CHAPTER 2.
CLONING, EXPRESSION AND PURIFICATION OF
GLUCOSE 6-PHOSPHATE DEHYDROGENASE
FROM Brugia malayi
1. INTRODUCTION

The pentose phosphate pathway (PPP) represents an alternative route for glucose oxidation in cells. The major products of its oxidative phase, NADPH and ribose 5-phosphate, are essential for cell survival, lipid and nucleotide biosynthesis, as well as regulation of the redox state of the cell (Wood, 1986). Glucose 6-phosphate dehydrogenase (β-D glucose 6-phosphate: NADP oxidoreductase EC 1.1.1.49) catalyzes the first step of the PPP through the reaction:

\[
\beta-D \text{ glucose 6-phospate} + \text{NADP}^+ = 6\text{-phosphogluconolactone} + \text{NADPH} + H^+
\]

Under in vivo conditions, the reaction is irreversible due to the hydrolysis of the lactone by lactonase (Levy, 1979). The activity of glucose 6-phosphate dehydrogenase (G6PD) is the main control site for the flux of glucose 6-phosphate (G6P) toward the PPP. In this sense, the concentration of NADP⁺ is a primary factor governing such activity (Wood, 1986). The enzyme shows a wide spectrum of specificity for the nicotinamide coenzyme (Levy, 1979).

Evidence for the occurrence of the phosphogluconate pathway (also called hexose monophosphate shunt or pentose phosphate pathway) has been reported in parasitic nematodes (Rothstein et al., 1970; Murfitt et al., 1976) and the activities of G6PD and 6-phosphogluconate dehydrogenase in crude extracts has been shown (Smith and Brown, 1977; McManus and Smyth, 1982; Titanji et al., 1988; Bhandary et al., 2006). In addition to its critical involvement in determining the flux of glucose into the PPP, G6PD activity is also essential for NADPH-dependent processes. Therefore, knowledge of the properties of G6PD in nematodes is essential for understanding its role in cell metabolism. The present chapter reports the purification and characterization of G6PD from the human filarial parasite, *Brugia malayi*.

2. MATERIALS AND METHODS

2.1. Materials

Na₂HPO₄, NaH₂PO₄, Sodium chloride, Imidazole, Acrylamide, Ammonium persulphate, \( N, N\text{-Methylene-bis-acrylamide} \), Sodium dodecyl sulphate (SDS), Proteinase K, Protease inhibitor cocktail, Commassie brilliant blue R-250 stain, CaCl₂, Ethidium bromide (EtBr), Phenyl methyl sulfonyl fluoride, Glycerol, Bovine serum albumin, Ampicillin, β-mercaptoethanol, Triton X-100, Bradford reagents, Skimmed milk, 3,3′-Diamino benzidine, Hydrogen peroxide, Glutaraldehyde, phenol: chloroform:
isoamyl alcohol (25: 24: 1) and anti-mouse IgG HRP coupled were procured from Sigma (St Louis, MO, USA). QIAprep Spin Miniprep plasmid kit, QIAquick Gel Extraction kit, AntiHis IgG and Ni-NTA Agarose matrix were purchased from Qiagen, Germany. Nitrocellulose membrane (Schleider & Schuell, Pore size 0.45 µm) and Centriprep (Amicon) were purchased from Millipore USA. pGEM®-T Easy (T/A cloning) vector from Promega and expression vector pTriEx-4 from Novagen, Darmstadt, Germany. Agarose, DNA ladders 1Kb & 500 bp and restriction enzymes BamHI & EcoRI were obtained from Bangalore Genei, India. Revert Aid™ H Minus first strand cDNA synthesis kit, T4 DNA ligase, protein marker (Page Ruler™), prestained protein ladder and isopropyl-thio-galactoside (IPTG) were from MBI fermentas, Hanover, Maryland, USA. K$_2$HPO$_4$, KH$_2$PO$_4$, KCl, Glycine and Tris-HCl, were from USB, Cleveland, USA. Luria-Bertani medium and Agar powder (certified) were from HiMedia, India. All other chemicals were of analytical grade. Standard molecular weight markers for size exclusion chromatography like ovalbumin, albumin, aldolase, apoferritin and Superdex 200HR column, were purchased from GE health care, Singapore. All solutions were prepared in Millipore water.

