

## Research Articles

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# ‘DEAD-box’ helicase from *Plasmodium falciparum* is active at wide pH and is schizont stage-specific

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### Abstract

**Background & objectives:** DNA helicases catalyse unwinding of duplex DNA in an ATP-dependent manner and are involved in all the basic genetic processes. In order to study these important enzymes in the human malaria parasite we have recently cloned the first full-length ‘DEAD-box’ helicase gene from *Plasmodium falciparum* (3D7). In the present study, we report some of the important activities of the encoded protein.

**Methods:** We have expressed the *P. falciparum* helicase in *Escherichia coli* and characterised the encoded biochemically active helicase protein. The characterisation of the protein was carried out using radioactively labeled substrate and the standard strand displacement assay. The localisation of the enzyme was studied using immunofluorescence assay.

**Results & conclusion:** *P. falciparum* helicase gene is 1551 bp in length and encodes for a protein consisting of 516 amino acid residues with a predicted molecular mass of 59.8 kDa. The protein is designated as *Plasmodium falciparum* DEAD-box helicase 60 kDa in size (PfDH60). Purified PfDH60 showed ATP and Mg<sup>2+</sup> dependent DNA unwinding, ssDNA-dependent ATPase and ATP-binding activities. Interestingly, this is a unique helicase because it works at a wide pH range (from 5.0–10.0). The peak expression of PfDH60 is mainly in schizont stages of the development of *P. falciparum*, where DNA replication is active. The cell-cycle dependent expression suggests that PfDH60 may be involved in the process of DNA replication and distinct cellular processes in the parasite and this study should make an important contribution in our better understanding of DNA metabolic pathways in the parasite.

**Key words** DNA helicase – malaria – p68 – *Plasmodium falciparum* – unwinding activity

### Introduction

Duplex DNA unwinding is catalysed by a group of enzymes called DNA helicases, which act in an ATP-dependent fashion to produce the ssDNA template<sup>1</sup>. By catalysing the unwinding of duplex DNA, helicases play an essential role in many cellular processes such as DNA replication, repair, recombina-

tion and transcription<sup>2–5</sup>. Mostly, helicases from the variety of organisms contain about nine short conserved amino acid sequence fingerprints (designated as Q, I, Ia, II, III, IV, V and VI), called ‘helicase motifs’<sup>6–9</sup>. Because of the presence of the sequence DEAD or DEAH or DEXH in motif II the helicase family is also called ‘DEAD-box’ protein family. In this family of proteins, the core, which harbours the

conserved motifs, is usually flanked by specific amino- and carboxy-terminal extensions that vary widely in length and sequence<sup>10,11</sup>. Multiple DNA helicases have been isolated from both prokaryotic and eukaryotic systems but not much work has been done on helicases from malaria parasites. It is interesting to note that the genomic sequence of *P. falciparum* shows the presence of multiple putative 'DEAD-box' helicase genes.

*P. falciparum* causes most problematic disease in humans as a result of its prevalence, virulence and drug resistance. The pathogenicity of this parasite mainly results from its rapid rate of asexual reproduction in the host and its ability to sequester in small blood vessels. There are a limited number of drugs in widespread use for the treatment of *P. falciparum* malaria and a lack of new affordable drugs<sup>12</sup>. *P. falciparum* slowly has developed resistance to nearly all available antimalarial drugs<sup>13</sup>. The search for novel effective, safe and affordable antimalarial drugs for *P. falciparum* malaria is one of the most important tasks to pursue. In the case of *P. falciparum*, based on differential gene expression study in the presence of chloroquine, it has been predicted that RNA helicase like proteins may be involved in antimalarial action of the drug<sup>14</sup>. In order to find alternate ways to control malaria through inhibitors specific for DNA/RNA helicases, we have initiated a systematic study of helicases from malaria parasites.

Recently, we have purified and characterised an eIF-4A homologue, which is the prototype of the DEAD-box family of helicases, from *Plasmodium cynomolgi*<sup>15,16</sup>. Here we report our recent work on the first full-length helicase gene from *P. falciparum* (3D7) and the detailed characterisation of the encoded functionally active helicase protein. Our studies show that this enzyme contains DNA helicase, ssDNA-dependent ATPase and ATP-binding activities. It is highly homologous to p68, a member of the 'DEAD-box' protein family, which has well conserved orthologues from yeasts to humans<sup>17-21</sup>. The nuclear 'DEAD-

box' protein p68 was identified because of its immunological relations to the SV40 large tumor antigen, it has been shown to contain RNA helicase activity and the human p68 protein has been shown to undergo dramatic change in nuclear location during the cell cycle<sup>21-23</sup>. The immunofluorescence assay studies of PfDH60 revealed that its peak expression is mainly in the schizont stages of the development of *P. falciparum*, where DNA replication is active.

