Invest Clin 52(2): 121 - 139, 2011

A combined proteomic and immunologic approach for the analysis of *Schistosoma mansoni* cercariae and adult worm protein extracts and the detection of one of the vaccine candidates, Sm28GST, from a Venezuelan parasite isolate.

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Key words: *Schistosoma*, proteome, Sm28GST.

Abstract. Understanding the mode of *Schistosoma mansoni* larval invasion and the mechanism of immune evasion utilized by larvae and adult worms is essential for a rational development of vaccines or drugs to prevent or cure the disease. This parasite has a very complex molecular organization in all parasite stages, and identifying the major parasite proteins would give clues to schistosome metabolism and to the interaction of the parasite with the host immune system. Our goal was the evaluation of the protein parasite repertoire using a proteomic approach, and the characterization of protein extracts from two different parasite stages of a Venezuelan isolate, such as cercariae and adult worms, previously performed by other authors in some other strains. A comparison among authors was made. Besides, we aimed to identify different isoforms of one of the vaccine candidates, the gluthation-S-transferase protein (Sm28GST), by 2D SDS-PAGE and mass spectrometry, and to achieve its immunologic detection using sera from rabbits immunized with synthetic peptides derived from the Sm28GST protein. These techniques allowed the identification of some of the target molecules of the protective immune response that are being evaluated as potential members of a multi-component and multi-stage anti-*S. mansoni* vaccine and to clarify if the selected peptides induce antibodies that are able to recognize different isoforms of the Sm28GST.

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Aproximación al análisis proteómico e inmunológico de extractos proteicos de cercaria y verme adulto de *Schistosoma mansoni* y detección de uno de los candidatos a vacuna, Sm28GST, de un aislado venezolano.

Invest Clin 2011; 52(2): 121 - 139

Palabras clave: *Schistosoma*, proteómica, Sm28GST.

Resumen. Es esencial comprender la forma como las larvas de *Schistosoma mansoni* invaden y los mecanismos de evasión inmune utilizados por larvas y adultos, para el desarrollo racional de vacunas o drogas para prevenir o curar la esquistosomiasis. Este parásito tiene una organización molecular muy compleja en todos sus estadíos, por lo que la identificación de las proteínas más importantes es clave para investigar el metabolismo del esquistosoma y la interacción del parásito con el sistema inmune del hospedero. El objetivo de este trabajo fue evaluar el repertorio proteico del parásito utilizando una aproximación proteómica y la caracterización de extractos proteicos de dos estadios parasitarios diferentes de un aislado venezolano, como la cercaria y el verme adulto, previamente realizado por otros autores en otras aislados. Se realizó una comparación entre autores. Además, se identificaron diferentes isoformas de uno de los candidatos a vacuna, la glutation S transferasa (Sm28GST) por 2D SDS-PAGE y espectrometría de masas y se logró su detección inmunológica, usando sueros de conejos inmunizados con péptidos sintéticos derivados de la proteína Sm28GST. Estas técnicas permitieron identificar algunas de las moléculas blanco de la respuesta inmune protectora que están siendo evaluados como miembros potenciales de una vacuna multi-estadio y multi-componente y aclarar si los péptidos seleccionados indujeron anticuerpos capaces de reconocer diferentes isoformas de la Sm28GST.

Received: 21-01-2010. Accepted: 10-02-2011

INTRODUCTION

The trematode *Schistosoma mansoni,* that affects approximately 200 million persons worldwide, can survive many years in the human porto-hepatic system due to a variety of immunologic and biochemical adaptive mechanisms. Therefore, it is very important to understand the molecular organization of each parasite stage, especially those that are more vulnerable to the immune attack and chemotherapy. The identification of the major parasite proteins would give clues to the schistosome metabolism and to target molecules involved with the host immune system (1). One of the major problems we face for the development of efficient anti-schistosome vaccines is the lack of information about the degree of homology among vaccine candidate molecules from parasites of different geographic origins, since very few parasite isolates and strains have been immunochemically and biochemically characterized. Based on this, our group initially tried to circumvent that limitation evaluating if selected regions from potential protective molecules were conserved worldwide, by

demonstrating if sera from mice infected with three different *S. mansoni* isolates, as well as four other schistosome species (*S. rodhaini, S. guineensis, S. bovis, S. haematobium*) (2) were able to recognize the selected peptides. The recognition of the different peptides by all of these tested sera was the guarantee that we were working with conserved regions of target molecules. One of them was the well studied Sm28GST protein, which was recognized by the sera of mice infected with three different *S. mansoni* isolates (2).

In the current study, the protein parasite repertoire was studied using a proteomic approach of two different parasite stages, the cercaria and adult worms from a Venezuelan isolate (JL). As far as we know, previous works were performed with the well known Puerto Rican strain (3-7) and an Egyptian strain (8), while others did not identify the parasite origin (9-11). It was important to analyze other parasite isolates in order to have a more comprehensive picture of its protein composition, studying worms from different geographic regions.

A brief review of the application of proteomics to the identification of novel schistosome candidate vaccines was recently published (12). In our study, particular emphasis was given to the identification of the gluthation S transferase protein (Sm28GST) (13) by 2D SDS-PAGE and mass spectrometry, in parallel with its immunologic detection using serum from rabbits immunized with synthetic peptides derived from the Sm28GST protein. This molecule, localized in the tegument, protonephridia and the female reproductive system (13), is one of the schistosome vaccine candidates (14) under evaluation in our laboratory as part of a multi-component synthetic peptide vaccine. Being aware that one of the strategies for survival of parasites under different conditions is the redundancy of key molecules that could allow them to evade the host immune response (15, 16), we combined a proteomic and an immunological approach to confirm if a potential protective epitope synthesized chemically is present in different Sm28GST isoforms of isolated parasites from different geographic origin.

