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Mapping the proteome of *Leishmania Viannia* parasites using two-dimensional polyacrylamide gel electrophoresis and associated technologies

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In this study we have demonstrated the potential of two-dimensional electrophoresis (2DE)-based technologies as tools for characterization of the *Leishmania* proteome (the expressed protein complement of the genome). Standardized neutral range (pH 5-7) proteome maps of *Leishmania (Viannia) guyanensis* and *Leishmania (Viannia) panamensis* promastigotes were reproducibly generated by 2DE of soluble parasite extracts, which were prepared using lysis buffer containing urea and nonidet P-40 detergent. The Coomassie blue and silver nitrate staining systems both yielded good resolution and representation of protein spots, enabling the detection of approximately 800 and 1,500 distinct proteins, respectively. Several reference protein spots common to the proteomes of all parasite species/strains studied were isolated and identified by peptide mass spectrometry (LC-ES-MS/MS), and bioinformatics approaches as members of the heat shock protein family, ribosomal protein S12, kinetoplast membrane protein 11 and a hypothetical *Leishmania*-specific 13 kDa protein of unknown function. Immunoblotting of *Leishmania* protein maps using a monoclonal antibody resulted in the specific detection of the 81.4 kDa and 77.5 kDa subunits of paraflagellar rod proteins 1 and 2, respectively. Moreover, differences in protein expression profiles between distinct parasite clones were reproducibly detected through comparative proteome analyses of paired maps using image analysis software. These data illustrate the resolving power of 2DE-based proteome analysis. The production and basic characterization of good quality *Leishmania* proteome maps provides an essential first step towards comparative protein expression studies aimed at identifying the molecular determinants of parasite drug resistance and virulence, as well as discovering new drug and vaccine targets.

Key words: proteomics, proteome, *Leishmania*, two-dimensional polyacrylamide gel electrophoresis.

Mapa del proteoma de parásitos de *Leishmania Viannia* a partir de electroforesis de geles de poliácramida en dos dimensiones y otras técnicas asociadas

En este estudio demostramos el potencial de la electroforesis en dos dimensiones (2DE) como herramienta para la caracterización del proteoma de *Leishmania* (expresión proteica complementaria del genoma). Los proteomas por 2DE en el rango neutro (pH 5-7) de extractos solubles de promastigotes de *Leishmania (Viannia) guyanensis* y *Leishmania (Viannia) panamensis* fueron reproducibles usando tampón de lisis de urea y nonidet P-40. Con la tinción de azul de Coomassie y nitrato de plata se detectaron, con buena resolución, 800 y 1.500 puntos de proteínas, respectivamente. Entre las proteínas de referencia comunes, aisladas de los proteomas de las cepas estudiadas, se identificaron por medio de espectrometría de masa de péptidos (LC-ES-MS/MS) y métodos bioinformáticos, proteínas de choque térmico, proteína ribosomal S12, proteína de membrana del cinetoplasto 11 y una proteína hipotética específica de *Leishmania* de 13 kDa con función desconocida. Por *immunoblot* y utilizando un anticuerpo monoclonal, se detectaron específicamente las proteínas paraflagelar 1 y 2 de 81,4 kDa y 77,5 kDa, respectivamente. La expresión proteica diferencial encontrada en los distintos clones de parásitos fueron reproducibles al ser comparados por medio de un programa

analizador de imágenes. Estos datos demuestran el poder de resolución del análisis de proteomas por 2DE. La producción y caracterización de mapas proteicos básicos de *Leishmania* con buena calidad constituye un paso esencial en los estudios proteómicos comparativos orientados a la identificación de factores moleculares involucrados en la resistencia a drogas, virulencia de parásitos y el descubrimiento de blancos para nuevas drogas y vacunas.

Palabras clave: proteómica, proteoma, *Leishmania*, electroforesis de geles de poliacrilamida en dos dimensiones.

Proteomics examines the dynamics of cellular protein synthesis, and provides a novel approach to studies of gene expression and protein function that complements genomic information. Direct profiling and characterization of the proteome (all of the proteins produced from the genetic information of a cell) is advantageous since proteins are both the functional cellular components that determine biological phenotype and the targets of most therapeutic agents (1,2).

