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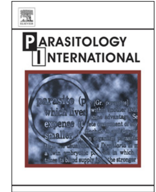

Proteomic analyses of membrane enriched proteins of *Leishmania donovani* Indian clinical isolate by mass spectrometry

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ABSTRACT

Visceral leishmaniasis (VL) is a major fatal disease prevalent in North-East India, caused by a protozoan parasite *Leishmania donovani*. The parasite multiplies and thrives inside mammalian macrophages and is transmitted by the bite of the sandfly. Due to the unsatisfactory treatment measures, increasing drug resistance and the advent of HIV-*Leishmania* co-infection there has been an urgent need to develop novel drug/vaccine targets against VL. Target identification is the key step in drug discovery and proteomics seems to be a suitable strategy for it due to the availability of *Leishmania major*, *Leishmania infantum*, *Leishmania braziliensis*, *Leishmania donovani*, *Leishmania mexicana* and *Leishmania tarentolae* genome sequence. Since, majority of proteome analyses of *Leishmania* have, so far, been performed on whole-cell extracts; this study is dealing with the sub-proteome analysis of the membrane-enriched protein (MEP) fractions of *L. donovani*. The analysis of 95 protein spots of the MEPs from two dimensional (2-D) gel image through matrix asserted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) endorsed the identification of various relevant functional proteins. Out of 95 the MEP spots 72 have been identified and were classified on the basis of their biological function. Several proteins of unknown function that belong to different classes like cell signaling, transmembrane receptors, and transporters have been identified which could be the new potential therapeutic targets against VL in future. The proteome array of the MEPs contributes to further elucidation of the biological system of *L. donovani* as well as host–parasite relationships which may be further investigated for their crucial biological role in *L. donovani* for disease management.
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1. Introduction

Visceral leishmaniasis (VL), the most overwhelming vector borne infectious disease transmitted by Phlebotomine sandflies, is caused by *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi*. The *Leishmania* parasite develops to an infective form — promastigotes inside the gut of the sandfly and is injected together with saliva into a mammalian host during blood feeding. Components present in promastigotes have been shown to contain proteins responsible for virulence and pathogenicity that may act as potential drug/vaccine targets [1]. Modern genomics-based approaches such as proteomics offer increased hope for the discovery of promising new drug targets by virtue of their ability to characterize complex parasite biology and biochemistry [2]. Proteomics, being widely employed in the study of *Leishmania* seems to be a suitable strategy as the availability of annotated sequenced genome of *Leishmania major* has opened the door for dissection of both protein expression/regulation and function. Advances in proteomic technologies have enabled the enhancement of an understanding of virulence/pathogenicity/host–pathogen interactions, and drug resistance thereby defining novel therapeutic/vaccine targets.

Using the proteomics approach, preliminary efforts made in this direction included the generation of a 2-D gel map of *L. donovani* promastigote membrane enriched protein (MEP) fraction through matrix asserted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) which shall be beneficial to identify proteins that are expressed in a stage-specific manner and should accelerate our understanding of key processes related to *Leishmania* biology [3,4]. Although the amastigote form of the parasite is responsible for clinical manifestations, but proteomic studies on the promastigote form are also relevant because about 90% of the proteomes remain qualitatively unchanged throughout the life cycle of the parasite [5]. *L. donovani* is a well-studied pathogen but the comprehensive proteome profile of its membrane proteins has remained unexplored. As we know the membrane proteins mediate many vital cellular processes. They transfer metabolites, transmit signals between cells and their environment, facilitate electron

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transport and act in the uptake and efflux of small molecule ligands. The membrane proteins primarily mediate many fundamental biological processes including the host–parasite interactions, virulence and invasiveness, or induction of immune response of the infected organism. Due to the immense importance of the membrane proteins in the cellular physiology, the present study was focused on the preparation and proteomic analysis of the fraction enriched in the membrane proteins of *L. donovani*. This research communication deals with the membrane sub-proteome mapping of sodium antimony gluconate (SAG) sensitive isolate of *L. donovani* that will be able to highlight some of the relevant information regarding the *Leishmania* proteomics.

2. Materials and methods

2.1. Culture of L. donovani

The *L. donovani* clinical isolate (SAG sensitive strain 2001) was grown in RPMI-1640 medium (in-vitro) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, USA) at 25 °C as described previously [6,7].

2.2. Chemicals used

Acrylamide, agarose, bis-acrylamide, biolytes, coomassie brilliant blue (CBB), dithiothreitol (DTT), iodoacetamide, IPG strips, mineral oil and other 2-D standards were purchased from Bio-Rad, USA; trypsin, glycerol, thiourea, protease inhibitor cocktail (PIC), CHAPS, EDTA, TEMED, ammonium persulfate (APS), sodium chloride, mercaptoethanol, glucose, sorbitol, urea, Tris, SDS, Tri fluoro acetic acid (TFA), Tri butyl phosphine (TBP), acetonitrile (AcCN), Triton X-100 from Sigma Chemical Co., USA; α-cyano-4-hydroxycinnamic acid (CHCA), and peptide calibration standard mixture (Cal mix.) from Applied biosystem, USA.