MATERIALS AND METHODS

Preparation of parasite material

Free swimming cercariae from the Venezuelan isolate JL, kindly provided by Dr. Italo Cesari (Instituto Venezolano de Investigaciones Científicas, IVIC, Venezuela), were shed in water from *Biomphalaria glabrata* snails infected 45 days before. These larvae were frozen and afterwards lyophilized. The cercarial protein extract (CE) was achieved solubilizing the lyophilized cercariae in 7M Urea, 2M Thiourea, 4% CHAPS, 20 mM Tris pH 8, 65 mM DTT (IEF buffer). Male and female adult worms from the same isolate were collected from experimentally infected outbred golden hamsters after liver perfusion. Worms were washed and homogenized in phosphate-buffered saline (PBS) containing protease inhibitors (1mM PMSF; 1mM EDTA) in an ice bath, and centrifuged at 12,000 g for 2 h at 4°C. The supernatant was lyophilized and considered as the adult worm protein extract (AWE), which was solubilized in IEF buffer. Both extracts were processed using one precipitation with 100% acetone (v/v), overnight –20°C, followed by a wash of the pellet with 80% acetone (v/v). AWE was additionally cleaned through a Sephadex G-25 micro column for desalinization.

Two dimensional electrophoresis

After quantification using the Bradford protein determination method (17) with bovine serum albumin (Fraction V, Sigma)

as a standard, 75 μg of CE and 66,2 μg of AWE were used for bidimensional electrophoresis in a Bio Rad IEF system (18). For first evaluations, IEF was performed with 7 cm strips with a 3-10 non-linear pH range. A 5-8 pH linear range was used afterwards for better resolution. IEF was carried out at 250 V for 15 min, 4,000 V for 2h, and then gradually increasing to a total of 20,000 V/h at 20°C, at a maximum current of 50 μA/strip. After IEF, strips were reduced in equilibration buffer (6M urea, 2% SDS, 0.375M Tris HCl pH 8.8, 20% glycerol, 0.025% (w/v) bromophenol blue) containing 130 mM DTT for 15 min and were then alkylated in equilibration buffer containing 135 mM iodoacetamide for 20 min. The second dimension was performed in 12% acrylamide gels and the gels were silver stained (19). Spots were analyzed by the PDQuest Program (Bio Rad).

Mass spectrometry

Spots were excised and processed for mass spectrometry in the Plateforme Proteomique de l'Esplanade, Institut de Biologie Moleculaire et Cellulaire, Strasbourg, France.

In gel digestion. For protein identification, stained protein spots were picked out from the corresponding gels. The gel digestion procedure was carried out as described by Rabilloud et al. (20). Selected spots have been washed with 2 cycles of 100 μL of 25 mM ammonium carbonate buffer (NH_4HCO_3) / 100 μL of acetonitrile (ACN) / dehydration. Between these two cycles, reduction was achieved by 45 min treatment with 10 mM DTT in NH_4HCO_3 buffer (100 μL) at 56°C and alkylation reaction was performed by addition of 100 μL of 25 mM iodoacetamide in 25 mM NH_4HCO_3 buffer for 45 min at room temperature. All treatments were performed under shaking. The final dried spots were rehydrated with three volumes of trypsin (Promega, V5111), 12.5 ng/μL, in 25 mM NH_4HCO_3 buffer (freshly diluted) and the digestion was performed at room temperature overnight.

Afterwards, 5 μL of 35% H_2O / 60% ACN / 5% HCOOH were added and the mixture vigorously agitated for 15 min in order to extract tryptic peptides.

MALDI mass spectrometry. MALDI mass measurement was carried out on an Biflex III (Bruker-Daltonics GmbH, Bremen, Germany) matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) used in reflector positive mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% water / 50% acetonitrile was used as a matrix. Mass spectra were internally calibrated with trypsin autolysis peaks (m/z = 842.510 and m/z = 2211.105). Monoisotopic peptide masses were assigned and the peak list transferred through MS BioTools™ program (Bruker Daltonics, Bremen, Germany) as input to search against NCBI non-redundant protein sequence database. Variable modifications (methionine oxidation and cysteine carbamidomethylation) were taken into account and the peptide mass error was limited to 50 ppm. Identifications were performed after MALDI-TOF analysis and searches on MASCOT (MatrixScience) over databanks without any restricted taxonomy.

Synthetic peptides

Peptides were manually synthesized using Merrifield's protocol (21) for the t-Boc based solid-phase peptide synthesis, modified by Houghten et al. (22) for the simultaneous multiple peptide synthesis. Glycine and cysteine amino acids were introduced at both carboxy and amino termini to allow polymerization (23, 24). Two polymerizable synthetic peptides were constructed after the sequence from the protein Sm28GST: IMT-232, (aa: 115-130) (25, 26), and IMT-234 (the carboxi-terminal region aa:

190-211) (27, 28). Both epitopes correspond to the border of the slot from the active site of the enzyme.

Rabbit immunization

Two New Zealand rabbits were immunized subcutaneously with these peptides (250 μg / dose / rabbit) emulsified (1:1) in Complete Freund adyuvant (CFA) in the first dose and in Incomplete Freund adyuvant (IFA) in the second and third boosts (days 15 and 30). Rabbits were bled 10 days post third immunization, under anesthesia (ketamine chlorhydrate: 10 mg/Kg). Their maintenance and manipulations were carried out according to legislation and guidelines established by the Asociación Venezolana de Bioterios. All sera were stored at –20°C until use.