## Material & Methods

*Isolation of genomic DNA and PCR: P. falciparum* (strain 3D7) was cultured using human erythrocytes with 5% haematocrit in RPMI media from Gibco supplemented with 10% human serum using a protocol described earlier<sup>24</sup>. The genomic DNA from this parasite culture was isolated using standard protocol<sup>25</sup>. The DEAD-box helicase gene was amplified using *P. falciparum* genomic DNA as template and oligonucleotide primers PfH3F-5'-GGGATCCATGGAAAGGCAAATCTA-3' and PfH3R-5'-CC-AAGCTTTCATCTT GAATAAGCTAT-3'. The primers were synthesised from Microsynth (Microsynth GmbH, Balgach, Switzerland). These primers contain *Bam*HI and *Hind*III sites for cloning (the recognition sequence for restriction enzymes is written in italics). The PCR conditions used for primer pair PfH3F and PfH3R were 95°C for 1 min, 54°C for 1 min and 72°C for 2 min. This was repeated for a total of 35 cycles and at the end one elongation was done at 72°C for 10 min. The PCR products were analysed by agarose gel electrophoresis. A single band of ~1.5 kb was obtained. This product was further validated by using the primer pair PfH3M-5-GTTATTGATGAAGCTGATCGT-3' and PfH3R and same PCR conditions. A single band of expected size (~700 bp) was obtained. The PCR product of 1.5 kb was gel purified and cloned into pGEM-T vector (Promega) to generate pGPfH clones. The DNA clones obtained were sequenced by dideoxy sequencing reactions. The DNA band was excised using *Bam*HI and *Hind*III enzymes and gel purified for

subcloning into the expression vector.

**Southern blotting:** For Southern blotting the *P. falciparum* genomic DNA was digested with *Bam*HI and *Nde*I. The digested DNA was electrophoresed on a 0.8% agarose gel and transferred to Nylon membrane according to standard protocol<sup>25</sup>. The longer PCR fragment was labeled using Random priming kit (Invitrogen, CA, USA) and  $\alpha^{32}\text{P}$  dCTP and was used as a probe for hybridisation after purification. The membrane was pre-hybridised in 6 XSSC (SSC is 0.15 M NaCl, 0.15 M sodium citrate), 5 X Denhardt's solution for 3 h at 60°C. Hybridisation was done overnight in the pre-hybridisation buffer containing the labeled probe at 55°C. The blot was washed twice for 5 min at 25°C with 5X SSC, 0.1% (w/v) SDS and then twice at 50°C for 15 min with 2 XSSC and 0.1% SDS. The bands were visualised by autoradiography.

**Expression and purification of protein:** The complete open reading frame of *P. falciparum* helicase was subcloned into an expression vector pET-28a at *Bam*HI/*Hind*III sites. The expression clones were transformed into *E. coli*. Bacteria were grown in LB medium to  $A_{600\text{nm}} = 0.6$ . The expression of recombinant protein was induced by 1mM IPTG for 4 h. To purify the recombinant protein, the harvested bacterial cells were subjected to three freeze/thaw cycles at -70°C. After suspension in lysis buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% Triton and 0.5% Tween 20), the bacterial cells were further disrupted by sonication. After centrifugation at 4°C, the soluble bacterial lysates were allowed to bind to Ni-NTA (Qiagen, GmbH, Germany) in binding buffer (250 mM NaCl, 20 mM Tris-HCl pH 8.0) supplemented with 20 mM imidazole and protease inhibitor cocktail at 4°C for 1 h (Sigma). The column was extensively washed sequentially with binding buffer supplemented with 50 mM and 100 mM imidazole and protease inhibitor cocktail. The recombinant His-tagged proteins were eluted with 250 mM imidazole in protein buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10% glycerol and protease inhibitor cock-

tail). The protein was further dialysed against the same buffer without any imidazole or NaCl. The final purified protein was checked for purity on 10% SDS-PAGE. This purified protein was used for all the assays described in the following sections.

