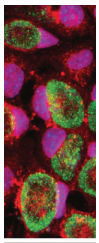

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SHORT COMMUNICATION

Mass spectrometry-based proteomic analysis of *Leishmania donovani* soluble proteins in Indian clinical isolate

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This article provides a comprehensive 2D PAGE-based mass spectrometric analysis of the soluble proteome of a clinical isolate of *Leishmania donovani*, the cause of visceral leishmaniasis.

Keywords
Leishmania donovani; clinical isolate; SLD; 2-DGE; MALDI-TOF/TOF-MS.

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Abstract

Leishmania donovani, a causative organism of visceral leishmaniasis (VL), is responsible for high mortality throughout the world. Due to the unsatisfactory treatment measures and increasing drug resistance, there has been an urgent need to develop novel drug/vaccine targets against VL. The aim of this study was to identify novel targets in soluble *L. donovani* (SLD) protein. SLD protein was isolated and resolved by two-dimensional gel electrophoresis and analyzed through MALDI-TOF/TOF-based mass spectrometry. Proteomic results identified several proteins as drug targets, Th1 stimulatory, novel, and hypothetical proteins which could have crucial biological functions in *Leishmania* pathogenesis.

Leishmania donovani, a protozoal parasite, is the etiologic agent of a chronic and fatal disease visceral leishmaniasis (VL; Sundar *et al*., 2006). Unfortunately, the prevalence of parasites becoming resistant to the first-line drug pentavalent antimony (Sb^V) is increasing in several parts of the world (Thakur *et al*., 2004). Therefore, need for the identification of novel and effective targets is imperative. Significance of *Leishmania* parasite as a human pathogen has made it in focus of numerous studies in areas such as host–parasite interactions, cell differentiation, and mechanisms of drug resistance. The usefulness of large amount of *Leishmania* genome sequencing information can be utilized for the investigation of protein identification (Ivens *et al*., 2005). To achieve this purpose, one such way is two-dimensional gel electrophoretic (2-DGE) separation of the protein molecules, coupled with mass spectrometry (MS) and bioinformatics, which provides the foundation for simultaneous identification of novel proteins and potential drug targets (Biron *et al*., 2005).

Earlier, we have identified a fraction of the soluble *L. donovani* (SLD) proteins of Indian clinical isolate (2001) ranging from 68 to 97.4 kDa (Gupta *et al*., 2007). In this consequence, it was necessary to take an attempt to identify whole SLD proteins with the help of reference map of all known *Leishmania* spp. sequences in the public database. Present research work is the extension of our previous work and an effort for the mapping of whole SLD proteins by 2-D gel and MS-based proteomics approaches for the identification of large number of proteins as well as new targets in the same clinical isolate of *L. donovani*.

Leishmania donovani Indian clinical isolate (strain 2001) was grown in RPMI-1640 medium as described by Garg *et al.* (2006). SLD protein was prepared as per method described previously (Scott *et al*., 1987). Protein estimations were carried out by Lowry method (Lowry *et al*., 1951). SLD protein samples were precipitated in trichloroacetic acid and finally prepared for 2-DGE (Gupta *et al*., 2007). 2-DGE of SLD was performed according to the

manufacturer's manual (Bio-Rad). Briefly, 1.5 mg of SLD fractions was solubilized in rehydration buffer, immobilized on dry strips, pI 3–10, 17 cm (BioRad) separately, and allowed to rehydrate for 18–22 h. Isoelectric focusing (IEF) was performed at 20 °C using the Protean IEF cell (BioRad) according to the manufacturer's instructions after rehydration step. After IEF, the strip was equilibrated and SDS-PAGE was performed according to the Laemmli method (1970). The gel was stained with Coomassie Brilliant Blue (CBB, G-250; Bio-Rad). CBB-stained gel was scanned, and images were acquired by gel imaging system (Investigator™ ProPic; Genomic Solution). The protein spots of interest were manually excised from the gels and stored at −80 °C. The in-gel trypsin digestion of proteins and purification of peptides from gel was carried out according to the manufacturer's manual (Applied Biosystems). To extract the tryptic-digested peptides, 50% AcCN and 0.3% TFA solution were added, and finally, peptides were purified with C-18 reversed-phase mini column, Zip-Tip C18 (Millipore, Bedford, MA).

The resulting peptides were mixed with matrix α-cyano-4-hydroxycinnamic acid (Applied Biosystems) in 50% AcCN, 0.1% TFA and spotted onto a MALDI sample plate of MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems 4700 Proteomics Analyzer, MA) for MS and MS/MS analysis (Kumar *et al.*, 2010). Database searching for protein identifications was performed with MS or MS/MS data using Global Proteome Server v3.5 software (Applied Biosystems) equipped with MASCOT (http://www.matrixscience.com) search engine. Data were searched against the SWISS-PROT (http://www.expasy.ch/sprot), NCBInr (http://www.protein.sdu.dk/gpmaw/NCBInr/ncbinr.html), and TriTryp (http://www.tritrypdb.org/tritrypdb) databases. The maximum peptide precursor tolerance was set at 40 μg mL^{-1}, 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 variable modification. Tandem MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF-MS data. Confidence interval percent for the best ion score should be above 95 (significance level $P < 0.05$) for the determination of the false discovery rate. Localization of identified proteins was done by sosui (http://www.bp.nuap.nagoya-u.ac.jp/sosui) and WoLF-PSORT (http://www.wolfpsort.org) programs.