Western blots

The immunized rabbit sera were tested against AWE and CE in 1D SDS-PAGE. AWE and CE 2D gels were transferred to PVDF membranes and exposed to the anti-IMT-232 and 234 rabbit serum at a 1:100 dilution. Anti-rabbit IgG conjugated to horseradish peroxidase was used at a 1:2,000 dilution and Western blots were developed with TMB Membrane Peroxidase Substrate (KPL; Gaithersburg MD, USA).

***Schistosoma mansoni* database**

Search of the genes corresponding to Sm28GST was performed using SchistoDB database (www.schistodb.net) in order to investigate the gene copy number responsible for the expression of the different isoforms of this protein.

BLASTp analysis for homology

The degree of homology between cross reactive proteins was performed using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Proteomic analysis

In the 2D SDS-PAGE of adult worm protein extract (AWE) (Fig. 1a), a total of 172 individualized spots are shown, but only the most prominent or the strongest signals (47 spots, 27.3%), based in the highest concentration of proteins in each silver stained spot, were excised, processed and analyzed by MS. Several spots were identified (Table I): probable ER-60 luminal cystein protease precursor, superoxide dismutase (Cu-Zn), thioredoxin peroxidase, actin-2, enolase, fructose biphosphate aldolase, Sm28GST, GST-Omega, 30 kD glycoprotein, among others. Among the spots analyzed, few of them were separated sequentially, suggesting the occurrence of post translational modifications in the following proteins: actin-2 molecules (19 spots), superoxide dismutases (Cu-Zn) (2 spots), enolases (2 spots), disulfide-isomerase ER-60 precursors (2 spots), Sm28GST (5 spots), fructose-biphosphate aldolases (2 spots), thioredoxin peroxidase (3 spots) and 30-kDa glycoprotein (4 spots).

In the 2D SDS-PAGE of CE (Fig. 2a), the resolution of the spots was higher than in the AWE. In fact, at least 257 individualized spots were observed, compared to the 172 observed to the mature adult worm preparation, which is expected to be composed of a more complex and rich array of functional molecules. The most prominent spots were identified as (Table I): thioredoxin peroxidase 2, actin (Sj), HSP70 (Sj), a disulfide isomerase homologue, chaperonin, ATP synthase, enolase, prohibitin, serpin, glyceraldehyde-3-phosphate-dehydrogenase (GA3PDH) mayor larval surface antigen, citrate synthase, 14-3,3 protein, Sm21.7, 28K antigen, GST- Omega, and others. They correspond to 33 of the total spots detected (12.8%). The spots sep-