2.2. Designing of Primers

For cloning of BmG6PD gene primers were designed on the basis of the _B. malayi_ G6PD protein sequence available at National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The complete nucleotides sequence was used to design specific primers using web available Oligotech programme. Restriction sites in the forward and reverse primers were selected based on the restriction summary corresponding to BamHI and EcoRI sites respectively to facilitate cloning in expression vector in correct orientation since both restriction sites were absent in the complete ORF of _Brugia malayi_ G6PD. Restriction summary was obtained from web-based tool Sequence Manipulation Suite available at http://www.ualberta.ca/~stothard/javascript/. Sequences used for protein multiple alignment, were retrieved from www.ncbi.nlm.nih.gov. Web-based clustalW (Des Higgins) tool was used for protein multiple alignments available at http://bioweb.pasteur.fr/seqanal/interfaces.

Forward Primer-  5’ GGATCC GAT GTC GCA TGA AAA TTC GCC GA 3’  
Reverse Primer-  5’ GAATTC ACG TTC AGT GTT TGG AAT CCA TTT G 3’  
BamHI and EcoRI sites are underlined in the nucleotide sequence of forward and reverse primer respectively.
2.3. Extraction of Total RNA and cDNA synthesis

Total RNA was isolated by the TRIzol method. Briefly, 0.02g of *B. malayi* parasites were crushed in 1.0 ml of TRIzol reagent with the help of pestle in eppendorf tube and left for 10 min and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected into fresh RNase free eppendorf tube and 0.2 ml of chloroform was added. The tube after gently mixing for 15 sec was incubated at 25 °C for 5 min. The mixture was centrifuged at 14,000 rpm for 20 min at 4 °C and the aqueous phase was transferred into a fresh RNase free eppendorf tube. RNA was precipitated by adding 0.5 ml isopropanol and centrifugation at 12,000 rpm for 10 min at 4°C after incubation for 10 min at 25 °C. The supernatant was discarded and pellet was washed with 1.0 ml of 75% ethanol and centrifuged at 12,000 for 5 min at 4°C. The pellet was air dried and resuspended in 20 µl of DEPC treated water by heating for 10 min at 60°C and stored at -80 °C for 2 to 3 days. The quality of isolated RNA was checked on 1% agarose gel. Undegraded RNA preparation showing two distinct bands of 28S and 18S RNA was used for downstream applications. Total RNA preparation was subjected to synthesis of first strand cDNA (suitable for PCR Amplification) using Revert Aid\textsuperscript{TM} H Minus first strand cDNA synthesis kit. The reaction mixture composed of RNA template, oligo dT primer, dNTPs, Reverse transcriptase, RNase inhibitor etc. 50-100 ng of total RNA was used per reverse transcription reaction. The cDNA synthesized was used for PCR and stored according to the instructions provided in the kit manual.

2.4. PCR Amplification

*Brugia malayi* G6PD (BmG6PD) gene was PCR amplified using the forward and reverse primers mentioned above. The PCR reaction contained 1X PCR buffer, 2.5 mM MgCl\textsubscript{2}, 400 µM dNTPs, 2.5 units of Taq DNA polymerase (all from Promega), 25 pmole of forward and reverse primers each, (Inschem) and 50-100 ng of *B. malayi* cDNA. The PCR was performed in thermocycler (eppendorf Mastercycler\textsuperscript{®}/Mastercycler\textsuperscript{®} gradient) with initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 68°C for 1 min and 72°C for 2 min with a final extension at 72°C for 10 min. PCR amplified products were analyzed through electrophoresis on 1% agarose gel in Tris-Acetate-EDTA (TAE buffer) at 10 volts/cm.
2.4.1. Agarose Gel Electrophoresis

The PCR amplified product was analyzed using horizontal submarine gel electrophoresis as described by Sambrook et al. (2001). The 1% gel was prepared by adding appropriate amount of agarose powder in 1X TAE buffer and melting in microwave oven for 2-3 min. Melted agarose was cooled under tap water to 40°C and ethidium bromide (0.5 µg/ml) was added before casting it into a tray fitted with a comb. Solidified gel was placed in an electrophoresis tank filled with 1X TAE buffer and DNA samples were mixed with gel loading dye and loaded into the wells. The electrophoresis was carried out at constant voltage of 60 V. After the required amount of separation, DNA was visualized under UV light. The electrophoresed DNA was analyzed on Gel Documentation system (Amersham) and photographed.