2.3. Fractanation of L. donovani membrane enriched proteins (MEPs)

The MEP fraction was isolated from the late log phase of promastigotes of *L. donovani* using carbonate extraction method described by Pávková et al. in 2005 [8]. Briefly, the log phase of promastigotes (10^9) of *L. donovani* culture was disintegrated using freeze–thawing in liquid nitrogen and then lysed by ultrasonication in TNE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) with protease inhibitor cocktail (Sigma, USA) and undisrupted parasites were eliminated by centrifugation. The lysate was then incubated with ice-cold 0.1 M sodium carbonate (pH 11) and stirred for 1 h at 4 °C. Finally, the MEPs were collected by ultracentrifugation in a Beckman ultracentrifuge (CA, USA) at 120,000 *g* for 1 h at 4 °C in the pellet and cytoplasmic proteins (CPs) in the supernatant [9]. Protein estimations were done by Lowry method [10].

2.4. Western blot analysis

Equal amounts of *L. donovani* CPs, MEPs and whole cell lysate (WCL) proteins (200 μg) were separated on 12% SDS-PAGE. Then, the proteins were transferred to a PVDF membrane (Millipore, USA) and the membrane was blocked in 5% non-fat milk in TBST (150 mM NaCl; 10 mM Tris, 0.1% Tween 20 pH 7.4). Then, the membrane was washed three times (10 min each) with TBST and incubated with mouse anti-Leishmania-gp63 antibody at 1:500 dilution (LifeSpan BioSciences, USA) followed by horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Millipore, USA) (1:5000 dilution) to visualize the marked antigens. Immunoblots were exposed to X-ray film after reaction with ECL kit (Amersham Biosciences, Singapore) of enhanced chemiluminescence. The same blot was stripped off and re-probed with mouse monoclonal anti-tubulin antibody (Sigma, USA) at 1:2000 dilution followed by HRP-conjugated anti-mouse secondary antibody (Millipore, USA) at 1:5000 dilutions. The films were scanned and processed for result.

2.5. Protein sample preparation for two dimensional gel electrophoresis (2-DGE)

The MEPs of *L. donovani* were treated with trichloro-acetic acid (TCA) to a final concentration of 10% w/v separately and washed three times with acetone to eliminate contaminants like nucleic acid and salts (by employing PBS or Tris–HCl buffer). The dry pellet of the MEPs was solubilized in a rehydration buffer [7 M urea, 2 M thiourea, 2 mM Tri butyl phosphine (TBP), 40 mM Tris, 4% w/v CHAPS, 1% v/v biolyte (3–10), and 1% v/v Triton X-100] Dissolved MEP fraction was vortexed and centrifuged at 10,000 *g* for 10 min to remove insoluble material.

2.6. Two dimensional gel electrophoresis

2.6.1. First dimension/iso-electric focusing (IEF)

2-DGE of MEP fractions was performed as described previously with minor modifications according to the manufacturer's manual (Bio-Rad, USA). Briefly 1.5 mg of MEP fractions were solubilized in rehydration buffer, immobilized on dry strips, *pI* 3–10, 17 cm (BioRad, USA) separately and allowed to rehydrate for 18–22 h. IEF was performed at 20 °C using the Protean IEF cell (BioRad, USA) according to the manufacturer's instructions after rehydration step. The IEF run was carried out using the following conditions: (i) conditioning step for 20 min at 250 V, (ii) liner voltage ramping for 2 h and 30 min at 10,000 V, (iii) for the final focusing step 10,000 V constant until 40 kVh, and the current did not exceed 50 μA/strip.

2.6.2. Second dimension

After IEF, the strip was equilibrated in solution A (0.375 M Tris, pH 8.8 containing 6 M urea, 2% SDS, 20% glycerol, 2% w/v DTT) and B (solution A without DTT, but with 2.5% w/v iodoacetamide) for 20 min at room temperature and inserted on to a 12% 2-D SDS-PAGE gel (20 x 22 cm) with wide range prestained protein molecular weight marker (Fermentas, USA). The gel was sealed with 1% agarose and SDS-PAGE was performed at 16 mA/gel for the initial 30 min and then at 24 mA/gel at 14 °C until the running dye reached the bottom. The gel was removed from the apparatus then it was stained.

2.7. Gel staining, image analysis and protein spot excision

The gel was stained either up to 4 h or overnight with (0.25 g/100 ml) colloidal coomassie brilliant blue [CBB, G-250 (Bio-Rad, USA)]. The stained gel was scanned and images were acquired by gel imaging and spot picking system (Investigator™ ProPic, Genomic solution, USA). The protein spots of interest were manually excised from the gels and transferred to micro-centrifuge tubes, labeled properly and stored at −80 °C.