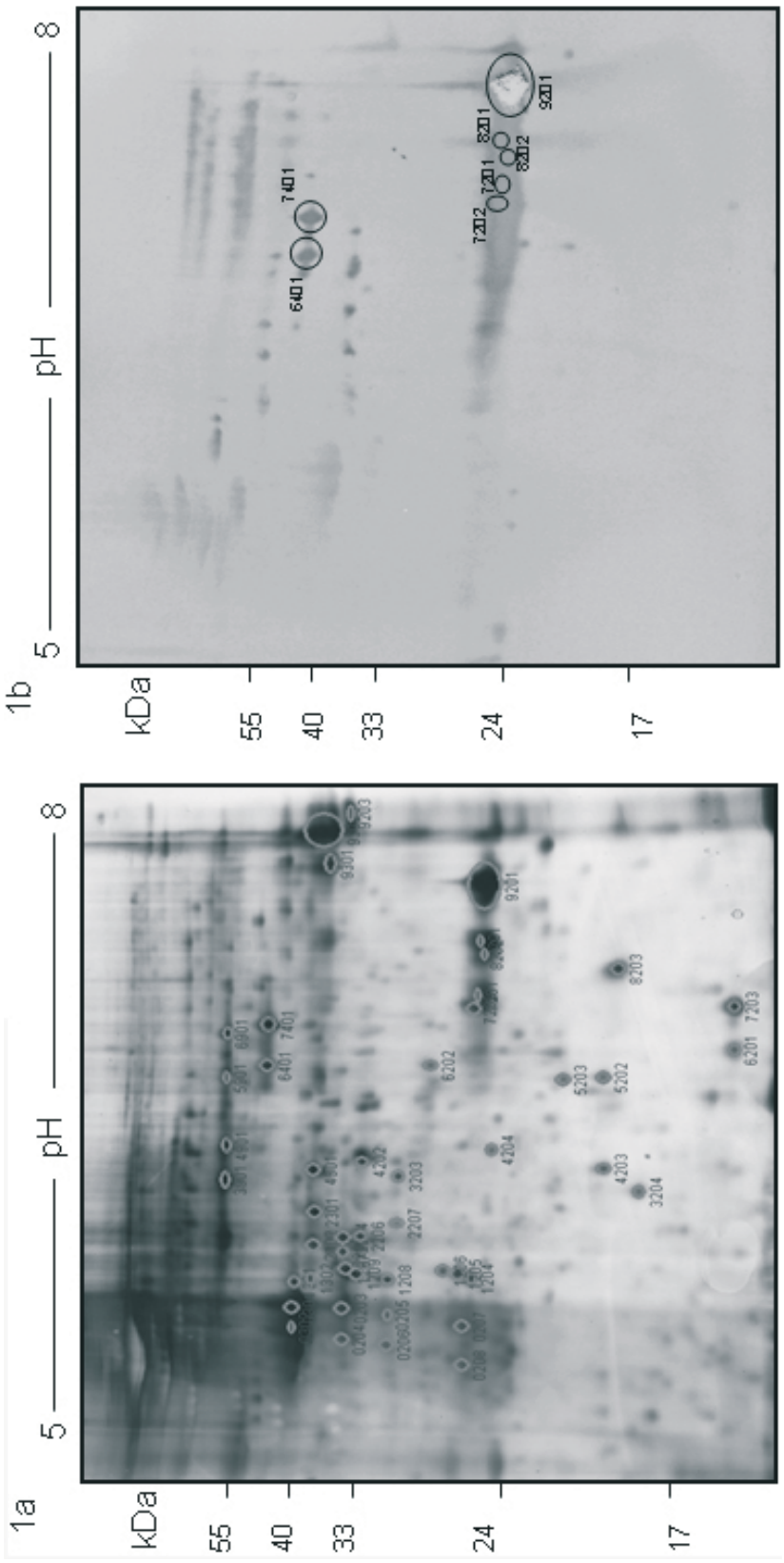

Fig. 1. Adult worm protein extract (AWE) from *Schistosoma mansoni* JL strain was run in: 1a) 7 cm IEF strip, pH 5-8, 12% silver stained SDS-PAGE with spots selected for MS; 1b) 7 cm IEF strip, pH 5-8, 12% SDS-PAGE blotted onto PVDF membrane, incubated against rabbit serum anti Sm28GST synthetic peptides IMT-232, 234 (1/100 dilution) and anti rabbit IgG conjugate (1/2,000 dilution).

TABLE I
MS RESULTS OF AWE AND CE PROTEIN SPOTS FROM *Schistosoma mansoni* JL STRAIN

Sample Number	Stage extract	Identification	Theoretical Mw/pI	NCBI Accession N°	Score Mascot	Error (ppm)	% cover
1302	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	173	26	45
4204	AWE	glutathione S-transferase omega [*Schistosoma mansoni*]	27827/5.90	28628851	266	33	64
1206	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	70	21	30
2204	AWE	30 kDa glycoprotein	18273/7.81	632506	65	64	54
5203	AWE	thioredoxin peroxidase [*Schistosoma mansoni*]	21909/6.08	5163492	156	26	63
3203	AWE	CDP-glucose 4,6-dehydratase [*Vibrio fischeri* ES114]	41308/5.47	59710790	83	49	23
6202	AWE	hypothetical protein V12B01_21691 [*Vibrio splendidus* 12B01]	41308/5.47	59710790	60	54	22
206	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	143	33	40
5202	AWE	thioredoxin peroxidase [*Schistosoma mansoni*]	21909/6.08	5163492	136	16	63
2302	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	153	40	55
3204	AWE	thioredoxin peroxidase [*Schistosoma mansoni*]	21909/6.08	5163492	153	22	63
204	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	117	41	35
208	AWE	Actin-2 [*Schistosoma mansoni*] - C-terminal fragment	41999/5.30	1703114	124	26	46
1204	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	145	32	47
205	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	111	17	33
4202	AWE	30 kDa glycoprotein	18273/7.81	632506	108	36	82
2205	AWE	30 kDa glycoprotein	18273/7.81	632506	94	26	82
1205	AWE	Actin-2 [*Schistosoma mansoni*] - C-terminal fragment	41999/5.30	1703114	124	32	39
1208	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	135	29	49
6201	AWE	Superoxide dismutase [Cu-Zn]	15883/6.09	267013	133	30	77
1209	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	162	39	51
7202	AWE	Glutathione S-transferase 28 kDa (GST 28) (SM28 antigen)	23861/6.56	121700	183	24	69

TABLE I (*Continuation*)

Sample Number	Stage extract	Identification	Theoretical Mw/pI	NCBI Accession N°	Score Mascot	Error (ppm)	% cover
2206	AWE	30 kDa glycoprotein	18273/7.81	632506	118	26	82
203	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	127	18	33
301	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	226	37	64
207	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	156	30	46
5901	AWE	Probable protein disulfide-isomerase ER-60 precursor (ERP60)	54785/6.30	729434	293	25	55
6901	AWE	Probable protein disulfide-isomerase ER-60 precursor (ERP60)	54785/6.30	729434	313	22	55
8201	AWE	Glutathione S-transferase 28 kDa (GST 28) (SM28 antigen)	23861/6.56	121700	217	14	76
2301	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	167	31	50
1301	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	197	31	59
302	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	216	40	65
1207	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	163	28	47
4301	AWE	Actin-2 [*Schistosoma mansoni*]	41999/5.30	1703114	189	22	63
8202	AWE	Glutathione S-transferase 28 kDa (GST 28) (SM28 antigen)	23861/6.56	121700	167	9	52
6401	AWE	Enolase	47421/6.18	3023710	207	28	62
9203	AWE	SJCHGC06124 protein [*Schistosoma japonicum*]	36902/8.83	56758570	91	29	33
7203	AWE	Superoxide dismutase [Cu-Zn]	15883/6.09	267013	124	33	69
7201	AWE	Glutathione S-transferase 28 kDa (GST 28) (SM28 antigen)	23861/6.56	121700	189	26	60
7401	AWE	Enolase	47421/6.18	3023710	308	24	69
9301	AWE	Fructose-bisphosphate aldolase	39963/7.63	1703248	289	13	76
9302	AWE	Fructose-bisphosphate aldolase	39963/7.63	1703248	290	13	77
9201	AWE	Glutathione S-transferase 28 kDa (GST 28) (SM28 antigen)	23861/6.56	121700	209	71	83
1303	CE	actin [*Xanthophyllomyces dendrorhous*]	41938/5.38	1150540	82	41	28
8203	CE	thioredoxin peroxidase 2 [*Schistosoma mansoni*]	21909/6.08	10281263	165	38	76

