Characterization of a novel amastin-like surface protein (ALSP) of *Leishmania donovani*, a probable lipase

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ABSTRACT

In the current study, a novel putative protein of *Leishmania donovani*, amastin-like surface protein (ALSP) has been characterized. The gene was cloned in a bacterial system and the protein was overexpressed. A polyclonal antibody was developed against the protein, which detected a 10kDa band in the *L. donovani* amastigote. ALSP mRNA was detected in *L. donovani* amastigote, which was not expressed in the promastigote. ALSP mRNA was unexpressed in either morphological forms of *Leishmania major*. MALDI-TOF confirmed the molecular weight of ALSP as 10 kDa. I-TASSER predicted the function of ALSP as a lipase, which we confirmed in preliminary *in vitro* experiments using amastigotes of *L. donovani*. ALSP has GAS amino acid sequences, which might act as the active site for its lipase activity. The selective expression of ALSP in amastigotes probably makes it important in virulence mechanisms such as survival in the phagolysosome and modulation of its membrane and other metabolic functions, necessary for parasite survival in the human host. ALSP can act as a peptide vaccine target and maybe detected in the peripheral blood or urine as a molecular biomarker of active disease in visceral leishmaniasis.

Keywords: amastin, lipase, host-parasite, *Leishmania donovani*, parasitism, lipid organelle.

INTRODUCTION

Leishmaniasis is an insect-borne global scourge caused by the protozoan parasite Leishmania. The morphology of different species of Leishmania alternates between two distinct morphs: an extracellularly located flagellated promastigote swarming in the gut of wide species of female phlebotomine sandflies and morphologically distinct intracellular located non-flagellated amastigote transmitted to the blood of the mammalian host (Leifso et al., 2007; Pulvertaft and Hoyle 1960). World Health Organization (WHO) reports that 12 million individuals are affected by leishmaniasis around the globe, among which 20,000–40,000 people are decimated by the disease every year (Alvar et al., 2012). Limited drugs such as antimonials, amphotericin B and miltefosine are available to treat visceral leishmaniasis (kala azar) but they are limited in their efficacy due to toxic effects, nonspecific modes of action, increasing drug resistance and rising cost (Sundar and Agarwal, 2016). There are no vaccines as well against the parasite (Ghosh and Bandopadhyay, 2003, Zutshi et al., 2019).

Kinetoplast organisms like *Leishmania donovani* and other *Leishmania* species, *Trypanosoma brucei* (causing sleeping sickness) and *Trypanosoma cruzi* (causing Chagas disease) expresses a wide family of surface glycoprotein called amastins. *In-silico* prediction studies have highlighted a predicted low molecular weight analog called amastin-like surface protein (ALSP) (Gene ID: LdBPK_301490.1) (Aslett M et al., 2010; Sengodan et al, 2014). Whether at all ALSP is related to amastin is hitherto unknown. Previous studies have shown that the immunogenicity of amastin sequences are furthermost than all surface antigens of *Leishmania* in mice (Stober et al., 2006) and show intense immunogenicity with human visceral leishmaniasis (Rafati et al., 2006). A low molecular weight protein may be an efficient target for vaccine candidacy (Khan et al., 2020). Diverse amastin protein are translated in *L. donovani* amastigotes in individuals with visceral leishmaniasis (Rochette et al., 2005; de Paiva et al., 2015). Our preliminary studies of structural predictions highlighted a lipase-like role for ALSP. Some previous studies have identified lipases as a key virulence factor for the live amastigote in the host phagolysosome and coordination of complex metabolism. The lipid turnover renders the macrophage rather effete in eliminating the amastigote (Wassef et al., 1985, Bouazizi-Ben Messaoud et al., 2017, Rabhi et al., 2012). Lipases may also modulate membrane properties and tissue modelling to favor widespread dissemination, as seen in visceralization of the parasite. (O'Neal et al., 2020, Ramakrishnan et al., 2013, Biagiotti et al., 2017, Beach et al., 1979).

In the current study, we characterized the novel amastin-like surface protein (ALSP), including expressing its gene in a bacterial system, overexpressing the protein in vitro, testing its molecular weight and expression patterns in both the promastigote and amastigote of *L. donovani* and examining its predicted function as a lipase.

MATERIALS AND METHODS

Culture of cells and parasites

Promastigotes of *L. donovani* (AG83) were maintained at 23 °C in Medium199 (M199), with 10% FBS (Fetal Bovine Serum), penicillin (50 U/mL) and streptomycin (50 µg/mL) (Gibco, US). Centrifugation at 1000*g* for 10 min were used to obtain promastigotes in their late log phase (10 million promastigotes/mL). Then, it was washed with phosphate buffer saline (PBS) at pH 7. Human acute monocytic leukemia cells THP1 was maintained in RPMI (Gibco, US) media, with added FBS and the antibiotics penicillin and streptomycin. Invitro conditions changes the promastigotes into amastigotes within the THP1 cells at a ratio of 1:10 (cell: parasite). The amastigotes were isolated by procedures optimized in the laboratory, following the methods described by Moreno, M.L.V et al., (1998) with some modifications.

Accession numbers

The IDs (Identities) and annotations of the novel protein sequences are as follows:

(CBZ37742.1 LDBPK_342650), GeneDB[i] (LdBPK_301490.1.1: pep), Gene ID: 13392833

Prediction of ALSP 3D structure and function through iterative threading assembly refinement algorithm (I-TASSER)

ALSP amino acid sequence was uploaded to the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang and Zhang, 2015). A large ensemble of structural conformations, called decoys is generated by I-TASSER stimulations for each target. The SPICKER program uses the decoys to select the best five final models based on pair-wise structure similarity. The confidence score (C-score) is used to determine the structure quality, which varies between -5 to 2. Higher C-score defines a model as more significant than a lower one. Template modeling score (TM-score0 and Root Mean Square Deviation (RMSD) are estimated by confidence score (C-score) and protein length following the correlation between these parameters.

Based on the I- TASSER structure prediction the function of the ALSP has been annotated

using COFACTOR and COACH. COFACTOR infers protein functions (ligand-binding sites, EC and GO) using structure comparison and protein-protein networks. COACH uses a meta-server approach that combines multiple function annotation results from the COFACTOR, TM-SITE and S-SITE programs.

Model evaluation

The predicted 3D structure of ALSP was assessed by PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK) and ProQ (https://proq.bioinfo.se/cgi-bin/ProQ/ProQ.cgi) web servers. PROCHECK is used to test the stereochemical quality and correctness of the 3D protein structure (Laskowski et al., 1993), and the validation of generated models was further accomplished by ProQ (Wallner and Elofsson, 2003).

Lipase assay

Triglyceride lipase is an enzyme that hydrolyses triglycerides into glycerol and fatty acid (Gupta et al., 2003). The enzyme activity of ALSP in whole lysate was checked by Lipase Assay Kit ($R_{EAI}G_{ENE}$, US) in Unit/µg. The Unit/µg (U/µg) is defined as 1µg protein-producing 1uMol glycerol/fatty acid in 1min at 37 ^{0}C.

The 125 µM/mL of oleic acid standard solution was diluted to 62.5, 31.25, 15.625, 7.8125 and 3.9 µM/mL with anhydrous ethanol to make a standard curve at 710 nm absorbance.

Selection of Antigen as Vaccine Candidate efficiency

The antigenicity of ALSP was checked through ANTIGENPro (Magnan et al, 2010) and VaxiJen 2.0 online tools (Doytchinova and Flower, 2007).

Cloning the ALSP gene

The ALSP gene (Gene ID: CBZ37742.1) was PCR (polymerase chain reaction) amplified in 50 uL reaction volume containing 100 ng of purified genomic DNA of *L. donovani* promastigotes. 100 pmol forward (EcoRI->5'GT C GAATT CGTATG CAT ATG CGT GTA CTT GTG CGT 3') and reverse gene-specific primer (XhoI->5'TTCTCGAG TCA GCA CGG AAA GGA ACG CGA 3'), 200 uM dNTP (Deoxynucleotide triphosphates), 3U Taq DNA polymerase. The fragment was inserted into the pGEX4T2 vector at its multiple cloning site, utilizing the restriction sites of *EcoRI* and *XhoI*. The pGEX4T2-ALSP hybrid construct was used to transform the *Escherichia coli* DH5α and BL21 respectively.

