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Proteomic analysis of the excretory-secretory products from *Strongyloides venezuelensis* infective larvae: new insights for the immunodiagnosis of human strongyloidiasis

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Abstract

Serodiagnosis of human strongyloidiasis is a practical alternative to parasitological methods due to its high sensitivity. However, cross-reactivity with other helminth infections limits its utility, and this problem is due to the use of homologous or heterologous somatic extracts of the parasite as an antigen source. Excretory-secretory (E/S) products from *Strongyloides* infective larvae can be used to improve the serodiagnosis. The combined use of western blot and proteomics became an interesting strategy to identify immunological markers for the serodiagnosis of strongyloidiasis. The present study describes the proteomic analysis of the antigenic components from E/S products of *S. venezuelensis* infective larvae that were recognized by IgG antibodies from patients with strongyloidiasis. Our results showed that IgG antibodies from patients with strongyloidiasis recognized between 15 and 16 antigenic bands in the E/S products from *S. venezuelensis* that were incubated in PBS or in RPMI culture medium, respectively. Overall, antigenic bands of low and high molecular weight were more specific than those of intermediate molecular weight, which were cross-reactive. A 36-kDa antigenic band was 93% sensitive and 100% specific (a probably arginine kinase of 37 kDa), while other antigenic bands were highly sensitive but low specific. Proteomic analysis revealed differences between the protein profiles from E/S-RPMI and E/S-PBS since only one-third of all proteins identified were common in both types of E/S products. Bioinformatic analysis showed that more than 50% of the proteins from E/S products are secreted within extracellular vesicles and only a small percentage of them are actually released by the classical secretory pathway. Several components from the E/S products were identified as plasminogen-binding proteins, probably used as an immune evasion mechanism. The data provided here provide valuable information to increase understanding of E/S products from *S. venezuelensis* infective larvae. This may help us to find new targets for the immunodiagnosis of human strongyloidiasis.

Keywords *Strongyloides* · Excretory-secretory products · Western blot · Antibody · Antigen · Proteomics

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Introduction

Human strongyloidiasis is a parasitic disease caused predominantly by the nematode *Strongyloides stercoralis* with a worldwide distribution, especially in tropical and subtropical regions including Africa, Southeast Asia, and Latin America. Human strongyloidiasis is still considered a neglected tropical disease, and it is estimated that approximately 600 million individuals are infected worldwide (Nutman 2017; Buonfrate et al. 2020).

S. stercoralis infection begins when the infective third-stage filariform larvae (iL3) penetrate the host skin and migrate to reach the lungs and then the digestive tract, where they become parasitic female adults in the small

intestine, producing eggs by parthenogenesis; these eggs develop into rhabditiform larvae that are excreted along with the feces into the environment where they develop either into iL3 forms (homogonic route) or into free-living adult stages (heterogonic route). These adult forms will mate to produce eggs which then develop into rhabditoid larvae and then into iL3. The possibility of host autoinfection exists when rhabditoid larvae are retained in the intestine and become iL3 while still in the human host. Therefore, massive re-penetration and larval migration may result in disseminated infection or hyperinfection syndrome (Nutman 2017).

Human *Strongyloides* infection is usually chronic, asymptomatic, and longstanding. However, the use of corticosteroids as part of immunosuppressive therapies after solid organ transplantation or previous history of HTLV-I infection can lead to hyperinfection syndrome with a mortality rate from 15 to 87% (Vasquez-Rios et al. 2019).

Definitive diagnosis of *Strongyloides* infection using conventional parasitological methods to detect larvae in feces may be difficult due to low or irregular parasitic load in the feces, resulting in high rates of false-negative results (Requena-Méndez et al. 2013; Vasquez-Rios et al. 2019). For these reasons, detection of antibodies by immunoserological tests was developed and used as complementary alternatives to parasitological diagnosis, and these include indirect immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA), and western blot (WB) (Requena-Méndez et al. 2013).

Among them, ELISA is the most used and preferred test due to its high sensitivity and usefulness in seroepidemiological studies (Casado et al. 2019; Salvador et al. 2020). However, a critical limitation is the occurrence of crossreactivity with other helminth infections, probably due to the use of somatic extracts of the parasite (Conway et al. 1993a; Requena-Méndez et al. 2013). On the other hand, WB has been used for the recognition of *Strongyloides* antigens by serum antibodies from patients with strongyloidiasis with high sensitivity and specificity (Sato et al. 1990; Conway et al. 1993b; Sudré et al. 2007). When the WB was used together with proteomic analysis became an important tool to identify potential immunological markers for the serodiagnosis of human strongyloidiasis (Rodpai et al. 2016, 2017; Corral et al. 2017).

Strongyloides iL3 release a wide range of excretory-secretory (E/S) products that play a fundamental role in the host-parasite interaction, including recognition, invasion, and immune evasion, making them main targets for activation of host immune response. Due to limitations in obtaining a continuous source of *S. stercoralis* iL3, other species such as *S. venezuelensis* offer an alternative source of E/S products to be applied to the serodiagnosis of human strongyloidiasis (Cunha et al. 2017; Roldán Gonzáles et al. 2021).

Recently, we have tested the usefulness of E/S products from *S. venezuelensis* for the detection of IgG antibodies in patients with strongyloidiasis by ELISA, obtaining high values of sensitivity and specificity, when compared with different somatic extracts of the parasite (Roldán Gonzáles et al. 2021). On the other hand, secretomes of *S. ratti* and *S. venezuelensis* were characterized from E/S products obtained in serum-free culture media, showing a wide variety of molecules involved in facilitating host invasion and migration, nutrient acquisition, antioxidant activity, and immune evasion or suppression, enabling their survival into the host (Soblik et al. 2011; Maeda et al. 2019). However, there are no studies related to the antigenic characterization of the secretome from *S. venezuelensis* to search for potential markers to improve the serodiagnosis of human strongyloidiasis.

The present study describes the proteomic analysis of the antigenic components of E/S products from *S. venezuelensis* iL3 that were recognized by serum IgG antibodies from patients with human strongyloidiasis.

Materials and methods

Patients and serum samples

A total of 260 serum samples were analyzed, including 71 patients with laboratory diagnosis of *S. stercoralis* infection (presence of larvae in stool samples) and 105 serum samples from apparently healthy individuals with negative results for parasites in stool samples and no history of *Strongyloides* infection. In order to determine the rate of cross-reactivity, 84 serum samples from individuals with other helminth infections were used (17 patients infected with *Ascaris lumbricoides*, 20 patients infected with *Trichuris trichiura*, 2 patients infected with *Enterobius vermicularis*, 7 patients infected with hookworms, 13 patients infected with *Hymenolepis nana*, one patient infected with adult *Taenia solium*, 11 patients with neurocysticercosis (larval stages of *T. solium*), 5 patients infected with *Diphyllobothrium pacificum*, 2 patients infected with *Schistosoma mansoni*, and 6 patients with *Fasciola hepatica*). Positive and negative control sera were made from a mixture of sera from a patient with intestinal strongyloidiasis and sera from healthy individuals, respectively.

Production of S. venezuelensis iL3

The strain of *S. venezuelensis* used in this study was maintained by serial passages in male Wistar rats (*Rattus norvegicus*) at the Instituto de Medicina Tropical de São Paulo, USP, São Paulo, Brazil (Comitê de ética no uso de animais da FMUSP, protocol N° 0356A). Male Wistar rats (30 days

of age) were inoculated subcutaneously with approximately 30,000 iL3 in the abdominal region. Six days post-infection, the feces containing *Strongyloides* eggs were collected and moistened with tap water and then mixed with animal bone charcoal; the mixture was placed in wide glass jars and incubated at 28 °C for three days. After this procedure, iL3 were recovered according to the modified Baermann technique (Lok 2007), treated with sodium hypochlorite 0.25% for 5 min and then washed four times with sterile distilled water at 4000 × g for 1 min.