Proteomics has the unique capacity to resolve and identify multiple protein forms produced from a single gene by mRNA processing and/or post-translational modifications (e.g., glycosylation, phosphorylation), which are critical in modulating protein function (2,3); these mechanisms generate a diversity of protein forms and functions that cannot be predicted by genomic analysis alone. By facilitating comparative analyses of protein expression profiles in cells of different genetic, physiological and developmental backgrounds, proteomics has multiple applications in infectious diseases research (1,2).

In the case of *Leishmania*, the protozoan parasite that gives rise to the cutaneous, mucocutaneous and visceral forms of leishmaniasis currently affecting 12-15 million people worldwide (4,5), proteomics has high potential as a tool for identifying the molecular determinants of parasite drug resistance and virulence, and for the discovery of new therapeutic targets.

The key technical aspects of experimental proteomics are the solubilization, separation and detection of proteins via two-dimensional

polyacrylamide gel electrophoresis (2DE) and staining, examination of the resulting proteome maps using advanced image analysis software, and the identification of individual proteins principally by biological mass spectrometry coupled with bioinformatics tools.

The objective of this study is to demonstrate the use of 2DE-based technologies in the construction and partial characterization of reference proteome maps of parasites of the *Leishmania* (*Viannia*) subgenus, which are the main causative agents of (muco)cutaneous infections in the New World including Colombia (4,5). This type of mapping and analysis will form an important basis for phenotype-based investigations of the *Leishmania* proteome.

Materials and methods

Chemicals

Acrylamide, bis-acrylamide, Coomassie blue R-250 and Servalyte™ ampholytes for isoelectric focusing were purchased from Serva GmbH (Heidelberg, Germany). Carbamalyte™ isoelectric point (pI) standards and broad range molecular weight (M_r) standards were obtained from Amersham-Pharmacia Biotech (Uppsala, Sweden) and Bio-Rad (USA), respectively. All other reagents were of electrophoresis grade.

Biological material

Promastigotes of *Leishmania* (*Viannia*) *guyanensis* (WHI/BT/78/M5313) including genetically-distinct clones with highly-metastatic or non-metastatic phenotypes (6,7) and *L. (V.) panamensis* (MHOM/CO/86/1166) were cultured at 26 °C in Schneider's *Drosophila* medium containing 10% heat-inactivated foetal bovine serum (Vecol, Bogotá, Colombia), 1% glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Logarithmic phase parasites were harvested at 72 h after subculture by centrifugation (3,000 x g, 10

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min), washed in cold phosphate-buffered saline (PBS; 13.6 mM NaCl, 0.27 mM KCl, 0.15 mM KH_2PO_4 , 0.4 mM Na_2HPO_4 , pH 7.4) and either solubilized immediately or stored at -70°C until required.

Preparation of *Leishmania* protein extracts

Protein extracts of promastigotes were routinely prepared by solubilizing 10^8 parasites in 100 μl of a buffer comprising 9 M urea, 4% Nonidet P-40 (NP-40) detergent, 2% 2-mercaptoethanol (2-ME) and 5% of Servalyte™ ampholytes pH 9-11 (8, 9); solubilization was assisted by passing the extract two to three times through a 21.5 gauge needle using a Hamilton syringe. We have previously demonstrated that this methodology provides optimal representation and resolution of *Leishmania* proteins on 2DE gels compared to other protocols (9).

Following solubilization, samples were centrifuged (14,000 $\times g$, 2 min) to remove residual insoluble material. The protein concentration of the final soluble extract was determined using a modification of the Bradford protein assay (10) to enable standardization of sample loading on 2DE gels.

Protein separation by 2DE. Solubilized proteins were separated according to isoelectric point (pI) in the first dimension by isoelectric focusing (IEF), and subsequently by subunit molecular weight (M_r) using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. IEF and SDS-PAGE were performed using the IsoDalt 2DE system (Argonne National Laboratory, Illinois, USA) (11,12).

