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# *Plasmodium falciparum*: Enhanced soluble expression, purification and biochemical characterization of lactate dehydrogenase

Ritu Berwal [a], Natarajan Gopalan [a,*], Kshitij Chandel [a], G.B.K.S. Prasad [b], Shri Prakash [a]

[a] *Defence Research and Development Establishment, Jhansi Road, Gwalior 474002, Madhya Pradesh, India*
[b] *Jiwaji University, Gwalior 474002, Madhya Pradesh, India*

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ABSTRACT

*Plasmodium* lactate dehydrogenase (pLDH), owing to unique structural and kinetic properties, is a well known target for antimalarial compounds. To explore a new approach for high level soluble expression of *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) in *E. coli, Pf*LDH encoding sequence was cloned into pQE-30 Xa vector. When transformed *E. coli* SG13009 cells were induced at 37 °C with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) concentration, the protein was found to be exclusively associated with inclusion bodies. By reducing cell growth temperature to 15 °C and IPTG concentration to 0.25 mM, it was possible to get approximately 82% of expressed protein in soluble form. Recombinant *Pf*LDH (r*Pf*LDH) was purified to homogeneity yielding 18 mg of protein/litre culture. r*Pf*LDH was found to be biologically active with specific activity of 453.8 μmol/min/mg. The enzyme exhibited characteristic reduced substrate inhibition and enhanced $k_{cat}$ [$(3.2 \pm 0.02) \times 10^4$] with 3-acetylpyridine adenine dinucleotide ($APAD^+$). The procedure described in this study may provide a reliable and simple method for production of large quantities of soluble and biologically active *Pf*LDH.

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$A_{260}$, absorbance at 260 nm
$APAD^+$, 3-acetylpyridine adenine dinucleotide
*E. coli*, *Escherichia coli*
HMW, high molecular weight
IPTG, isopropyl β-D-thiogalactoside
$k_{cat}$, catalytic rate constant
$K_m$, Michaelis constant
LB, Luria–Bertani
LDH, lactate dehydrogenase (EC 1.1.1.27)
LMW, low molecular weight
Ni–NTA, nickel–nitrilotriacetic acid
ORF, open reading frame
*Pf*LDH, *P. falciparum* lactate dehydrogenase
pLDH, *Plasmodium* lactate dehydrogenase
r*Pf*LDH, recombinant *Pf*LDH
SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

## 1. Introduction

Malaria counts among mankind's worst scourges. There is a worldwide increasing concern about malaria, not only because of high morbidity and mortality, but also because of the progressive increase of its prevalence in tropical areas, where control measures were effective in the past. Global figures for deaths caused by malaria range from 1.5 to 2.7 million each year, most of whom are children under 5 years of age and pregnant women (Breman, 2001; Phillips, 2001). Almost all these deaths are caused by *Plasmodium falciparum,* one of the four species of malaria parasites in humans. The efficacy of conventional antimalarial drugs and insecticides in controlling falciparum malaria outbreaks is declining with increasing resistance of parasites and their vectors (Price and Nosten, 2001; Wellems and Plowe, 2001; Greenwood and Mutabingwa, 2002; Bernes and White, 2005; Casimiro et al., 2006). Thus, renewed efforts are required to develop novel and affordable antimalarials to overcome the detrimental effects of drug resistance, particularly in developing countries.

During asexual intraerythrocytic cycle, malaria parasite depends extensively upon anaerobic glucose metabolism for ATP generation. Glucose uptake is 30- to 50-folds higher than that of the uninfected

* Corresponding author. Fax: +91 751 2341148.
*E-mail address:* natagopalandrde@gmail.com (N. Gopalan).

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erythrocyte and the major product of glucose metabolism is L-lactate (Seheibel and Pflaum, 1970; VanderJagt et al., 1990). *Plasmodium* lactate dehydrogenase (pLDH; EC 1.1.1.27), the terminal enzyme of anaerobic glycolysis, is very essential for parasite survival as it regenerates $NAD^+$ from NADH for continued use in glycolysis. It has been previously reported that pLDH has unique structural and kinetic properties as compared to its mammalian counterpart making it as a good antimalarial drug target (VanderJagt et al., 1981; Bzik et al., 1993; Makler and Hinrichs, 1993; Makler et al., 1993; Dunn et al., 1996; Gomez et al., 1997; Sessions et al., 1997). It includes lack of substrate inhibition, ability to utilize the NADH analog 3-acetylpyridine adenine dinucleotide ($APAD^+$) as cofactor and a distinctive insertion of five amino acid residues in the active site loop region. The suitability of *P. falciparum* lactate dehydrogenase (*Pf*LDH) as an antimalarial target was further confirmed with the elucidation of the crystal structure of the enzyme which revealed the inserted amino acids formed a cleft adjacent to the active site that might be suitable for binding inhibitors (Dunn et al., 1996). Further apart from antimalarial target, pLDH is a well known diagnostic target owing to presence of unique epitopes as compared to human lactate dehydrogenase (LDH) isoforms (Piper et al., 1999; Fogg et al., 2008).