Preparation of E/S products

In our latest study, we have observed that *S. venezuelensis* iL3 is able to release antigenic proteins after incubation in 0.01 M phosphate buffer saline, pH 7.2 (PBS) (Roldán Gonzáles et al. 2021). Therefore, we decided to compare the antigenic profile of the E/S products from iL3 after incubation in RPMI 1640 medium and in PBS. Approximately 400,000 living iL3 larvae were placed in sterile polypropylene tubes containing either 5.0 mL of RPMI 1640 culture base medium (LGC Biotecnologia Ind., Cotia, SP, Brazil) or phosphate-buffered saline 0.01 M, pH 7·2 (PBS), both supplemented with a mixture antibiotics (Penicillin 100 U/mL, Streptomycin 100 U/mL, Gentamicin 50 μg/mL, and Amphotericin B 2.5 μg/mL), and incubated at 37 °C under 5% CO_2 for 24 h to obtain E/S products. After incubation, the supernatants were collected and mixed with a cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich Chemical Co., Saint Louis, MO, USA) and stored at −20 °C. All the supernatants were mixed and concentrated 100-fold using an Amicon YM10 membrane (EMD Millipore, Billerica, MA, USA) and then dialyzed against distilled water, centrifuged at 13,000 × g for 30 min at 4 °C, filtered through a membrane of 0.22 μm (EMD Millipore, Billerica, MA, USA), and stored at −20 °C until use. In those conditions, two types of antigenic preparations were obtained: E/S-RPMI and E/S-PBS, respectively. The protein content was determined by using the Bio-Rad DC protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA).

SDS–polyacrylamide gel electrophoresis and electroblotting of E/S products

E/S products (E/S-RPMI or E/S-PBS) were resolved according to their molecular weights by vertical SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Briefly, the E/S products were treated with a 5 × sample SDS-PAGE buffer (0.01 M Tris–HCl, pH 6.8, 1% SDS, 6% urea, 3.2% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue), heat-denatured at 75 °C for 15 min, and loaded on a 12% SDS–polyacrylamide gel at a ratio of 2.5 μg of protein per millimeter of gel.

Electrophoresis was performed in a Mini-Protean II apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 80 V for 10 min and then at 150 V for 140 min. Precision Plus Protein™ All Blue Standards (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used as electrophoresis running control. The apparent molecular weight (MW) of each component from E/S products was calculated following the method described by Matsumoto et al. (2019).

SDS–polyacrylamide gel containing separated E/S products were stained with a silver staining protocol compatible with mass spectrometry described by Shevchenko et al. (1996) or electro-transferred to 0.45 μm pore size, 7.5 cm × 8 cm nitrocellulose (NC) sheets in a Mini Transblot apparatus II (Bio-Rad Laboratories Inc.) at constant 100 V for 120 min at 4 °C. The NC sheets were washed for 30 min with PBS containing 0.1% Tween-20 (PBS-T) at room temperature (RT), cut into 3.5-mm-wide strips, and then stored at −20 °C until use.

Detection of human IgG antibodies against Strongyloides E/S products by western blot

NC strips were blocked with 5% nonfat milk diluted in PBS-T for 1 h at RT and then incubated with serum samples diluted 1:100 in blocking solution overnight at 4 °C in constant agitation. NC strips were washed three times for 5 min with PBS-T and then incubated with peroxidase-conjugated goat anti-human IgG (Fc specific) antibodies (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) diluted 1:2000 in blocking solution for 2 h at RT. After three washes with PBS-T as described above, NC strips were incubated with a chromogenic-substrate solution of 3,3′-diaminobenzidine containing cobalt salts (SIGMA*FAST*™ DAB with Metal Enhancer, Sigma-Aldrich Co. St. Louis, MO, USA) for 2 min at RT, and the enzymatic reaction was stopped by rinsing four times with tap water. A positive reaction on the NC strips was determined by visualizing defined black brown bands. All stages of this procedure were carried out under constant agitation using a volume of 800 μL for all reagents, and positive and negative control sera were used as reaction controls in all assays.

Proteomic analysis of antigenic bands by mass spectrometry

Antigenic bands that showed strong reactivity with sera from infected patients were located on silver-stained polyacrylamide gels and sliced into small fragments with the aid of a disposable scalpel and then deposited in 1.5 mL plastic tubes. The gel fragments were washed for 15 min in ultrapure water and then incubated in a silver destaining solution (equal parts of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate pentahydrate) until the stain

disappears and then washed for 15 min in ultrapure water. The gel fragments were washed three times with 50 mM ammonium bicarbonate buffer containing 40% acetonitrile (ACN) for 30 min; the gel fragments were dehydrated with 100% ACN for 15 min. The gel fragments were reduced with 10 mM dithiothreitol at 37 ℃ for 30 min and then alkylated with 55 mM iodoacetamide for 30 min in the dark. The gel fragments were dehydrated with 100% acetonitrile, and the remaining ACN was evaporated by vacuum centrifugation. Dehydrated gel fragments were hydrated for 45 min in sequencing grade trypsin solution (300 ng per fraction; Sigma-Aldrich Co. St. Louis, MO, USA) on ice. The hydrated gel pieces were covered with additional 50 mM ammonium bicarbonate and incubated overnight at 37 ℃. Following incubation, the enzymatic digestion was stopped by adding 10% formic acid and all the supernatants containing the tryptic peptides were transferred into new 1.5 ml plastic tubes. The gel pieces were then extracted three times with 50 mM ammonium bicarbonate containing 40% acetonitrile and then pooled with their respective supernatant. The extracted solutions were evaporated by vacuum centrifugation and then dissolved in 2% ACN prior to analysis.

All the peptide suspensions were analyzed by liquid chromatography-mass spectrometry in an ESI-IT-TOF instrument coupled to a UPLC 20A Prominence (Shimadzu, Kyoto, Japan). Samples (15 µL aliquots) were loaded into a C18 column (Kinetex C18, 5 µm; 50×2.1 mm) and fractionated by a binary gradient employing as solvents 0.1% formic acid (A) and ACN containing 0.1% formic acid (B). An elution gradient of 0–45% B was applied for 120 min at a constant flow of 0.2 mL/min after initial isocratic elution for 5 min. The eluates were monitored by a Shimadzu SPD-M20A PDA detector before being injected into the mass spectrometer. Raw LCD LCMS solution Shimadzu data were converted into an mzXML file by the LCM Solution (PRIDE) tool and then loaded into Peaks Studio V 7.0 (BSI, Canada). The false discovery rate was adjusted to $\leq 0.5\%$, and only proteins with a peptide confidence score $(-10\lg P) \geq 20$ and containing at least 1 unique peptide were considered in this study. For more details, see https://www.bioinfor.com/wp-content/uploads/2016/12/PEAKS7Manual.pdf.

Bioinformatic analysis of the identified proteins

Proteins were identified by Peaks Studio V 7.0 (BSI, Canada) using the UniProtKB database (Taxid: 75,913 – *Strongyloides venezuelensis*) and then were categorized according to their predicted molecular function, biological process, and cellular component using both the UniProtKB database (http://www.uniprot.org/) and QuickGO (http://www.ebi.ac.uk/QuickGO/).

SignalP 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) was used to predict the presence of an N-terminal signal peptide typical from the classical secretory pathway and TMHMM 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) to predict the presence of transmembrane helices in the first 60 amino acids.

OutCyte 1.0 (http://www.outcyte.com) was used to predict potential unconventional pathways of protein secretion (UPS) and check the presence of transmembrane helices. This tool was chosen because it showed improved performance when compared to the well-known SecretomeP 2.0 (Zhao et al. 2019).