The IsoDalt system enables protein samples to be analysed simultaneously in multiples of 20 under identical electrophoretic conditions thus ensuring reproducibility and comparability of proteome maps. General procedures for the preparation and electrophoresis of first- and second-dimension gels using the IsoDalt were essentially as described (11,12).

First dimension (IEF). Polyacrylamide IEF tube gels (22 cm \times 1.5 mm) were prepared containing an equal mixture (50:50) of pH 5-7 and pH 3-10 carrier ampholytes (Servalyte™) in order to give

optimal separation and resolution of proteins in the neutral range (pH 5-7) using 10 mM phosphoric acid as the anodal electrolyte and 20 mM sodium hydroxide as the cathodal electrolyte. Pre-focusing of the gels (at 200 V for 1 h) was performed to establish the linear pH gradient via migration of the ampholytes to their pI. The pH gradients of IEF gels were calibrated by the addition of Carbamalyte™ carbamylated protein standards of known pI to selected *Leishmania* samples prior to IEF (13). IEF gels were loaded with *Leishmania* samples containing 350 μg of total protein for Coomassie Blue staining or 100 mg of protein for silver staining. Individual proteins were subsequently focused to their pI by electrophoresis at 800 V for 17.5 h (a total of 14,000 Volt-hours). Focused gels were equilibrated (in 125 mM Tris-HCl pH 6.8, containing 10% glycerol, 2% SDS and 1% 2-ME), and then either transferred directly to SDS-PAGE gels or stored at -70°C until used.

Second dimension (SDS-PAGE). IEF gels were loaded onto 16 \times 18 cm vertical polyacrylamide slab gels (comprising a 9-18% acrylamide gradient) and protein subunits were separated by electrophoresis at 600 mA for 20 h (10) in SDS running buffer (25 mM Tris base, 0.2 M glycine, 0.1% SDS). SDS-PAGE gels were calibrated using broad range M_r protein standards.

Protein detection

Protein profiles were revealed by post-electrophoretic staining of SDS-PAGE gels with protein dye reagents. Standard methods (14,15) were employed for staining with silver nitrate. Coomassie blue staining was achieved essentially as described (16), except for the use of modified staining reagent (0.25% Coomassie Blue R-250, 50% methanol, and 4% phosphoric acid) which gave optimal resolution of *Leishmania* proteins.

Destaining of gels was achieved using successive 30 min washes in 20% ethanol, until optimal reduction of background staining was achieved. Digital images of the protein maps were recorded using the GelDoc 2000 gel documentation system and associated software (BioRad).

Proteome map analysis. Comparative analysis of digitized proteome maps was performed using the

image analysis software Melanie III (version 3.08, Geneva Bioinformatics S.A.; <http://www.expasy.ch/melanie/>) (17). A minimum of five proteome images for each *Leishmania* species or clone were aligned and matched to generate a composite map image. Landmark proteins were located and annotated, and gel images were calibrated with respect to pI and M_r, enabling determination of the physical parameters of proteins of interest. Significant differences between protein patterns and spot intensities (i.e., differentially expressed proteins) were determined by pairwise comparisons of spots between gel images. Additional quantitation of differential spot intensities was performed by densitometric analysis using Melanie III and Quantity One software (BioRad) (results not shown).

Mass spectrometry and bioinformatics. Biological mass spectrometry was employed (at the University of Lausanne) to identify well-expressed landmark proteins, which provide key reference points within the *Leishmania* proteome map. Individual protein spots (1.5 mm in diameter) were excised directly from Coomassie blue-stained SDS-PAGE gels. Proteins were then digested in gel with trypsin essentially as described (18), except that digestion was performed in 96 well plates using a total 20 µg trypsin (Promega) per plate and a ProGEST robotic system (Genomics Solutions, Ann Arbor, Michigan). Peptide products were recovered from the supernatant of tryptic digests and analysed by liquid chromatography-electrospray tandem mass spectrometry (LC-ES-MS/MS) using a SCIEX QSTAR Pulsar hybrid quadrupole time-of-flight (Q-ToF) instrument (Concord, Ontario, Canada), equipped with a nanoelectrospray source and interfaced with a LC-Packings Ultimate HPLC system (Amsterdam, Holland). Separation of peptides was performed using a PepMap reversed-phase capillary C18 column (75 mm internal diameter x 15 cm length) at a flow rate of 200 nl/min along a 52 min gradient of acetonitrile (0-40%). Peak detection and selection of sequentially eluting peptides for collision-induced fragmentation was controlled using instrument-associated software.