TABLE I (*Continuation*)

Sample Number	Stage extract	Identification	Theoretical Mw/pI	NCBI Accession N°	Score Mascot	Error (ppm)	% cover
2202	CE	similar to NM_011967 proteasome (prosome. macropain) subunit. alpha type 5 in *Mus musculus* [*S. japonicum*]	27476/5.22	29841012	84	24	38
6302	CE	PREDICTED: similar to Actin-87E isoform 1 [*Apis mellifera*]	42157/5.30	66509793	68	39	25
9304	CE	SJCHGC05011 protein [*Schistosoma japonicum*] = similar to T-complex protein 1 subunit gamma (BLAST)	24514/9.41	76154176	70	29	32
1302	CE	unknown [*Schistosoma mansoni*] = Serpin (BLAST)	29100/5.05	2623846	108	33	45
602	CE	protein disulfide isomerase homologue [*Schistosoma mansoni*]	54463/4.92	312018	203	37	47
603	CE	protein disulfide isomerase homologue [*Schistosoma mansoni*]	54463/4.92	312018	113	26	27
2301	CE	unknown [*Schistosoma mansoni*] = Serpin (BLAST)	29100/5.05	2623846	89	46	33
601	CE	protein disulfide isomerase homologue [*Schistosoma mansoni*]	54463/4.92	312018	161	38	38
2602	CE	HSP70 [*Schistosoma japonicum*]	71613/5.12	2829289	75	28	19
8301	CE	glutathione S-transferase omega [*Schistosoma mansoni*]	27827/5.90	28628851	212	24	56
4302	CE	actin [*Schistosoma japonicum*] - C terminal fragment	41999/5.30	6979994	118	24	35
2601	CE	SJCHGC09424 protein [S. japonicum] = ATP synthase subunit beta. mitochondrial precursor (BLAST)	56074/5.85	56758584	167	25	39
305	CE	14-3-3 epsilon [*Schistosoma mansoni*]	28850/4.85	6649234	187	25	60
302	CE	14-3-3 protein	28468/4.74	790658	113	24	40
9305	CE	Glyceraldehyde-3-phosphate dehydrogenase (Major larval surface antigen) (P-37)	36640/8.16	120709	253	28	67
7301	CE	SJCHGC06488 protein [*Schistosoma japonicum*] = prohibitin protein (BLAST)	30259/5.54	56755505	160	31	54
4601	CE	SJCHGC09129 protein [*Schistosoma japonicum*] = GroEL protein	62614/9.51	56753359	115	47	27

TABLE I (*Continuation*)

Sample Number	Stage extract	Identification	Theoretical Mw/pI	NCBI Accession N°	Score Mascot	Error (ppm)	% cover
5402	CE	actin [*Schistosoma japonicum*]	41999/5.30	6979994	236	50	69
4401	CE	actin [*Schistosoma japonicum*]	41999/5.30	6979994	236	27	63
1601	CE	SJCHGC09424 protein [*Schistosoma japonicum*] = ATP synthase subunit β. mitochondrial precursor (BLAST)	56074/5.85	56758584	178	19	39
2401	CE	actin [*Schistosoma japonicum*]	41999/5.30	6979994	171	20	51
9401	CE	SJCHGC00653 protein [*Schistosoma japonicum*] = citrate synthase (BLAST)	52487/7.98	56759284	96	24	27
301	CE	14-3-3 protein	28468/4.74	790658	149	20	50
9201	CE	Antigen Sm21.7	21789/6.85	417776	96	15	44
8501	CE	enolase	47421/6.18	1002616	219	20	53
1602	CE	SJCHGC09424 protein [S. japonicum] = ATP synthase subunit β. mitochondrial precursor (BLAST)	56074/5.85	56758584	157	19	39
9301	CE	28K antigen [*Schistosoma mansoni*]	23861/6.56	10164	180	31	59
9501	CE	enolase	47421/6.18	1002616	248	39	61
9302	CE	28K antigen [*Schistosoma mansoni*]	23861/6.56	10164	196	21	65
5401	CE	actin [*Schistosoma japonicum*]	41999/5.30	6979994	96	171	40
9402	CE	SJCHGC00653 protein [*Schistosoma japonicum*] = citrate synthase (BLAST)	52487/7.98	56759284	113	21	28

Note: Unidentified spots are not shown.
AWE: Adult worm protein extract.
CE: Cercarial protein extract.
Theoretical Mw/pI: Theoretical molecular weight/Isoelectric point.
% cover: proportion of the sequence that fit to the masses of the peptides submitted for identification.