**Western blotting:** For Western blotting, the proteins were separated on SDS-PAGE and electrophoretically transferred to nitrocellulose membrane as described<sup>26</sup>. The membranes were blocked in 2% non-fat milk in Tris-buffered saline (TBS) and incubated with the appropriate primary antibody (Penta-His, Qiagen, GmbH, Germany or anti-pea p68 polyclonal antibody raised in rabbit (a kind gift from Dr. Narendra Tuteja, ICGEB, New Delhi, India) for 3 h at room temperature. The blot was washed and incubated for 1 h with the appropriate secondary antibody (Sigma) coupled to alkaline phosphatase. The blots were developed using BCIP and NBT (Sigma) according to the manufacturer's instructions.

**Preparation of substrate and DNA helicase assay:** The DNA helicase substrate used in the DNA unwinding assay was prepared in the same way as described earlier<sup>27</sup>. The oligodeoxynucleotides were synthesised from Microsynth (Microsynth GmbH, Balgach, Switzerland). The regular substrate used in this study consisted of a  $^{32}\text{P}$ -labeled 47-mer DNA oligodeoxynucleotide [5'-(T)<sub>15</sub>GTTTTCCAGTCACGAC(T)<sub>15</sub>-3'] annealed to M13mp19 phage ssDNA to create a partial duplex. At both the 5' and 3' ends, this oligodeoxynucleotide contains 15 bp of non-complementary region. About 10 ng of this oligodeoxynucleotide was labeled at 5-end with T4 polynucleotide kinase (5U) in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine and 1.85 MBq of [ $\gamma^{32}\text{P}$ ] ATP (Specific activity 222 TBq/mmol). The labeled oligodeoxynucleotide was then annealed with 2.5  $\mu\text{g}$  of single-stranded circular M13mp19 (+) DNA in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 100 mM NaCl and 1 mM DTT by heating at 95°C for 1 min, transferring immediately to 65°C for 2 min and then

cooling slowly to room temperature. Non-hybridised oligodeoxynucleotide was removed by gel filtration through a 1 ml Sepharose 4B column (Pharmacia, Sweden) with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 100 mM NaCl.

The helicase assay measures the unwinding of  $^{32}\text{P}$  labeled DNA fragment from a partially duplex DNA molecule. The reaction mixture (10  $\mu\text{l}$ ) containing 20 mM Tris-HCl (pH 8.0), 8 mM DTT, 1.0 mM  $\text{MgCl}_2$ , 1.0 mM ATP, 10 mM KCl, 4% (wt/vol) sucrose, 80  $\mu\text{g/ml}$  BSA,  $^{32}\text{P}$  labeled helicase substrate (~1000 cpm) and the helicase fraction to be assayed was incubated at 37°C for 60 min (unless otherwise indicated). The reaction was terminated by the addition of 0.3% SDS, 10 mM EDTA, 5% glycerol and 0.03% bromophenol blue. After further incubation at 37°C for 5 min, the substrate and products were separated by electrophoresis on a 12% non-denaturing polyacrylamide gel. The gel was dried and exposed to hyper film with an intensifying screen for autoradiography. DNA unwinding was quantitated as described previously.

*DNA-dependent ATPase assay and affinity labeling with [ $\alpha$ - $^{32}\text{P}$  ATP]:* The hydrolysis of ATP catalysed by PfdH60 was assayed by measuring the formation of  $^{32}\text{P}$  from [ $\gamma$ - $^{32}\text{P}$ ] ATP. The reaction condition was identical to those described for the helicase reaction, except that the  $^{32}\text{P}$ -labeled helicase substrate was replaced by a mixture of [ $\gamma$ - $^{32}\text{P}$ ] ATP (specific activity 222 TBq/mmol) and cold ATP (1 mM). The reaction was performed for 2 h at 37°C both in the presence and absence of 100 ng of M13 mp19 ssDNA, followed by thin layer chromatography and quantitation as described earlier<sup>28</sup>.