Uninterpreted peptide mass spectra data were used for direct interrogation of the MSDB or NCBItr

protein sequence databases using Mascot software (www.matrixscience.com). Where direct database searches failed to yield statistically significant matches, the MS/MS spectra were manually interpreted to derive sequence tags (of 5 to 10 amino acids in length) for each protein. These sequence tags were then combined into a unique search string and used for error tolerant homology searches in the NCBItr database with the MS-BLAST algorithm (<http://dove.embl-heidelberg.de/Blast2/msblast.html>) (19).

Associated homology searches and bioinformatics analyses were performed using the SwissProt/ (Tr)EMBL database of the Swiss Institute of Bioinformatics and the European Bioinformatics Institute (EBI) (<http://expasy.ch>), the EBI genomic database (<http://www.ebi.ac.uk>) and the *Leishmania major* genomic and expression sequence tag (EST) databases (<http://www.sanger.ac.uk>).

Immunodetection. Immunoprobings of *Leishmania* proteome maps was performed using monoclonal antibody (mAb) 2E10 which recognizes *Leishmania* paraflagellar rod proteins 1 and 2 (PFR-1/-2), and was generously provided by Dr. Diane McMahon-Pratt (20). Two dimensional protein arrays were transferred from SDS-PAGE gels to PVDF transfer membrane Hybond-P (Amersham-Pharmacia Biotech) by electroblotting at 100 V for 1.5 h. Membranes were subsequently incubated overnight at 4 °C in blocking buffer (PBS containing 5% BSA, 0.2% horse serum, and 0.3% Tween-20), washed twice (5 min, 25 °C) in wash buffer (PBS/0.05% Tween), and then incubated (1 h, 25 °C) with mAb 2E10 at a dilution of 1:500 in wash buffer. Following two further washes (as above), blots were incubated (30 min, 25 °C) with universal peroxidase-labeled secondary antibody (DAKO Corporation, California), washed twice (as above) and then immunoreactive spots were visualized colourimetrically by incubating blots in 3,3'-diaminobenzidine substrate for peroxidase (Vector Laboratories, California) according to manufacturer's instructions.

Results

The described 2DE methodology yielded highly reproducible proteome maps of the neutral pI range

of soluble extracts of *L. Viannia* promastigotes with good representation and resolution of protein spots (figure 1a, 1b).

Approximately 800 protein spots were routinely detected using Coomassie blue staining and 1,500 spots using silver nitrate, thereby verifying the advantageous greater sensitivity of the latter method (14,15). Nevertheless, Coomassie blue is known to produce staining intensities that are proportional (within limits) to protein abundance, whilst silver staining can give rise to disproportionate intensities resulting in under- or overestimation of protein quantity. This differential staining of individual proteins results from variations between the respective chemical interactions of the two dye reagents with proteins, although the latter have not been fully defined (21). Additionally, silver stain reagent interferes considerably with mass spectrometric analyses (22), and so Coomassie blue-stained protein spots were used exclusively for peptide sequencing via mass spectrometry.