ExoPred web server (http://imath.med.ucm.es/exopred/) was used to predict potential protein secretion within exosomes. Proteins that had neither signal peptide, UPS, nor evidence of being secreted within exosomes were considered intracellular or non-secreted proteins.

NetNGlyc 1.0 (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0) and NetOGlyc 4.0 (https://services.healthtech.dtu.dk/service.php?NetOGlyc-4.0) were used for N-glycosylation and O-glycosylation site predictions, respectively.

The antigenic density of each identified protein was estimated by means of its abundance of antigenic regions (AAR) value using the Secret-AAR tool (http://microbiomics.ibt.unam.mx/tools/aar/toolaar.php). The AAR value is the ratio between the sequence lengths to the number of predicted antigenic regions (Cornejo-Granados et al. 2019). Proteins with an AAR value ≤ 50 were considered potentially antigenic.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5.0 program. Sensitivity, specificity, and predictive values for WB were calculated using 2×2 tables with a confidence interval of 95%. To compare the degree of concordance between the two tests, the kappa index (k) was used. Differences were considered statistically significant at a level of 5%.

Results

Western blot analysis

Sixteen antigenic bands were recognized in E/S-RPMI by the positive control serum and were then grouped into low-molecular-weight bands (LMW: 30, 32, 36, 39, 41, 45, 47), intermediate-molecular-weight bands (IMW: 55, 60, 66, 71, 89), and high-molecular-weight bands (HMW: 94 kDa, 105–115 kDa, 179 kDa, 224 kDa). With the exception of the 179-kDa antigenic band, all the same bands were recognized

in ES-PBS, although there was a weaker reactivity in some antigenic bands from the LMW group (30 kDa, 41 kDa, 45 kDa, and 47 kDa) (Table 1 and Fig. 1a).

Sera from patients with strongyloidiasis recognized the 16 antigenic bands from E/S-RPMI and the 15 antigenic bands from E/S-PBS, showing a predominant reactivity with bands of 47 kDa (97.2%), 36 kDa (92.9%), 224 kDa (83.1%), 55 kDa (80.3%), 60 kDa and 179 kDa (76.1%), and 41 kDa and 105–115 kDa (73.2%). On the other hand, sera from healthy individuals recognized the bands of 60 kDa (72.4%) and 55 kDa (18.1%) (Table 1).

Sera from patients with other helminthiasis recognized bands of 60 kDa (42.9%), 55 kDa (33.3%), and 66 kDa (21.4%), followed by bands of 47 kDa and 105–115 kDa (11.9%), and the band of 224 kDa (10.7%). There was an unexpected recognition of two bands of 15 kDa (8.3%) and 136 kDa (4.8%) in both types of E/S products that were not recognized by the sera from strongyloidiasis patients. Bands of 36 kDa, 71 kDa, and 94 kDa were not recognized by this group of sera (Table 1).

Patients infected with *A. lumbricoides* recognized the bands of 55 kDa (76.5%), 60 kDa (64.7%), and 66 kDa (52.9%); patients infected with *T. trichiura* recognized the bands of 60 kDa (35%), but also with an unexpected band of 136 kDa (20%); patients infected with *E. vermicularis* recognized the band of 60 kDa (50%); patients infected with hookworms recognized the band of 60 kDa (28.6%), but also to a lesser extent with the bands of 55 kDa and 224 kDa (14.3%); patients infected with *H. nana* recognized the bands of 32 kDa, 39 kDa, 55 kDa, and 60 kDa (30.8%), 41 kDa and 45 kDa (15.4%), and 30 kDa (7.7%); patients with neurocysticercosis recognized the bands of 55 kDa and 66 kDa (54.5%), but also with the bands of 89 kDa, 105–115 kDa, and 179 kDa (45.5%). An unexpected band of 15 kDa was also recognized by 54.5% of these patients, as well as the only patient infected with *T. solium*. Patients infected with *D. pacificum* recognized predominantly the band of 60 kDa (80%), followed by the bands of 66 kDa and 89 kDa (60%). All the patients infected with *S. mansoni* recognized the bands of 60 kDa and 224 kDa, and half of them recognized the bands of 47 kDa and 55 kDa; all the patients infected with *F. hepatica* recognized the band of 224 kDa, and half of them recognized the bands of 32 kDa, 60 kDa, and the wide band of 105–115 kDa (Fig. 1b). A perfect concordance was observed in both types of E/S products for the detection of IgG antibodies in the three groups of sera (kappa index = 1).

Based on the WB results, three antigenic fractions named LMW (from 30 to 47 kDa), IMW (from 55 to 89 kDa), and HMW (from 94 to 224 kDa) from both E/S products were chosen to be analyzed by mass spectrometry (Fig. 1a). Tables 2 and 3 show all the proteins identified in the three selected fractions from E/S-RPMI and E/S-PBS, respectively.

Mass spectrometry and bioinformatic analysis from the selected antigenic fractions of E/S-RPMI

A total of 44 proteins were identified by mass spectrometry in the three selected fractions from E/S-RPMI: 8 proteins (18.2%) had a signal peptide to be released through the classical secretory pathway, 24 proteins (54.5%) had evidence of being secreted within exosomes, and 12 proteins (27.3%) were considered intracellular. Twenty-eight proteins (63.6%) were predicted to be O-glycosylated (12 proteins in fraction HMW, 6 proteins in fraction IMW, and 10 proteins in fraction LMW), and 6 proteins (13.6%) were predicted to be N-glycosylated (5 proteins in fraction HMW and 1 protein in fraction LMW). There were 4 proteins with both types of glycosylation (3 proteins in fraction HMW and 1 protein in fraction LMW). With regards to predicted antigenicity, a total of 28 proteins (63.6%) had AAR ≤ 50 and were considered potentially antigenic (Table 2).

Gene ontology analysis classified the identified proteins according to the cellular component, biological process, and molecular function in a total of 40 categories. In terms of

Table 1 Reactivity of sera from patients with strongyloidiasis, healthy individuals, and patients with other helminth infections against the different antigenic bands from *Strongyloides venezuelensis* excretory-secretory (E/S) products determined by western blot

Fraction	E/S products antigenic band (kDa)	% Positivity		
		Strongyloidiasis patients (n=71)	Healthy individuals (n=105)	Other helminthiases (n=84)
HMW	224	83.1	0	10.7
	179*	76.1	0	6
	105–115	73.2	0	11.9
	94	64.8	1.9	0
IMW	89	42.2	0	9.5
	71	35.2	1.9	0
	66	42.2	0	21.4
	60	76.1	72.4	42.9
	55	80,3	18.1	33.3
LMW	47	97.2	3.8	11.9
	45	46.5	1.9	7.1
	41	73.2	3.8	7.1
	39	45.1	0	4.8
	36	92.9	0	0
	32	26.8	0	8.3
	30	33.8	0	1.2

*Because the 179-kDa antigenic band was not present in E/S-PBS, there was no reactivity in the three groups of sera

