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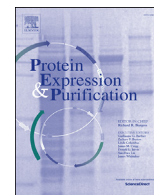

Molecular cloning, purification and characterization of *Brugia malayi* phosphoglycerate kinase

Ranjeet Kumar [a], Pawan Kumar Doharey [b], Jitendra Kumar Saxena [b], Sushma Rathaur [a,*]

[a] Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi 221005, India
[b] Division of Biochemistry, CSIR – Central Drug Research Institute, Lucknow 226001, India

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Phosphoglycerate kinase (PGK) is a glycolytic enzyme present in many parasites. It has been reported as a candidate molecule for drug and vaccine developments. In the present study, a full-length cDNA encoding the *Brugia malayi* 3-phosphoglycerate kinase (*Bm*PGK) with an open reading frame of 1.3 kb was isolated and PCR amplified and cloned. The exact size of the *Bm*PGK's ORF is 1377 bps. The *Bm*PGK gene was subcloned into pET-28a (+) expression vector, the expressed enzyme was purified by affinity column and characterized. The SDS–PAGE analysis revealed native molecular weight of recombinant *Brugia malayi* 3-phosphoglycerate kinase (r*Bm*PGK) to be ~45 kDa. The enzyme was found sensitive to temperature and pH, it showed maximum activity at 25 °C and pH 8.5. The K_m values for PGA and ATP were 1.77 and 0.967 mM, respectively. The PGK inhibitor, clorsulon and antifilarial drugs albendazole and ivermectin inhibited the enzyme. The specific inhibitor of PGK, clorsulon, competitively inhibited enzyme with K_i value 1.88 μM. Albendazole also inhibited PGK competitively with K_i value 35.39 μM. Further these inhibitory studies were confirmed by docking and molecular simulation of drugs with enzyme. Clorsulon interacted with substrate binding site with glutamine 37 as well as in hinge regions with aspartic acid 385 and valine 387 at ADP binding site. On the other hand albendazole interacted with asparagine 335 residues. These effects were in good association with binding interactions. Thus current study might help in designing and synthesis of effective inhibitors for this novel drug target and understanding their mode of interaction with the potent anthelmintic drugs.

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1. Introduction

Lymphatic filariasis is an infectious tropical parasitic diseases, caused by tissue dwelling nematodes e.g. *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. It is commonly known as elephantiasis and is a painful, profoundly disfiguring disease usually acquired in childhood that has major social and economic impact in Asia, Africa, Western pacific and America. This disease is identified as the second leading cause of long-term and permanent disability, with an estimated morbidity at 5.5 million Disability-adjusted life years (DALYs) [1]. At present, combinatorial administration of diethylcarbamazine (DEC), albendazole and ivermectin are being practiced as control measurement of lymphatic filariasis [2]. Since these drugs are not microfilaricidal, they are not recommended in chronic pathology. DEC has been available for treatment of filarial disease for almost 50 years and is still in use. Since the disease treatment is currently based on only a limited number of drugs. A potent macro/microfilaricidal drug is urgently needed to combat the disease. To develop new drugs it is essential to look for specific targets in the filarial worms that can be exploited.

Phosphoglycerate kinase (PGK) seems to be interesting potential drug target due to its importance in energy metabolism of parasites ATP synthesis. Phosphoglycerate kinase (EC 2.7.2.3) produces ATP after catalyzing 1, 3-bisphosphoglycerate to 3-phosphoglycerate. It is used for production of ATP using magnesium as a cofactor and is marked as the first substrate-level phosphorylation reaction in the glycolytic pathway [3]. These reactions occurs during energy generation through glycolytic process and is present in both aerobic and anaerobic metabolisms, which are characteristic for early and late developmental stages of parasites, respectively [4]. In wide range of organisms, the PGK enzyme is highly conserved, the tertiary structure of yeast and horse PGKs are determined by crystallography [5,6]. After vaccination with such an antigens, the probability of eliciting an autoimmune response is high. However,

* Corresponding author.
E-mail address: surathaur@rediffmail.com (S. Rathaur).

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this is reported that parasitic *Fasciola hepatica* PGK has different antigenic epitopes as compared to host PGK, so antibodies produced against parasitic PGK do not reacts with host PGK [7]. The PGK has already been explored in helminths such as *Fasciola hepatica* as potential vaccine candidate for controlling fasciolosis [8].

An antigenic PGK of molecular mass 48 kDa having two isoenzymes, has been purified & characterized in *Plasmodium falciparum* [9]. In *Clonorchis sinensi,* PGK has been localized in different part of parasite by immunoelectron microscopy suggesting that the enzyme play important role in movement via muscle contraction [10]. Further studies were shown that *Clonorchis sinensi* PGK could be used as serodiagnostic reagent for clonorchiasis [11]. Recombinant PGKs of parasites, *Clonorchis sinensi*, *Schistosoma japonicum* and *Plasmodium falciparum* were expressed in *Escherichia coli* exhibited enzymatic activity [11–13]. In contrast three distinct PGK encoded by three different genes have been reported in case of *Trypanosoma brucei* [14]. PGK also influences DNA replication and repair in the mammalian nucleus and a host factor necessary for in vitro mRNA synthesis in *Sendai* virus has been identified [15,16].

The aim of the present study was to clone and express *Brugia malayi* phosphoglycerate kinase (*Bm*PGK) and to perform biochemical, molecular and bioinformatic characterization of recombinant *Bm*PGK. These results will augment our knowledge for understanding the biological role played by PGK in *B. malayi*.

We further extended our work to detect the binding capacity with inhibitor and antifilarials drugs in the PGK sequence of the *Brugia malayi*. A tertiary structure of enzyme was built to dock with clorsulon a specific inhibitor of PGK, antifilarial drugs albendazole and ivermectin to get an insight into their inhibition mechanisms. The information of predicted binding map of *Bm*PGK from this study would aid in structural analysis & screening and designing of new antifilarial drugs.

