Summary

Diseases produced by protozoan parasites are one of the main cause of morbidity and mortality around the world, affecting millions of people. Leishmaniasis is a disease complex caused by the species of haemoflagellate protozoan parasites belonging to the genus Leishmania. This disease ranges in severity from skin lesions to serious disfigurement and fatal systemic infection. WHO has classified the disease as emerging and uncontrolled and estimates that the infection results in 2 million new cases a year. There are 12 million people currently infected worldwide, and disease threatens 350 million people in 88 countries. Current treatment is based on chemotherapy, which relies on hand full of drug with serious limitations such as high cost, toxicity, difficult route of administration and lack of efficacy in endemic areas. Till date no vaccine is available.

There are two major forms of this disease are reported, visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) as well as various other cutaneous manifestations include mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and Post kala-azar dermal leishmaniasis (PKDL).

Visceral Leishmaniasis (VL) is a serious health problem in eastern part of India i.e. almost all districts of Bihar, four districts of Jharkhand, five districts of Uttar Pradesh and ten districts of West Bengal. Standard drugs against VL are being compromised. Therefore there is urgent need to develop new line of drug and drug targets. The pentavalent antimonials such as meglumine antimoniate and sodium stibogluconate have been recommended for the treatment of leishmaniasis for over 70 years. The emergence of drug resistance against these drugs has detoriated the situation and there is no prevention measures are available till date. Miltefosine is the first recognized oral treatment for leishmaniasis, but resistance to miltefosine has also been observed. Unfortunately very little information is available on biochemical and molecular mechanism of emergence of resistance against standard drugs. Therefore there is urgent need to develop a new class of drug against leishmaniasis.

The unusual localization of glycolytic enzymes in glycosomes of kinetoplastida supposedly endows them with specific structural features, and the crucial importance of glycolysis for the parasites energy supply makes the glycosome and its constituting proteins ideal targets for specific drugs against leishmaniasis. In the present thesis the triosephosphate isomerase (TIM) enzyme of glycolytic pathway has been studied. Triosephosphate isomerase is an enzyme of glycolytic pathway which interconverts glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Till date there have been no reports of cloning, overexpression and characterization of TIM from *Leishmania donovani.* Keeping in view the importance of triosephosphate isomerase for trypanosomatids, efforts have been made to characterize TIM from *L. donovani.*

The complete ORF of 753 bp TIM gene was PCR amplified from the *L. donovani* (Dd8) genomic DNA and cloned in pGEMT easy cloning vector. Recombinant clone was sequenced in both direction using T7 forward and SP6 reverse primers to confirm the sequence of amplicon. The nucleotide sequence of LdTIM has been deposited in GenBank under Accession No. DQ649411. The DNA sequence for coding region was found enriched in G+C residues (61.9%) in comparison with A+ T residues (38.1%). Sequence analysis showed that the amino acid of *L. donovani* TIM was highly homologous to *L. major* TIM with 93 % identity. It exhibited 92 % and 88.10 % identity with *L.infantum* and *L.mexicana* respectively. The signature sequence AYEPVWAIGTG (166-176) as well as residues involved in catalytic mechanism i.e lysine 13, asparagine 12, glutamic acid 168 and histidine 96 were found conserved. Potent differences were observed in LdTIM amino acid sequence as compared with HTIM. The LdTIM has cysteine residue at position 15 and glutamate at position 66 while the corresponding residues in HTIM are methionine and glutamine respectively. These may be targeted to find agents that selectively inhibit the parasite enzyme.

Leishmania proteins are generally insoluble in nature and tend to form aggregate and appears as an inclusion bodies upon expression in prokaryotic hosts. In practice, it is worthwhile to test several different vector/host combinations to obtain the best possible yield of protein in its functional form. The LdTIM ORF was subcloned in pET-28(a) expression

vector and conditions were optimized for the high expression of active LdTIM in *E. coli*. The protein was purified using Ni-NTA and traditional methods using gel filtration followed by ion exchange chromatography. Approximately 7 µg purified LdTIM was obtained by Ni-NTA chromatography with specific activity of 5090 units/mg. Further, several attempts were made to increase the amount of protein but the desired protein failed to bind with the Ni-NTA column, suggesting that the His-tag was not free for the matrix. It may be possible that protein was buried inside during the folding process hence the classical method of gel filtration (Sephadex G- 100) and ion exchange chromatography (Q- Sepharose) was followed. The recombinant LdTIM has 27.6 kDa in size, dimeric in nature and found functional. The specific activity of purified LdTIM was 8.7 x 10^3 µm/min/mg. The yield of recombinant LdTIM was ~ 17.5 mg/L of *E. coli* culture. Biochemical studies revealed that the K_m and V_{max} for the substrate glyceraldehyde 3- phopshate were 328 µM and 2011 µM/ min. The optimum pH of LdTIM is ranging from pH 7.2-9.0 and temperature of 25 °C. LdTIM was found sensitive towards urea and GdmCl as manifested by loss of enzyme activity. The protein looses 75 % and 85 % activity at the concentration of 2 M solution of urea and GdmCl respectively and the complete loss of enzyme activity was observed at 3 M concentration of urea and GdmCl. The effect of increasing concentrations of SDS on the enzymatic activity of LdTIM showed that the enzyme looses 6.26 % and 83.89 % activity at concentration of 0.3 mM and 0.45 mM respectively. However, at concentration of 0.60 mM, a complete loss of enzyme activity was observed.