HMW, high molecular weight; *IMW*, intermediate molecular weight; *LMW*, = low molecular weight
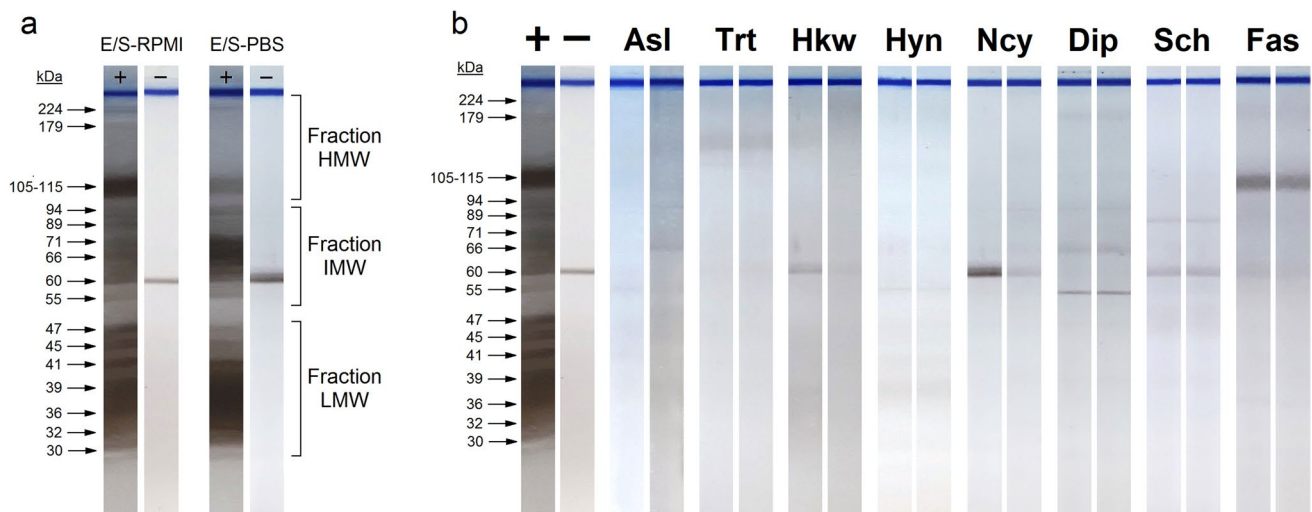
Fig. 1 Antigenic components from *S. venezuelensis* iL3 E/S products (E/S-RPMI and E/S-PBS), obtained by Western blot. (**a**) Using a positive control serum (pool of sera from patients with strongyloidiasis) and as control of reactivity, negative control serum (pool of sera from healthy individuals). The high molecular weight fraction (HMW), intermediate molecular weight fraction (IMW), and low molecular weight fraction (LMW) were shown. (**b**) Western blot results show-ing the cross-reactivity of E/S products from *S. venezuelensis* iL3 obtained in RPMI medium (E/S-RPMI) with a representative panel of serum samples from individuals with other helminthiases, including infections caused by *A. lumbricoides* (Asl), *T. trichiura* (Trt), hookworms (Hkw), *H. nana* (Hym), neurocysticercosis (Ncy), *D. pacificum* (Dip), *S. mansoni* (Sch), and *F. hepatica* (Fas). Control positive (+) and negative (−) sera were included as a comparison

cellular components, these proteins were mainly related to cytoplasm (38.6%), cell membrane (22.7%), extracellular region (13.6%), and nucleus (9.1%). In terms of biological processes, these proteins were involved mainly in protein transport (11.4%), larval development (9.1%), biosynthetic process (9.1%), oxidative stress (9.1%), and carbohydrate metabolic process (6.8%), among others. In terms of molecular function, these proteins were involved mainly in metal ion binding (22.7%), ATP binding (18.2%), protein binding (13.6%), nucleic acid binding (11.4%), transferase activity (11.4%), kinase activity (9.1%), and peptidase activity (9.1%), among others (Fig. 2, Supplementary Table 4).

In fraction HMW, a total of 16 proteins were identified by mass spectrometry (5 proteins had a signal peptide, 7 proteins were secreted within exosomes, and 4 proteins were considered intracellular) and 10 of these proteins were considered potentially antigenic (AAR ≤ 50). Among these proteins that can be highlighted because their potential antigenicity and their similar MW with the WB results are Degenerin unc-8 (94.56 kDa; pI = 6.05), two peroxidasin-like proteins of 107.66 kDa (pI = 7.04) and 114.6 kDa (pI = 9.64), calsyntenin-1 (111.15 kDa; pI = 4.99), and two uncharacterized proteins of 169.075 kDa (pI = 6.47) and 220.3 kDa (pI = 5.03).

In fraction IMW, a total of 9 proteins were identified (6 proteins were secreted within exosomes and 3 proteins were intracellular) and 6 of these proteins were considered potentially antigenic (AAR ≤ 50). Among these proteins that can be highlighted because their potential antigenicity and

their similar MW with the WB results are actin, alpha cardiac muscle 1 (55.68 kDa; pI = 6), phosphoglycerate mutase (57.56 kDa; pI = 5.89), glucose-6-phosphate isomerase (62.19 kDa; pI = 6.52), phosphoenolpyruvate carboxykinase (73.94 kDa; pI = 6.03), and calpain-2 catalytic subunit (89.93 kDa; pI = 4.87).

In fraction LMW, a total of 19 proteins were identified (2 proteins had a signal peptide, 11 proteins were secreted within exosomes, and 6 were considered intracellular) and 12 of these proteins were considered potentially antigenic (AAR ≤ 50). Among these proteins that can be highlighted because their potential antigenicity and their similarity with the WB results are two CAP domain-containing proteins of 30.5 kDa (pI = 9.79) and 40.67 kDa (pI = 10.33), 14–3-3 protein zeta (33.74 kDa; pI = 4.81), arginine kinase (37.04 kDa; pI = 7.57), adenylate kinase isoenzyme 1 (38.7 kDa; pI = 7.51), fructose-bisphosphate aldolase (39.49 kDa; pI = 6.32), malate dehydrogenase (40.96; pI = 8.91), two isoforms of actin, alpha cardiac muscle 1 of 41.79 kDa and 41.8 kDa (pI = 5.3), uncharacterized protein of 44.3 kDa (pI of 7.43), isocitrate dehydrogenase (46.015 kDa; pI = 6.04), and 2-phospho-D-glycerate hydro-lyase (47.17 kDa; pI = 6.04).

Mass spectrometry and bioinformatic analysis from the selected antigenic fractions of E/S-PBS

A total of 44 proteins were also identified by mass spectrometry in the three selected fractions from the E/S-PBS: 7

Table 2 Proteins identified in the three antigenic fractions from E/S-RPMI

Fraction	Uniprot	MW (kDa)	pI	Protein description	SignalP	Exopred	TMHMM	Predicted glycosylation	AAR value
HMW	A0A0K0F3W6	220.295	5.03	Uncharacterized protein	-	Yes	-	O	47.44
	A0A0K0FPQ6	186.767	5.9	DNA polymerase	-	Yes	-	O	53.53
	A0A0K0FZN1	169.075	6.47	Uncharacterized protein	Yes	-	-	N, O	36.64
	A0A0K0EVB3	152.102	5.8	Amidohydro-rel domain-containing protein	-	Yes	-	O	33.56
	A0A0K0FYR1	149.357	9.43	RNase NYN domain-containing protein	Yes	-	Yes	O	60.71
	A0A0K0F456	143.255	6.54	Protein RIC1 homolog	-	Yes	-	O	63
	A0A0K0FH30	136.095	6.18	Neuroglian	Yes	-	Yes	N, O	33.56
	A0A0K0FZT7	125.57	9.42	Histone-lysine N-methyltransferase	-	Yes	-	O	51.86
	A0A0K0F830	118.003	6.68	Adenylate cyclase	-	-	Yes	O	61.12
	A0A0K0F465	114.595	9.64	Peroxidasin-like protein	Yes	-	-	N	37.54
	A0A0K0F0E3	111.147	4.99	Calsyntenin-1	Yes	-	Yes	N	40.12
	A0A0K0F3T7	109.957	6.45	Aminopeptidase EC 3.4.11	-	Yes	-	-	53.72
	A0A0K0FL11	107.659	7.04	Peroxidasin-like protein	Yes	-	-	N, O	48.4
	A0A0K0FAE3	103.459	6.66	Uncharacterized protein	Yes	-	Yes	-	37.29
	A0A0K0FG75	102.918	6.82	Tudor domain-containing protein	-	Yes	-	O	35.28
	A0A0K0EV85	94.563	6.05	Degenerin unc-8	-	-	Yes	O	41.45
IMW	A0A0K0F9R3	89.934	4.87	Calpain-2 catalytic subunit	-	Yes	-	O	33.24
	A0A0K0FK28	81.849	4.93	Uncharacterized protein	-	Yes	-	O	59.08
	A0A0K0F4D9	78.412	5.94	Tr-type G domain-containing protein	-	-	Yes	-	41.18
	A0A0K0G0S7	73.944	6.03	Phosphoenolpyruvate carboxykinase (GTP)	-	Yes	-	O	32.9
	A0A0K0FYM2	65.394	7.56	Uncharacterized protein	-	Yes	-	O	63.22
	A0A0K0G292	62.193	6.52	Glucose-6-phosphate isomerase	-	Yes	-	-	39.57
	A0A0K0F6Y2	58.413	7.16	X-ray repair cross-complementing protein 6	-	-	-	O	123.75
	A0A0K0F9F9	57.563	5.89	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)	-	Yes	-	O	37
	A0A0K0F6C3	55.676	6	Actin, alpha cardiac muscle 1	-	-	-	-	36.14