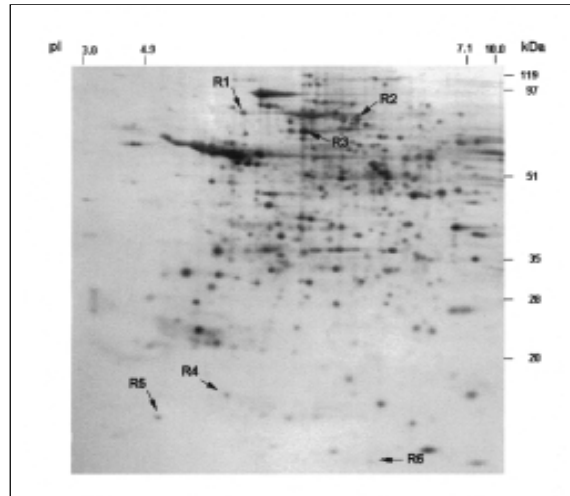
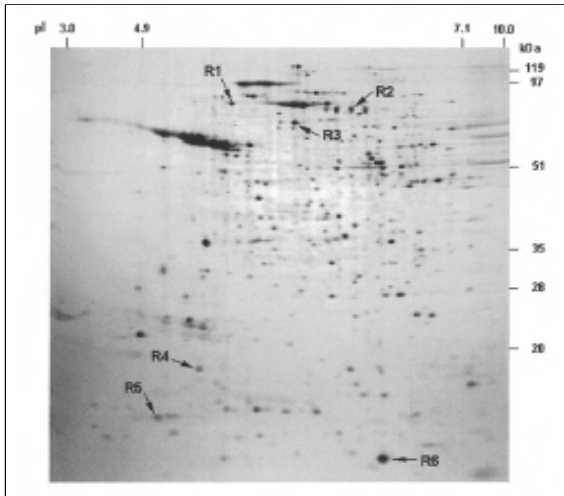
The strongly expressed reference proteins R1 to R6 were consistently observed in all maps of both *Leishmania* strains investigated, and were isolated and identified by mass spectrometry and

bioinformatics analyses in order to establish landmarks for the proteome.

Consistent data was obtained via mass spectrometric sequencing of peptides derived from multiple samples of the R1 to R6 protein spots. Peptide sequences generated from each reference protein by LC-ES-MS/MS displayed high amino acid identities with specific database sequences of proteins from other *Leishmania* species, allowing unequivocal identification of R1 to R6 as members of the heat shock protein family, kinetoplast membrane protein-11, ribosomal protein S12 and a hypothetical, *Leishmania*-specific 13 kDa protein of unknown function (table 1).

The capacity of 2DE to capture variations in protein expression profile is illustrated in figure 2, which shows marked differences in the expression profiles of four proteins (designated α , β , γ and δ) in the soluble proteomes of two genetically distinct *L. guyanensis* clones with differing metastatic capacities.

The power of immunoblotting as a tool for protein identification in proteome maps was demonstrated by the specific detection of the *L. guyanensis* PFR-1 and PFR-2 proteins using mAb 2E10 (figure 3).



Figures 1a and 1b. Reference soluble proteome maps (pH 5-7) of promastigotes of a) *L. guyanensis* and b) *L. panamensis* showing landmark proteins identified by mass spectrometry. Soluble proteome maps of promastigotes were produced by IEF (with amplification of the neutral region pH 5-7) in the first dimension and 9%-18% gradient SDS-PAGE in the second dimension followed by protein staining with a) Coomassie blue and b) silver nitrate, respectively (see Materials and methods). The identities of reference proteins R1-R6 are presented in table 1.

Table 1. Identities and physical properties of reference proteins R1 to R6 of *L. guyanensis* promastigotes.

Protein code	Identity ^a	Accession No. of homologue (Species) ^b	No. of matching peptides	Physico-chemical properties subunit M _r (kDa) ^c	pI ^d
R1	Heat shock protein 70 kDa (4)	SwissProt P12077 (<i>L. major</i>)	4	76.3 ± 3.5 (5)	5.4 ± 0.07 (5)
R2	Heat shock protein 70 kDa (1)	SwissProt P12076 (<i>L. major</i>)	3	73.1 ± 4.3 (5)	6.1 ± 0.07 (5)
R3	Heat shock protein 60 kDa (Chaperonin)	(Tr)EMBL O61118 (<i>L. brasiliensis</i>)	7	66.0 ± 2.4 (5)	5.7 ± 0.07 (5)
R4	Ribosomal protein S12	(Tr)EMBL Q9GRJ6 (<i>L. major</i>)	4	18.1 ± 1.0 (5)	5.2 ± 0.05 (5)
R5	Hypothetical protein (13 kDa)	(Tr)EMBL Q9BLU8 (<i>L. major</i>)	1	15.1 ± 0.7 (5)	4.9 ± 0.02 (5)
R6	Kinetoplast membrane protein 11	(Tr)EMBL O43974 (<i>L. brasiliensis</i>)	1	12.9 ± 1.0 (5)	6.3 ± 0.04 (5)

^a Proteins identified by mass spectrometry and bioinformatics analyses (see Materials and methods).