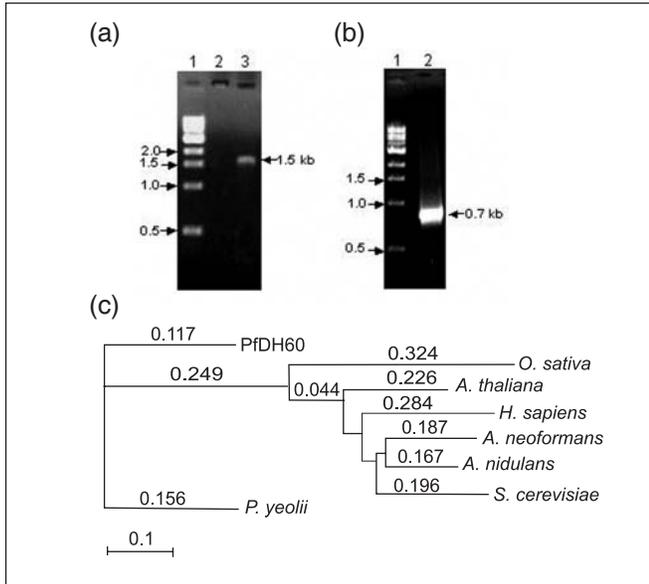
The affinity labeling reaction was carried out in ice with 20 ng of pure PfdH60 in 10  $\mu\text{l}$  buffer containing 15 mM HEPES pH 7.9, 60 mM KCl, 1 mM  $\text{MgCl}_2$ , 6 mM DTT and 2.22 MBq of  $\alpha$ - $^{32}\text{P}$  ATP (specific activity 222 TBq/mmol) in the presence of 3  $\mu\text{g ml}^{-1}$  M13ssDNA. The mixture was irradiated with a

254 nm UV light at a distance of 5 cm for 30 min in ice and then incubated for 10 min without irradiation at room temperature. The products were separated by 10% SDS-PAGE and radio labeled protein was visualised by autoradiography.

*Immunofluorescence assay:* For this assay smears of parasitised red blood cells of different developmental stages were prepared and fixed. For blocking, the slides were incubated in 10% fetal calf serum in phosphate buffered saline (PBS). The slides were washed with PBS and incubated with purified IgG of anti-PfdH60 antibodies at 1 : 200 dilutions in PBS containing 10% foetal calf serum. After washing, the slides were incubated for 1 h at 37°C with secondary antibody (fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma). After washing the slides were incubated in 4',6'-di-amidino-2-phenylindole dihydrochloride (DAPI) (2  $\mu\text{g/ml}$  in PBS) for nuclear staining. The slides were washed and mounted with anti-fade reagent (Fluoroguard, BioRad, USA) and viewed under oil immersion. Confocal images were collected using a Bio-Rad 2100 laser-scanning microscope attached to a Nikon TE 200U microscope and the figures were prepared using Adobe Photoshop.

## Results

*Cloning of DEAD-box DNA helicase from *P. falciparum* and southern blotting:* In order to clone the first 'DEAD-box' helicase from *P. falciparum*, the genome of *P. falciparum* was searched using 'DEAD-box' motif as query. A number of positive hits were found. Out of these, some were chosen and analysed further. The sequence with complete reading frame upstream and downstream of 'DEAD-box' motif, which also contained all the other helicase motifs including the 'Q' motif was chosen for further studies. Accordingly the primers Pfh3F and Pfh3R were synthesised and used for PCR using *P. falciparum* genomic DNA as template. A single band of ~1.5 kb was obtained which agreed with the size of the gene (Fig. 1a). To confirm the validity of the gene, the primer pair



**Fig. 1:** (a) and (b) – PCR amplification of DEAD-box helicase of *P. falciparum*. Panel (a) shows the PCR product using primers PfH3F and PfH3R. Panel (b) shows the PCR product using primers PfH3M and PfH3R. The numbers in both the panels indicate the size of the bands in kb; and (c) – Comparison of amino acid sequence of PfdH60 protein with other DEAD-box helicases. Phylogenetic guide tree using Clustal W program based on the multiple alignment. Amino-acid sequence alignment of PfdH60 protein with other p68 homologues from *P. yeonii* (EAA15859), *C. neoformans* (EAL20020), *A. thaliana* (NP974985), *O. sativa* (XP462826), *A. nidulans* (EAA57794), *H. sapiens* (NP006377) and *S. cerevisiae* (CAA36874). Multiple alignment was done using Clustal W program and MacVector 7.2.2 software. The accession numbers are written in brackets. A value of 0.1 corresponds to a difference of 10% between two sequences

PfH3M and PfH3R was used with the product of the first PCR as template. A product of expected size of ~700 bp was obtained (Fig. 1b).