Table 2 (Continued)

Fraction	Uniprot	MW (kDa)	pI	Protein description	SignalP	Exopred	TMHMM	Predicted glycosylation	AAR value
LMW	A0A0K0FKU3	47.168	6.04	2-phospho-D-glycerate hydro-lyase (Gamma-Enolase)	-	Yes	-	-	31.07
	A0A0K0EX00	46.015	6.04	Isocitrate dehydrogenase [NADP]	-	Yes	-	-	40.9
	A0A0K0G262	44.313	7.43	Uncharacterized protein	Yes	-	-	O	49.25
	A0A0K0FV38	41.81	5.3	Actin, alpha cardiac muscle 1	-	-	-	O	37.7
	A0A0K0FL48	41.792	5.3	Actin, alpha cardiac muscle 1	-	-	-	O	37.7
	A0A0K0FYF9	40.957	8.91	Malate dehydrogenase	-	Yes	-	-	37.5
	A0A0K0G1T0	40.668	10.33	CAP domain-containing protein	Yes	-	-	N,O	34.73
	A0A0K0FPH6	40.636	9.14	Inositol polyphosphate 5-phosphatase	-	-	-	O	69
	A0A0K0F7B1	39.485	6.32	Fructose-bisphosphate aldolase	-	Yes	-	O	28.08
	A0A0K0FTY1	38.707	7.51	Adenylate kinase isoenzyme 1	-	-	Yes	O	34.7
	A0A0K0EZR7	37.039	7.57	Arginine kinase	-	Yes	-	O	36.89
	A0A0K0FH91	36.262	6.8	L-lactate dehydrogenase	-	Yes	-	-	67
	A0A0K0FZ76	34.501	9.4	Uncharacterized protein	-	Yes	-	-	58.6
	A0A0K0FW12	34.273	6.99	60S acidic ribosomal protein P0	-	Yes	-	O	52.5
	A0A0K0FYU3	33.738	4.81	14–3-3 protein zeta	-	Yes	-	-	25.08
	A0A0K0FDI0	32.832	9.11	CCHC-type domain-containing protein	-	Yes	-	O	58.2
	A0A0K0F5G7	31.489	6.32	S-formylglutathione hydrolase	-	Yes	-	-	69.75
	A0A0K0FXA8	30.495	9.79	CAP domain-containing protein	Yes	-	-	O	36.75
	A0A0K0G0W1	30.150	8.33	Col_Cuticle_N domain-containing protein	-	-	Yes	-	74.25

HMW, high molecular weight; *IMW*, = intermediate molecular weight; *LMW*, low molecular weight pI indicates isoelectric point. SignalP means proteins containing a signal peptide sequence for the classical secretory pathway. Exopred indicates proteins with evidence of being secreted within exosomes. TMHMM indicates proteins containing transmembrane helices in the first 60 amino acids. N indicates proteins containing sites for N-glycosylation. O indicates proteins containing sites for O-glycosylation. AAR indicates the abundance of antigenic regions

proteins (15.9%) had a signal peptide to be released through the classical secretory pathway, 29 proteins (65.9%) had evidence of being secreted within exosomes, and 8 proteins (18.2%) were considered intracellular. Thirty proteins (68.2%) were predicted to be O-glycosylated (10 proteins in fraction HMW, 13 proteins in fraction IMW, and 7 proteins in fraction LMW), and 8 proteins (18.2%) were predicted to be N-glycosylated (2 proteins in fraction HMW, 3 proteins in fraction IMW, and 3 proteins in fraction LMW). There were 7 proteins with both types of glycosylation (1 protein in fraction HMW, 3 proteins in fraction IMW, and 3 proteins in fraction LMW). With regards to potential antigenicity, a total of 30 proteins (68.2%) had AAR ≤ 50 and were considered potentially antigenic (Table 3).

Gene ontology analysis classified the identified proteins according to the cellular component, biological process, and molecular function in a total of 31 categories. In terms of cellular components, these proteins were mainly related to cytoplasm (31.8%), nucleus (20.4%), cell membrane (13.6%), and cytoskeleton (13.6%). In terms of biological processes, the proteins were involved mainly in the catabolic process (15.9%), biosynthetic process (15.9%), phosphorylation (13.6%), carbohydrate metabolic process (6.8%), proteolysis (6.8%), apoptotic process (6.8%), and larval development (4.5%), among others. In terms of molecular function, the proteins were involved mainly in ATP binding (25%), kinase activity (22.8%), metal ion binding (20.5%), protein binding (18.2%), transferase activity (6.8%), peptidase activity (6.8%), and dehydrogenase activity (6.8%), among others (Fig. 2, Supplementary Table 5).

In fraction HMW, a total of 11 proteins were identified by mass spectrometry (2 proteins had a signal peptide, 4 proteins were secreted within exosomes, and 5 proteins were considered intracellular) and 7 of these proteins (AAR ≤ 50) were considered potentially antigenic. Among these proteins that can be highlighted because their potential antigenicity and their similar MW with the WB results are an uncharacterized protein of 102.4 kDa (pI = 6.04), calsyntenin-1 (111.15 kDa; pI = 4.99), protein transport protein sec16 (204.68 kDa; pI = 5.17), and non-specific serine/threonine protein kinase (208.13 kDa; pI = 8.83).

In fraction IMW, a total of 15 proteins were identified (2 proteins had a signal peptide, 12 proteins were secreted within exosomes, and 5 proteins were considered intracellular) and 12 of these proteins (AAR ≤ 50) were considered potentially antigenic. Among these proteins that can be highlighted because their potential antigenicity and their similar MW with the WB results are retinal dehydrogenase 2 (55.11 kDa; pI = 5.78), alanine aminotransferase 1 (55.57 kDa; pI = 8.35), actin, alpha cardiac muscle 1 (55.67 kDa; pI = 6), phosphoglycerate mutase (57.56 kDa; pI = 5.89), glucose-6-phosphate isomerase (62.19 kDa; pI = 6.52), uncharacterized proteins of 66.2 kDa (pI = 6.88),

septin-2 (67.28 kDa; pI = 8.68), malic enzyme (71.99 kDa; pI = 8.91), phosphoenolpyruvate carboxykinase (73.94 kDa; pI = 6.03), and neuroglian (77.24 kDa; pI = 8.04).