2.4.2. Purification of PCR Amplified Product

The amplified PCR product was visualized on a 1% agarose gel stained with ethidium bromide (EtBr) and the gel slice containing the amplified gene was excised out and purified using a QIAquick GEL Extraction Kit. Briefly, the excised gel slice was weighed and 3 volumes of buffer QG was added and heated at 50°C for 10 minutes until the gel slice was completely dissolved and PCR product was precipitated with isopropanol and applied to the QIAquick column for binding of DNA. After washing, the DNA was eluted in 50 µl of Nuclease free water (autoclaved triple distilled water).

2.4.3. Cloning of BmG6PD Gene in pGEM®-T Easy (T/A cloning) vector

The eluted product was ligated onto linearised pGEM®-T Easy vector (Figure 2.1) using T4 DNA ligase. The vector takes advantage of the 3’A overhang at each end of the PCR product, added by Taq DNA polymerase. The linearized pGEMT Easy cloning vector contains the 5’ddT overhang at both ends and is ligated to the PCR product with high specificity.
In a 10 µl ligation reaction, following components were added in a 0.5 ml tube.

Table 2.1 : Ligation reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation buffer</td>
<td>2 X</td>
<td>5 µl</td>
</tr>
<tr>
<td>pGEMT Easy cloning vector</td>
<td>50 ng/µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Amplified PCR product</td>
<td>75 ng/µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>3 units/µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>--</td>
<td>Up to 10 µl</td>
</tr>
</tbody>
</table>

The ligation mixture was incubated overnight at 4°C.

Figure 2.1: Restriction map of pGEM®-T Easy Vector
2.4.3.1. Preparation of competent *Escherichia coli* cells

The competent cells were prepared by the method described by Cohen. (Cohen, 1972). A single colony of *E. coli* DH5α cells was picked from Luria-Bertani (LB) agar plate and inoculated aseptically into a 5ml LB tube and incubated overnight at 37°C in a shaker incubator at 200 rpm. 150 µl of the overnight grown culture was inoculated into 25 ml LB medium. The cells were grown to the log phase at 37°C, 200 rpm till the OD600 reached approximately 0.3-0.4 and harvested at 5000 rpm for 7 min at 4°C. The cell pellet was resuspended in 10 ml of ice cold 0.1 M CaCl2 solution and placed on ice for 1 hr. The cells were centrifuged for 4000 rpm at 4°C for 5 min and resuspended in 2 ml of chilled 0.1 M CaCl2 +15% glycerol solution. Finally aliquots of 200 µl of competent cells were stored at -70°C until used.

2.4.3.2. Transformation

A test transformation was setup with 4ng of pGEM®-T Easy control plasmid to check the transformation efficiency by heat shock treatment as described by (Hanahan, 1983). For this process, the control plasmid was added to the chemically competent *E. coli* DH5α cells and the mixture was incubated on ice for 20 minutes, after which it was transferred rapidly to a preheated 42°C circulating waterbath for 90 sec without shaking. Reaction mixture was then transferred rapidly onto ice for 2 min, followed by immediate addition of 800 µl of C-medium (Fermentas) at room temperature and incubated at 37°C for 1 hr with agitation (~200 rpm) to allow the cells to recover. About 100 µl of recovered cells were plated onto appropriate antibiotic selection agar plates and incubated overnight in inverted position at 37°C. The transformation efficiency was calculated and when it was found approximately 104 CFU µg-1 DNA, remaining competent cells were used to transform the ligation mix. Ligated mixture was transformed according to the above mentioned procedure. After recovery, the transformants were incubated overnight at 37°C and selected by plating onto selective LB Ampicillin (100 µg/ml) with X-gal (40 µg/ml) plates. Positive white colonies were picked up for further screening.

2.4.3.3. Conformation of positive clones

2.4.3.3.1. Preparation of master plate and screening of positive transformants by colony PCR

Master plate was prepared by streaking the colonies picked from Amp/X-gal plate with pipette and incubated for overnight at 37°C. The positive transformants were screened by colony PCR to identify colonies harboring recombinant construct. Briefly,
single colony from LB agar plate was inoculated in 5 ml LB broth and grown at 37°C, with shaking (180 rpm) till the OD$_{600nm}$ reached 0.4-0.6. Approximately 5 µl of this grown culture was used as template in same PCR conditions as used previously for the same gene mentioned above. Plasmids were isolated from PCR positive colonies by QIAprep Spin Miniprep kit.