2.8. In-gel digestion of protein spots

The in-gel digestion of proteins and purification of peptides from gel was carried out according to the manufacturer's manual (Applied Biosystem, USA). Briefly, 2-DGE protein spots were excised and transferred into microcentrifuge tubes. Gel pieces were rinsed three times with Milli-Q water (Millipore, USA) to remove SDS and buffer salts. Followed by 50% v/v acetonitrile (AcCN) in 50 mM ammonium bicarbonate (ABC), pH 8.0 was added and the gel pieces were crushed using pestle. After centrifugation, supernatant was discarded, and the above step was repeated 2–3 times. The gel pieces were shrunk by dehydration by adding 100 μl AcCN and vacuum dried. Gel pieces containing proteins were rehydrated by adding 10–20 μg/ml digestion buffers containing sequencing grade modified 10 μg/ml trypsin (Sigma, USA). After 15 min, 25 μl of 50 mM ammonium bicarbonate

(ABC) was added to keep the gel pieces wet during tryptic cleavage (37 °C, overnight). To extract the tryptic peptides, 50% of AcCN/ 0.3% TFA solution was added, and the samples were incubated for 15 min and then vortexes properly. The separated liquid was dried under vacuum and the peptides were again dissolved in 10 μl 0.1% TFA. The peptides were purified with C-18 reversed-phase mini column filled in a micropipette tip, Zip-Tip C18 (Millipore, Bedford, MA, USA).

2.9. Peptide derivatization

The resulting peptides were mixed with a double volume of matrix, α-cyano-4-hydroxycinnamic acid (Applied Biosystem, Farmingham, USA) of 10 mg/ml in 50% AcCN, 0.1% TFA. α-Cyano-4-hydroxycinnamic acid was always freshly prepared.

2.10. MALDI-TOF/TOF MS analysis

The derivatized digests of peptide were desalted and spotted onto a MALDI sample plate. MS and MS/MS spectra were acquired in the positive ion mode on a MALDI-TOF/TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Framingham, MA, USA). Spectra were obtained by accumulation of 1000 and 4000 consecutive laser shots respectively in MS and MS/MS mode and laser intensity used were in the range of 5000 to 6000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems, USA), a standard mixture of six peptides des-Arg1-Bradykinin (904.4681), Angiotensin I (1296.6853), Glu1-Fibrinopeptide B (1570.6774), ACTH [clip 1–17] (2093.0867), ACTH [clip 18–39] (2465.1989) and ACTH [clip 7–38] (3657.9294). Mass calibration for MS/MS spectra was performed by fragment masses of precursor Glu1-Fibrinopeptide B (1570.6774). Peak harvesting was carried out using 4000 Series Explorer™ Software (Applied Biosystems, USA) for MS or MS/MS data.

2.11. Database search for protein identification

Database searching for protein identifications was performed with MS or MS/MS data using a Global Proteome Server v3.5 software (Applied Biosystems, USA) equipped with MASCOT — Matrix Science (www.matrixscience.com) search engine. Only monoisotopic masses were used for searching against the SWISS-PROT (http://www.expasy.ch/sprot), NCBInr (http://www.protein.sdu.dk/gpmaw/NCBInr/ncbinr.html), TriTryp (http://tritrypdb.org/tritrypdb) databases with a minimum number of matched masses set at 4. The maximum peptide precursor tolerance was set at 40 ppm and MS/MS fragment tolerance was defined as 0.2 kDa. At the most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification and methionine oxidation as a variable modification. Tandem MS i.e. MS/MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF/MS data. The identification criteria with MS/MS data were that peptide count should not be less than two and confidence interval % for the best ion score should be above 95 (significance level $P < 0.05$).

2.12. Localization and classification of identified proteins

Localization of identified proteins was done by the protein localization prediction programs SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui) and WoLF-PSORT (http://wolfpsort.org). Identified proteins of *L. donovani* MEP fractions were also classified on the basis of their cellular localization as reported by SOSUI and WoLF-PSORT bioinformatics programs.

3. Results

We fractionated the MEPs of *L. donovani* by sonication and ultracentrifugation for its further proteomic analyses. The enrichment of *L. donovani* membrane proteins was confirmed by Western blotting. A band of ~63 kDa corresponding to gp63 was visible in the membrane enriched sample, but not in the cytoplasmic protein fraction of *L. donovani* (Fig. 1). Cytoskeleton is a structure tightly linked to the plasma membranes and very difficult to completely exclude the cytoskeleton proteins from cellular fractions [11]. It is not easy to monitor the tubulin in cellular fractions or homogenates and it may be found in nuclear, cytoplasmic and membrane fractions [12]. Therefore, we observed the occurrence of nearly an equal amount of α-tubulin in CP and MEP fractions and more in WCL fraction (Fig. 1). This result was somewhat expected like earlier published reports [11–13]. After that, the MEPs were separated by 2-DGE with minimal streaking as shown in Fig. 2 and the proteins identified therein are listed in Table 1. The reproducibility of the 2-D patterns was confirmed and considered final when three consecutive runs produced identical patterns with the same MEP fraction. In total, 95 well resolved protein spots in the MEPs of *L. donovani* of varying intensities were detected in the CBB stained gel within the pI range of 3 to 10 (Fig. 2). Most of these spots were detected around 4 to 8 *pI* i.e. acidic to slight basic pH range. The predicted molecular masses of identified proteins ranged from 10 to 110 kDa, with majority of proteins exhibiting a molecular mass between 20 and 80 kDa. All 95 protein spots excised from a CBB stained gel of MEP fraction were analyzed and 72 (76%) proteins were identified from spots by MS and MS/MS analyses. Minor identification failures could be due to a sample amount, specific peptide characteristics and extensive post-translational modifications (PTM) or significant divergence from sequenced strains [14]. A significant correlation between theoretical and experimental physicochemical properties of identified proteins (*Mr* and *pI*) was obtained, though some discrepancies existed which could be due to heavily prevalent post-translational protein modifications in protozoan parasites. In addition, it was also noticed that the mass and charge of several proteins were different from those predicted by the *Leishmania* genome, which is hitherto reported to be a common feature of most proteomic analyses, probably reflecting the effect of protein 'maturation' events including PTM [14]. The identification of the same protein at more than one location within the same gel was observed like actin, tubulin, and heat-shock proteins. The multiple spots identifying same protein can be accounted for post-translational modifications which might have taken place either in the living system or presence of truncated/degraded portions of the proteins, as previously reported [5,14].