Confirmatory analysis of Cloning through colony PCR, double digestion of the cloned Plasmid, and DNA sequencing

ALSP-Forward and Reverse primers were used to do colony PCR with transformed and untransformed DH5α and BL21 cells, following the same PCR program. The restriction-digestion of the ALSP-GST construct was done after isolating the plasmid from the transformed DH5α cells using a plasmid isolation mini kit (GCC Biotech). The isolated plasmid was dissolved in 20 µl of Diethyl pyrocarbonate (DEPC)-treated water and used for Sanger sequencing, performed (Xcelris Labs, Bangalore, India). Nucleotide-nucleotide basic local alignment search tool (BLASTn) was performed for the chromatogram to check the identity with the available sequence of Leishmania species in the National Center for Biotechnology Information (NCBI) database.

Production of anti ALSP antibody

Antisera were developed against the ALSP by an optimal peptide epitope domain of a protein (SSPFSSTRSSSSSRS –C, the cysteine residue addition at the C terminal end is required for keyhole limpet hemocyanin (KLH) conjugation, applied by a bioinformatics tool [BioBharati LifeScience Pvt. Ltd. Kolkata, India]. New -Zealand white rabbit was immunized with this conjugation. The serum was accumulated from the rabbit in both cases such as before as well after immunization and it was done up to the 6th booster dosage. The final antisera were obtained at 2 and half months.

Antibody titers were obtained by indirect ELISA. 500 pg of strain ALSP per well was applied to study their titer of different dilutions factor of antisera containing antibody of both the batches (1:500, 1:1000, 1:2000, 1:5000, 1:10000, 1:20000, 1:400000 dilutions). To determine the specificity of antibody, the pre-immune sera were diluted very uniformly.

Affinity-based immune-precipitation assay for the native protein purification

$1x10^6$ cells of promastigote and amastigote were resuspended in 1x PBS (PH-7.4) solution and for incubation the cells were kept at 4 ^{0}C for 10 minutes. Followed by rapid freezing – thawing technique and after centrifugation at 200 g and 100 g the supernatant was collected in both the cases promastigote and amastigote. Then the collected supernatants were used for immunoprecipitation with the pre immune sera and antisera of New Zealand White rabbit. The immunoprecipitation was carried out with Protein A- Sepharose bead, which were added to the pre-immune sera and antisera in a 1:2 volume ratio and incubated at 4 ^{0}C overnight with gentle inversion rotation. Unbounded pre-immune sera and antisera were washed out by

using of 1xPBS (PH-7.4). This process was done for repeatedly three times at room temperature and then centrifuged at 200 g for promastigote and incase of amastigote centrifuged at 100 g. The soluble supernatants of both forms were added to the specific antibody loaded Protein A- sepharose column and incubated overnight at 4 ^{0}C with gentle inversion rotation. The unbound protein was removed similarly like before. The bound protein with antisera and pre-immune sera through Protein A- sepharose bead in both forms was separated by boiling with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 15 min at 100 ^{0}C and collected by centrifugation. The elutes were analyzed by immunoblotting after incubation with the same anti-ALSP polyclonal antibody.

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight)

The protein dialysate was subjected to MALDI-TOF to detects its molecular weight. The solubilized protein was added on a target MALDI plate matrix using α-cyano-hydroxycinnamic acid (CHCA). This was examined by MALDI-TOF mass spectrometer (Applied Biosystems, USA). The resultant spectra was visualized. The central instrumentation facility at Council of Scientific & Industrial Research-Indian Institute of Chemical Biology (CSIR-IICB) Kolkata was used for this study.

Determination of native protein through sequencing

The MALDI-TOF-MS/MS analysis was done using trypsin-digested ALSP to elaborate its sequence (100 ppm).

Two-step reverse transcription PCR

ALSP transcripts in both forms of *L. donovani* was examined using isolated whole-cell RNA with TRI Reagent (Sigma T9424). RNA was reversed transcribed to cDNA at 42 °C for 60 min. Forward and reverse ALSP gene-specific primers were used. Polymerase Chain Reaction (PCR) was used to detect the amplified expression of ALSP.

Fluorescence microscopy

L. donovani digenic forms were obtained by centrifugation (4000g for promastigotes and 100 g for amastigotes). The pellets were washed twice with phosphate buffer saline. After fixation with chilled methanol for 2 min, the cells were treated with permeabilization buffer for 30 sec-1 min. The pellets were rewashed with phosphate buffer saline and conjugated with

preimmune sera and immune sera targeted against ALSP (1:25 dilution) in the presence of blocking agent 3% bovine serum albumin for 45 min. The cells were assessed with fluorescein isothiocyanate (FITC)-conjugated goat-derived IgG (Thermo Fisher Scientific, Waltham) (at 1:500 dilution) for 30 min. Cells were viewed after cover slipping with mounting media plus DAPI and viewed with a fluorescence microscope (Zeiss, UK) at 10×. Pre-immune sera incubation was used as negative controls. Appropriate optical filters were used for 4′,6-diamidino-2-phenylindole (DAPI) (λ = 461 nm) and Fluorescein isothiocyanate (FITC) (λ = 591 nm) respectively.

Localization of ALSP in Leishmania major

The promastigotes of *L. major* were cultivated in a T-25 flask supplemented with M199 media plus 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at 25 ^{0}C. On the other hand, amastigotes of *L. major* were grown in a T-75 flask in RPMI media at 37 ^{0}C in 5% CO_2. The same protocol for microscopy was followed here as was done for *L. donovani*.

RESULTS

Prediction of 3D structure and function of ALSP and evaluation of the Model by PROCHECK and ProQ

Figure 1.a showed the Helix (H), Coil (C) and Strand (S) regions of the predicted secondary structure of ALSP and their solvent accessibility.

The 3D structure of the ALSP protein was projected by homology modeling with its amino acid sequence on the I-TASSER. The range of the C-score is -5 to 2, where a model is highly significant with a magnificent value of c and conversely. C-score of top 5 models were -3.58, -4.36, -4.77, -4.56, -4.95 respectively. Model 1 contains a higher C-score, as shown here (**Figure 1.b**).

The function of ALSP was determined *in-silico* by COFACTOR and COACH softwares, derived from the I-TASSER projection. The likely function of ALSP was triglyceride lipase, (GO: 0004806). The biological process is associated with cytosolic lipid metabolism (GO: 0016042).

The lipase enzyme active site of the ALSP may be GAS amino acid' sequence (**Figure 1.c**).

Confirmatory study of the ALSP 3D structure was done by construction of Ramachandran

plots through PROCHECK. The result revealed that residues of the ALSP model fall within 79.1% and 19.4% in the most favored and additional allowed regions independently, and there was only 1.5% of residues that fall within disallowed regions (**Figure 1.d**). In broad-spectrum, a score of about 100% indicates the best stereochemical quality of the model (Reddy et al., 2006). Consequently, the PROCHECK result proposes that the predicted model quality of ALSP is satisfactory. The predicted 3D structure quality was also checked via ProQ and it is evident that the predicted LG score was 2.193, which is greater than 1.5. As per the ProQ database, if the LG score of a model is than 1.5, then it is a fairly good model.

The antigenicity of ALSP was 0.3821 and 0.4294, calculated by using VaxJen and AntigenPro software.

Pilot study of In-vitro functional analysis of ALSP through Lipase assay

The amino acid sequence of ALSP was examined with the I-TASSER software for predicting its probable rôle (**Figure 2.a**). Based on the *in-silico* data, the function of ALSP was examined through *in-vitro* analysis by Lipase assay (**Figure 2.b**). Oleic Acid was used as a standard solution. ALSP produced glycerol in *L. donovani* amastigote, while the promastigotes almost did not show any such activity. 2.41 mg/ml crude cell lysate from amastigotes produced 11.82 µM/mL glycerol. The enzyme activity was calculated for ALSP in the amastigotes form, and it was estimated to be 48.96 U/µg (**Figure 2.c**).

