division, cell signaling and transportation, and regulation of muscular contraction were revealed (Table S2; Fig. 2). Most proteins were associated with enzymes involved in the metabolic process and energy generation, and oxidation–reduction (16 protein spots) (Table 1), which could be used to keep the parasite alive. Five spots (nos. 18, 19, 20, 213, and 214) were identified as aconitate hydratase. It is an enzyme involved in the tricarboxylic acid cycle (Krebs cycle or the citric acid cycle) which catalyzes the reversible isomerization of citrate and isocitrate. Four spots (nos. 18, 20, 210, and 213) were identified as propionyl-CoA carboxylase alpha chain. Propionyl-CoA carboxylase is a biotin-dependent enzyme (GO:0004075), a Krebs cycle intermediate. Spot nos. 12 and 32 were identified as NADH-ubiquinone oxidoreductase or NADH dehydrogenase (mitochondrial complex I), which is a respiratory chain enzyme involved in ATP synthesis-coupled electron transport (GO:0042773) and the oxidation–reduction process (GO:0055114). These proteins are also involved in the aging of *C. elegans*, as RNAi knockdown of specific NADH-ubiquinone oxidoreductase has been shown to extend lifespan (Lee et al. 2003). Two spots (nos. 187 and 188) were identified as enolase involved in phosphopyruvate hydratase activity in the glycolytic process. Enolase might be used as a vaccine candidate and in immunodiagnosis, as recombinant *Ascaris suum* enolase can

Table 1 Summary of the immunoreactive protein spots from *Strongyloides stercoralis* L3 extract recognized by human strongyloidiasis sera. All of the identified proteins were matched to the *S. stercoralis* database in the UniProtKB and WormBase ParaSite using peptide sequences from LC–ESI–MS/MS and MASCOT search

No. of spot	Protein description	Theoretical Mr (kDa)/ p*I*	UniProt entry
2	14-3-3 protein zeta	27.933/4.80	A0A0K0DYN8
		33.725/4.90	A0A0K0DYP0
		26.571/4.99	A0A0G4E6L1
10, 11, 177	Actin, alpha cardiac muscle 1	41.810/5.30	A0A0K0DXX5
		68.761/5.71	A0A0K0ENM6
		41.792/5.30	A0A0K0EIA0
12	NADH-ubiquinone oxidoreductase 75-kDa subunit, mitochondrial	80.242/6.20	A0A0K0EKC5
18, 20, 210, 212	Propionyl-CoA carboxylase alpha chain, mitochondrial	81.083/8.23	A0A0K0ER14
18, 19, 20, 213, 214	Aconitate hydratase, mitochondrial	84.105/7.88	A0A0K0DWW2
25, 187, 188	Calponin repeat	42.603/6.29	A0A0K0DVS8
25, 206	Paramyosin, long form	104.229/5.44	A0A0K0DSA2
32	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	33.298/7.06	A0A0K0EKU9
36, 179, 180	Calponin repeat (Unc-87 protein)	42.096/6.93	A0A0K0DYR6
36	Acetyl-CoA hydrolase/transferase, acetyl-CoA hydrolase/transferase C-terminal domain	53.373/8.88	A0A0K0EMU7
136, 140	Galectin	65.398/9.11	A0A0K0ESR6
138	Guanine nucleotide-binding protein (G protein) subunit beta-4	37.410/5.71	A0A0K0E586
139, 140	Troponin I	32.298/5.13	A0A0K0E4K7
146	Fructose-bisphosphate aldolase 1	39.450/6.27	A0A0K0DWR6
154	Probable arginine kinase F46H5.3	42.063/7.57	A0A0K0DZN6
179, 180	Probable citrate synthase, mitochondrial	51.813/8.55	A0A0K0E9Q4
180	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	45.393/8.72	A0A0K0DVU6
187, 188	Enolase	47.162/5.92	A0A0K0EI07
206	Myosin heavy chain	227.069/5.55	A0A0K0DZH9
206	Troponin T, skeletal muscle	49.057/5.04	A0A0K0DT39

immunize mice to responses against *A. suum* infection (Chen et al. 2012; Wang et al. 2012). One spot (no. 180) was identified as a medium-chain specific acyl-CoA dehydrogenase enzyme possibly involved in the metabolism of fatty acids, as has been reported in *A. suum* (Komuniecki et al. 1985). One spot (no. 36) was identified as belonging to the acetyl-CoA hydrolase/transferase family. The acetyl-CoA hydrolase/transferase C-terminal domain-containing enzyme participates in pyruvate metabolism (GO:0006084). Two spots (nos. 179 and 180) were identified as citrate synthase, a key enzyme to catalyze the first reaction of the citric acid cycle (GO:0006099) that catalyzes the condensation of oxaloacetate and acetyl-CoA to form citrate. One spot (no. 154) was identified as arginine kinase F46H5.3, which belongs to the ATP:guanido phosphotransferase family. The structure and function of this group are related to enzymes that involve the transfer of phosphate between ATP and various phosphogens. One spot (no. 146) was identified as fructose-bisphosphate aldolase 1. This enzyme functions in gluconeogenesis and glycolysis pathways (GO:0006096), which are related to a reversible reaction of the aldol and fructose 1,6-bisphosphate. Four of these metabolic proteins (propionyl-CoA carboxylase alpha chain, enolase, citrate synthase, and arginine kinase) have been identified in *S. stercoralis* L3 using proteomic analysis approaches (Marcilla et al. 2010). Moreover, these proteins have also been identified as being immunoreactive in nematode infection, such as that of *Trichostrongylus colubriformis* (Kiel et al. 2007).