Out of 72 identified the membrane proteins, a number of proteins were integral membrane proteins (e.g. cytochrome b, cytochrome c, various transporters), few belong to organelle membrane proteins (e.g. glyceraldehyde-3-phosphate dehydrogenase, 60S ribosomal protein L23a), some were membrane associated proteins (e.g. LPG2, tubulin, gp63) and some soluble proteins (e.g. elongation factors, HSPs) because a fraction enriched in the membrane proteins still

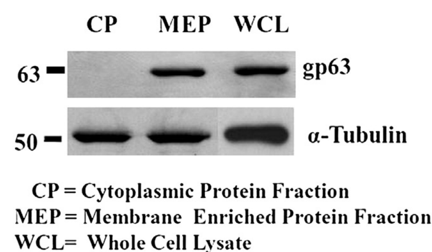

Fig. 1. Western blot showing the of expression level of gP63 (63 kDa) and α-tubulin (50 kDa) in membrane enriched proteins (MEPs) and cytosolic proteins (CPs) fractions of *L. donovani*.

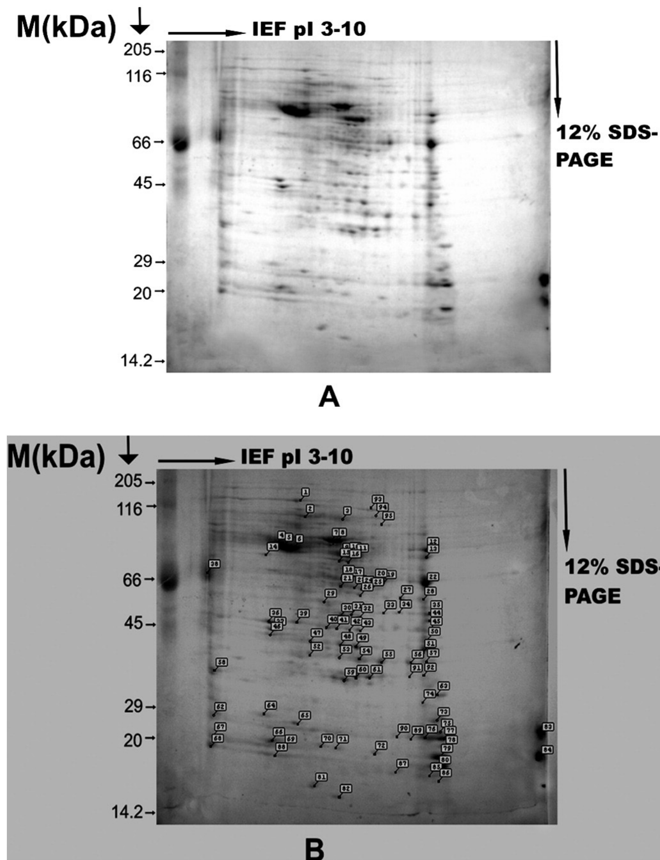

Fig. 2. (A) Representative CBB stained 2-D gel map of the fraction enriched in membrane proteins of *L. donovani*. The MEPs were loaded onto IPG strip *pI* 3–10 followed by SDS-PAGE (12%). (B) Numbers in gel indicate the protein spots that have been processed for MALDI-TOF/MS analysis and listed in Table 1.

contain a number of non-membrane proteins and this fact cannot be neglected or ignored [13]. The membrane proteins are usually predicted according to the presence of alpha-helical transmembrane domains, which often exhibit positive hydropathy values [15]. On the other hand, the membrane proteins often span the membrane as beta-barrel structures and have negative hydrophobicity values similar to those of cytoplasmic proteins [16]. Moreover, many proteins, such as lipoproteins or glycoproteins, can be associated with membranes by PTM [17, 18]. In Table 1 among all the identified proteins, the membrane proteins regarded according to their name or the name of their homolog protein or prediction of their localization by the programs SOSUI and WoLF-PSORT were shown. SOSUI is a very accurate and reliable program for the classification and secondary structure prediction of the membrane proteins [19]. WoLF-PSORT is able to do protein sub-cellular localization prediction [20]. On the other hand, the usage of prediction programs was pivotal for the categorization of proteins classified as hypothetical or unknown.