Overexpression of the leishmanial complete gene (CBZ37742.1) of ALSP and confirmation

The ligated product of pGEX-4T-2 vector and ALSP gene was transferred on LB-AMP plate with DH5α competent cells (**Figure 3.a**). Thereafter, the cloning of the ALSP gene was confirmed through colony PCR, restriction digestion, and sequence analysis. The colony PCR with colony 1 and colony 2 visualized a sharp band for each near 255bp (**Figure 3.b**). Consequently, those two colonies were used for restriction digestion with *EcoRI* and *XhoI* restriction enzymes, and the restriction digestion result was exhibited an intense band close by 255bp and 4970bp for the ALSP gene and pGEX-4T2 vector in respect of 1kb DNA Marker. The chromatogram confirmed the cloning of ALSP in the pGEX-4T2 vector with 89% identity (**Figure 3.c & 3.d**) in respect to the *L. donovani* database. The target gene was expressed in *E. coli* BL21 strain and used for protein purification in abundant quantity.

Production of antiserum against epitopic ALSP sequence

The highly probable antigen from KLH conjugated ALSP was used for immunization of NZ

White Rabbit. Antibody concentrations were calculated. Affinity purification of antisera was performed by indirect ELISA. Both batches demonstrated a diminishing titer upon and increasing dilution. The concentration of pre-immune sera remained insignificant in comparison with the immunized batches of animals, which implied that the developed antisera was complementary against the antigen. The Batch 1 showed higher titer than Batch 2 (**Figure 4.a**). Batch 1 was used to purify ALSP-antibody through true affinity purification (**Figure 4.b**).

The specificity was checked using Indirect ELISA (**Figure 4.a and 4.b**) and Immunoprecipitation (IP) (**Figure 4.e**). The immunoprecipitation was obtained with purified induced and uninduced ALSP-GST fusion protein. This was pulled down with antisera and preimmune sera of a NZ White Rabbit, using primary antibody and anti-rabbit-HRP (Horse Radish Peroxidase) conjugate secondary antibody. A band was seen at 35kDa for purified ALSP-GST fusion protein (**Figure 4.e**, Lane 4). **Figure 4.c and 4.d** represented the purification and confirmatory data of ALSP-GST fusion protein.

Digenic Expression of ALSP gene and protein in Leishmania donovani

The gene transcription of ALSP in the promastigote and amastigotes of *L. donovani* were analyzed by reverse transcription PCR. GAPDH gene (496bp) was used as a positive control (**Figure 5.a**, L2). PCR for the ALSP gene revealed a band at 255 bp (**Figure 5.A, L4**) for the amastigotes. No band was seen for the lysate derived from the promastigotes (**Figure 5.A, L3**). This demonstrated that the ALSP mRNA expression was exclusively in the amastigotes.

Fluorescence microscopy revealed the expression of ALSP protein in amastigotes but not in promastigotes. (Figure 5a and b).

Digenic expression of ALSP in L. major amastigotes and promastigotes forms

The transcript level expression of the ALSP gene was checked in both structural forms of *L. major* to confirm its stage specificity. It was done through RTPCR, using complimentary DNA acquired from the RNA of both the vertebrate and invertebrate forms of *L. major* with respect to a positive control (GAPDH, 496bp). The PCR amplified products of ALSP with the gene-specific primers were ran on a 1% agarose gel, which exhibited no band for amastigotes and promastigotes (**Figure 6.a**, L5 & L6) of *L. major*, but the amastigotes of *L. donovani* showed a clear band about 255bp (**Figure 6.b**, L4) for ALSP.

Fluorescence microscopy did not show expression of ALSP in either promastigote or

amastigote of *L. major* (**Figure 6.b**).

Purification and sequence analysis of ALSP from Leishmania donovani amastigotes

The antisera against ALSP was used to purify the native protein from the amastigotes' lysate. Both the antisera and preimmune sera were cross- linked with protein A-Sepharose bead (Invitrogen-101041). The purification was performed with another negative control, albumin-like protein. Both the negative controls could not detect the protein. When probed, the lysate of amastigotes reveals a signal at 10 kDa (Figure 7.a). The precise molecular weight obtained by MALDI-TOF MS was 10.147 kDa (Figure 7.b). MALDI-TOF-MS-MS analysis showed the sequence, using the peptide fragments of the trypsin-digested protein (from amastigotes) (Figure 7.c).

DISCUSSION

The results of the current study preliminary suggest that a novel protozoan parasitic protein, amastin-like surface protein (ALSP), act as a lipase enzyme. The function of the protein was predicted through *in-silico* analysis by I-TASSER. The in-vitro analysis with lipase assay showed it produced glycerol in amastigotes. Further, to characterize the protein, it was cloned in a bacterial system and a polyclonal antibody was produced against the 10kDa protein, which was present in *L. donovani* amastigote. Immunofluorescence show the expression of amastin-like surface protein in amastigotes but not in the promastigotes. The protein was unexpressed in *L. major*.

While evaluating the function of our novel protein of interest amastin-like surface protein (ALSP), the I-TASSER software highly predicted the role of the putative protein as a triacylglycerol lipase. Closer examination of the peptide sequence shows this protein as serine-rich, the signature of Class III serine lipases belonging to numerous *Leishmania* species. In our preliminary experiments, we could demonstrate the lipase activity of the amastin-like surface protein. This protein did have the spaced trypsin like catalytic triad, Ser-His-Asp (Brady et al., 1990), though we did not have the scope to examine its secondary structure in details. Closer examination of the peptide sequence also did not reveal the commonly described sequence of glycine-x1-serine-x2-glycine (x usually is histidine or tyrosine), the common catalytic sequence present in a wide variety of lipases. However, a GAS domain was present in ALSP. GHS domains were present in eleven other putative lipases of *Leishmania donovani* reported in the GenDB database (data not shown). The

amastin-like surface protein (ALSP) does have serine richness and also have histidine and glycine distribution, as well as terminal serines characteristic of lipases.

Lipases are an important class of extracellularly secreted enzymes, contributing to the exoproteome of multiple *Leishmania* species. Earlier, such a lipase was examined in *Leishmania* major [Freidlin] (Shakarian et al., 2010). In the current study, either the script or the mature ALSP protein in *L. major* was not observed. Our cross-examination of multiple lipases from *L. donovani* from the GenDB database did not show major overlap and sequence alignment with ALSP, likely supporting our claim that ALSP is a novel lipase of *L. donovani*. The GenDB database additionally shows lipases present in multiple other species of *Leishmania* and other kinetoplastids including multiple species of *Trypanosoma*. These extracellularly secreted lipases are also present in many fungi, as well prokaryotic organisms like *Yersinia, Pseudomonas cepacia* and *Helicobacter pylori* (Slomiany and Slomiany., 1992; Konig et al., 1996; Straus et al., 1992).

What it means to be a functional lipase in *Leishmania* is far from clear but there is few precedence as to the role of the lipases. These are simple hydrolases generating glycerol and fatty acids, which are important substrate for metabolic processes. Furthermore, these are important membrane components and lysis of membrane may be one of first major steps in insinuation of a deflagellated promastigote into the host phagolysosome to form an amastigote to accomplish its digenic lifecycle. This however may not be the role for ALSP, as ALSP expression was seen minimal to non-existent in the promastigote form in the present study. A previous preliminary study also could not detect any low molecular protein below 20 kDa in *L. donovani* promastigotes (Mitra, 2015) Earlier though it was reported that the lipase *LdLip3* was present in both the promastigote and the amastigote forms of *L. major* (Shakarian et al., 2010), where it might contribute to vertebrate host cell entry, as well as contribute to virulence mechanisms like membrane remodelling of phagolysosome.

Parasites rely on a complex system of uptake and synthesis mechanisms to satisfy their lipid needs. Parasites like *Leishmania* have adopted complex mechanisms of host lipid harvesting (Hart and Coombs 1982). The glycerol from hydrolysed lipid is transported across to the parasite from the host cell through aquaporin AQP1-like aquaglyceroporin channels, contributing to the complex energy metabolism of the parasite (Frezard et al., 2014). Glycerol-transport proteins have been predicted to be present in *L. donovani* (Ranjan et al., 2020). Fatty acids derives from the host are transported back to the parasite by complex mechanisms (Berman et al., 1987, Ramakrishnan et al., 2013). Apart from supporting parasite

energy generation, the fatty acids may be importantly involved in neo synthesis of special membrane lipids like phosphorylceramide (Bouazizi-Ben Messaoud et al., 2017). Recently, a preliminary study has demonstrated a role for albumin-like protein in uptake of fatty acids by the intracellular form of *L. donovani* (Laha et al., 2019). Importantly, these channel proteins are significantly targeted by antimonials, one of the most important chemotherapeutic avenues for treating advanced leishmanial diseases.