In fraction LMW, a total of 18 proteins were identified (3 proteins had a signal peptide, 13 proteins were secreted within exosomes, and 2 proteins were considered intracellular) and 11 of these proteins (AAR ≤ 50) were considered potentially antigenic. Among these proteins that can be highlighted because their potential antigenicity and their similar MW with the WB results are peptidase S1 domain-containing protein (30.56 kDa; pI = 8.6), 14–3-3 protein zeta (33.74 kDa; pI = 4.81), an uncharacterized protein of 33.83 kDa (pI = 6.19), two isoforms of arginine kinase of 37.04 kDa (pI = 7.57) and 40.54 kDa (pI = 8.37), two isoforms of fructose-bisphosphate aldolase of 39.5 kDa (pI = 6.32 and 7.98), malate dehydrogenase (40.96 kDa; pI = 8.91), two isoforms of actin, alpha cardiac muscle 1 of 41.79 kDa and 41.81 kDa (pI = 5.3), and 2-phospho-D-glycerate hydro-lyase (47.17 kDa; pI = 6.04).

Fourteen proteins were in common in both types of E/S products (1 protein with signal peptide, 11 proteins secreted within exosomes, and 2 intracellular proteins), of which 12 proteins were considered potentially antigenic (AAR ≤ 50) (Tables 2 and 3).

Discussion

Our results showed that several components of E/S products from *S. venezuelensis* iL3 were recognized by serum IgG antibodies from patients with human strongyloidiasis, but also by sera from individuals with other helminth infections and healthy individuals. In general, the antigenic bands of the LMW and HMW fractions were more specific than those of the IMW fraction, which had cross-reactivity and were therefore unspecific.

Brindley et al. (1988) were the first to characterize the E/S products from *S. stercoralis* iL3 using SDS-PAGE and immunoprecipitation, showing antigenic bands of 240 kDa, 90 kDa, 66 kDa, 60 kDa, 54 kDa, 50 kDa, 40 kDa, 35 kDa, 33 kDa, 30 kDa, 25 kDa, and 12 kDa. It is interesting to note that most of the antigenic bands reported by them agreed with the MW of the antigenic bands from E/S products of *S. venezuelensis* iL3, in particular those of the LMW and IMW groups.

Among the different components of the LMW group, the antigenic band of apparent MW of 36 kDa was 93% sensitive and 100% specific and without evidence of cross-reactivity. Interestingly, this antigenic band matches with an arginine kinase of 37 kDa (Uniprot A0A0K0EZR7), which lacks glycosylation sites and possesses a high antigenic potential (AAR = 38.9), as determined following the bioinformatic analysis. Furthermore, there is no other identified protein

**Table 3** Proteins identified in the three antigenic fractions from E/S-PBS

Fraction	Uniprot	MW (kDa)	pI	Protein description	SignalP	Exopred	TMHMM	Predicted glycosylation	AAR value
HMW	A0A0K0EXN8	208.133	8.83	Non-specific serine/threonine protein kinase	-	-	-	O	39.19
	A0A0K0EW98	204.682	5.17	Protein transport protein sec16	-	Yes	-	O	35.82
	A0A0K0EUC6	191.875	5.01	Uncharacterized protein	Yes	-	Yes	N, O	38.87
	A0A0K0G111	172.431	7.66	ELYS-bb domain-containing protein	-	-	Yes	O	60.44
	A0A0K0G049	131.877	6.89	Short transient receptor potential channel 4	-	-	Yes	O	46.16
	A0A0K0FYF3	123.604	6.62	Delphilin	-	Yes	-	O	39.96
	A0A0K0FB18	112.967	8.62	Anchor cell fusion failure-1	-	-	Yes	O	75.38
	A0A0K0F0E3	111.147	4.99	Calsyntenin-1	Yes	-	Yes	N	40.12
	A0A0K0F9S9	103.181	8.77	[Heparan sulfate]-glucosamine N-sulfotransferase	-	-	Yes	O	63.14
	A0A0K0FXN2	102.390	6.04	Uncharacterized protein	-	Yes	Yes	O	49.78
	A0A0K0G3F0	96.050	6.31	Doublecortin-like and CAM kinase-like protein	-	Yes	-	O	60.64
IMW	A0A0K0FCT6	82.694	6.38	Receptor protein serine/threonine kinase	-	-	Yes	N, O	51.57
	A0A0K0G523	77.240	8.04	Neuroglian	Yes	-	Yes	N, O	49.07
	A0A0K0G0S7	73.944	6.03	Phosphoenolpyruvate carboxykinase (GTP)	-	Yes	-	O	32.9
	A0A0K0F7R7	73.643	5.59	Uncharacterized protein	-	Yes	-	O	45.64
	A0A0K0FD75	71.998	8.91	Malic enzyme	-	Yes	-	O	39.88
	A0A0K0FB44	67.288	8.68	Septin-2	-	Yes	-	O	36.69
	A0A0K0FUU8	66.197	6.88	Uncharacterized protein	-	Yes	-	O	46.33
	A0A0K0EX33	62.853	8.8	Pct-1	-	Yes	-	O	55.7
	A0A0K0G292	62.193	6.52	Glucose-6-phosphate isomerase	-	Yes	-	-	39.57
	A0A0K0EZM4	58.664	8.19	Uncharacterized protein	-	Yes	-	O	41.15
	A0A0K0EZX9	58.524	9.15	Phosphate-regulating neutral endopeptidase	Yes	-	Yes	N, O	52.5
	A0A0K0F9F9	57.563	5.89	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)	-	Yes	-	O	37
	A0A0K0F6C3	55.676	6	Actin, alpha cardiac muscle 1	-	-	-	-	36.14
	A0A0K0FUR4	55.573	8.35	Alanine aminotransferase 1	-	Yes	-	O	45.55
	A0A0K0FTW4	55.112	5.78	Retinal dehydrogenase 2	-	Yes	-	O	42.58

Table 3 (Continued)

Fraction	Uniprot	MW (kDa)	pI	Protein description	SignalP	Exopred	TMHMM	Predicted glycosylation	AAR value
LMW	A0A0K0FKU3	47.168	6.04	2-phospho-D-glycerate hydro-lyase (Gamma-Enolase)	-	Yes	-	-	31.07
	A0A0K0G2Q6	45.306	8.97	Arginine kinase	-	Yes	-	-	50.38
	A0A0K0FXC6	42.046	9.47	Metalloendopeptidase	Yes	-	-	N, O	63.83
	A0A0K0FV38	41.810	5.3	Actin, alpha cardiac muscle 1	-	-	-	O	37.7
	A0A0K0FL48	41.792	5.3	Actin, alpha cardiac muscle 1	-	-	-	O	37.7
	A0A0K0FYF9	40.957	8.91	Malate dehydrogenase	-	Yes	-	-	37.5
	A0A0K0FGG6	40.544	8.37	Arginine kinase	-	Yes	-	O	40.11
	A0A0K0G127	39.501	7.98	Fructose-bisphosphate aldolase	-	Yes	-	-	45.88
	A0A0K0F7B1	39.485	6.32	Fructose-bisphosphate aldolase	-	Yes	-	-	28.08
	A0A0K0EZR7	37.039	7.57	Arginine kinase	-	Yes	-	O	36.89
	A0A0K0FH91	36.262	6.8	L-lactate dehydrogenase	-	Yes	-	-	67
	A0A0K0FXE7	34.073	9.21	Histone-lysine N-methyltransferase SET-MAR	-	Yes	-	-	58
	A0A0K0EYE0	33.828	6.19	Uncharacterized protein	-	Yes	-	-	41.57
	A0A0K0FYU3	33.738	4.81	14-3-3 protein zeta	-	Yes	-	-	25.08
	A0A0K0EWQ2	33.296	7.06	Galectin	Yes	-	-	N, O	52
	A0A0K0F8N8	31.520	9.46	Uncharacterized protein	-	Yes	-	-	269
	A0A0K0F5G7	31.489	6.32	S-formylglutathione hydrolase	-	Yes	-	-	69.75
	A0A0K0G0W4	30.567	8.6	Peptidase S1 domain-containing protein	Yes	-	-	N, O	42.57