2.4.3.3.2. Isolation of plasmid DNA from transformed E. coli (Mini Prep)

Plasmid DNA was isolated from the overnight grown culture by using QIAprep spin miniprep kit as per manufacturer’s protocol. Briefly, the cells harbouring the recombinant plasmid were harvested by centrifugation at 6000 rpm for 5 min and resuspended in 250µl of buffer P1 (containing RNAse) followed by addition of 250µl P2 and mixed by inverting the tubes 4-6 times. The mixture was then precipitated by adding 350µl of buffer N3 and the contents of the tube were mixed by inverting the tubes 10 times. The supernatant was obtained by centrifugation at 13000 rpm for 10 min and applied to the QIAprep spin column. After washing the columns with 750µl of buffer PE the plasmid was eluted in 30µl of nuclease free water (Sigma). The isolated plasmids were visualized on 1% agarose gel by electrophoresis.

2.4.3.3.3. Restriction Digestion of Plasmid DNA

Clone confirmation was further assessed by restriction digestion of the isolated plasmid with BamHI and EcoRI (Promega) restriction enzymes in 10X buffer and 0.1 mg/ml BSA supplied with the enzymes. Nearly 1 µg of Plasmid DNA was digested in a 20 µl reaction volume for 3 hrs at 37°C temperature. Reaction was terminated by addition of 10 mM EDTA or heating the reaction mixture at 70°C for 10min. Restriction digestion was analyzed by agarose gel electrophoresis using DNA molecular weight markers.

2.4.3.3.4. Sequencing of BmG6PD gene

One positive clone was selected and sequenced by automatic sequencer ABI Prism (Version 3.0.Model 3100) at Department of Biochemistry, University of Delhi, South campus (UDSC), New Delhi.

2.4.4. Sub Cloning of BmG6PD Gene in pTriEx-4 Expression Vector

The BmG6PD gene was further sub cloned into pTriEx-4 Multisystem Expression Vector (Figure 2.2) to produce G6PD protein with histidine tag at both the N-terminus and C-terminus. pGEM®-T Easy (T/A cloning) vector containing BmG6PD gene was digested with BamHI and EcoRI. pTriEx-4 vector was also digested with the
same restriction enzymes. The digested product (vector and insert) was run on 1% agarose gel followed by extraction of desired DNA bands from agarose gel slices and purified from agarose gel by using gel extraction and purification kit (Qiagen) as described earlier. Gel purified vector and insert DNA, in an optimized molar ratio of 1:10, were subjected to ligation in a total volume of 10 µl of reaction mixture as mentioned in Table 2.2.

**Table 2.2 : Ligation reaction mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation buffer</td>
<td>10 X</td>
<td>1 µl</td>
</tr>
<tr>
<td><em>BamHI/EcoRI</em> digested pTriEx-4</td>
<td>25 ng/µl</td>
<td>3 µl</td>
</tr>
<tr>
<td><em>BamHI/EcoRI</em> digested pGEM®-T Easy + Insert</td>
<td>75 ng/µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1 Weiss unit/µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>--</td>
<td>Up to 10 µl</td>
</tr>
</tbody>
</table>

The ligation mixture was incubated overnight at 4°C.

**Figure 2.2 : Restriction map of pTriEx-4 expression vector.**
2.4.4.1. Transformation in *E. coli C41* (DE3) cells

Ligated mixture was added to 200 µl of *E. coli C41* (DE3) competent cells and the mixture was incubated on ice for 30 minutes. Heat shock at 42°C for 90 sec was given to the mixture and it was immediately chilled on ice again for 5 minutes. After 5 minutes, 800 µl of LB medium was added immediately to the transformed cells and incubated for 1 hr at 37°C with shaking (200 rpm) for recovery. Following recovery, the transformants were selected by plating onto selective ampicillin agar plates and incubated overnight at 37°C.

2.4.4.2 Clone confirmation

The positive clones were confirmed by colony PCR and restriction digestion using *BamHI* and *EcoRI* restriction enzymes as described earlier. Positive clones were used for protein expression.