Though chemotherapy options like antimonials, amphotericin B and miltefosine remain the main stay for treating visceral leishmaniasis, emerging drug resistance, non-availability of drugs and toxicity remain major obstacles in obtaining complete remission and significantly contributes to sustained mortality of visceral leishmaniasis. Thus, attempts for developing vaccines have been aimed.

ALSP does not have KDEL sequence, making it unlikely to dock to a cell membrane or an organelle membrane and shall function as an extracellularly secreted lipase. Normally, lipases are relatively bulky protein with approximate molecular weight of 60 kDa (Cygler et al., 1993). The predicted molecular weight of the putative lipases of *L .donovani* were around 70 kDa (As seen in UniProt). *LdLip3* molecular weight is 33kDa. Recombinant *Leishmania* antigens (single peptides/polypeptides) were used to produce second-generation vaccines. Among different trials, promising results in phase I were shown by GLA-SE adjuvant tagged LEISH-F3, a multicomponent vaccine, to produce immune response in healthy subjects (Coler et al., 2015). The molecular weight examination of ALSP revealed a rather relatively small protein of 10kDa. This makes ALSP an important target as a peptide vaccine component. Preliminary examination with VaxiJen v2.0 and ANTIGENPro shows the immunoinformatic feasibility of eliciting cell-based immunity against this peptide target. Our cloning studies reveal the potential of generating the ALSP protein in large quantities in vitro. This remains our goals for future studies. Cumulatively, the results of the present study show that the novel 10kDa ALSP supports *Leishmania donovani* parasitism by its lipolytic activity in the amastigote form.

Because *Leishmania* are obligate parasites, they must procure macromolecules like glycerol and fatty acids to facilitate their opportunistic survival. Previous studies have shown the preference of amastigotes in using fatty acids as their carbon source via beta oxidation, apart from utilizing glucose and proline (Tielens and van Hellemond., 2009; McConville et al., 2007, Berman., 1987; Hart and Coombs., 1982;). *LdLip3* was shown to be active in both promastigote and amastigote of *L. donovani* (Shakarian et al., 2010), whereas ALSP activity

was negligible in promastigotes of *L. donovani*. ALSP may facilitate the survival of the parasite by remodelling of the phagolysosome membrane and may induce tissue inflammation when secreted extracellularly in the human host. Earlier studies have shown that lipase precursor-like protein confers the oral drug alkylphosphocholine miltefosine (but not sodium antimony gluconate and amphotericin B) resistance in *L. donovani* by enhancing macrophage infectivity and increasing IL-10/TNFα ratio (Deep et al., 2018). The solitary expression of ALSP in amastigotes rather than promastigotes is an exceptional phenomenon, as only a percentage of proteins are differentially expressed between these morphological forms. For most part, all proteins are constitutively expressed in both promastigotes and amastigotes, making it non-selective for the parasite to survive in the invertebrate and human host (Leifso et al., 2007; Ranjan et al., 2020). Additionally, bioinformatics prediction and the current study indicates differential expression of ALSP in different species of *Leishmania* (*donovani vs. major*), which also is an intriguing observation.

Surprisingly, there is significant overlap between diverse lipases and esterase enzymes acetylcholine esterase, carboxylesterase and cholesterol esterase (Cygler et al., 1993). Though the geometry of the active size varies, as also seen with ALSP, there is overlap between these classes of enzymes. The nomenclature of amastin-like surface protein (ALSP) does not suggest similarity with amastin proteins, a significant class of surface proteins widely expressed in all leishmanial species, *Trypanosoma brucei*, responsible for sleeping sickness and *Trypanosoma cruzi*, the inducer of Chagas disease. Preliminary sequence alignment did not show overlap with alpha, beta, gamma or delta amastins (data not shown). Though amastin proteins are such widely distributed, detailed investigation into the function of amastins are only scant. Secondary structure comparison of amastins has shown its similarity to claudins, important cell-cell junction proteins (Jackson. 2009). Preliminary reports suggest that the amastigote form of *T. cruzi* develop cell synapses before the insinuation in the secondary host cell (Bonfim-Melo et al., 2018). Whether ALSP performs such roles remains unresolved in the current study. Intriguingly, lipases are related to some cell adhesion proteins like *Drosophila* neurotactin (de la Escalera et al., 1990).

Advanced leishmanial disease often present with bleeding diathesis, making it difficult to obtain tissue biopsies for establishing diagnosis. Examination of ALSP in the peripheral blood may aid as a diagnostic tool under such circumstances. Though initial vaccination strategies have not received robust outcomes, it may be appreciated that efforts to develop new vaccines are critical. HIV-leishmanial coinfections are important emerging infections

across the globe in both rural and urban areas. These important theranostic implications elevate the significance of further examining the novel protein ALSP in future studies.

Conflict of interest

The authors declare that there is no conflict of interest regarding scientific or financial matter.

Author Contributions

BB, Performed majority of experiments drafted first version of manuscript; BL, coordinated MALDI-TOF experiments; AC, reviewed literature, interpreted data, drafted introduction and discussion; MG, Conceptualized the study, obtained funding, supervised experiments, coordinated drafting of manuscript.

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Legends

Figure. 1 ***Structure prediction and structure-based function annotation of ALSP through I-TASSER.*** (a) Predicted secondary structure and solvent accessibility of ALSP (b) Predicted best model of ALSP by I-TASSER where C-score is -3.58, estimated TM-score =0.32Â±0.11, and estimated RMSD-score=11.6Â±4.5Ã. (c) Exhibited GAS region of ALSP from its amino acids sequence (d) Validation of ALSP predicted 3D model through Ramachandran plot where 79.1% of ALSP residues fall within the most favoured and 19.4% in the additional allowed regions independently, and only 1.5 % of residues that fall within disallowed regions

Figure. 2 ***Function of ALSP as triglyceride lipase***. (a) The function of ALSP predicted through *in-silico* analysis using its amino acids sequence in I-TASSER online tool. It was

found to act as a lipase enzyme with other possible roles. (b) Depicts a standard curve between different concentration of Oleic Acid and Absorbance of Oleic Acid at 710nm. (c) Lipase activity of ALSP in amastigotes and promastigote forms. It showed 48.96 U/mg enzyme activity for amastigotes and very minimal for promastigotes (0.009 U/mg).

Figure. 3 ***Confirmation of the cloning in a prokaryotic system with transformed DH5α competent cells*** **through colony PCR, restriction digestion, and sequencing analysis.** (2.a) Transferred ligated pGEX-4T-2 vector and Gene of Interest (ALSP) on LB-AMP plate with E.coli DH5α competent cells (2.b) Colony PCR of cloned plasmids of colony 1 (Lane 2) and colony 2 (Lane 3) and diagram of restriction digestion of those cloned plasmids with EcoRI and XhoI restriction enzymes exhibited an intense band near 255 bp for each colony in respect of the 1kb DNA ladder at Lane 1. (2.c and 2.d) Chromatogram of pGEX4T2 vector with the 255bp long ALSP insert and Sequencing analysis of the ALSP gene through Nucleotide BLAST against Leishmanial gene data bank.

Figure 4 ***Evaluation of polyclonal antibody against Leishmania donovani ALSP protein***. (3.a) Binding dynamics of polyclonal antibodies evaluated by indirect ELISA. The titer of preimmune serum is highlighted by the green and blue traces. The binding strengths of antisera in batch 1 and batch 2 are projected by red and black traces respectively. (3.b) Affinity purification of ALSP-antibody from batch 1. (3.c) Purification of ALSP-GST protein on 15% SDS-PAGE. 10-250 kDa molecular mass Marker (Lane 1). Lane 2 shows purified BL21 cells with empty vector; Lane 3 shows purified BL21 cells without Construct; Lane 4, 5 and 6: first, second, and third eluates of ALSP-GST. (3.d) Purified ALSP-GST fusion protein (about 35 kDa) in induced (Lane 2) and uninduced form (Lane 3). Western Blotting was performed with Anti-GST-tagged antibody. (Lane 1). (3.e) Immunoprecipitation of ALSP-GST fusion protein. ALSP-GST in induced and uninduced forms pulled down with antisera/preimmune sera of an NZ White Rabbit by probing customized anti-amastin-like surface protein antibody and anti-rabbit-horse radish peroxidase conjugated secondary antibody. Lane 2 and 3: Antisera/preimmune sera with the uninduced fusion protein; Lane 4 and 5: Antisera/preimmune sera with the purified fusion protein; and Lane 1: 10-250 kDa molecular mass marker.