Over-production of therapeutic and diagnostic target protein plays an important role to provide materials for crystallography, inhibition kinetic studies, immunization and screening assays. Over expression in *Escherichia coli* (*E. coli*) is generally accepted as the best way to produce large quantities of protein. However, one of the major drawbacks associated with the high level expression of heterologous gene products in *E. coli* is the inability of many expressed proteins to reach a native conformation and their tendency to accumulate with in refractile aggregates known as inclusion bodies (Baneyx and Mujacic, 2004). From earlier reports (Bzik et al., 1993; Turgut-Balik et al., 2001) of *Pf*LDH expression using pKK223-3 vector, it has been observed that expression of the protein in *E. coli* is associated with the problem of protein aggregation. Another *Pf*LDH expression report (Gomez et al., 1997) using pTrc99 vector has not mentioned about the relative distribution of expressed protein in soluble/insoluble fractions and yield of the protein. To alleviate problems associated with soluble expression of *Pf*LDH, we have cloned and overexpressed *Pf*LDH and its biochemical characterization was carried out in our efforts to find inhibitors of malaria parasite.

## 2. Materials and methods

*E. coli* host strain SG13009 harboring the pREP4 plasmid encoding the lac I repressor, the expression vector pQE-30 Xa, QIAamp® DNA Blood mini kit, HRP conjugated anti-His antibodies, Factor Xa Protease, Factor Xa removal resin and gel extraction kit were from Qiagen (Valencia, CA, USA). HRP conjugated anti-rabbit antibodies were from Dako A/S, Denmark. PCR kit, all DNA modifying enzymes, protein molecular weight markers were from MBI, Fermentas Inc. (USA). All chemicals, dialysis bags and Gen Elute™ Plasmid Mini Prep kit were procured from Sigma chemicals (S. Louis, MO, USA). Luria–Bertani (LB) broth for preparation of bacterial growth media was obtained from Difco (BD, NJ, USA). Trans-Blot transfer nitrocellulose membranes, PVDF membranes and silver staining kit were from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Amicon ultra centrifugal filter devices were from Millipore Corporation (Bedford, MA, USA). Ni-Sepharose 6 fast flow resin, XK16/20 column, Superdex 200 10/300 GL column, low molecular weight (LMW) and high molecular weight (HMW) gel filtration calibration kits were from Amersham Biosciences (Sweden).

### 2.1. Blood samples

Blood samples (0.5–1.0 ml) were collected from patients with fever who were attending malaria clinics at the primary health care centres at Jind and Delhi, India after obtaining prior permission from the authorities and informed consents from the patients. Thick and thin blood smears were prepared, stained with giemsa and examined for the presence of malaria parasite by light microscopy. *Plasmodium* infected blood samples were stored at 4 °C till further use.

### 2.2. Isolation of P. falciparum genomic DNA and amplification of PfLDH gene

*Plasmodium falciparum* positive blood samples were processed for extraction of genomic DNA. Plasma and buffy coat of leucocytes were removed by centrifugation at 200*g* for 10 min and RBCs (200 μl) were processed for the isolation of *P. falciparum* genomic DNA by using a QIAamp® DNA Blood mini kit as per manufacturer's instructions. Absorbance at 260 nm ($A_{260}$) was measured and DNA concentration was calculated as $A_{260} \times 50\,(\mu g/ml) \times$ dilution factor. The integrity of DNA samples was monitored by agarose gel electrophoresis.

Oligonucleotide primers NGF-5′-ATGGCACCAAAAGCAAAA ATCG-3′ and NGR-5′-TTAAGCTAATGCCTTCATTCTC-3′ corresponding to *Pf*LDH open reading frame (ORF) were constructed based on *Pf*LDH gene sequence (K1 strain) (GenBank: XM_001349953). PCR was performed in 25 μl reaction volume containing 10 pmol of each primer, 1.25 mM $MgCl_2$, 200 μM of dNTPs, 100 ng of *P. falciparum* genomic DNA and 2.5 U of *Pfu* Polymerase. Reaction conditions were as follows: initial denaturation of 5 min at 94 °C, 35 cycles of 60 s at 94 °C, 45 s at 60 °C, 60 s at 72 °C and a 10 min final extension at 72 °C. The amplicon was purified by agarose gel electrophoresis and recovered from gel with gel extraction kit as per manufacturer's instructions.

### 2.3. Cloning of PfLDH ORF into pQE-30 Xa

The commercially available *E. coli* expression vector, pQE-30 Xa, was linearized with StuI restriction enzyme, dephosphorylated with calf intestinal phosphatase and gel purified. The purified amplicon and digested vector were subjected to blunt end ligation. The resulting plasmid, designated pQE-30 Xa*Pf*LDH, was transformed into *E. coli* host strain SG13009 by $CaCl_2$ heat shock method and plated on LB agar plates containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The resultant colonies were screened by direct PCR using insert specific primers. Further, orientation of insert in positive colonies was confirmed using one vector specific primer and one insert specific primer.

### 2.4. Expression of recombinant PfLDH (rPfLDH)

After expression screening of PCR positive clones, plasmid was extracted from the clone showing maximum expression using Gen Elute™ Plasmid mini prep kit and subsequently confirmed by sequencing (Microsynth, Switzerland) in both directions. Expression studies (incubation temperature, IPTG concentration, post-induction time) were performed to identify the best conditions for high level soluble expression of r*Pf*LDH. Samples collected were sonicated and analysed for soluble expression of r*Pf*LDH by determining relative percentage distribution of expressed protein in soluble and insoluble fractions through densitometric analysis using AlphaEase® FC software version 4.1.0 (Alpha Innotech Corp., CA, USA). For expression of *Pf*LDH, mother culture was set up by inoculating 10 ml LB broth supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) with 10 μl glycerol stock of *E. coli* SG13009 cells containing the expression construct pQE-30 Xa*Pf*LDH. The culture was grown overnight at 30 °C, while shaking at 200 rpm. One litre LB broth with appropriate antibiotics was inoculated with 10 ml of overnight culture

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