SLD proteins were separated by 2-DGE as shown in Fig. 1a, and the numbered spots (Fig. 1b) indicate the identified/unidentified proteins listed in Supporting Information, Table S1. Proteome-specific information of identified proteins, such as accession numbers, molecular mass, pI, number of peptides matched of PMF data/percentage of sequence coverage, MOWSE score ($P < 0.05$), is mentioned in Table S1. The gels prepared with preparative protein loadings displayed good resolution with only minimal streaking. The reproducibility of the 2-D patterns was confirmed and considered final when three consecutive runs produced identical patterns with same SLD fraction. In total, 184 well-resolved protein spots of varying intensity in CBB-stained gel were detected and 132 (71.7%) proteins were identified (Table S1). Many of the identified proteins have either reported as drug targets, vaccine candidates or diagnostic markers in other species (Table S1). Eighteen novel proteins have been identified from SLD fraction, which are very noteworthy proteins (boldface in Table S1). Few identified proteins (29) could not be classified into any category because they were hypothetical proteins. Their functional properties are yet to be deciphered.

Identified proteins in SLD fraction were classified on the basis of their biological function into 17 functionally related groups (Fig. 1c). The identified proteins were also classified on the basis of their subcellular localization (Table S1) with the help of very accurate and reliable bioinformatics programs such as sosui and WoLF-PSORT (Gardy *et al.*, 2003). Many proteins showed a good correlation in predicted and obtained values in terms of their Mr and pI, while many others showed a considerable variation. The predicted molecular masses of identified proteins ranged from 10 to 140 kDa, with majority of proteins exhibiting a molecular mass between 20 and 70 kDa. 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 (Sinha *et al.*, 2002; McNicoll *et al.*, 2006). Well-resolved protein spots were detected within the pI range of 3–10 in Fig. 1a. Of these spots, majority were detected around 4–8 pI. At the same time, some proteins having almost same molecular weight displayed a shift in their pI. A significant correlation between theoretical and experimental physicochemical properties of identified proteins (Mr and pI) was made, although some discrepancies existed which could be due to heavily prevalent post-translational modifications (PTMs) of protein in protozoan parasite (McNicoll *et al.*, 2006). Minor identification of protein failures could be due to sample amount, specific peptide characteristics and extensive PTMs or significant divergence from sequenced strains (McNicoll *et al.*, 2006). The identification of the same protein at more than one location within the same gel was also observed (e.g. actin, tubulin, calreticulin). These observations suggested that PTMs are widely prevalent in this organism. In this study, more than one protein was detected in a single spot, for example alpha-tubulin, tubulin-beta chain, gp63, heat shock 70-related protein1, pyruvate kinase, enolase. Some more examples are listed in Table S1.

Previously, we have identified promastigote form of SLD protein fraction ranging from 68 to 97.4 kDa that induces Th1 responses (Gupta *et al.*, 2007). This study provided large-scale protein profiling of *L. donovani* SLD fraction. Taken together, proteomics data presented here enable the identification of novel drug targets and vaccine development through the identification of antigenic or immunogenic proteins for vaccine development. It is not easy to identify the best vaccine candidates, Th 1 stimulatory proteins, and drug targets from a pool of *Leishmania* proteins by traditional research methods (Kumari *et al.*, 2008). Proteome analysis by 2-D gel electrophoresis together with MALDI-TOF/MS is a powerful approach for the resolution