Figure. 5 ***Digenic expression of ALSP gene and protein in Leishmania donovani promastigotes and amastigotes.*** A. Reverse transcription-based amplicon of ALSP transcripts of *L. donovani*. Lane 1, 1 kb DNA marker; Lane 2, RT-PCR product of total RNA. GAPDH used as a positive control Lane 3 & 4, RT-PCR amplicons of RNA from both forms of the parasites respectively. Lane 4 shows a band at 255 bp, while no band was seen in Lane 3; Lane 2 exhibits a dense band near 496 bp. B. ALSP protein localization in promastigote (a) and amastigote (b), by fluorescence microscopy.

Figure. 6 ***Digenic expression of ALSP gene and protein in Leishmania major promastigotes and amastigotes*****.** A. RT-based enhancement of ALSP transcripts by total RNA acquired from both morphological forms of the parasite *L. major*. Lane 1, 1 kb DNA marker; Lane 2, RT-PCR product of whole-cell RNA of GAPDH, used as a positive control, and Lane 3 & 4, RT-PCR products of whole-cell RNA from both structural forms of *L. donovani* respectively and Lane 5 & Lane 6 contain RT-PCR yields of total-cell RNA of *L. major* in both respective shapes. Lane 4 contains a band near 255 bp; no detection of bands in Lane 3, Lane 5, and Lane 6; Lane 2 displays a clear band nearby 496 bp. B**.** Fluorescence microscopy did not detect ALSP in the promastigotes and amastigotes of *trypa L. major*.

Figure. 7 ***Western blot of ALSP expression and determination of molecular weight of ALSP by MALDI-TOF***. (**a**) Western blotting was carried out with antiserum and anti-rabbit horse radish peroxidase conjugated secondary antibody. Lane 1, molecular mass marker (10-250 kDa); Lane 2, purified protein expressed in promastigotes, used as negative control; Lane 3, ALSP band at 10 kDa in amastigotes; Lane 4, albumin-like protein expressed in amastigotes, used as negative control. (**b**) The M peak at 10.147 kDa revealed the accurate molecular mass of ALSP by MALDI mass spectrometry. (**c**) Sequencing of the purified protein was done by MALDI-TOF-MS-MS analysis after tryptic digestion. The sequencing data of ALSP after MALDI-TOF-MS-MS analysis showed 100% similarity with leishmanial amastin-like surface protein (ALSP) when uploaded in the NCBI for BLASTn

a

Predicted Secondary Structure

```
                    20                  40                  60                  80
                     |                   |                   |                   |
Sequence    MHMRVLVRACTLSRGSPWLSPIPHSPPSALLSSSVAPSRLPCGSLCCAPPLHLSQHFGARFRRWGASSPFSSTRSSSSSRSFPC
Prediction  CCSSSSSSSSSCCCCCCCCCCCCCCCCHHHHHHCCCCCCCCCCCCCCCCCCCCHHHHHHHHHHHHHHCCCCCCCCCCCCCCCCCCCC
Conf.Score  9424314321104688565668999827776334474558766511068235888770787731368986544456555667 89
            H:Helix; S:Strand; C:Coil
```

Predicted Solvent Accessibility

```
                    20                  40                  60                  80
                     |                   |                   |                   |
Sequence    MHMRVLVRACTLSRGSPWLSPIPHSPPSALLSSSVAPSRLPCGSLCCAPPLHLSQHFGARFRRWGASSPFSSTRSSSSSRSFPC
Prediction  7423211321324444422441444334322444234442313320133333135313431442347442444454555462 48
            Values range from 0 (buried residue) to 9 (highly exposed residue)
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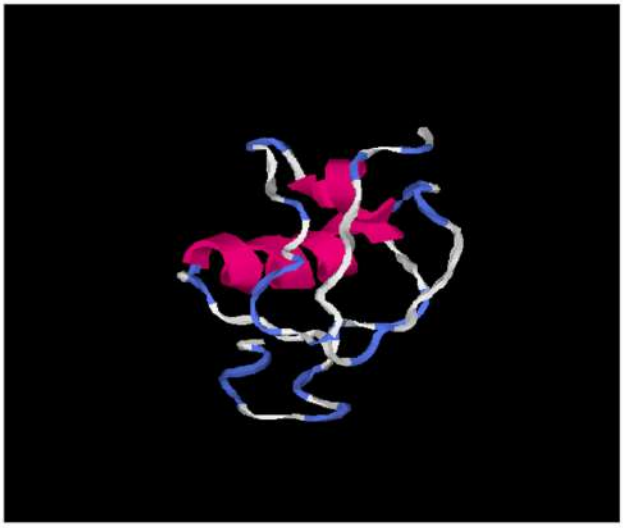
b



c

```
>tr|E9BR63|E9BR63_LEIDB Amastin-
like surface protein, putative
OS=Leishmania donovani (strain
BPK282A1) OX=981087
GN=LDBPK_342650 PE=4 SV=1
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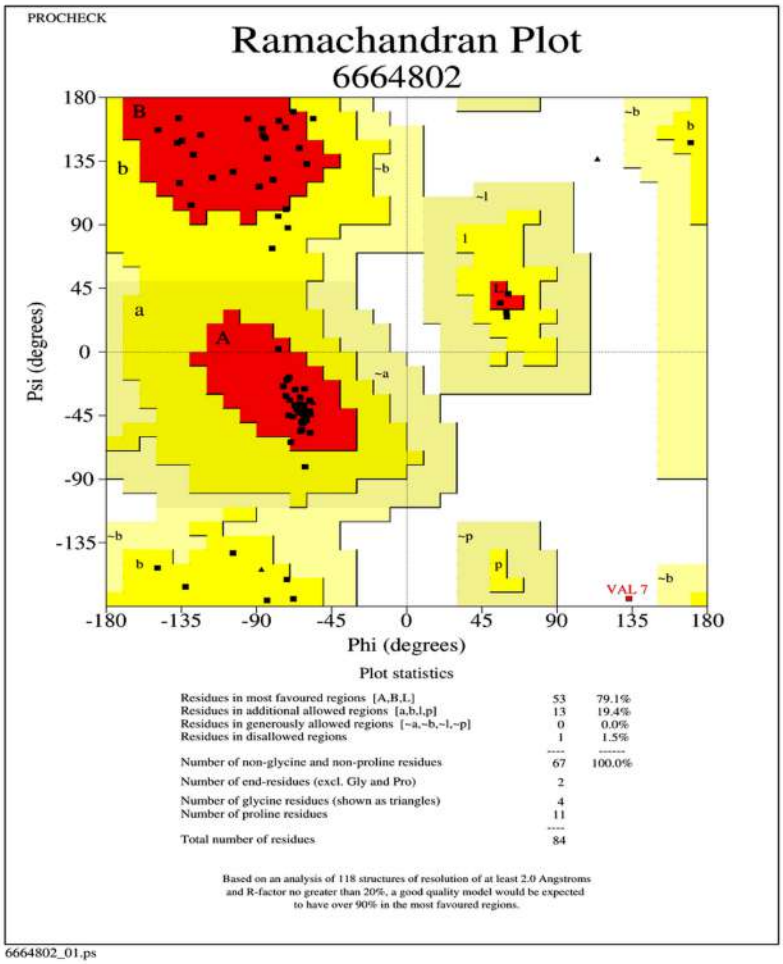
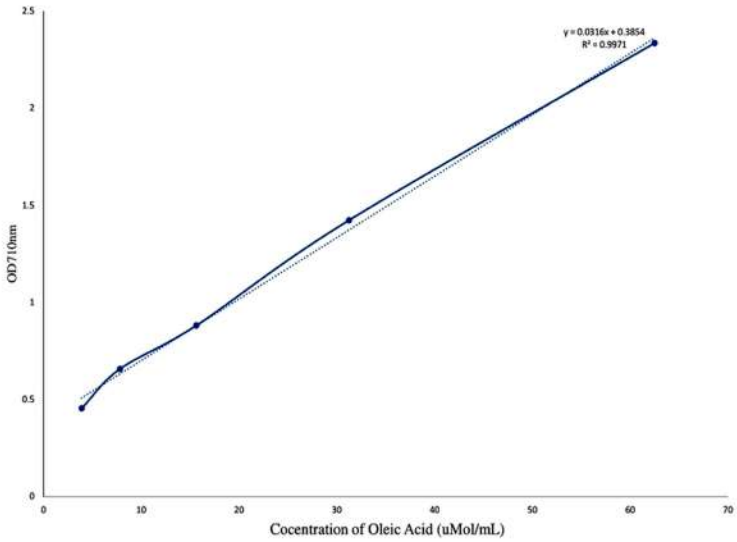