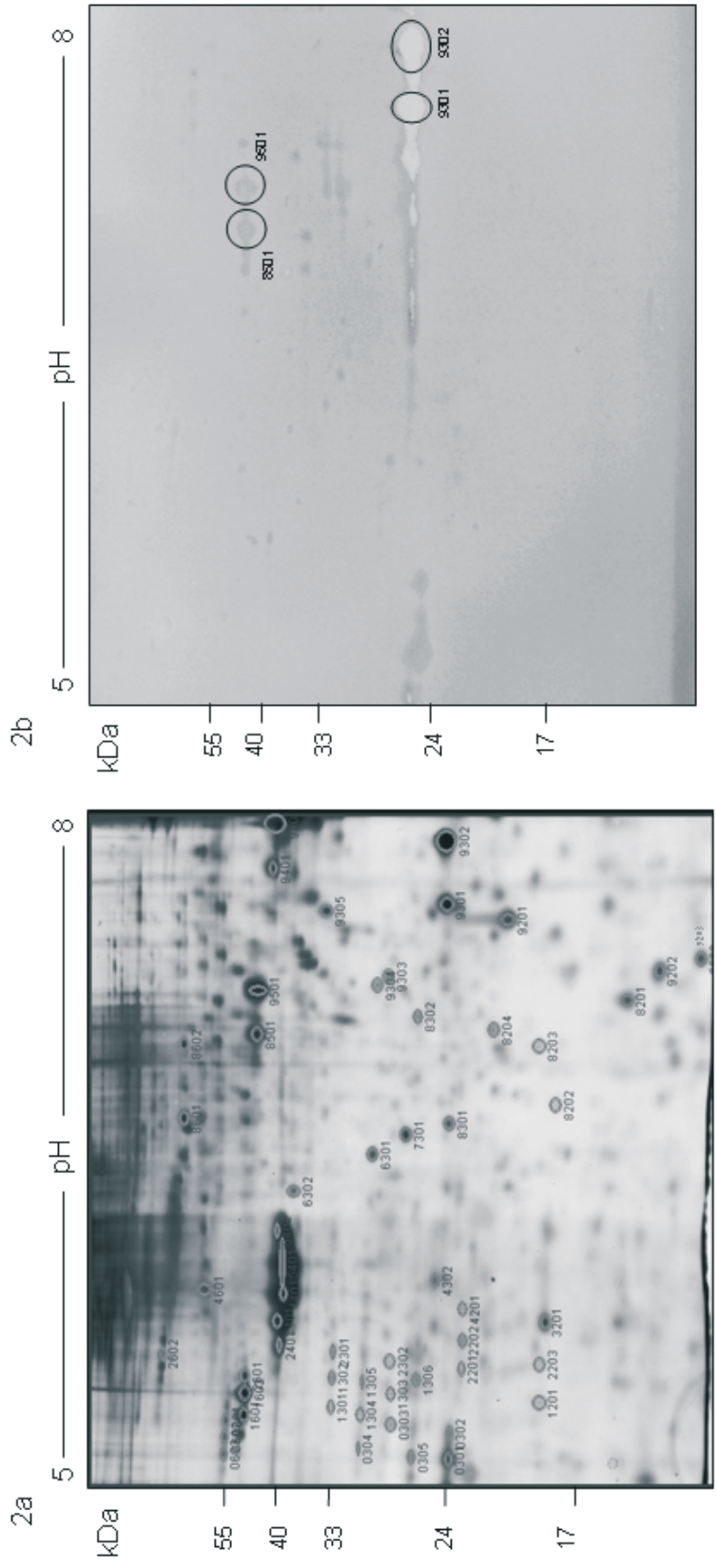

Fig. 2. Cercarial protein extract (CE) from *Schistosoma mansoni* JL strain was run in: 2a) 7 cm IEF strip, pH 5-8, 12% silver stained SDS-PAGE with spots selected for MS; 2b) 7 cm IEF strip, pH 5-8, 12% SDS-PAGE blotted onto PVDF membrane, incubated against rabbit serum anti Sm28GST synthetic peptides IMT-232, 234 (1/100 dilution) and anti rabbit IgG conjugate (1/2,000 dilution).

arated sequentially correspond to: disulfide isomerase homologue (3 spots), *S. japonicum* actin (4 spots), enolase (2 spots), *S. japonicum* citrate synthase (2 spots), serpin (2 spots), 28K antigen (2 spots) and ATP synthase (3 spots).

The theoretical isoelectric points and molecular masses were obtained from the MASCOT software.

The Table II shows the proteins found in this study and compares them with those found by other authors in previous studies. Differences will be stressed in the Discussion.

Immunochemistry

When rabbit antisera against the synthetic peptides derived from the Sm28GST molecule were tested against AWE and CE in 1D SDS-PAGE and Western blot (data not shown), it was observed a strong signal in AWE and a less intense in CE, in the range of 28 kDa. But when these rabbit sera were used against 2D gel electrophoresed AWE and CE blotted onto PVDF membranes, we observed the recognition of at least 5 sequential spots to AWE (Fig. 1b) and 2 to CE (Fig. 2b) in the range of 28 kDa and of some less intense spots, corresponding to higher molecular weights. Among them, two spots corresponding to enolase were identified in both parasite preparations.

***Schistosoma mansoni* database**

Once it was evident that some spots were arranged in a sequential distribution on the 2D gels, we investigated if they corresponded to different isoforms of the Sm28GST and if they were originated from a single copy gene or a multi-gene family. Search on the genome of *S. mansoni* demonstrated that Sm28GST is a single copy gene.

BLAST analysis for homology

Based on the fact that rabbit immune anti- Sm28GST peptide sera also recognized enolase, it was carried out the analysis of the homology between these cross reactive proteins. It was found that the only region of both proteins that had a significant degree of homology (42%) contained 13 out of 22 aa of the Sm28GST peptide IMT-234.

DISCUSSION

A limited number of studies have been carried out on the proteomic analysis of the larval and adult stages of schistosomes (11, 12, 29, 30, 31) and it is noteworthy to point out that only two international *S. mansoni* strains, the Puerto Rican (3-7) and an Egyptian strain (8), were analyzed so far. Therefore, it is necessary to evaluate if other *S. mansoni* strains and isolates from different geographic origins are homogeneous from the molecular point of view, since recent studies have shown molecular diversity and polymorphism occurrence in this parasite, as reported in mucin proteins (32).