The identified proteins were classified on the basis of their biological function into ten functionally related groupings — Carbohydrate metabolism, Cell Signaling, Chaperons, Cytoskeletal organization, Dephosphorylation, Fatty acid synthesis, Protein synthesis, RNA processing, Transport, and Unknown biological function (Fig. 3). Glyceraldehyde-3-phosphate dehydrogenase [21], gp63 [22], and Cytochrome c [23] were advocated as DT. Some other proteins were also identified in the membrane-enriched protein of *L. donovani* which has been reported as a potent DT and listed in Table 1. Activated protein kinase C receptor homolog [24], Promastigote surface antigen (PSA) [25], HASPA Protein [25,26], and Variant surface glycoprotein phospholipase C [27] were proposed as VCs. *L. donovani* p36 LACK DNA vaccine has been found to be highly immunogenic but not protective against experimental VL [28]. The most noteworthy and interesting proteins among the identified proteins in the membrane fraction of *L. donovani* are the presence of transporter proteins particularly ATP-binding cassette (ABC) proteins which are known to be involved in drug resistance in parasitic protozoans [29]. The role of ABC transporters in drug targeting was confirmed by Castanys-Munoz et al. [30]. 10 novel proteins have been identified in the MEP fraction of *L. donovani* and reported in bold in Table 1. Many of the identified proteins in MEP raction have either described as drug targets (DTs) or vaccine candidate (VCs) in many species (Table 1). The identified proteins were classified on the basis of their cellular localization (Table 1). Many identified proteins could not be classified into any category because they were hypothetical proteins and their functional and metabolic roles are yet to be deciphered.

4. Discussion

Proteome analysis by 2-DGE together with MALDI-TOF is a powerful approach for the resolution and identification of proteins in complex biological samples. *L. donovani* is a well-studied pathogen that causes fatal VL, but the comprehensive proteome profile of its membrane proteins has remained unexplored so far. The proteome analysis of the cell membrane is considered a daunting task, mainly due to the problems encountered at two levels: (a) isolation of the membrane-enriched protein and (b) solubilization of the membrane proteins in a manner amenable to isoelectric focusing [15]. The membrane sub-proteome analyses of some *Leishmanis* spp. have been analyzed recently. In an earlier study by our group, identification of differentially overexpressed MEPs as well as CPs in sodium antimony gluconate (SAG) sensitive and resistant clinical isolates of *L. donovani* was carried out [9]. Yao et al. in 2010 performed proteomic examination of *L. chagasi* plasma membrane proteins for contrasting studies between avirulent and virulent parasite forms [31]. Very recently, Brotherton et al. in 2012 reported the proteome analysis of the MEPs in *L. infantum* promastigotes and axenic amastigotes [13]. We have isolated the MEP fractions with utmost care and we were able to identify several membrane proteins of *L. donovani*. The present investigation focused on the preparation of the fraction enriched in the membrane proteins of *L. donovani* and provides a comprehensive mass spectrometry based proteomic dissection of its membrane sub-proteome. Such a prefractionation of the proteins reduces the complexity of 2-D maps by removing most non-membrane proteins [15]. Notably, the proteomic study presented here documents a range of novel proteins including unknown/hypothetical proteins (Table 1) in the membrane fraction of *L. donovani* which was hitherto not reported earlier. Our data matched with other *Leishmania* species proteins which showed the relevance of the result. A match with other *Leishmania* species is authentic and reliable because they are close with *L. donovani*.

At present, in ~33.6 Mb genome 8300 protein coding genes of *L. major* [32] (http://www.genedb.org/leish/index.jsp), *L. infantum* (www.genome.jp/kegg-bin/show_organism?org=lif) and other *Leishmania* sp. have been sequenced. In consequence, it was necessary to identify *L. donovani* proteins by using reference of all known *Leishmania* spp. sequences in the public database. This strategy has been applied with success for other *Leishmania* species that lack a fully sequenced genome [33]. Among all identified proteins in the MEP fraction of *L. donovani* some proteins (e.g. gp63, LPG, PSA, LACK, HSPs) have already been detected and studied in the case of *Leishmania* spp. But some proteins like Multidrug resistance protein, p-glycoprotein-like protein, Leishmanolysin C1, Surface membrane glycoprotein GP46/M-2, Amino acid transporter, Hydrophilic acylated surface protein B, Hexose transporter, Maoc family dehydratase like protein, GPI mannosyltransferase 1, Ubiquinone biosynthesis protein-like protein, and Ubiquinone biosynthesis protein COQ4 homolog have been identified for the first time in *L. donovani* membrane sub-proteome analysis. The role

Table 1
Proteins identified in membrane-enriched fraction of *L. donovani*.