a

Consensus prediction of GO terms

Molecular Function	GO:0004806	GO:0008810	GO:0016162	GO:0004674	GO:0019900	GO:0009055	GO:0005506	GO:0016903	GO:0051539	GO:0030976
GO-Score	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.07
	triglyceride lipase activity	cellulase activity	cellulose 1,4-beta-cellobiosidase activity	protein serine/threonine kinase activity	kinase binding	electron transfer activity	iron ion binding	Oxidoreductase activity	4 iron, 4 sulfur cluster binding	thiamine pyrophosphate binding

b



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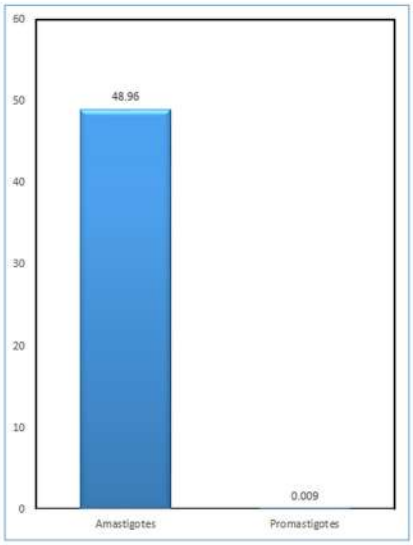

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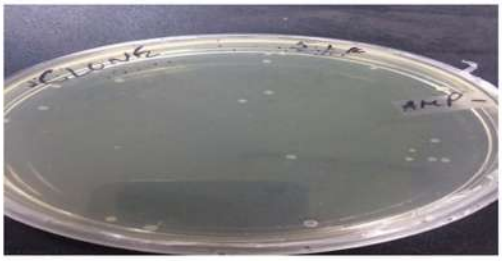

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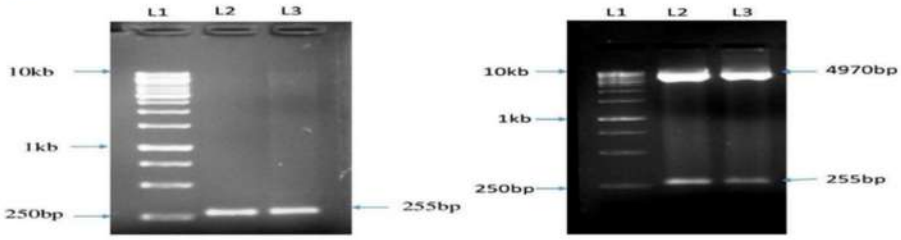

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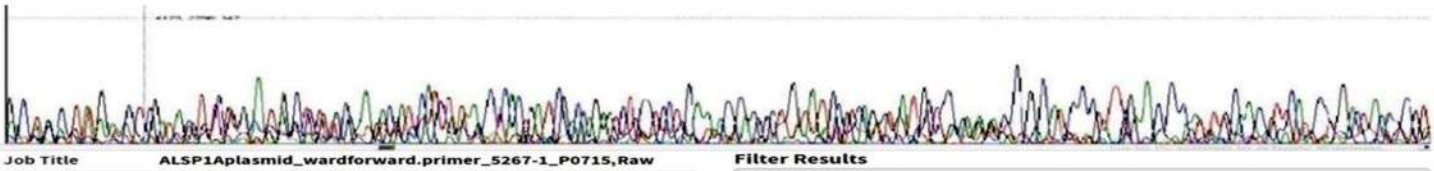

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Filter Results

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Leishmania donovani amastin-like surface protein, putative (LDBPK_342660), partial mRNA	292	292	27%	2e-74	89.12%	XM_003864377.1

d

Leishmania donovani amastin-like surface protein, putative (LDBPK_342660), partial mRNA