Particular emphasis was laid on the Sm28GST, since synthetic peptides derived from this protein are one of the protective peptides evaluated in outbred mice in our laboratory (unpublished results). The presence of antioxidants, such as Sm28GST are crucial in the detoxification and antioxidant mechanisms of the helminths and they are probably implicated in protection against oxidative stress, specially originated by the heme group (9) in adult worms. The importance of this identification lies in the fact that the production of neutralizing antibodies against the Sm28GST activity is related to the reduction of eggs (antifecundity effect) in tissues and feces (14, 33, 34), and reduction of the viability of mature eggs (27). Additionally, the previous demonstration of antigenic community among different schistosome species and different vaccine candidate molecules including Sm28GST, also at the epitopic

TABLE II

REVIEW TABLE COMPARING RESULTS OBTAINED IN THIS STUDY WITH ADULT AND CERCARIAL PREPARATIONS FROM *S. mansoni* JL STRAIN AND PREVIOUS WORKS

Protein	MW/pH	AWE JL strain	CE JL strain	Curwen et al., 2004 [3] AS PR strain	Knudsen et al., 2005 [5] CS PR strain	van Balkom et al., 2005 [10] AWT ND strain	Braschi et al., 2006 [6] AWT PR strain	Curwen et al., 2006 [4] CS PR strain	Delcroix et al., 2007 [9] GC ND strain	Hansell et al., 2008 [7] CS PR strain	El Ridi & Tallima, 2009 [8] SS Egypt strain
HSP70 (*S. japonicum*)	71,613/5.12		X	X EGG	X	X	X			X	
SJCHGC09129 protein (*S. japonicum*) = chaperonin	62,614/9.51		X			X					
SJCHGC09424 protein (*S. japonicum*) ATP synthase	56,074/5.85		X		X	X	X				
Probable protein disulfide-isomerase ER-60 precursor (ERP60)	54785/6.30	X				X					
Disulfide isomerase homologue	54,463/4.92		X			X	X				
SJCHGC00653 protein (*S. japonicum*) Citrate synthase	52,487/7.98		X		X	X	X				
Enolase	47,421/6.18	X	X	X	X	X	X				
Actin 2	41,999/5.30	X	X	X	X	X		X			Similar X
paraFructose 1,6 biphosphate aldolase	39,963/7.63	X		X	X	X	X	X		X	X
SJCHGC06124 (*S. japonicum*)	36,902/8.83	X									
GA3PDH	36,640/8.16		X	X LUNG, WORM	X	X					
SJCHGC06488 protein (*S. japonicum*) = prohibitin	30,259/5.51		X			X					
Serpin	29,100/5.05		X	X CERCARIA		X		X		X	
14-3-3 epsilon	28,850/4.85		X	X		X					
14-3-3 protein	28,468/4.74		X	X	X	X	X				X

TABLE II (*Continuation*)

Protein	MW/pH	AWE JL strain	CE JL strain	Curwen et al., 2004 [3] AS PR strain	Knudsen et al., 2005 [5] CS PR strain	van Balkom et al., 2005 [10] AWT ND strain	Braschi et al., 2006 [6] AWT PR strain	Curwen et al., 2006 [4] CS PR strain	Delcroix et al., 2007 [9] GC ND strain	Hansell et al., 2008 [7] CS PR strain	El Ridi & Tallima, 2009 [8] SS Egypt strain
Glutation S transferase omega	27,827/5.90	X	X			X					
SJCHGC05011 protein (*S. japonicum*) = chaperonin	24,514/9.41		X			X					
28 kDa antigen Glutathione S-transferase (*S. mansoni*)	23,861/6.56	X	X	X	X	X		X		X	X
Thioredoxin peroxidase 2	21,909/6.08		X		X	X					
Sm21.7 antigen	21,789/6.85		X	X LUNG, WORM	X	X	X			X	
Thioredoxin peroxidase 1	21,315/6.10	X				X					X
30 kDa glycoprotein	18,273/7.81	X									
Cu/Zn superoxide dismutase	15,883/6.09	X		X LUNG, WORM	X		X		X		

AWT: adult worm tegument. CS: Cercarial secretions. SS: Schistosomula secretions. GC: gut content.
AS: All stages. AWE: Adult worm protein extract. CE: Cercarial protein extract. PR strain: Puerto Rican strain.
ND strain: Non described strain.

level, argues in favor of the relevance of this conserved protein (2).

Pérez-Sánchez et al. (35, 36) afforded valuable information about the protein composition of the tegument of adult *S. bovis* male and female worms. A range of tegumental and soluble proteins was identified using 2D immunoblots (known as immunome), including orthologs of the vaccine candidate Sm28GST. For that reason, a comparative analysis of the proteome from different studies is summarized in Table II and demonstrates the degree of homology among different parasite strains. It is noteworthy that different extracts and also different techniques for protein extraction are compared and protein representation may reflect these differences. That is the case of the studies carried out by

Curwen et al. (3), van Balkom et al. (10), and Braschi et al. (6), even when they identified some of the proteins found in this study in the *S. mansoni* tegument (Table II), also found others not observed in this work.