Spot[a] no.	Protein identified[b]	Species[c]	Accession no.[d]	Mol. mass[e] (pr Ed.)	pI[f] (prEd.)	Pm/% of Sc/Ms[g]	Function[h]	Cellular localization[i]	Class/family[j]	Remarks[k]
1	ABC protein	Lmj	LmjF33.0310	161.7	6.0	9/23/156	TP	MP	AT	DT
2	Hypothetical protein	Lmj	A2QGE4_ASPNC	39.4	6.6	10/8/102	UN	MP	Unknown	??
3	Heat shock protein 100	Li	LinJ.29.1360	96.9	7.2	12/10/91	CH	MT	clpA/clpB	??
4	Tubulin beta chain	Lmx	TBB_LEIME	50.0	4.7	17/14/98	CO	CS	Tubulin	Th1
5	Tubulin beta chain	Lmx	TBB_LEIME	50.0	4.7	19/15/99	CO	CS	Tubulin	Th1
6	Tubulin beta chain	Lmx	TBB_LEIME	50.0	4.7	15/11/103	CO	CS	Tubulin	Th1
7	Alpha tubulin	Lb	LbrM.13.0190	53.5	4.9	11/17/123	CO	CS	Tubulin	Th1
8	Alpha tubulin	Lb	LbrM.13.0190	53.5	4.9	9/14/109	CO	CS	Tubulin	Th1
10	**Multidrug resistance protein**	**Lt**	**MDR_LEITA**	**172.0**	**5.9**	**1 3/11/129**	**CS**	**MP**	**Unknown**	**DT, DR**
11	Protein tyrosine phosphatase	Ld	Q0PEE2_LEIDO	55.2	9.6	14/16/142	DP	CP	Hydrolase	IP
12	**p-Glycoprotein-like protein**	**Li**	**LinJ.26.2700**	**139.2**	**6.9**	**9/12/104**	**CS**	**MP**	**Unknown**	**DT, DR**
13	Hypothetical protein	Li	LinJ.32.0380	106	6.3	12/14/117	UN	MP	Unknown	??
14	ABC transporter	Lmj	LmjF.32.3080	77.3	6.2	9/15/99	TP	MP	AT	DT
15	Mucin-associated surface protein (MASP)	Tc	Q4DU24_TRYCR	27.5	4.1	18/12/111	UN	MP	Unknown	VC
16	Hypothetical protein	Li	LinJ.33.1170	82.9	5.2	15/9/117	UN	MP	Unknown	??
17	RNA binding protein	Li	LinJ.29.1470	70.9	9.5	9/12/107	RP	CP	Unknown	DT
18	Putative MAP kinase kinase	Lmx	Q9Y074_LEIME	41.4	5.2	9/10/98	UN	CP	Protein kinase	DT
19	Coronin	Lmj	Q4QB38_LEIMA	56.6	6.6	9/11/96	AB	CP	Unknown	??
22	Heat shock protein 83	Ld	HSP83_LEIDO	52.6	5.4	15/19//125	CH	CP	Hydrolase	Th1
24	ABC1 transporter	Li	LinJ.11.1220	72.7	5.8	9/12/111	TP	MP	AT	DT
26	Probable proton ATPase 1A	Ld	ATXA_LEIDO	107.0	5.2	6/8/82	CS	MP	Hydrolase	??
28	**Amino acid transporter**	**Lmj**	**Q4Q6N1_LEIMA**	**58.4**	**7.6**	**13/19/112**	**TP**	**MP**	**Unknown**	**DT**
29	Hypothetical transmebrane protein	Tb	Q8WPU4_9TRYP	20.6	10.4	9/11/79	UN	MP	Unknown	??
30	**Leishmanolysin C1**	**Lmx**	**GP63_LEIME**	**69.0**	**6.2**	**11/9/88**	**CS**	**MP**	**Hydrolase**	**??**
31	gp63, Leishmanolysin	Lb	A4H626_LEIBR	62.5	4.7	15/23/137	CS	MP	Hydrolase	DT
32	gp63, Leishmanolysin	Lb	A4H626_LEIBR	62.5	4.7	11/19/122	CS	MP	Hydrolase	DT
33	Surface antigen protein 2	Li	A4HV45_LEIIN	43.9	5.1	19/21/117	CS	MP	Unknown	VC
34	LPG2	Ld	Q25266_LEIDO	37.1	9.7	6/9/77	TP	MP	ST	VC
35	**GPI mannosyltransferase 1**	**Li**	**LinJ.30.2030**	**49.3**	**9.3**	**17/13/121**	**TP**	**ERM**	**Transferase**	**DT**
36	p36 LACK protein	Li	LinJ.28.2970	34.3	6.5	6/21/106	CS	MP	Unknown	VC, DT
37	Hypothetical protein	Lb	LbrM.25.1880	51.9	8.8	8/11/107	UN	MP	Unknown	??
38	Small GTP-binding protein	Lb	LbrM.05.0030	21.7	8.4	10/15/121	TP	CP	GTP-binding	DT
40	**Surface membrane glycoprotein GP46/M-2**	**La**	**GP46_LEIAM**	**50.1**	**10.7**	**11/65/102**	**CS**	**MP**	**Hydrolase**	**??**