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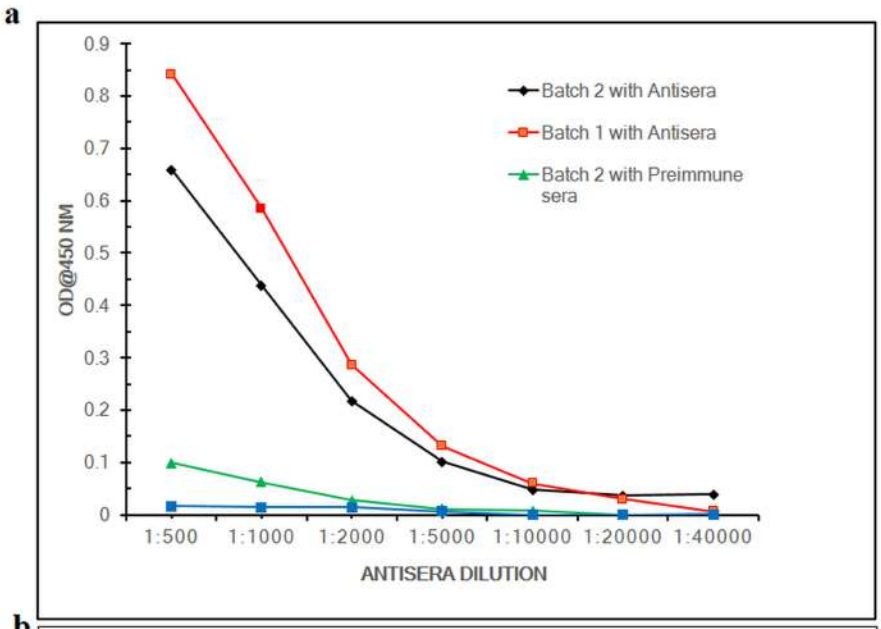
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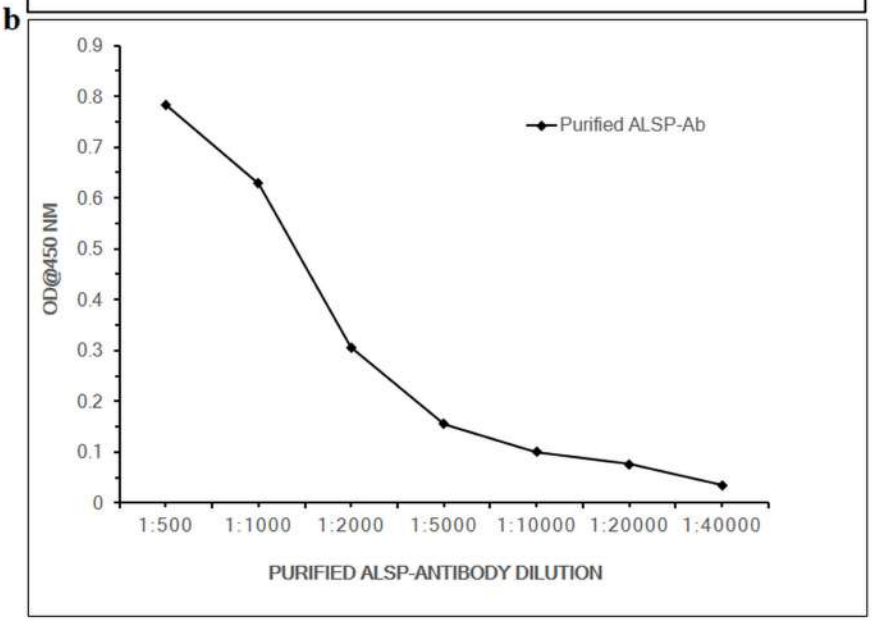
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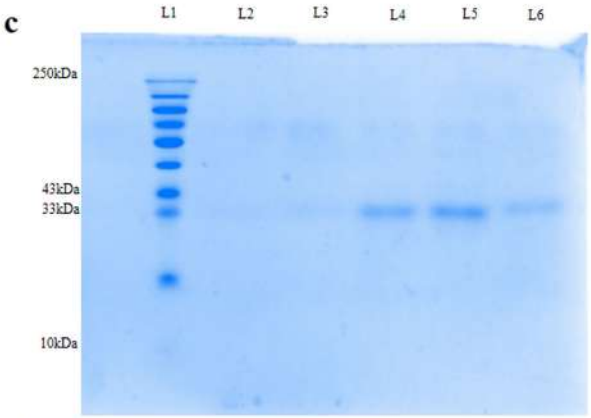
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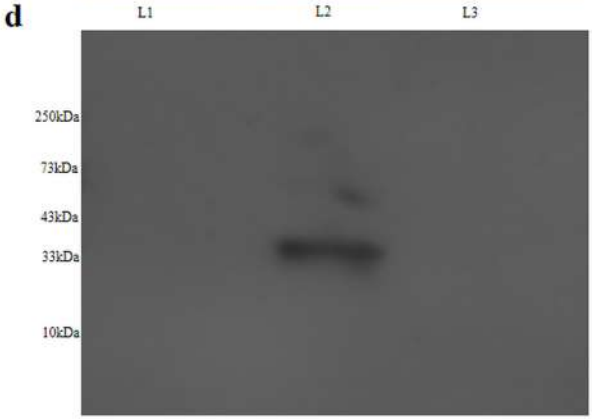
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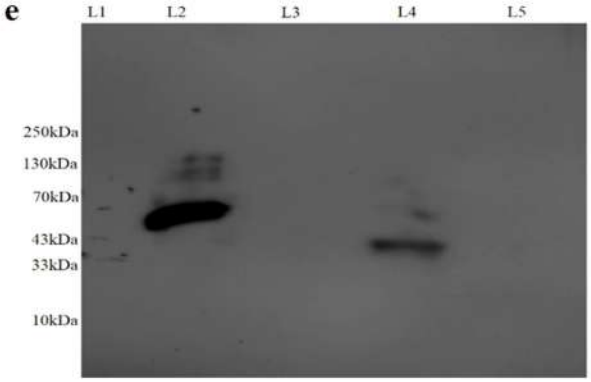
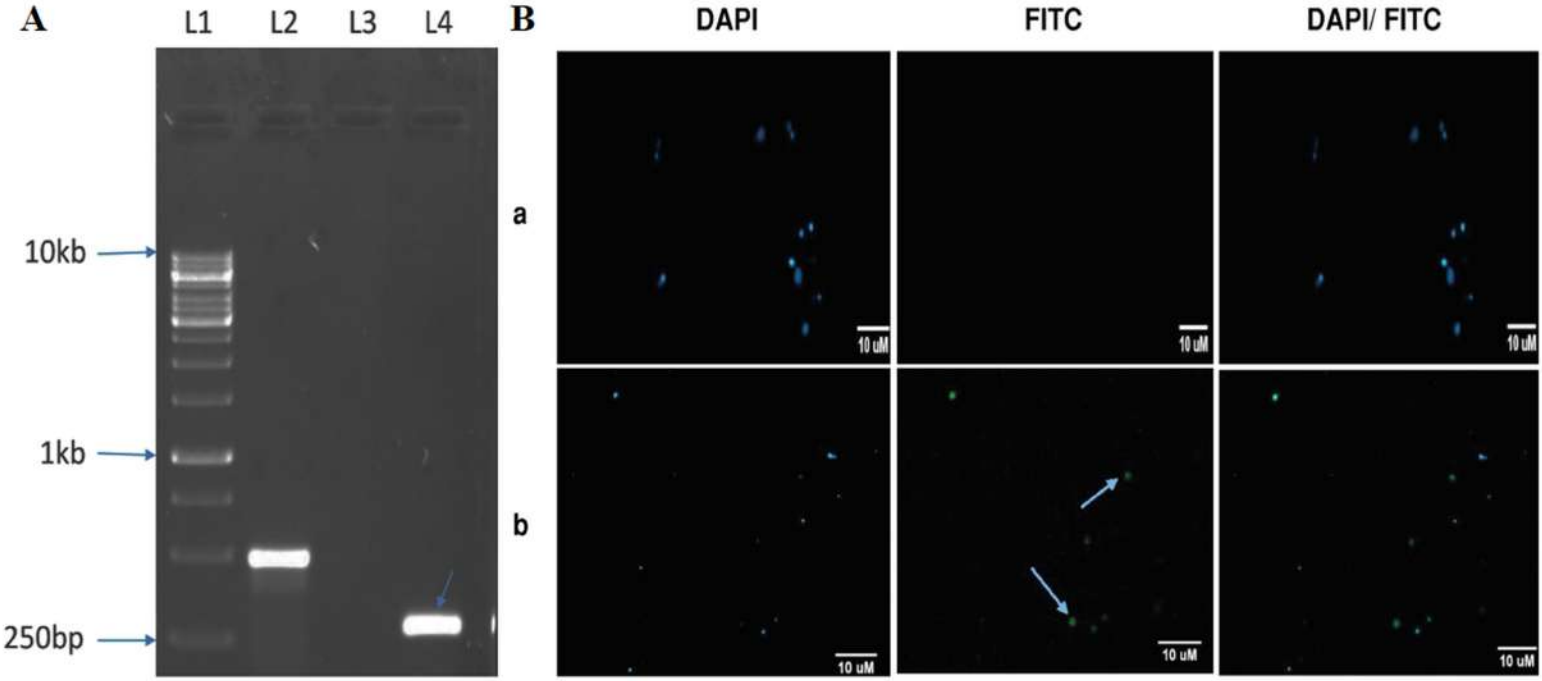
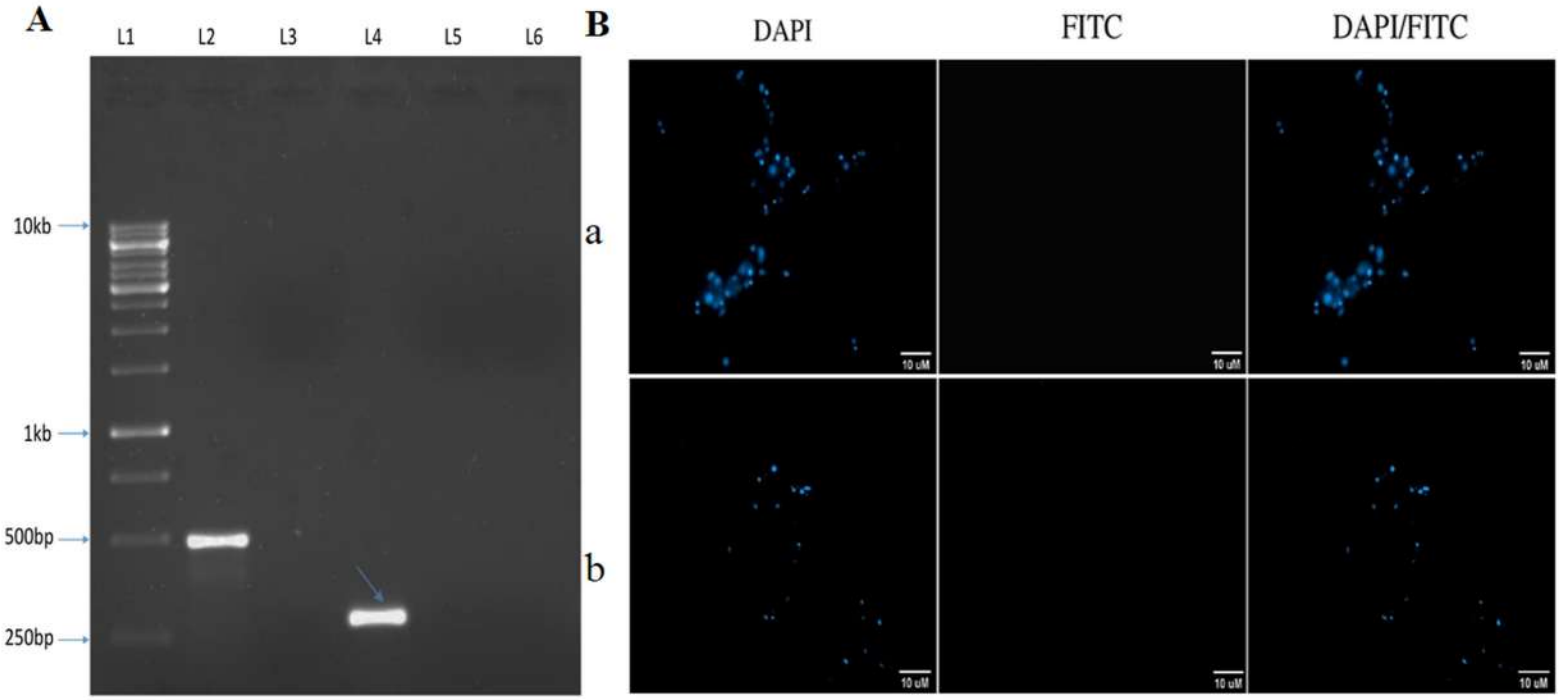
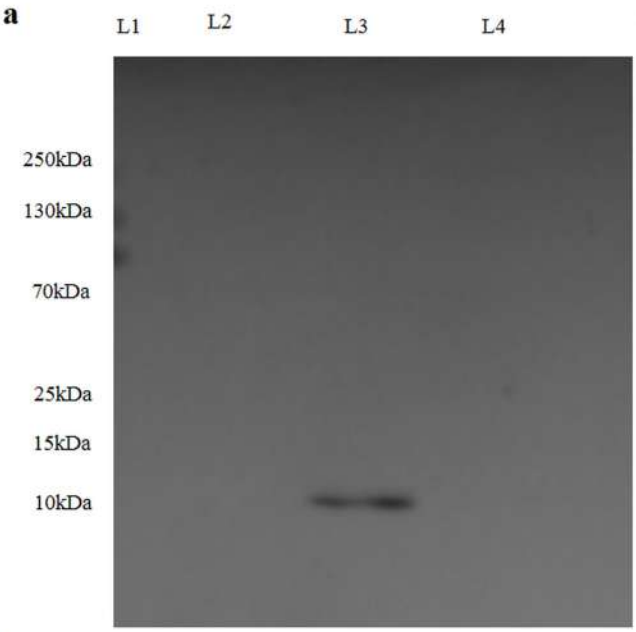
10kDa


