CHAPTER 6.
SUMMARY AND CONCLUSION
1. SUMMARY

Lymphatic filariasis is a mosquito-borne disease that is endemic in 81 countries worldwide with 1.2 billion people at risk and an estimated 120 million are infected. The filarial nematode *Wuchereria bancrofti*, one of the three causative agents in humans, accounts for 91% of infections throughout the world. *Brugia malayi* and *Brugia timori* are responsible for the remaining infections and they are restricted to South and Southeast Asia. The disease is the second leading cause of permanent and long-term disability with over 40 million infected people suffering from clinical disease manifested mainly as lymphoedema, hydrocoele and elephantiasis. The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was established in 1999 with the objective of interrupting transmission of the parasites in all endemic countries by 2020. The current WHO strategy for GPELF includes the delivery of antifilarial drugs, either alone or in combination (diethylcarbamazine or ivermectin monotherapy, or either drug in combination with albendazole), to as many people as possible at yearly intervals until transmission has been interrupted. Available chemotherapy for lymphatic filariasis is far from satisfactory. The antifilarials currently being used for chemotherapy are basically microfilaricidal and exert little effect on adult worms. Hence identification of macrofilaricidal, embryostatic agents with microfilaricidal activity is required. The filarial parasites mainly depend upon carbohydrate for their energy requirements. Pentose phosphate pathway (PPP) is an important metabolic pathway for yielding reducing power in the form of NADPH and production of pentose sugar needed for nucleic acid synthesis. Glucose-6-phosphate dehydrogenase (G6PD) is the first enzyme of the PPP that converts α-D-glucose-6-phosphate into D-glucono-1,5-lactone-6-phosphate and is involved in crucial role in maintaining the NADPH/NADP⁺, GSH/GSSG ratio, protection from redox stress induced apoptosis and in cell growth. The tightly controlled concentrations of ROS and fluctuations in redox potential are important mediators of signaling processes, stress response, development and aging. Inhibition of G6PD activity causes accumulation of 6-phosphogluconate, which inhibits glycolysis by competitively inhibiting the glucose-6-phospho isomerise. Therefore decrease in G6PD activity and as a result NADPH level will impair the entire antioxidant system. Due to its key role in energy metabolism, antioxidant system as well as its enzymatic and structural features, G6PD of pathogenetic organisms is considered as a potential drug target.
The cloning of *Brugia malayi* G6PD was carried out by isolating total RNA from the parasite and synthesis of cDNA. G6PD gene was PCR amplified from cDNA using specific primers and cloned in pGEM®-T Easy cloning vector. The positive clones were confirmed by restriction digestion and sequencing of the clones. These clones were sub cloned in the pTriEx-4 expression vector. Recombinant G6PD clone was transformed in *E. coli* C-41 cells for expression of protein. Recombinant protein was purified by Ni-NTA affinity chromatography and expression was confirmed by the western blotting. The subunit molecular mass of the BmG6PD was found to be ~75 kDa on 10% SDS-PAGE. The exhibited size of purified recombinant BmG6PD protein was also confirmed by Western blotting using anti-His antibodies. The native molecular weight of the recombinant protein was found to be ~300 kDa, indicating the tetrameric nature of the BmG6PD. Tetramer formation was further confirmed by interchain cross-linking of the BmG6PD monomer using glutaraldehyde as cross-linker. Recombinant BmG6PD resembles human G6PD showing tetrameric nature. Parasitic G6PD was degraded to dimer and monomer forms at higher pH, which are catalytically inactive, whereas dimer form of human G6PD has been shown to be catalytically active.