2.5. Analysis of BmG6PD Expression in *E. coli* Cells

2.5.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To study the expression of recombinant BmG6PD, plasmid constructs containing BmG6PD gene in pTriEx-4 were transformed in T7 RNA polymerase encoding *E. coli* strain C-41 (DE3) cells. A single positive colony was inoculated to 5 ml LB tubes supplemented with 100 µg/ml ampicillin. Cells were grown for 14-16 h at 37 °C with shaking at 200 rpm. Subsequently, 100 ml LB broth tubes containing suitable antibiotic were inoculated with 1% (v/v) of 14-16 h grown culture and incubated at 37 °C with shaking at 180 rpm till the OD_{600nm} reached 0.4-0.5, the culture was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma). For the determination of optimum conc. of isopropyl thiogalactoside (IPTG), temperature and time for maximum expression of protein, mother culture was divided in two sets. One set was induced with different conc. (0.2mM, 0.5 mM, 0.8 mM and 1.0 mM) of IPTG and other uninduced culture served as control. These sets were allowed to grow at 37°C, 25°C and 20°C for 3, 6, 9 and 12 h. After each given time interval 1 ml culture from both induced and uninduced sets were harvested and pelleted by centrifugation at 8000 x g for 10 min at 4°C. Cells were resuspended in 100 µl PBS and lysed by sonication on pulse-rest cycle (4-5 cycles; 10 sec pulse at 25W with 1 min interval after each pulse). Soluble fraction was separated from insoluble fraction by centrifugation at 12,000 rpm for 30 min at 4 °C. Soluble fraction was taken in a separate microfuge tube and insoluble fraction was resuspended in 100 µl of PBS. Protein in both fractions was
determined by Bradford’s method (Bradford, 1976) using BSA as standard. Equal amount of insoluble and soluble fractions from induced and uninduced cultures were load on SDS-PAGE gel. Equal amount (80-100 µg protein) of insoluble and soluble fractions from induced and uninduced cultures were mixed with Laemmli dye (Laemmli, 1970) and heated in boiling water bath for 5 min. These samples were then analyzed on a discontinuous vertical SDS-polyacrylamide gel with a 5% (w/v) stacking and a 10% (w/v) resolving gel. Gels were run in a Laemmli buffer at a constant current at 15 mA. Protein molecular weight markers were run concurrently on the gels for appropriate analysis. The expression of BmG6PD was visualized by staining the gel with commassie brilliant blue R-250 stain.

2.5.2. Western Blotting

In order to detect recombinant BmG6PD in E. coli lysates (80-100 µg) were resolved on 10% SDS-PAGE (Laemmli, 1970). The resolved proteins were electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Pore size 0.45 µm) at 40 volts for 3 hrs in transfer buffer using wet transfer method (Sambrook, 1989). Membrane was incubated with 5% skimmed milk in PBS for overnight at 4°C followed by incubation with anti-His antibodies at a dilution of 1:500 in blocking solution (5% skimmed milk in PBS) for 3 h at 25°C. Primary antibody was removed by four-five washings with PBS containing 0.05% Tween-20. Membrane was then incubated with anti-mouse IgG coupled to horseradish peroxidase (HRP) at a dilution of 1:1000 in blocking solution (5% skimmed milk in PBS) for 2 h at 25°C. Secondary antibodies were removed; and membrane was washed with PBS containing 0.05% Tween-20 followed by development in 10 ml PBS containing 8.8 mM H$_2$O$_2$, 3,3’ diaminobenzedine and imidazole both at a concentration of 10 mg/ml. Development was carried out till bands of desired intensity appeared (approximately 2-3 min).

2.6. Purification of Recombinant BmG6PD

For recombinant protein purification 1% (v/v) inoculum of overnight grown culture was inoculated in fresh LB media supplemented with ampicillin (100µg/ml) and after shaking at 37°C till the OD$_{600nm}$ reached to 0.4-0.5, the culture was induced with 0.8 mM IPTG concentration and incubated again for 14-16 h at 20°C with constant shaking at 180 rpm. After that, the culture was centrifuged at 8000 rpm for 10 minutes and the pellet was resuspended in 30 ml of lysis buffer (50 mM NaH$_2$PO$_4$, 600 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, pH 7.0) containing protease inhibitor
cocktail (Sigma) and 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) and lysed by sonication with pulse-rest cycle (25 cycles; 15 sec pulse at 25W with 40 sec interval after each pulse). The lysate was centrifuged at 12,000 rpm for 30 min at 4 °C and the supernatant was collected. All further steps were performed under cold conditions. The supernatant obtained after centrifugation was loaded on Ni²⁺-NTA column preequilibrated with lysis buffer and contaminating proteins were removed by washing with the same buffer containing 50 mM imidazole, 0.1% (v/v) Triton X-100 and 1 mM β-mercaptoethanol respectively. Recombinant protein was eluted as fractions of 1.0 ml by 12 ml of lysis buffer containing 300 mM NaCl and 250 mM imidazole. The protein containing fractions were pooled and dialyzed against dialysis buffer pH 8 (50 mM NaH₂PO₄ and 200 mM NaCl). After dialysis protein was concentrated with centrifprep (Millipore). The purity of the eluted protein was checked by SDS-PAGE and its concentration was estimated by Bradford method (1976) using BSA as standard.