The limitations of the different proteomic studies based on the different protocols used for the preparation of the parasite material were demonstrated by Curwen et al. (4) and Knudsen et al. (5) who have shown that most cercarial proteins are secreted from the acetabular glands, but only when they artificially induced the invasive behavior *in vitro*. Knudsen et al. (5) found that uninduced free swimming cercaria released relatively small number of non-acetabular gland proteins including enolase, Sm28GST and actin (7), while the major secreted proteins were histolytic serine proteases that likely facilitate degradation of host skin tissue barrier, and factors that may contribute to immune evasion. In our work, we also found a schistosome serpin (serine protease inhibitor) that could possibly correspond to that previously reported in cercarial secretions (4, 7). Heat shock proteins, like HSP70 had also been identified as a major component of acetabular gland secretion (4). Sm21.7, a component of the schistosome surface or sub adjacent tegument, and Sm28GST, were released from the parasite in studies conducted by Hansell et al. (7). We also identified a group of schistosome glycolitic enzymes and kinases, known to be abundant in the cytoplasm of larval cells. These are presumably released as holosecretions from the acetabular cells (4, 5) and included: GA3PDH, citrate synthase, ATP-synthase and enolase. Hansell et al. (7) and Salter (37) have identified elastase (SmCE) isoforms (not identified in our study, probably because we did not work with excretory and secretory products), proteins associated with calcium function and cytosolic proteins such as heat shock protein chaperones.

Curwen et al. (3) reported the *Schistosoma mansoni* soluble proteome across the four different life-cycle stages. They showed a high degree of quantitative and qualitative similarities in spot patterns that were greater between adjacent stages. Their list included several of the first generation vaccine candidates such as triose phosphate isomerase, gluthation S transferase and fatty acid binding protein. They concluded that most of them were cytosolic enzymes.

In our study, the AWE lacks important excretory and secretory proteins that are poorly represented, such as the enzymes present in the vomit of this trematode, like the aspariginil endopeptidase (Sm32), cathepsin B (Sm31), cathepsin D, etc., which are only enriched when the adults worms are *in vitro* cultured (38). It is also possible that some of the excretory-secretory molecules, like the ones excreted by the cercarial cephalic or acetabular glands, are not represented in a detectable level by this CE preparation.

Comparing the results obtained with the JL isolate and the other two studied strains, only two proteins: SJCHGC09398 (*S. japonicum*) and a 30 kDa glycoprotein, were not previously reported. So far, the relevance of these two proteins remains unknown. Some spots in the 2D gel were identified as *Schistosoma japonicum* (Sj) proteins. The common identity of *S. japonicum* and *S. mansoni* proteins by MS is a confirmation that these parasites share conserved protein sequences.

It is noteworthy to mention the presence and abundance of 19 actin-2 spots in the AWE. Actin is a major component of the schistosome spines of the tegument (39, 40) and it seems to be one of the targets of Praziquantel (41). The abundance of spots identified as actin-2 might be the result of processing by tegument proteases and some of these fragments could play a major role in the host-parasite interaction.

A combined immunologic and proteomic approach allowed the identification of five spots corresponding to Sm28GST proteins of similar molecular weight in AWE, while in CE were observed only two Sm28K antigens. Both proteins, Sm28GST and Sm28K, have identical amino acid sequences. Since it has been demonstrated previously that there is only one copy of gene for the Sm28GST (42), the abundance of isoforms of the Sm28GST could be the result of post- translational modifications. The fact that the different isoforms were recognized by rabbit sera of animals immunized with synthetic peptides indicates that the selected peptides include a conserved region that is homologous to the original protein. Nevertheless, it is necessary to sequence those proteins using MS/MS techniques, since they could be different proteins sharing similar antigenic regions. On the other hand, there is a Sm28GST omega protein that is observed in the gel but is not recognized by rabbit serum. Searching in the protein databank, it seems a totally different protein from Sm28GST (43).

The less intense signals seen in the immunoblots, corresponding to higher molecular weights, could be nonspecific cross-reactions or precursors. Two of them were identified as enolase isoforms. Protein sequence homology search of GST and enolase was performed by BLASTp. It was found that the only region in both protein sequences that had a significant degree of homology could explain a partial cross-reactivity of the serum observed in the figures 1b and 2b.

We conclude that the sequence contained in the polymeric synthetic peptides used for rabbit immunization was able to imitate a well known antigenic region from the original Sm28GST protein. The existence of these similar proteins could only be detected by 2D SDS-PAGE. It could be interesting to investigate if the same epitopes are present in Sm28GST isoforms of the Puerto Rican and the Egyptian *S. mansoni* strains performing a Western blot of 2D gels using protein extracts of these parasites and the antisera against the Sm28GST synthetic peptides.

The production of different isoforms for this protein may be the expression of one of the multiple evasion strategies of this very complex parasite, since it could protect itself from the immune attack using alternative isoforms when recognized by the immune system. Also, they might have different functional capabilities under the multiple environments and conditions the different parasite stages are exposed to. Therefore, a plausible explanation is that the redundancy of this protein could allow that some of the isoforms might be involved in an immune evasion mechanism.

The proteomic approach certainly allows the identification of some potential target proteins of the protective immune

response that are being evaluated as members of a future anti-S. mansoni vaccine. And also to demonstrate if selected peptides could induce antibodies able to recognize different isoforms of these proteins. Since there are few isolates of this species available, the results presented herein enriches the information about the composition of this rather sophisticated parasite, allowing to foresee the potential efficacy of vaccine candidates against parasites from different geographic regions.

ABBREVIATIONS

2D SDS-PAGE, two dimensional sodium dodecyl sulfate poliacrilamide gel electrophoresis; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, Ditiotreitol; IEF, isoelectric focusing; EDTA, ethylenediaminetetraacetic acid; MALDI, Matrix-Assisted Laser Desorption/Ionization; NCBI, National Center for Biotechnology Information; IMT, Instituto de Medicina Tropical; PVDF, Polyvinylidene Fluoride.

ACKNOWLEDGEMENTS

This work was funded by FONACIT-CNRS Project N° 2004000012, FONACIT LANPIP Project N° 2000001639 and FONACIT Project G-2005000387. We thank the anonymous referees whose comments helped to improve the manuscript.

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