41	ATP synthase	Lmj	Q4QC61_LEIMA	54.0	5.6	14/18/132	TP	MP	ATPase	DT
42	Amino acid permease, putative	Li	LinJ.31.1850	65.5	7.4	9/14/108	TP	MP	Permease	??
43	Hypothetical protein	Lb	LbrM.31.0700	92.9	8.9	9/11/125	UN	MP	Unknown	??
44	Promastigote surface antigen (PSA)	Ld	Q4JI42_LEIDO	40.7	6.0	11/14/121	CS	MP	Unknown	VC
45	Promastigote surface antigen (PSA)	Ld	Q4JI42_LEIDO	40.7	6.0	13/19/127	CS	MP	Unknown	VC
47	Protein P18	Lt	P18_LEITA	50.2	12.2	8/14/135	CS	MT	Hydrolase	??
48	Cytochrome c	Lb	LbrM.161360	12.2	10	14/12/92	TP	CP	OR	DT
51	Hypothetical protein	Lmj	Q9N853_LEIMA	54	4.9	9/15/138	UN	MP	Unknown	??
52	**Hydrophilic acylated surface protein B**	**Lmx**	**Q9U1F9_LEIME**	**18.6**	**4.4**	**14/18/132**	**TP**	**MP**	**Unknown**	**??**
53	Activated protein kinase-C receptor homolog (LACK)	Ld	O43942_LEIDO	30.6	6.6	9/11/101	CS	MP	AR	VC
54	**Ubiquinone biosynthesis protein COQ4 homolog**	**Lb**	**LbrM.091490**	**21.2**	**9.7**	**10/15/104**	**UN**	**MT**	**Unknown**	**??**
55	Glycosomal membrane protein-like protein	Li	LinJ.28.2420	24.3	9.8	17/12/129	CS	MP	Unknown	??
56	Hypothetical protein	Li	LinJ.36.6640	30.1	10.7	9/16/116	UN	MP	Unknown	??
58	Hypothetical protein	Lmj	Lmj.F25.2290	32.9	8.3	11/17/143	UN	MP	Unknown	??
59	Cytochrome b	Lt	CYB_LEITA	44.5	6.7	9/18/137	TP	MT	Cytochrome b5	DT,DR
60	Cytochrome b	Lt	CYB_LEITA	44.5	6.7	9/18/137	TP	MT	Cytochrome b5	DT,DR
61	Hypothetical protein	Lb	LbrM.18.0420	60.4	8.6	10/17/111	UN	MP	Unknown	??
62	Hypothetical protein	Li	LinJ.06.1060	24.6	9.7	11/17/143	UN	MP	Unknown	??
63	Hypothetical protein	Lmj	Lmj.F36.2970	19.3	10.1	9/14/103	UN	MP	Unknown	??
65	Hypothetical protein	Li	LinJ.36.5100	25.9	7.6	9/19/106	UN	MP	Unknown	??
66	Maoc family dehydratase like protein	Li	LinJ.07.0180	16.9	4.9	9/11/104	FS	SP	OR	??
67	Kinetoplastid membrane protein 11	Ld	KM11_LEIDO	11.2	6.1	13/21/107	CO	KM	Unknown	DT
68	Kinetoplastid membrane protein 11	Ld	KM11_LEIDO	11.2	6.1	12/16/99	CO	KM	Unknown	DT
69	Maoc family dehydratase like protein	Li	LinJ.07.0180	16.9	4.9	10/14/119	FS	CP	OR	??
71	Hypothetical protein	Lmj	Q4FW70_LEIMA	25.2	10.2	9/19/106	UN	MP	Unknown	??
72	Hypothetical protein	Lmj	Q4FW70_LEIMA	25.2	10.2	9/19/106	UN	MP	Unknown	??
73	Hypothetical protein	Lb	LbrM.34.0250	8.6	8.6	13/11/111	UN	MP	Unknown	??
74	Hypothetical protein	Lmj	LmjF36.2970	19.2	9.7	9/13/107	UN	MP	Unknown	??
77	Glyceraldehyde-3-phosphate dehydrogenase	Lmx	G3PG_LEIME	39.0	9.0	10/17/109	CM	GS	OR	DT
81	Hypothetical protein	Li	LinJ.25.2400	30.0	8.2	15/12/121	UN	MP	Unknown	??
82	**Ubiquinone biosynthesis protein-like protein**	**Li**	**LinJ.09.1500**	**21.3**	**8.3**	**11/15/110**	**UN**	**MT**	**Unknown**	**??**
86	Hypothetical protein	Lb	LbrM.34.3970	25.7	10.2	9/12/94	UN	MP	Unknown	??
87	60S ribosomal protein L23a	Li	LinJ06.0590	16.3	11.2	9/10/87	UN	RS	Unknown	??
90	Hypothetical protein	Lb	LbrM.04.0590	19.7	8.5	13/14/132	UN	MP	Unknown	??
91	Glycosomal membrane protein-like protein	Li	LinJ.28.2420	24.3	9.8	17/12/129	CS	GS	Unknown	??