2.7. Determination of Native Molecular weight of BmG6PD

2.7.1. Size Exclusion Chromatography

The native molecular weight of BmG6PD was determined with the help of calibration curve between elution volume and log molecular weight (kDa) of standard marker proteins by the Superdex 200 HR 10/300 column (manufacturer’s exclusion limit 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech, Sweden), at a flowrate of 0.5 ml/min. The protein from gel filtration column was eluted with 50 mM sodium phosphate buffer (pH 8) containing 200 mM NaCl at a flow rate of 1 ml/min. The following molecular weight markers viz., ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa) and ferritin (443 kDa) were used to obtain the values of elution volume required for this purpose.

2.7.2. Cross-linking Analysis

The native molecular weight of BmG6PD was further confirmed by cross-linking with glutaraldehyde (Migneault et al., 2004). For glutaraldehyde treatment, reaction mixtures containing 50 to 100 µg of BmG6PD in 100 µl of 20 mM sodium phosphate buffer (pH 7.5) were mixed with 5 µl of 2.3% freshly prepared solution of glutaraldehyde for 2 to 5 minutes at, 37°C. The reaction was terminated by addition of 10 µl of 1 M Tris-HCl, pH 8.0. Cross-linked proteins were solubilized by addition of an equal volume of Laemmli sample buffer 25 µl of 0.1% bromophenol blue and
electrophoresed on 5% to 8% SDS-polyacrylamide gels. The molecular weight of the cross-linked product was determined by SDS-PAGE.

3. RESULTS

3.2. PCR Amplification of BmG6PD

A putative G6PD gene was predicted and annotated (NCBI Gene ID: 6102236, http://www.ncbi.nlm.nih.gov) in the *B. malayi* genome. The BmG6PD has a complete open reading frame of 1.6 Kb capable of encoding a protein consisting of 526 amino acids with a calculated molecular weight of 58 kDa. Specific forward and reverse primers were designed from the cDNA sequence information obtained from NCBI (Gene ID: 6102236) and used for the amplification of complete ORF of BmG6PD by PCR using full length cDNA as a template. PCR specifically amplified a fragment of 1.6 Kb, consistent with the predicted size of BmG6PD (Figure 2.3). In contrast, no amplification has been found in the reaction in which the template is absent (Figure 2.3, lane 2)

![Figure 2.3: PCR amplification of BmG6PD gene. Lane 1: 500bp DNA ladder (B. Genei); Lane 2: PCR reaction without template; PCR reaction with template (Amplified product).](image-url)
3.3. Cloning of PCR Amplified Product in pGEM®-T Easy (T/A Cloning) Vector

The PCR amplified product (1.6 Kb) was directly cloned into pGEM®-T Easy (T/A cloning) vector and was subsequently transformed into competent DH5α cells. Transformants were screened for positive clones by colony PCR and restriction digestion analysis. The positive clones screened by colony PCR showed an amplified band at 1.6 Kb (Figure 2.4A). Digestion of these constructs with BamHI and EcoRI, resulted in appearance of 1.6 Kb DNA fragment along with linearized vector of 3.0 Kb, which was not present in undigested construct (Figure 2.4B). Cloning of BmG6PD gene was finally confirmed by the sequencing of the clones. Clone pGEMT-BmG6PD was sequenced by dideoxy chain termination method. Sequencing results showed Figure 2.4C conclusively suggested that the ORF of BmG6PD was successfully cloned without any mutation.