HMW, high molecular weight; *IMW*, intermediate molecular weight; *LMW*, low molecular weight
pI indicates isoelectric point. SignalP indicates proteins containing a signal peptide sequence for the classical secretory pathway. Exopred=indicates proteins with evidence of being secreted within exosomes. TMHMM indicates proteins containing transmembrane helices in the first 60 amino acids. N indicates proteins containing sites for N-glycosylation. O indicates proteins containing sites for O-glycosylation. AAR indicates the abundance of antigenic regions

Fig. 2 Gene ontology analysis of the identified proteins from *S. venezuelensis* iL3 E/S products obtained following 24-h incubation in RPMI medium (E/S-RPMI) or in PBS (E/S-PBS). The proteins were grouped into cellular component (green bars), biological process (blue bars), and molecular function (red bars) in accordance with their GO signatures
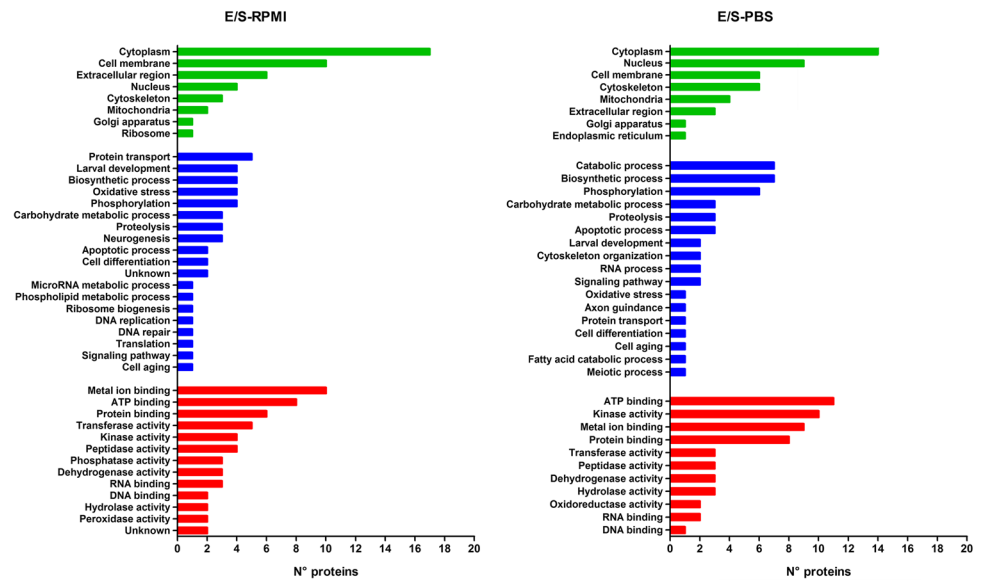
that has a similar molecular weight. Therefore, it is very likely that the 36-kDa antigenic band is indeed an arginine kinase, as it was present in both types of E/S products, according to the mass spectrometry results.

Arginine kinase belongs to the family of phosphagen kinases that are involved in energy homeostasis in a wide range of invertebrate species. Among helminths, only nematodes possess arginine kinase whereas cestodes and trematodes do not have this enzyme, although other types of phosphotransferases may be present (Barrett 2009). In the present study, up to three arginine kinase isoforms were identified in the E/S products of *S. venezuelensis* iL3 (Tables 2 and 3).

Some authors have evaluated the diagnostic potential of a recombinant arginine kinase from Toxocara canis as a marker of human toxocariasis, showing 100% sensitivity and minimal cross-reactivity with *Echinococcus granulosus*, *Toxoplasma gondii*, and *Entamoeba histolytica*. (Varghese et al. 2017). However, there are no studies so far on the diagnostic potential of this enzyme for the serodiagnosis of human strongyloidiasis and this arginine kinase of 37 kDa could be a candidate antigen for future research.

The antigenic band of 47 kDa was 97% sensitive but cross-reactive with sera from patients infected with *A. lumbricoides* and *H. nana*. Indeed, around 30% of sera from patients infected with *H. nana* reacted with most components of the LMW group. This antigenic band matches with a 2-phospho-D-glycerate hydro-lyase or enolase (Uniprot A0A0K0FKU3; MW = 47.17 kDa) which lacks glycosylation sites, is potentially antigenic (AAR = 31.1), and is identified in both types of E/S products. Enolase is a metabolic enzyme that belongs to the pathway glycolysis and is involved in the synthesis of pyruvate. Despite long being known as a cytosolic protein, enolase has been found on the surface of a variety of pathogenic bacteria, fungi, and

parasites acting as the main plasminogen-binding protein and possesses a high diagnostic potential (Gao et al. 2016; Ayón-Núñez et al. 2018; Ponce et al. 2018).

The antigenic band of 60 kDa (IMW group) was the most cross-reactive and unspecific of all antigenic bands of the E/S products from *S. venezuelensis* iL3, reacting with 43% of sera from individuals infected with other helminth infections, but also with 72% of sera from healthy individuals. This antigenic band matched with the Glucose-6-phosphate isomerase (Uniprot A0A0K0G292; MW = 62.2 kDa) which lacks glycosylation sites, is potentially antigenic (AAR = 39.57), and is identified in both types of E/S products. This metabolic enzyme has been found both on the surface and in the E/S products from different helminths and is involved in parasite development and/or in modulating the host-parasite relationship (Stadelmann et al. 2010; Diosdado et al. 2020).

Another cross-reactive and unspecific component from the IMW group was the antigenic band of 55 kDa, which matched with an Actin isoform of 55.7 kDa (Uniprot ID: A0A0K0F6C3) which lacks glycosylation sites, is potentially antigenic (AAR = 36.14), and is identified in both types of E/S products. Actin is a structural protein involved in many important cellular functions related to motility, division, shape, and signaling and also seems to be acting as a plasminogen-binding protein (González-Miguel et al. 2016).

Regarding the protein components of the HMW group, the antigenic band of 224 kDa was highly sensitive and did not react with sera from healthy individuals. However, there was cross-reactivity with sera from patients infected with trematodes. This antigenic band could be a non-specific serine/threonine protein kinase (Uniprot ID: A0A0K0EXN8; MW = 208 kDa) or an uncharacterized protein of 220 kDa (Uniprot ID: A0A0K0F3W6); both highly O-glycosylated

and potentially antigenic (AAR of 39.2 and 47.4, respectively). Likewise, the antigenic bands of 105–115 kDa and of 179 kDa also were sensitive and without reactivity in sera from healthy individuals, but were cross-reactive mainly with sera from patients with neurocysticercosis. Among the identified proteins that may match the antigenic band of 105–115 kDa are two Peroxidasin-like proteins of 107 kDa (Uniprot ID: A0A0K0FL11) and 115 kDa (Uniprot ID: A0A0K0F465) and the Calsyntenin-1 (Uniprot ID: A0A0K0F0E3; MW = 111 kDa; AAR = 40.1) which was identified in both types of E/S products. With regards to the antigenic band of 179 kDa, an uncharacterized protein of 169 kDa (Uniprot ID: A0A0K0FZN1; AAR = 40.1), which are highly O-glycosylated, may match this antigenic band. It is important to mention that the predicted MW of several identified proteins may vary since 68.2% and 70.4% of the proteins from E/S-RPMI and E/S-PBS, respectively, are glycosylated.