The product of the first PCR was cloned in pGEMT vector and positive clones were sequenced. The sequence analysis showed a complete genomic clone of 1551 bp with a methionine at the start and termination codon at the end. The deduced amino acid sequence revealed a protein consisting of 516 amino

acid residues with a predicted molecular mass of approximately 59.8 kDa. The sequence analysis also confirmed the presence of all the conserved domains of the DEAD-box protein family including the ‘Q’ motif<sup>7,9</sup>. Therefore, this protein is designated as *P. falciparum* DEAD-box helicase 60 kDa in size (PfdH60). It was observed that PfdH60 contains PTRELC in motif Ia in place of PTRELA. In domain II, PfdH60 contains VIDEAD in place of VLDEAD and in domain III: in place of commonly present sequence SAT, PfdH60 contains TAT<sup>29</sup>. The sequence RGLD is present in motif V of PfdH60. It has been speculated that this motif may function as an RNA-binding site in p68 family of proteins<sup>30</sup>.

A multiple alignment of amino acid sequence homology search using NCBI database revealed that PfdH60 aligned contiguously and showed highest homology with a putative ‘DEAD-box’ helicase from *P. yeonii* (~95%) and ~ 68–70 % similarity with p68 from various sources<sup>31</sup>. The blast analysis of PfdH60 also indicated that this gene is more homologous to p68 from plants. Phylogenetic tree reconstruction using ClustalW method showed that PfdH60 was evolutionary closest to a group of plant proteins including *O. sativa* Genbank entry 50900786 and *A. thaliana* entry 42573778 (Fig. 1c). These proteins have not been characterised but they are likely to share functionality with PfdH60. Next entry in the tree is *H. sapiens* p68 Genbank entry 38201710 and this protein has been well characterised and shown to contain helicase activity<sup>32</sup>. Another group of related proteins contain the Genbank entries 50257311 from *C. neoformans* and 40738604 from *A. nidulans*. These two entries are also hypothetical proteins but are likely to share functionality with PfdH60. The distantly related protein is the well-characterised p68 protein from *S. cerevisiae* Genbank entry 5272 (Fig. 1c)<sup>18</sup>. For Southern blot analysis the *P. falciparum* genomic DNA was digested with *Bam*HI and transferred to nitrocellulose as described in materials and methods. This DNA showed a single hybridising band at ~9.0 kb suggesting the existence of a single

copy of *PfDH60* gene in *P. falciparum* (Fig. 2a).

**Expression and purification of protein *PfDH60*:** For biochemical characterisation of *PfDH60*, the gene was cloned in the bacterial expression vector pET28a and the protein was produced after induction with IPTG. The SDS-PAGE analysis showed an additional polypeptide of ~60 kDa that is IPTG-induced in *E. coli* transformed with pET-*PfDH60*. *PfDH60* was purified using the soluble fraction only. The cytosolic extract was allowed to bind to Ni<sup>2+</sup>-NTA-agarose matrix and the bound *PfDH60* was eluted with 250 mM imidazole in protein buffer. The final purification step yielded a purified *PfDH60* enzyme, that showed a 59.8 kDa band on SDS-PAGE. The purity of this preparation was further confirmed by Western blotting using anti-His antibodies, that detected a single band of 59.8 kDa (Fig. 2b). This purification protocol routinely yielded ~0.5 mg of homogenous *PfDH60* from 1 litre bacterial culture. The purified

protein was checked for cross reactivity to anti-pea p68 by western blotting. The results show that *PfDH60* reacts with the polyclonal antibodies to pea p68 (Fig. 2c).

**DNA helicase, ATPase and ATP-binding activities of *PfDH60*:** The substrate for all the studies contained non-complementary tails of 15 nucleotides on both the 5' and 3' ends<sup>33</sup>. The reaction requirements of the enzyme are shown in Table 1. The enzyme was inactivated upon heating at 55°C for 5 min or after prolonged storage at 4°C. The enzyme activity was inhibited with EDTA (5 mM), ssDNA (10 µg/ml) and synthetic RNA (1 µg/ml) (Table 1). The helicase activity of *PfDH60* was checked over a pH range from 5.0–10.0. For checking the stability of the substrate,