Figure 2.4 : Cloning of BmG6PD gene in pGEM®-T Easy (T/A cloning) vector. (A) Screening of positive clones by colony PCR, Lane 1. DNA ladder 500 bp, Lanes 2-11. Positive clones (Clones no 1-10). (B) Screening of positive clone by restriction digestion analysis, Lane 1, molecular weight marker; DNA ladder 500 bp (5,000-500 bp, B. Genei); Lane 2, Undigested plasmid; Lane 3, Plasmid double digested with BamHI and EcoRI, which produced insert of 1.6 Kb and 3.0 Kb vector. (C) Sequencing results of pGEMT-BmG6PD clone by T7 P promoter primer.
3.4. Sub-cloning of BmG6PD Gene in pTriEx™ Transient Expression Vector

The BmG6PD gene was further sub-cloned into pTriEx-4 expression vector using BamHI and EcoRI restriction sites and the construct was named as pTriEx-BmG6PD. The sub-cloning was confirmed by digesting this construct with BamHI and EcoRI which resulted in liberation of 1.6 Kb insert along with the 5.2 Kb vector (Figure 2.5B, lane 3). The sub-cloning of BmG6PD gene in pTriEx-4 vector has led to the ~10 KDa additional amino acid residues including 6 histidine residues N-terminus and 8 histidine residues at C-terminus of BmG6PD.

Figure 2.5 : Sub-cloning of BmG6PD gene into pTriEx™ Transient Expression vector pTriEx™-4, (A) Screening of positive clones by colony PCR, Lane 1. DNA ladder 500 bp, Lanes 2, 3, & 5. Positive clones. (B) Lane 1. DNA ladder 1 Kb, Lane 2. Undigested plasmid, Lane 3. Double digestion of pTriEx-BmG6PD construct with BamHI and EcoRI, producing an insert of 1.6 Kb and vector of 5.238 Kb.
3.5. Expression of BmG6PD

To obtain the BmG6PD protein expression of cloned gene was carried out by transforming the construct pTriEx-BmG6PD in *E. coli* C-41 cells, which upon IPTG induction, showed over-expression of BmG6PD in soluble fraction whereas no expression was observed in the insoluble fraction. The exhibited size of the recombinant protein was found to be ~75 kDa, along with the ~10 kDa additional amino acids residues of expression vector (Figure 2.6A, lane 3). In contrast, no expression was observed in the soluble fraction of induced cells, which have only vector without insert and in the soluble fraction of uninduced cells. These results suggested that BmG6PD was over-expressed in transformation of pTriEx-BmG6PD into *E. coli* C-41 cells and its induction caused expression of soluble protein of expected size that was detected on Western immunoblotting using anti-His antibodies (Figure 2.6B).

**Figure 2.6**: Expression of BmG6PD in pTriEx-4 vector and its detection by Western blotting. (A) 10% SDS PAGE: Recombinant constructs were transformed in *E. coli* C-41 cells. Transformants were grown overnight at 37°C in LB broth and were subsequently used for inoculation of fresh LB broth. At OD$_{600}$ 0.4-0.6 cultures were induced with 0.8 mM IPTG. At completion of incubation cells were harvested and their soluble and insoluble fractions were prepared as described in ‘Materials and Methods’. Soluble and insoluble fractions were resolved on 10% SDS-PAGE. Lane 1. Pre-stained Protein ladder (Page Ruler™, MBI, fermentas); lane 2. Soluble fraction of uninduced cells; lane 3. Soluble fraction of induced cells; lane 4. Insoluble fraction of uninduced cells; lane 5. Insoluble fraction of induced cells. (B) Western Blotting. Lane 1. Pre-stained Protein ladder (Page Ruler™, MBI, fermentas); lane 2. Soluble fraction of uninduced cells; lane 3. Soluble fraction of induced cells; lane 4. Insoluble fraction of induced cells; lane 5. Insoluble fraction of induced cells.
3.6. Purification of Recombinant BmG6PD

The BmG6PD was successfully expressed and Nickel-affinity chromatography was used for single step purification of His-tagged BmG6PD protein. On SDS-PAGE, the subunit molecular weight of recombinant protein was found to be ~75 kDa (Figure 2.7A). The identity of recombinant protein was further confirmed by Western blotting using anti-His antibodies (Figure 2.7B).