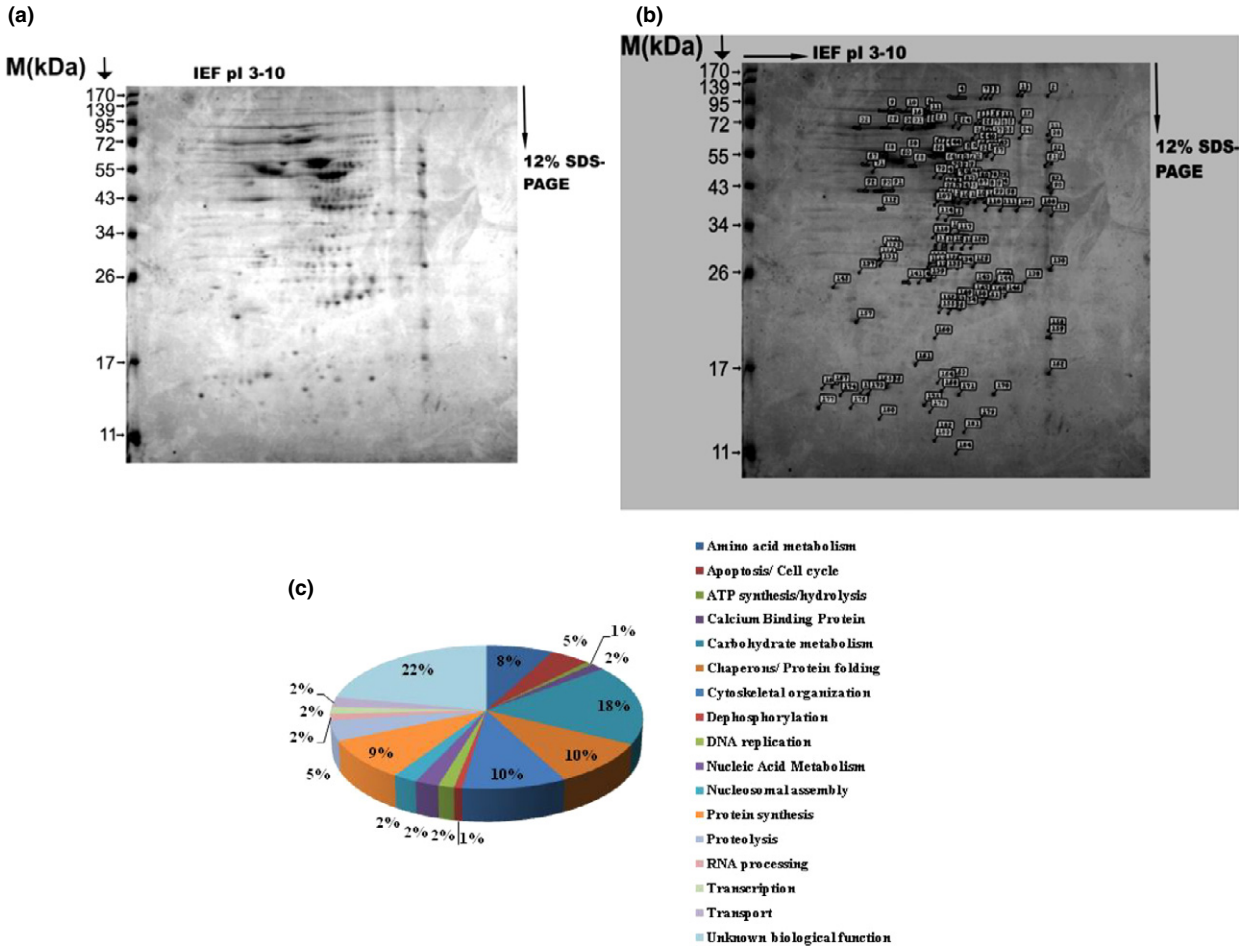

Fig. 1 (a) 2-D gel map of soluble proteins of *Leishmania donovani*. SLD proteins were loaded onto IPG strip pI 3–10 followed by SDS-PAGE (12%). (b) The numbered spots indicate the identified/unidentified SLD listed in Table S1. (c) Pie chart representing the percentage and biological categories of identified SLD proteins based upon their putative functions assigned using protein function databases.

and identification of proteins in complex biological samples (El Fakhry *et al.*, 2002; Drummelsmith *et al.*, 2003). This information can be instrumental in the development of novel drug targets/vaccine candidates for VL.

On the basis of results obtained here, a number of identified proteins can be targeted and expected to be far more effective outcome of the disease. Some novel proteins that were identified in SLD fraction were T-complex protein 1 gamma subunit, RNA pseudouridylate synthase, hypothetical ORF-2 protein, retrotransposon element, 60S ribosomal protein L17, cofactor-independent phosphoglycerate mutase, centromere/microtubule-binding protein cbf5, isoleucine tRNA synthetase, mitochondrial DNA polymerase I protein A, 60S acidic ribosomal protein P2-1, chaperone protein DNAJ, NAD-dependent deacetylase SIR2 homolog, DNA-directed RNA polymerase subunit, uridine kinase-like protein, possible RNA-binding protein, cell division-related protein kinase 2, ribosomal protein S12, mitochondrial DNA polymerase I protein A. They may play the pivotal role in disease progression and probably act as strong targets. These proteins have not been characterized in *L. donovani* and may serve as a novel target if its role is properly corroborated in VL. No detailed information about the biological functions of abovementioned proteins in SLD fraction with reference to *L. donovani* parasites is available till date. These new proteins should be explored for their possible role as novel drug targets/vaccine candidate against VL.

A large number of hypothetical proteins identified in this proteomics may represent as potential drug targets and putative vaccine candidates after doing extensive study over it. Characterization of identified novel and hypothetical proteins should be also in prime concern because these could be proved as novel panacea for drug development or putative vaccine candidates in future for VL. Because soluble proteins play vital roles in many cellular processes, they may become important targets for diagnosis and therapeutics. These proteins could be relevant to include in future studies of the proteins with specific functions such as intracellular survival, pathogenecity, disease control as well as in the understanding of drug resistance.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Proteins identified in soluble protein fraction of *Leishmania donovani*.