Kinetic properties of the recombinant BmG6PD showed significant differences as compared to host and other parasites enzyme. BmG6PD can efficiently utilize G6P as substrate and NADP⁺ as a cofactor. The Km values for G6P and NADP were 0.25 mM and 0.014mM respectively. The optimum pH and temperature for BmG6PD enzyme activity were found to be 8.5 and 40 °C respectively in addition to Kcat was 40 sec⁻¹. The specific activity of the BmG6PD was 0.535 U/mg. The SH-groups, 4 per mole were partially accessible in native BmG6PD. Some sulphhydryl groups were blocked by pCMB and the remaining thiols groups reacted at different rates with NEM. Blocking of SH group with SH-inhibitors, leads to activation of BmG6PD activity by N-ethylmaleimide. PEP showed allosteric inhibition of the enzyme and activity was reduced by 25% in the presence of 10mM PEP. The BmG6PD was inhibited by natural inhibitors viz; NADPH and ATP. The ki values of NADPH and ATP were 0.025mM and 6.5 mM respectively. ATP-γ-S, ATP-β,γ-NH, ADP-β-S, Na⁺, K⁺, Li⁺ and, Cu²⁺ ions were found to be strong inhibitors of BmG6PD. DHEA, 6-AN and steroids are strong inhibitor of mammalian, *Trypanosoma cruzi* and *Plasmodium* G6PD, however these steroids had no effect on *Leishmania* G6PD and BmG6PD. The specific interaction of DHEA, an uncompetitive inhibitor with an enzyme–substrate ternary
complex can increase metabolic intermediates to toxic concentrations inside cells. The formation of a ternary complex is essential for the binding of uncompetitive inhibitors. The lack of inhibition of the *Leishmania* G6PD and BmG6PD by DHEA and its derivatives is puzzling. It is feasible that this is due to a single but crucial substitution in the enzyme that prevents the binding of these compounds. The antifilarials showed little effect on BmG6PD activity, only suramin exhibited 75% inhibition. While CDRI compounds, inhibited the BmG6PD activity at 10 µM concentration. The structure of these compounds may be utilized for synthesis of novel and chemically diverse inhibitors for chemotherapy of filarial infection. The differences observed in kinetic properties and the inhibitory effect of suramin and compounds synthesized points towards the utilization of BmG6PD as putative drug target.

The crystal structure of human G6PD, clearly showed a structural NADP$^+$ molecule bound between the dimer interface and the C terminus, well separated from the catalytic coenzyme-binding site. On the other hand, NADP$^+$ not only serves as a substrate for the enzyme but also stabilizes G6PD, apparently by preventing the dissociation of the normally dimeric enzyme into inactive monomers. The effect of NADP$^+$ binding on the conformational properties of G6PD, the function of structural NADP$^+$ in oligomerization and refolding is concentration dependent. To see the effect of cofactor on tryptophan accessibility, stability and folding of the purified recombinant BmG6PD protein. The apoenzyme of BmG6PD was overexpressed and purified from affinity chromatography for the study of structural differences between apoenzyme and holoenzyme and on the cofactor binding and its contribution to enzyme stability. The presence of cofactor influences the tertiary structure of BmG6PD due to significant differences in the tryptophan microenvironment of holoenzyme. CD analysis indicated that BmG6PD is composed of 37% $\alpha$-helices and 26% $\beta$-sheets. However cofactor does not influence the secondary structure of the enzyme. The results indicate that in purified recombinant BmG6PD, each monomer contains one bound NADP$^+$ molecule, binding constant of NADP$^+$ and for NADPH dissociation in BmG6PD was $4.0 \times 10^{-5}$ and $2.9 \times 10^{-5}$ M respectively. The NADP$^+$ caused 90% quenching of the intrinsic fluorescence of BmG6PD. In contrast, G6P only quenched 10% of the intrinsic fluorescence of BmG6PD. A Stern-Volmer quenching constant was $K_{sv}= 3.5 \text{ M}^{-1}$ in acrylamide quenching experiments and plot showed tryptophan population is more exposed to polar solvent in the apoenzyme of BmG6PD. Furthermore, the Stern-Volmer constant
was very close to the holoenzyme stern-volmer constant, $K_{sv} = 3.4 \text{ M}^{-1}$, indicating that all the fluorophore are accessible to acrylamide. The Stern-Volmer quenching constants $K_{sv1} = 1.4$ and $K_{sv2} = 3.1$ in KI quenching, showed higher values than the one obtained for apoenzyme. These results indicate that in holoenzyme tryptophan residues are more exposed to the quencher. This might be due to the binding of NADP$^+$ to the apoenzyme that shifts tryptophan residues to a less hydrophobic microenvironment. Quenching with acrylamide indicated that two or more tryptophan residues become accessible upon cofactor binding whereas in KI quenching experiments, two type of tryptophan population were observed in the holoenzyme. The accessible tryptophan fraction was lower in the apoenzyme and only 90% of the fluorophores was accessible to acrylamide quenching. Fraction of tryptophan residue accessible to the charged (KI) quencher for apoenzyme and holoenzyme was 0.56 and 0.71 respectively. This result suggests that one or more of the seven tryptophan residues of BmG6PD becomes more accessible upon NADP$^+$ binding. It can be concluded that nearly all tryptophan residues of BmG6PD are on the surface of BmG6PD molecules and about 30% of them are located in negatively charged areas, where the tryptophan accessibility to the charged quencher is less as compared to the neutral quenchers. In addition, the effect of NADP$^+$ on the aggregation BmG6PD enzyme showed that NADP$^+$ induced oligomeric state of the enzyme was more stable. Unfolding and crosslinking study of BmG6PD showed that cofactor stabilizes the protein under high concentration of urea/GdmCl denaturants. The red-shift of tryptophan fluorescence to 357 nm in presence of GdmCl/urea indicated that upon denaturation the tryptophan moieties are exposed. The BmG6PD was treated with several denaturing agents, such as GdmCl and urea to obtain information about the structure flexibility of the protein. Efforts have been made to carry out a detailed characterization of the structural and functional changes associated with the GdmCl- and urea-induced unfolding of BmG6PD. The triphasic pattern observed in the GdmCl/urea-treated protein indicated formation of intermediate products. The enzyme activity was significantly inhibited at low concentration of GdmCl as compared to urea. Loss of BmG6PD activity by GdmCl/urea was found to be biphasic in nature.