Figure 2.7: SDS–PAGE and Western blot analysis of Ni-NTA purified recombinant BmG6PD. (A) 10% SDS–PAGE of purified recombinant BmG6PD lane 1, prestained protein ladder; lane 2, soluble fraction; lane 3, flowthrough from Ni–NTA column; lane 4, washing of Ni–NTA column with 50mM imidazole; lanes 5 & 6, protein eluted with 250mM imidazole. (B) Western blotting with anti-His antibodies and HRP conjugated anti-mouse IgG lane 1, prestained protein ladder; lane 2, uninduced BmG6PD; lane 3, induced BmG6PD.
3.7. Determination of Native Molecular weight (Mr) of BmG6PD

3.7.1. Size Exclusion Chromatography

Gel filtration experiments were carried out on Superdex™200 10/300GL (Manufacturer’s exclusion limit 600 kDa for proteins) with AKTA Fast Performance Liquid Chromatography (FPLC) Amersham Pharmacia Biotech, Sweden. Gel filtration of the native protein on the Superdex-200 column, calibrated with standard molecular weight markers, showed a single peak with retention volume of 11 ml (Figure 2.8A). The retention volumes obtained for various molecular weight markers viz., ovalbumin (43 kDa), albumin (67 kDa) aldolase (158 kDa), and ferritin (443 kDa) were 15.75 ml, 13.55 ml, 13.16 and 10.07 ml, respectively. When elution volumes of marker proteins were plotted as a function of log molecular mass, BmG6PD was found to have a molecular mass of ~300 kDa, suggesting the tetrameric nature of the protein (Figure 2.8B).

Figure 2.8 : Determination of native molecular weight of BmG6PD. (A) Size exclusion chromatography profile of BmG6PD along with standard molecular weight markers. (B) Elution volumes obtained in Fig. A are plotted as a function of log molecular weight.
3.7.2. Cross-linking Analysis

Tetramer nature of BmG6PD was further confirmed by interchain cross-linking of the monomeric subunits of BmG6PD using glutaraldehyde as cross-linker. The cross-linked product when analyzed by 5-8% gradient SDS-PAGE showed a band at ~300 kDa (Figure 2.9).

Figure 2.9 : SDS-PAGE profile of glutaraldehyde cross-linked BmG6PD. Lane 1. Pre-stained protein ladder (MBI, Fermentas); Lane 2. Uncross-linked BmG6PD protein; Lane 3. Glutaraldehyde cross-linked BmG6PD protein; Lane 4. Unstained high molecular weight protein ladder (Invitrogen).
4. DISCUSSION

G6PD is a NADP⁺ dependent first rate-limiting enzyme of the Pentose Phosphate Pathway which produces pentoses and NADPH. The activities of G6PD and 6-phosphogluconate dehydrogenase depend on the energy and redox potential of the cell (Igoillo-Esteve and Cazzulo, 2006). They are repressed by ATP, NADH and NADPH and control the expression of the genes of the PPP (Ibraheem et al., 2005).

Several genes have been cloned for different research purposes, and related proteins expressed in and purified from *Escherichia coli* cultures. Therefore recombinant DNA technology offers the best possible way to obtain large quantities of purified proteins and hence, this method was used for cloning, expression and purification of BmG6PD. In the present study, BmG6PD was successfully cloned in pTriEx-4 expression vector using *B. malayi* cDNA as template. Cloning was confirmed by sequencing. To evolve their functions, BmG6PD protein was expressed in the *E. coli* strains C41 (DE3) cells and confirm by western blotting, using penta His antibody. BmG6PD protein was purified in large quantity by affinity Ni-NTA chromatography. SDS-PAGE analysis demonstrated the aberrant mobility of the protein can be mainly accounted for by the electrophoretic mobility properties of the BmG6PD protein, however, the open reading frame (ORF) of the BmG6PD cDNA encodes only a 58 kDa protein. Thus the difference between the theoretical molecular weights and the apparent molecular weights of the BmG6PD was due to anomalous electrophoretic migration of the polypeptides by its negative charge (15% acidic amino acids) which is quite common and had been observed with other proteins (Klenova et al., 1997). The enzyme was tetramer (~300 kDa) with a subunit molecular weight of ~75 kDa. Oligomerization of BmG6PD protein was determined by gel filtration and glutaraldehyde crosslinking studies indicated the tetrameric nature of protein. G6PD has been reported to exist as monomer, dimer, tetramer and hexamer, however only dimeric and tetrameric form was found to be enzymatically active. Recombinant BmG6PD resembles human G6PD showing tetrameric nature (Wrigley et al., 1972). 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 (Wang and Engel, 2009).