^b Database accession numbers are indicated for homologous proteins/genes from other *Leishmania* species.

^c Subunit M_rs were determined via relative mobility calculations using standard marker proteins (BioRad). Data are presented as mean M_r ± S.D., with number of replicate 2DE gels in parentheses.

^d pI values were determined by calculating the mobilities of proteins relative to a series of carbamylated creatine phosphokinase standards (11). Data are presented as pI ± SD, with number of replicate 2DE gels in parentheses.

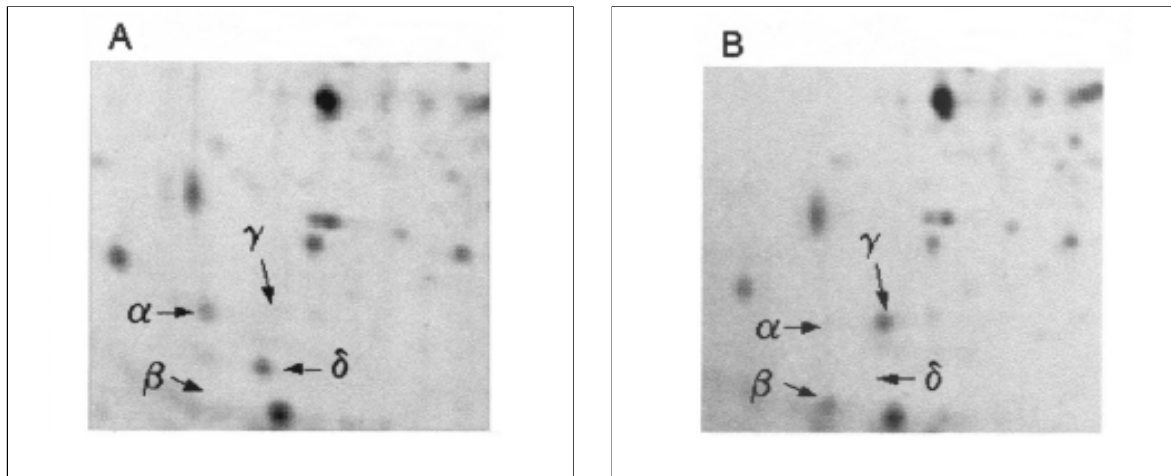


Figure 2. Detection of differential protein expression in genetically-distinct laboratory clones of *L. guyanensis*. Panels A and B show the same amplified regions of Coomassie blue stained soluble proteome maps (pH 5-7) of two distinct *L. guyanensis* laboratory clones, highlighting the differential expression of proteins α , β , γ and δ .

The physico-chemical properties of the immunoreactive protein spots (subunit M_{rs} of 77.5 and 81.4 kDa, and pIs of 5.93 and 6.02, respectively) correlated extremely well with those reported previously for the subunits of PFR-1 and PFR-2 (20).

Comparative analyses of spot coordinates were used to determine the location of the corresponding PFR-1 and PFR-2 spots in Coomassie blue-stained proteome maps produced in parallel with the immunoblot (figure 3). (The accuracy of localization of the PFR-2 spot was