The metabolic reactions, i.e., enzymes related to catabolism of lipid storage in *S. stercoralis* L3, are relatively understudied, despite their potential use in the design of enzymatic inhibitors that could provide novel chemotherapeutic interventions or drug targets. Metabolic pathways are essential for an organism's survival, which require extract energy from

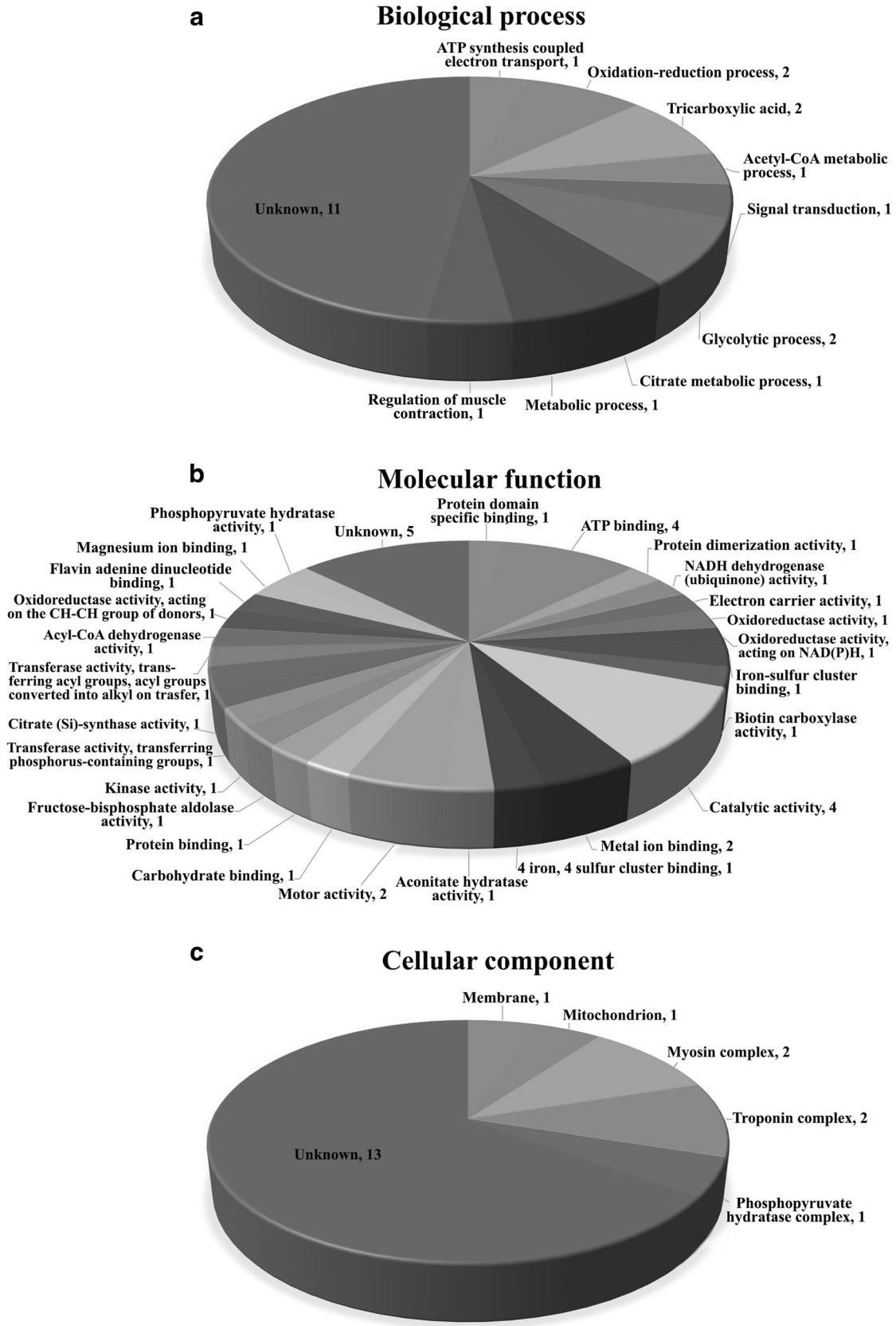

Fig. 2 Gene ontology (GO) terms of the 20 proteins of *Strongyloides stercoralis* third-stage larval extract. The identified proteins were classified into biological process (**a**), molecular function (**b**), and cellular component (**c**) using InterPro and QuickGO. The excess numbers of total annotated proteins are because some proteins were grouped in more than one functional category

carbon compounds. Therefore, inhibition of enzymes in these pathways likely shows a good choice of therapeutic strategy to resist parasitic infections (Timson 2016).