The purified BmG6PD of human filarial parasite was utilized for production of antibodies. The polyclonal antibodies produced in rabbit are mixture of various immunoglobulin molecules secreted against a specific antigen. These polyclonal antibodies can be used for finding out protein expression in tissues or to localize protein
within a cell by immunofluorescence microscopy. The antibodies generated in rabbit were highly specific as determined by ELISA and Western blotting. The titre of anti-BmG6PD antibody raised against recombinant protein was found to be 1:125,000 and used for western immunoblotting, recombinant BmG6PD as well as detecting presence of G6PD protein in mf and adult stages of parasite. The antiBmG6PD antibodies recognized a single band in both microfilariae as well as adult *B. malayi* lysate, conforming the expression of G6PD both in the microfilariae as well as in the adult stage of *B. malayi*. Fluorescence microscopic analysis indicated the cytosolic location of BmG6PD in adult *B. malayi*.

The amino acid sequence alignment of *B. malayi* G6PD with *Caenorhabditis elegans, Leishmania major, Trypanosoma brucei, Saccharomyces cerevisiae* and human G6PD was carried out by ClustalW to determine the extent of their homology. BLAST analysis showed that BmG6PD has maximum homology with *Caenorhabditis elegans* (69%). The alignment of the BmG6PD protein sequence, revealed the presence of three conserved regions: a nine-residue peptide (RIDHYLGKE, residues 209-217 of the BmG6PD), a nucleotide-binding fingerprint (GxxGGDLA, residues 45-52 of the BmG6PD) and the sequence EKPxG (residues 181-185 of the BmG6PD). With the aim to study structure-function relationship and to explore structural similarity with existing G6PD structure, a homology model of BmG6PD was constructed using human G6PD (PDB id: 2BH9) as template by the programme Modeler. The BmG6PD model is an average structure based on restraints derived from the coordinate sets of the template. The refined model was subjected to a series of tests for its internal consistency and reliability. The test was to compare the residue backbone conformation in BmG6PD model by inspection of psi/phi Ramachandran plot obtained from PROCHECK analysis. The stereochemical validation of the 3D model by PROCHECK program showed 92.1%, 7.1%, 0.9%, 0.0% of the residues lies in core, allowed, generously allowed and disallowed regions, respectively. Comparing this result with the human G6PD, used as a template for model building, we found that the backbone conformations of our BmG6PD model to be nearly as good as those of template. The overall fold of the monomer of BmG6PD is similar to that of Human G6PD. In homology modeling, some conserved relevant residues for NADP\(^+\) binding were correctly positioned in the obtained BmG6PD model. One single difference Arg\(^{52}\) instead of Lys\(^{45}\) was found in the nucleotide binding sites in BmG6PD model.
Superimposition of template and target model shows root mean square deviation (RSMD) of 0.346. Secondary structure composition of predicted structure of BmG6PD is 34.3% α-helix, 19.1% β-sheet and 46.6% random conformations. The secondary structure of BmG6PD showed that, it has 21 α-helices and 15 β-sheets connected by loops. NADP was docked to the coenzyme site and the structural NADP site. Both NADP conformations were positioned similar to NADP in the humanG6PD-NADP complex. The structural NADP site is seen in between the β-sheet and C-terminus of the monomer. The FlexX binding score of structural NADP is -10.96, less in the comparison with the coenzyme NADP that is -15.47. This score shows that, NADP bound less tightly in the structural site as compared with coenzyme site. In the NADP docking study, the NADP bound with the BmG6PD was similar to human G6PD. NADP was bound more strongly at the coenzyme binding sites as compared to the structural binding sites.