The two types of E/S products, obtained after incubation of iL3 in RPMI medium or in PBS, gave apparently similar antigenic profiles according to the WB results. However, proteomic analysis revealed that there were considerable differences between the protein profiles of E/S-RPMI and E/S-PBS since only one-third of all proteins identified by mass spectrometry were common in both types of E/S products. A possible explanation for this phenomenon is that the RPMI medium (rich in amino acids, glucose, vitamins, and minerals) may mimic better the host environment at 37 ℃ compared to PBS, which is a simple buffer solution that just mimics the physiological host pH. Using similar incubation conditions at 37 ℃, Maeda et al. (2019) observed a high mortality rate of *S. venezuelensis* iL3 and low yield in the production of E/S products after 12 h of incubation in PBS, while Dulbecco's modified Eagle's medium (DMEM) was able to stimulate much more the production of E/S products. Brindley et al. (1988) observed that *S. stercoralis* iL3 was able to incorporate amino acids or glucose from DMEM at 33 ℃, demonstrating that the parasite could be metabolically active "in vitro." Similarly, Tsuji et al. (1997) also observed that *S. venezuelensis* iL3 was able to incorporate [^{35}S]-methionine from the medium and synthetize proteins as a result of the temperature change during the "in vitro" transformation from free-living to the parasitic stage. These environmental differences could alter the protein profile that *S. venezuelensis* iL3 secretes "in vitro."

Host manipulation by helminth parasites is primarily due to the release of E/S products which are known to have immunomodulatory properties such as suppression of allergic responses, modulation of antigen-presenting cells, and induction of regulatory T-cell responses (Maizels et al. 2018). However, there is increasing evidence that helminths release a wide variety of proteins and microRNAs within extracellular vesicles, probably as a strategy to shape their immediate environment and modulate the host immunity (Drurey and Maizels 2021). Our results suggest that more than 50% of the proteins identified in the antigenic fractions of both types of E/S products from *S. venezuelensis* iL3 could be secreted within extracellular vesicles and only a small percentage of them are actually released by the classical secretory pathway. Marcilla et al. (2012) reported that more than 50% of the proteins from the secretomes of *Echinostoma caproni* and *Fasciola hepatica* are actually secreted within extracellular vesicles.

Proteins identified in the antigenic fractions of both types of E/S products from *S. venezuelensis* iL3 include metabolic and detoxifying enzymes, peptidases, proteins related to biosynthetic and signaling processes, cytoskeletal and nuclear proteins, calcium ion-binding proteins, and many of them were also identified in extracellular vesicles from other helminths such as *Ascaris suum* (Hansen et al. 2019), *Trichuris muris* (Tritten et al. 2017), *Fasciola hepatica* (Marcilla et al. 2012), and *Taenia pisciformis* (Wang et al. 2020), among others. The release of proteins to the host environment within extracellular vesicles by *S. venezuelensis* iL3 could explain the unusual presence of all these mentioned proteins.

Several studies have shown that metabolic enzymes from many species of parasitic protozoa and helminths can also be present extracellularly, being attached to the surface or being secreted within extracellular vesicles. At this extracellular level, these metabolic enzymes can also perform other additional functions, such as acting as ligands for a variety of components of the host. This binding would allow the parasites to participate in various important interactions such as adherence and invasion, modulation of host hemostatic and immune response, promoting angiogenesis, or acquiring molecules for nutrition (Gómez-Arreaza et al. 2014). In the present study, various metabolic enzymes including Phosphoenolpyruvate carboxykinase, Glucose-6-phosphate isomerase, Phosphoglycerate mutase, Enolase, Malate dehydrogenase, Fructose-bisphosphate aldolase, Adenylate kinase, and L-lactate dehydrogenase were found in both types of E/S products from *S. venezuelensis* iL3 and were previously reported as plasminogen-binding proteins (Gómez-Arreaza et al. 2014; González-Miguel et al. 2016; Ayón-Núñez et al. 2018; Diosdado et al. 2020). Likewise, other proteins such as actin, galectin, and 60S acidic ribosomal protein found in E/S products from *S. venezuelensis* iL3 have also been reported as plasminogen-binding proteins (Ayón-Núñez et al. 2018; Diosdado et al. 2020).

These findings show that *S. venezuelensis* iL3 could be manipulating the host fibrinolytic system through the recruitment of plasminogen (in a lysine-dependent manner) and its subsequent activation to plasmin by the host plasminogen activators as a survival mechanism, facilitating its migration through the different organs and host tissues.

An interesting group of proteins found in the LMW fraction of E/S-RPMI but not in E/S-PBS are two CAP domain (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins) proteins, of which one of them (A0A0K0FXA8; MW = 30.5 kDa; pI = 9.79) possesses 50% identity with the recombinant protein antigen NIE derived from *S. stercoralis* iL3 which is being used as a potential marker for the serodiagnosis of human strongyloidiasis. Maeda et al. (2019) have reported the presence of seven CAP domain proteins in E/S products from *S. venezuelensis* iL3, which includes the abovementioned protein. However, another CAP domain protein of 40.67 kDa (A0A0K0G1T0) was not reported by them and therefore constitutes the first report. CAP domain proteins are also known as sperm-coating protein/Tpx/ antigen 5/pathogenesis-related-1/Sc7 (SCP/TAPS) and share a common domain which adopts a unique α-β-α sandwich fold; these types of proteins have been reported in bacteria, plants, animals, and viruses and have been implicated in a variety of physiological contexts. CAP proteins were found in E/S products from hookworms and other helminths and are named as activation-associated secreted proteins (ASPs) or venom allergen-like proteins (VALs or VAPs). Although their molecular function is still somewhat enigmatic, recent studies have shown their participation as transporters of sterols or scavengers of leukotrienes. (Wilbers et al. 2018).

Another protein that deserves special attention is the 14–3-3 zeta protein (Uniprot ID: A0A0K0FYU3; MW = 33.7 kDa), which was identified in both types of E/S products from *S. venezuelensis* iL3. 14–3-3 proteins have been found within extracellular vesicles from several helminth parasites (Marcilla et al. 2012; Wang et al. 2020) and were able to bind the host mononuclear cell surface with modulatory effects on cytokine production, cell proliferation, and migration (Gadahi et al. 2016; Tian et al. 2018). A recombinant form of *S. stercoralis* 14–3-3 protein was produced and evaluated as a potential candidate for the serodiagnosis of human strongyloidiasis, showing 96% sensitivity and 93.8% specificity (Masoori et al. 2019).

Perhaps the main limitation of the present study was the lack of use of two-dimensional electrophoresis to separate the E/S products and then carry out the proteomic identification of those spots recognized by the IgG antibodies of patients with strongyloidiasis. However, the use of bioinformatics tools for the prediction of a signal peptide for protein secretion by the classical pathway, prediction of protein secretion by unconventional pathways including protein secretion within extracellular vesicles or exosomes, prediction of glycosylation sites, and estimation of the antigenic density helped to obtain more information on the nature and antigenic potential of E/S products from *S. venezuelensis* iL3.

All our results show that *S. venezuelensis* iL3 possesses proteins with immunomodulatory properties that can be released to the external environment using the classical secretory pathway or strategically secreted within extracellular vesicles or exosomes. Using mass spectrometry and bioinformatic analysis, we have been able to determine the biochemical characteristics of the antigenic components from the E/S products of *S. venezuelensis* iL3 identified by WB, showing that *S. venezuelensis* iL3 can release "in vitro" different protein profiles depending on the type of environment where they are exposed. Furthermore, our results also show that E/S products from *S. venezuelensis* iL3 have an interesting set of proteins with antigenic potential that could be explored as possible new markers for the immunodiagnosis of human strongyloidiasis, especially the arginine kinase of 37 kDa that matches with an antigenic band with high sensitivity and specificity. Future studies will be necessary to characterize and determine the specific functions of E/S products from *S. venezuelensis* iL3 and their role in the host-parasite interaction.

Declarations

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