Table 1 (*continued*)

Spot[a] no.	Protein identified[b]	Species[c]	Accession no.[d]	Mol. mass[e] (pr Ed.)	pI[f] (prEd.)	Pm/% of Sc/Ms[g]	Function[h]	Cellular localization[i]	Class/family[j]	Remarks[k]
92	Hypothetical protein	Li	LinJ.19.1630	18.9	10.2	15/11/127	UN	MP	Unknown	??
93	**Hexose transporter**	**Lmx**	**B1PLM1_LEIME**	**67.6**	**5.2**	**13/11/101**	**TP**	**MP**	**ST**	**DT**
94	Glucose transporter	Lmx	O76486_LEIME	66.1	7.7	12/10/91	TP	MP	ST	DT
95	Cysteine-leucine rich protein	Li	LinJ.34.0570	65.8	6.3	11/9/149	UN	CP	Unknown	??

The protein spots shown in Fig. 2 were identified using peptide mass fingerprint.
Note: protein spots analyzed but not identified: 9, 20, 21, 23, 25, 27, 39, 46, 49, 50, 57, 64, 70, 75, 76, 78, 79, 80, 83, 84, 85, 88 and 89.
[a] Protein spots no. indicated in Fig. 2.
[b] Name of identified protein.
[c] Species: La: *Leishmania amazonensis*; Lb: *Leishmania braziliensis*; Lc: *Leishmania chagasi*; Ld: *Leishmania donovani*; Li: *Leishmania infantum*; Lmj: *Leishmania major*; Lmx: *Leishmania mexicana*; Lt: *Leishmania tarentolae*; Tb: *Trypanosoma brucei*; Tc: *Trypanosoma Cruzin.*
[d] Accession numbers of protein according to NCBI and Swiss-Prot accession number.
[e] Predicted molecular mass in kDa.
[f] Predicted *pI*.
[g] No. of peptides matched (Pm)/percentage of sequence coverage (Sc)/MOWSE score (Ms).
[h] Function of identified proteins; AB: actin binding, CH: chaperon, CM: carbohydrate metabolism, CO: cytoskeletal organization, CS: cell signaling, FS: fatty acid synthesis, PS: protein synthesis, RP: RNA processing, TP: transport, and UN: unknown.
[i] Cellular localization of identified proteins; CP: cytosolic protein, CS: cytoskeletal, ERM: endoplasmic reticulam membrane, GS: glycosome, KM: kinetoplastid membrane, MP: membrane protein, MT: mitochondria, and RS: ribosomal surface.
[j] Class/family; AR: aldehyde reductase, AT: ABC transporter, OR: oxidoreductase, and ST: sugar transporter.
[k] Remarks; DR: drug resistance, DT: drug targets, IP: immunogenic protein, Th1: Th1 stimulatory, VC: vaccine candidate, and ??: not described previously.

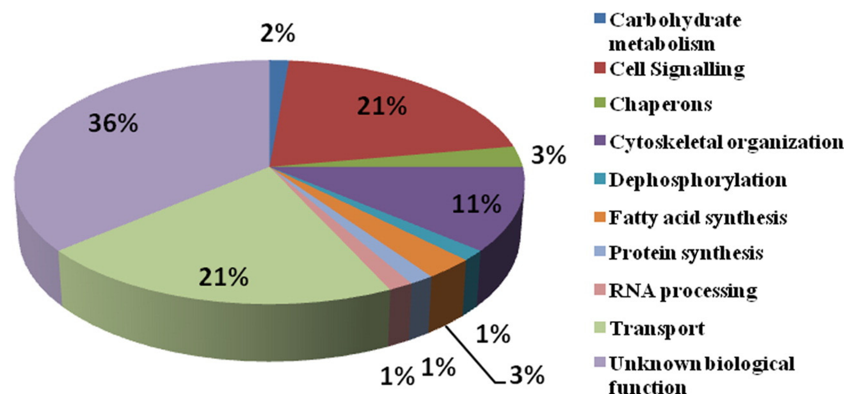

Fig. 3. Pie-chart representing the percentage and biological categories of identified membrane-enriched proteins of *L. donovani* based upon their putative functions, assigned using protein function databases.

of *p-glycoprotein-like protein* which was detected in *L. major* was suggested to be involved in multidrug resistance [34]. Joet et al. validated *the hexose transporter* as a novel drug target in *Plasmodium falciparum* [35]. The increasing rate of the development of drug resistance in these parasites also necessitates the need for identification and characterization of novel targets. These proteins considered to be novel drug/vaccine targets for VL, have not been characterized in *L. donovani*. A large number of hypothetical proteins were identified in this study. Characterization of identified hypothetical proteins should be also a prime concern because these may be represented as novel targets for VL. This investigation has thus revealed the identification of some novel proteins, which in the light of the increasing drug resistance could be proved as a panacea for the management of the disease.

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