2. CONCLUSION

G6PD gene of Brugia malayi was successfully cloned and expressed. The expressed protein was purified by use of affinity chromatography and biochemical and biophysical characterization was carried out. The catalytic activity was confirmed by the zymogram assay. The BmG6PD Km for NADP$^+$ is lower than that for G6P, suggesting the higher affinity of BmG6PD to NADP$^+$. Native molecular weight of BmG6PD as determined by FPLC and glutaraldehyde crosslinking indicated its tetrameric nature. NADPH is a natural inhibitor for G6PD. The BmG6PD was competitively inhibited by dinucleotide product NADPH with respect to NADP and G6P. The ATP, a noncompetitive inhibitor, showed Ki of 6.5mM. Non-hydrolyzable ATP and ADP analogs were effective inhibitors of BmG6PD. The SH-groups play an important role in the activity of NADP-specific BmG6PD and blocking of this group with SH-reagents leads to activation of BmG6PD activity by N-ethylmaleimide and inhibition of BmG6PD activity by p-chloromercuribenzoate. Alteration of the reactive thiols groups by blocking with chemical residues leads to an increase in the enzyme activity. This suggests that some thiols susceptible to NEM modification are less accessible to pCMB. Some of the thiols modified by NEM are apparently more directly involved in the catalytic activity of BmG6PD. BmG6PD does not show any effect in the activity by EDTA indicating Mg$^{++}$ have not significantly role in the activity of BmG6PD. DHEA, 6-aminonicotinamide and certain related steroids have been found to be potent
inhibitors of mammalian G6PD but the BmG6PD was not inhibited by DHEA and 6-
aminonicotinamide. It is feasible that this is due to a single but crucial substitution in
the enzyme that prevents the binding of these compounds. The presence of cofactor
influences the tertiary structure of BmG6PD due to significant change in the tryptophan
microenvironment of the protein. The purified recombinant BmG6PD, each monomer
contains one bound NADP$^+$ molecule, binding constant of NADP$^+$ in BmG6PD is
higher as compared to human G6PD. The BmG6PD NADP$^+$ binding sites shows lower
affinity for the coenzyme as compared to human G6PD. CD analysis indicated that
BmG6PD is composed of 37% α-helices and 26% β-sheets. Cofactor does not influence
the secondary structure of the protein. The NADP$^+$ caused 90% quenching of the
intrinsic fluorescence of BmG6PD. Quenching with acrylamide indicated that two or
more tryptophan residues become accessible upon cofactor binding whereas in KI
quenching experiments, two type of tryptophan population were observed in the
holoenzyme. Loss of enzyme activity of BmG6PD was found to be biphasic of GdmCl
and urea treated BmG6PD, which was found to be irreversible. The equilibrium
unfolding of BmG6PD by urea and guanidine hydrochloride proceeded through
stabilization of several unique oligomeric intermediates. In both GdmCl and Urea
induced unfolding of enzyme is a three state process. The effect of NADP$^+$ on the
aggregation of enzyme showed that NADP$^+$ induced oligomeric state of the enzyme
was more stable. The antibodies against BmG6PD were successfully raised as indicated
by ELISA and western blot. The expression of BmG6PD performed by Western
immunoblotting confirmed the expression of G6PD both in the microfilariae as well as
in the adult stage of B. malayi. Fluorescence microscopic analysis indicated the
cytosolic location of BmG6PD in adult B. malayi. A homology model of BmG6PD
was constructed using human G6PD as template. The backbone conformations of our
BmG6PD model to be nearly as good as those of template. The refined model was used
for the NADP$^+$ docking to the coenzyme site and the structural NADP site. Both NADP
conformations were positioned similar to NADP in the humanG6PD-NADP complex.
The differences observed in structure, kinetic properties and the inhibitory effect of
suramin and compounds synthesized points towards the utilization of BmG6PD as
putative drug target.