2. Materials and methods

2.1. Chemicals and reagents

Glyceraldehyde 3-phosphate dehydrogenases (GAPDH), Phosphoglyceric acid were purchased from Sigma (St. Louis, MO, USA). Ni-NTA agarose and Gel elution kit were purchased from Qiagen (Germany). IPTG, pre stained markers, restriction enzymes (*Bam*H1 and *Xho*I), cDNA synthesis kit and T4 ligase were purchased from MBI Fermentas (Hanover, MD, USA). pET-28a (+) expression vector was purchased from Novagen (Madison, WI, USA). pGEMT cloning vector and Taq DNA polymerase were purchased from Promega (Madison, WI, USA).

2.2. PCR amplification of B. malayi PGK gene and cloning

Trizol method was used for isolation of total RNA of *B. malayi* and the quality of isolated RNA was checked on 1.0% (w/v) agarose gel, undegraded RNA preparation showing two distinct bands of 28s and 18s was used for reverse transcription reaction as reported earlier by Pawan et al. [17]. About 20 ng RNA was subjected to cDNA synthesis using cDNA synthesis kit. PCR amplification of *B. malayi* PGK gene was carried out by using the forward primer 5′-GGATCCATGACGCTGAATAAATTATCGATCG-3′ (*Bam*HI site under lined) and the reverse primer 5′-CTCGAGTTAAGCTGGAGAAAGCGCATCAAC-3′ (*Xho*I site under lined). The accession no. of *Bm*PGK is XM_001891857.1. Primers were designed on the basis of sequence information available at http://www.ncbi.nlm.nih.gov/gene. PCR reaction was carried out in 50 μl reaction mixtures consisting of 1 × PCR buffer, 200 μM dNTPs, 10 pmol of each primer, 1.5 mM $MgCl_2$, 20–50 ng cDNA and 1.0 U of Taq DNA polymerase. The PCR reaction was performed for 30cycles: 4 min at 94 °C, 30 s at 94 °C, 1 min at 60.2 °C, 2 min at 72 °C with final extension for 10 min at 72 °C in a thermocycler (PTC-100 MJ Research, USA). The PCR amplification of gene coding for *Bm*PGK carried out using a temperature gradient from 56.5 to 62 °C. The amplicon was analyzed on 1% agarose gel containing 0.5 μg/ml of ethidium bromide and the band was visualized under UV light and documented using Alpha Innotech imaging system (Alpha Innotech Corp. USA). The PCR amplified product was purified from gel by gel extraction kit (QIAGEN, USA). The purified *Bm*PGK PCR product was ligated into pGEMT easy cloning vector at 4 °C overnight according to manufacturers' instructions. The *Bm*PGK–pGEMT easy construct was transformed into competent DH5α *E. coli* cells (made competent using Rapid Transit kit from Sigma) and plated on LB-ampicillin/IPTG/X-gal plates followed by incubation at 37 °C for about 20 h. The resulting colonies were screened for the presence of insert by colony PCR using the gene specific primers. The plasmid DNA was purified from overnight culture using QIAGEN Mini prep kit and the presence of insert was verified by *Bam*H1 and *Xho*1 restriction digestion of purified recombinant plasmid and sub cloned into a prokaryotic expression vector pET-28a (+) resulting in plasmid pET-*Bm*PGK. This plasmid was transformed in T7 RNA polymerase encoding *E. coli* BL21 strain. Cloning of complete *Bm*PGK ORF was confirmed by the sequencing of the clone.

2.3. Expression and purification of recombinant B. malayi PGK

The *E. coli* BL21 strain harbouring pET-*Bm*PGK plasmid was grown overnight in Luria–Bertini (LB) medium at 37 °C, supplemented with 30 μg/ml kanamycin. 1% (v/v) of cells were inoculated into the same fresh medium and cells were grown at 37 °C to mid log phase where optical density is equal to 0.5–0.6. Induction of expression was carried out by the addition of isopropyl β-*d*-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and cell growth was continued at 20 °C for 16 h. Cells were harvested by centrifugation at 8000×*g* for 5 min and pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme) pH 8.0 containing protease inhibitor cocktail (Sigma) and lysed by pulse sonication. The insoluble debris was removed by centrifugation at 12,000×*g* for 45 min at 4 °C. The histidine-tagged *Bm*PGK was purified from the soluble fraction using nickle nitrilotriacetic acid (Ni^{2+}-NTA) affinity resin. The bound protein was eluted using a stepwise imidazole gradient and fractions showing enzyme activity were dialyzed against 50 mM sodium phosphate buffer, pH 7.6, containing 200 mM NaCl, 1 mM EDTA, 8% glycerol and 1 mM DTT.

2.4. SDS-PAGE and MALDI-MS/MS analysis

The molecular mass of r*Bm*PGK was determined by 10% (w/v) SDS-PAGE according to the method of Laemmli [18]. The *Bm*PGK, which migrated as a single band in SDS-PAGE, was excised from the gel and further processed for MALDI mass sequencing as described earlier [19] at the Interdisciplinary School of Life Sciences-Department of Biotechnology, Banaras Hindu University (ISLS, DBT-BHU).

2.5. PGK assay

The activity of r*Bm*PGK was assayed as described by Bucher et al. [20] with minor modifications. The total reaction mixture of 1 ml containing 0.1 mM triethanolamine-chloride (pH 8.5), 10 mM magnesium chloride, 5 mM dithiothreitol (DTT), 1 mM protease inhibitor cocktail, 50 mM sodium chloride, 2 mM ATP, and 10 mM 3-phosphoglyceric acid and purified *Bm*PGK enzyme was incubated

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