Two spots (nos. 136 and 140) were identified as galectin, a protein which binds sugars with a specific affinity for β-galactoside. It has a variety of mediation functions including transmembrane signaling, cell–cell interactions, and cell–matrix adhesion. This protein is related to initiating host immune response and has implications for vaccine development (Vasta 2009).

One spot (no. 138) was identified as a guanine nucleotide-binding protein (G protein) subunit beta-4, which mediates the chemosensory signals that regulate *S. stercoralis* L3i activation in cyclic guanosine monophosphate (cGMP) pathway signaling. This G-beta subunit gene was expressed in all developmental stages (Stoltzfus et al. 2014).

Twelve immune reactive spots were identified as the locomotion protein components of the cytoskeleton in the nematode (Ono 2014) (i.e., actin, paramyosin and myosin heavy chain, troponin complex, and calponin repeat-containing protein). Three spots (nos. 10, 11, and 177) were identified as actin, the protein associated with muscle contractility in *C. elegans* (Ono 2014). Two spots (nos. 25 and 206) were identified as paramyosin and myosin heavy chain proteins, which are known for their role in muscle contraction in the nematode (Kagawa et al. 2007). Six spots (nos. 25, 36, 179, 180, 187, and 188) were identified as calponin repeat-containing protein. Calponin is a cytoskeletal protein that regulates actomyosin contractility and stabilizes actin filaments (Rozenblum and Gimona 2008). Identified immunoreactive spots (nos. 139, 140, and 206) are related to troponin T and troponin I, the tropomyosin subunits. The troponin complex is a heteromeric protein attached to tropomyosin that regulates muscle contraction in the nematode *C. elegans* (Kagawa et al. 2007). Tropomyosin, an invertebrate pan-allergen, has been reported to be a helminthic allergen, such as *Anisakis simplex*, that occurs in response to high IgE titer in asymptomatic *Anisakis*-infected patients (Asturias et al. 2000). The structural muscle proteins such as myosin, paramyosin, and calponin of *T. colubriformis* have been identified as immunoreactive in immune sheep (Kiel et al. 2007). Interestingly, the cytoskeletal proteins (actin, paramyosin, myosin heavy chain, and tropomyosin) have been identified as the most abundant transcripts encoding predicted excretory–secretory proteins of *S. stercoralis* L3, which are likely related to host–parasite interaction and host immune system regulation (Marcilla et al. 2012). One spot (no. 2) was identified as 14-3-3 protein zeta. The 14-3-3 proteins are acidic proteins, a large family of broadly expressed 24- to 33-kDa proteins, with a role in regulatory signal transmission related to cell migration, proliferation, and morphology changes which occur during the parasite life cycle (Siles-Lucas Mdel and Gottstein 2003). These proteins also are potential good targets for anthelmintic drugs and vaccines (Siles-Lucas Mdel and Gottstein 2003) and for diagnostic method development (Rodpai et al. 2016).

Among the 26 identified spots, we found that 10 spots (38.46%) contained multiple proteins (Table 1 and Table S2). Multiple proteins were likely detected in a single spot because of the high sensitivity of protein identification by LC–ESI–MS/MS (Lim et al. 2003) and the limited separation performance of 2D gels. We also found that 10 proteins (50%) of the 20 identified proteins were identified in multiple spots (Table 1 and Table S2). In this case, multiple spots indicated either conformational changes that result in segregation of proteins to different isoelectric points (Deng et al. 2012) or the presence of protein isoforms (Kiel et al. 2007).

This study suggests that a relatively large number of parasite antigens are involved in immune responses to *S. stercoralis* infection and helpful in understanding the molecular mechanisms that support host–parasite interaction. The identified antigenic proteins could possibly help in developing a strategy for the mass production of antigens using a recombinant technique. These could, in turn, be used in the development of immunodiagnostic tests for human strongyloidiasis. Nevertheless, the limitation of this experiment is that the entire smeary signal on the western blots is specific for *Strongyloides* patient sera possibly due to the nature of the *Strongyloides* L3 extract, i.e., glycoproteins, in spite of the purification through the 2-D Clean-Up Kit. However, next experiments need to be used for clarifications.

Acknowledgements We wish to acknowledge the support of the English Consultation Clinic at the Khon Kaen University Faculty of Medicine Research Affairs Division and the Khon Kaen University Publication Clinic at the Research and Technology Transfer Affairs Division for their assistance.

Compliance with ethical standards

Funding This study was supported by a TRF Senior Research Scholar Grant, Thailand Research Fund grant number RTA5880001; the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Thailand, through the Health Cluster (SHeP-GMS); and the Faculty of Medicine, Khon Kaen University (grant number TR57201) through WM and PMI. RR was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (grant no. PHD/0053/2556). OS was supported by a scholarship under the Post-Doctoral Training Program from the Research Affairs and Graduate School, Khon Kaen University (grant no. 58101).

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Khon Kaen University Ethics Committee for Human Research (HE591192) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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