In order to achieve specific inhibition of LdTIM a cysteine-reactive agent methylmethane thiosulphonate (MMTS) was used as probe, in order to test its effect on enzyme activity and structure. The MMTS inhibited 75% LdTIM enzyme activity within 15 minutes at 250 µM MMTS concentration. Higher concentrations of MMTS or longer preincubation time did not increase the extent of inhibition. LdTIM was found more sensitive to MMTS than reported for LmTIM and TbTIM. MMTS inhibited 80% of LmTIM and 90% of TbTIM activity at concentration of 1000µM and 400µM respectively. In TcTIM 100 % inactivation was achieved with in 15 minutes at 15 µM concentration. These results illustrates that LdTIM exhibit differences to MMTS with markedly similar proteins with identical or similar cysteine

contents. The K_m of LdTIM treated with 1mM MMTS and 3 mM MMTS concentration was 0.34 mM which were slightly different with respect to untreated LdTIM which has K_m of 0.328 mM suggesting MMTS didn't affect the rate of reaction.

The urea induced structural changes in LdTIM was monitored by intrinsic fluorescence. The fluorescence spectra LdTIM at 0 M urea concentration showed a peak at 338 nm indicating that tryptophan molecule(s) are not completely buried in the native protein but partially exposed to the solvent. As the concentration of urea increases from 0 M to 8 M, a shift in wavelength from 338 nm to 354.3 nm were recorded with decrease in fluorescence which shows that urea induces the exposure of the buried tryptophan residues.

The chromatogram of native LdTIM showed a single elution peak at 9.75 ml which corresponds to the molecular weight of ~54 kDa while SDS-PAGE analysis showed only a single band of 27.6 kDa, suggesting that the LdTIM is a homodimer. The LdTIM samples incubated with MMTS exhibited a second peak appearing at larger elution volume i.e 12.5 ml suggesting that MMTS dissociates the LdTIM dimer inducing the formation of a monomer.

The dye ANS has been used as a probe for the detection of non-polar surface patches and "molten globule" intermediates. The fluorescence emission spectrum of ANS was not perturbed in the presence of LdTIM, suggesting that ANS did not bind significantly to the native protein with maximum of the emission curve at 490 nm. On the other hand, increase in ANS fluorescence with shift in wavelength to 500 nm was observed in LdTIM incubated with MMTS as compared to native LdTIM. This indicates that MMTS induces the exposure of buried hydrophobic surfaces and /or cationic groups in LdTIM.

Further the effect of bovine serum albumin on the reactivation of LdTIM was investigated. The recombinant LdTIM expressed into pET-23a (+) expression vector was appeared as inclusion bodies. The available protein in inclusion bodies was solubilized in 6M guanidine hydrochloride and purified using Ni-NTA affinity chromatography. Denatured purified LdTIM was allowed to reactivate at protein concentrations, ranging from 1 to 20µg/ml in the

presence and absence of 10 µg/ml BSA in the reactivation media. The maximum reactivation of denatured protein, i.e., 3455 µM/min/mg was observed at 5 h with 10 µg/ml LdTIM concentration with BSA in reactivation buffer. The titration curve of different concentrations of BSA (0 - 80 µg/ml) on reactivation of LdTIM at 10 µg/ml showed the maximal effect of BSA was attained at concentration of 10 µg/ml in 5 h. The fluorescence emission spectrum of ANS for LdTIM at 5 h showed maximum of the emission curve at 510 nm while maximum of the emission curve was shifted toward shorter wavelength i.e. 490 nm with increase in fluorescence in LdTIM samples incubated for 5 hours in presence of 10 µg/ml BSA, suggesting that BSA induces the structural changes in denatured LdTIM. Here we conclude that the BSA assists in the refolding and regain of LdTIM enzyme activity by providing framework for structure formation

The energy minimized and refined 3D structure of the LdTIM was built by using homology modeling based on the known crystal structure of TIM from *L. mexicana* as a template. The RMSD difference between the template and target is found to be 0.128 Å only. The modeled LdTIM was validated by PROCHECK, ProSA II and WHATIF. The results showed that the backbone conformation (PROCHECK), the residue interaction (Prosa II) and, the residue contact (WHATIF) are well within the limits established for the reliable structures and these tests suggest that we have obtained a good model for LdTIM to further characterize its binding site and explore its interactions with the substrate. All the residues around the active site of TIM protein in *L. mexicana* are found to be conserved in LdTIM. Most of the non conserved residues belong to surface region of the protein. Secondary structure elements consist of 11 alpha helices and 8 beta sheets with intermittent loop regions

This is the first report of purification and characterization of recombinant TIM from *Leishmania donovani*. Studies on recombinant LdTIM will help in evaluating this enzyme as potential drug target for visceral leishmaniasis.