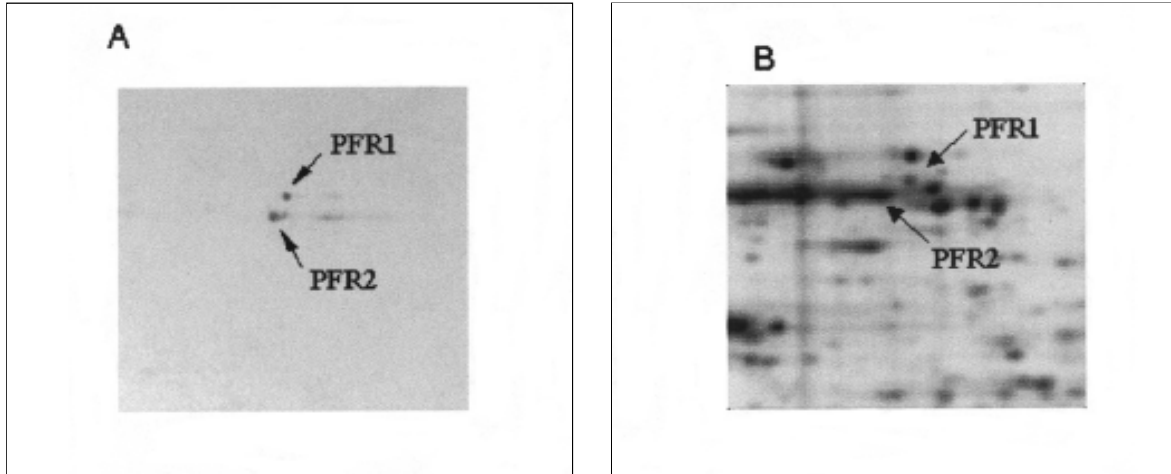


Figure 3. Specific immunodetection of paraflagellar rod proteins 1 and 2 in the proteome map of *L. guyanensis*. Immunoblot analysis of the soluble (pH 5-7) proteome of *L. guyanensis* promastigotes using mAb 2E10 was performed as described in Materials and methods. Panel A shows the immunoreactive subunits of PFR-1 ($M_r 81.4 \pm 0.7$ kDa; $pI 6.02 \pm 0.03$, $n=3$) and PFR-2 ($M_r 77.5 \pm 0.7$ kDa; $pI 5.93 \pm 0.01$, $n=3$), and panel B shows the corresponding region of a Coomassie blue-stained proteome map with the identified PFR-1 and 2 subunits arrowed.

subsequently confirmed by 2DE analysis of *L. guyanensis* extracts containing samples of pure recombinant PFR-2 protein, and the specificity of mAb 2E10 was also confirmed by one-dimensional SDS-PAGE and immunoblotting of promastigote extracts; data not shown).

Discussion

The successful production of reference proteome maps, identification of selected protein spots by mass spectrometry/bioinformatics and immunodetection, and capacity to detect differentially expressed proteins collectively confirm the utility of proteomics as a tool for phenotypic analyses of *Leishmania* parasites.

Although suitable for detailed expression analyses of high- and medium-abundance proteins, these baseline proteome maps are not entirely representative since numerous low-abundance proteins are not detected by Coomassie blue or even silver staining; this is a common limitation when performing whole proteome analysis by 2DE.

Another limitation is the high density of spots in the pH 5-7 region, which inevitably leads to a degree of overlapping of certain adjacent protein spots.

However, the representation and resolution of protein spots will be improved further by the use

of recently developed fluorescent protein stains (such as SYPRO Orange or Ruby) which offer higher sensitivity (with a lower limit of detection of 50 fmol of protein).

Furthermore, the complexity of the proteins in parasite extracts can be reduced via use of narrow range pH and acrylamide gradients in the first and second dimensions, respectively, and/or fractionation of protein samples prior to 2DE.

These modifications enable the amplification of specific regions of the proteome for more detailed analyses.

Leishmania is particularly amenable to proteomics-based studies due to its relatively small genome size: the *Leishmania major* genome is currently estimated to contain a maximum of 9,800 genes (23). Significantly, the proteins encoded by the majority (69%) of *L. major* genes sequenced to date remain unclassified with respect to putative biological function, and around 40% of these are potentially unique to *Leishmania* (23).

Proteomics will play an increasingly important role in linking genomics with functional analyses of both classified and unclassified *Leishmania* gene products, especially in relation to the molecular mechanisms of parasite virulence and drug

resistance, the identification of drug targets and vaccine antigens, and the analysis of host-parasite interactions.

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