e
L1
L2
L3
L4
L5
250kDa
130kDa
70kDa
43kDa
33kDa
10kDa

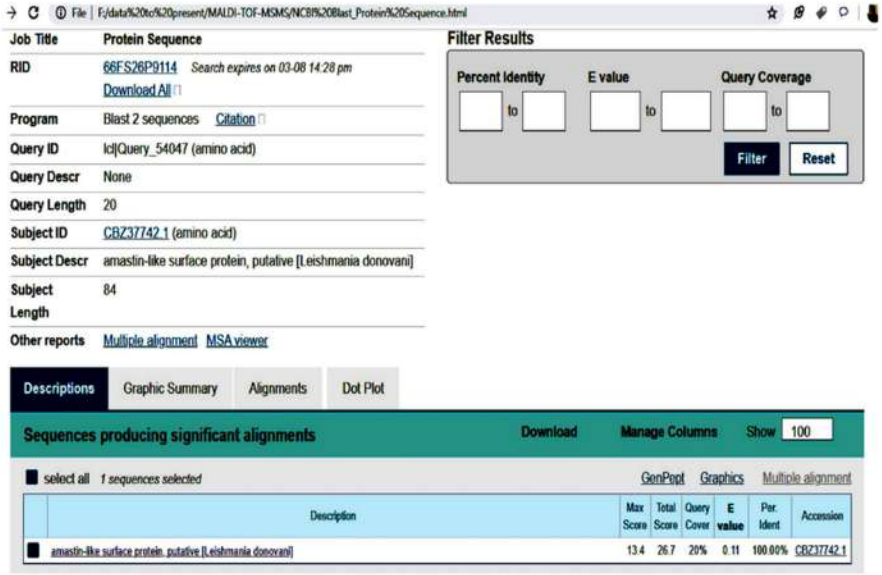

A
L1
L2
L3
L4
10kb
1kb
250bp
B
DAPI
FITC
DAPI/ FITC
a
b
10 uM


A
L1
L2
L3
L4
L5
L6
10kb
1kb
500bp
250bp
B
DAPI
FITC
DAPI/FITC
a
b
10 uM

a



c



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Job Title	Protein Sequence
RID	66FS26P9114 *Search expires on 03-08 14:28 pm* Download All
Program	Blast 2 sequences Citation
Query ID	lcl|Query_54047 (amino acid)
Query Descr	None
Query Length	20
Subject ID	CBZ37742.1 (amino acid)
Subject Descr	amastin-like surface protein, putative [Leishmania donovani]
Subject Length	84
Other reports	Multiple alignment MSA viewer

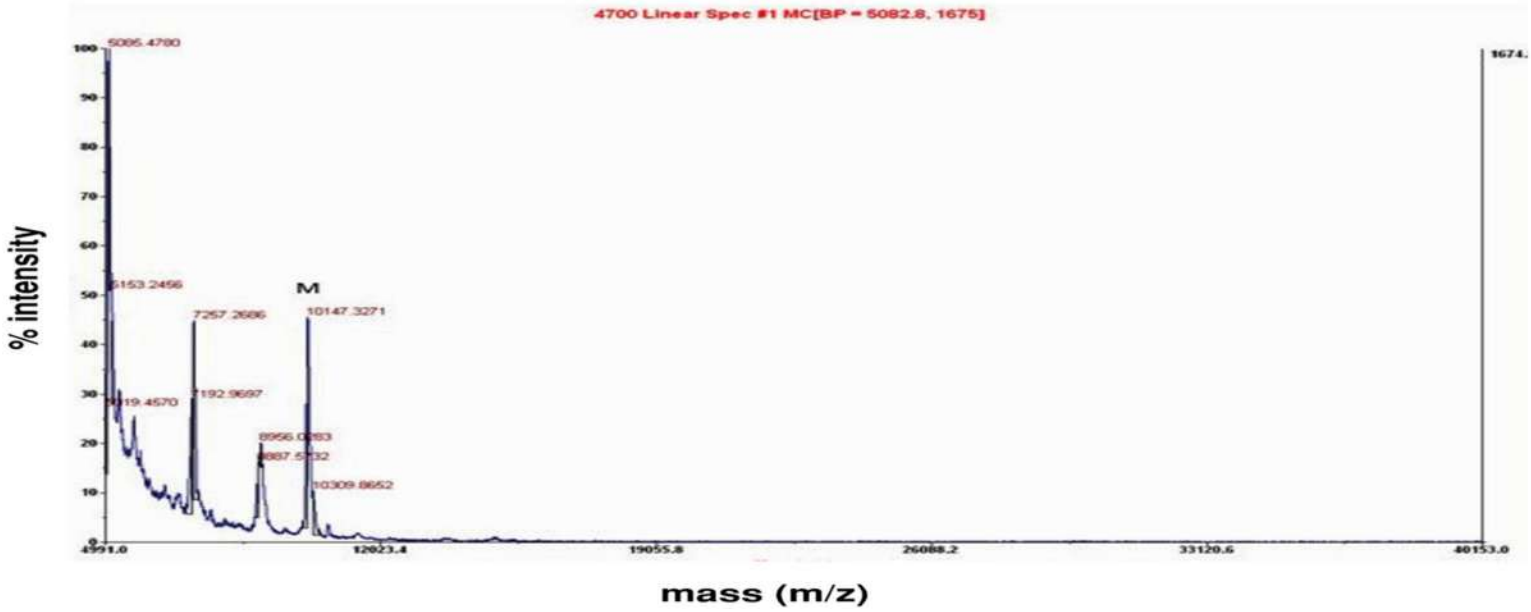
Filter Results

Percent Identity [ ] to [ ] E value [ ] to [ ] Query Coverage [ ] to [ ] Filter Reset

Descriptions | Graphic Summary | Alignments | Dot Plot

Sequences producing significant alignments — Download — Manage Columns — Show 100

select all *1 sequences selected* — GenPept Graphics Multiple alignment

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
amastin-like surface protein, putative [Leishmania donovani]	13.4	26.7	20%	0.11	100.00%	CBZ37742.1

b