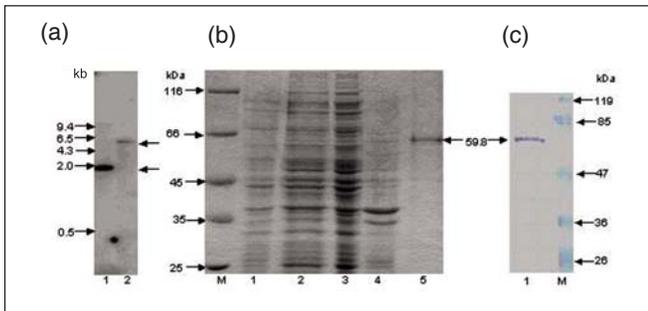


Fig. 2: (a) – Southern blot analysis of the DEAD-box helicase gene of *P. falciparum*. Genomic DNA was digested with *Bam*HI (lane 1) and *Nde*I (lane 2) restriction enzymes. Molecular weight markers in kilobases (kb) are shown on the left side of the autoradiogram and the arrows show the hybridised bands; (b) – Expression and purification of *PfDH60*. The proteins were separated by SDS-PAGE and visualised by Coomassie blue staining. Lane M is the molecular weight marker. Lane 1, uninduced bacterial cell lysate, lane 2, IPTG-induced bacterial cell lysate, lane 3, cytosolic fraction of cell lysate, lane 4, pellet of cell lysate, and lane 5, purified *PfDH60* after Ni<sup>2+</sup>-NTA agarose column; and (c) – Western blot analysis of the purified protein using anti-pea p68 antiserum. Only the pure *PfDH60* was checked for cross-reactivity

**Table 1. Reaction requirements of *PfDH60***

Reaction conditions	Helicase activity % unwinding
Complete reaction	80
–enzyme	<4
+heated enzyme (55°C, 5 min)	<4
–ATP	<4
–ATP + dATP (1 mM)	75
–ATP + ATP [γS] (1 mM)	<4
–ATP + CTP or dCTP (1 mM)	<4
–ATP + GTP or dGTP (1 mM)	<4
–ATP + dTTP (1 mM)	<4
–ATP + UTP (1 mM)	<4
–MgCl <sub>2</sub>	<4
–MgCl <sub>2</sub> + MnCl <sub>2</sub> (1 mM)	75
–MgCl <sub>2</sub> + AgCl <sub>2</sub> (1 mM)	<4
–MgCl <sub>2</sub> + CaCl <sub>2</sub> (1 mM)	<4
–MgCl <sub>2</sub> + CuCl <sub>2</sub> (1 mM)	<4
–MgCl <sub>2</sub> + NiCl <sub>2</sub> (1 mM)	<4
–MgCl <sub>2</sub> + ZnCl <sub>2</sub> (1 mM)	<4
+KCl (200 mM)	<4
+EDTA (5 mM)	<4
+M13 ssDNA (10 µg/ml)	<4
+M13 RF1DNA (10 µg/ml)	74
+Trypsin (1U)	<4
+Poly [C], or [C/G] (30 µM as P)	72
+Poly [A], or [U] (30 µM as P)	<10
+Synthetic RNA [1 µg/ml]	<20

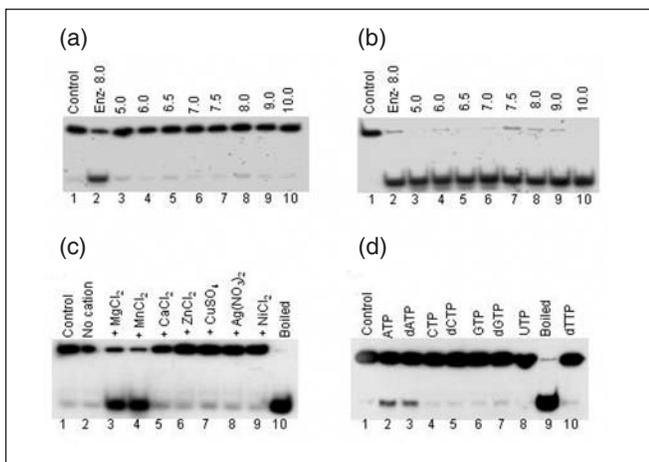
first the substrate alone was incubated for 1 h in standard assay conditions and it was observed that the partially duplex DNA substrate was stable at this wide pH (Fig. 3a).

Further it was observed that PfdDH60 was able to unwind the partially duplex DNA substrate over a wide pH range from 5.0–10.0 (Fig. 3b). But further characterisation of the helicase activity was carried out at pH 8.0. For optimum activity PfdDH60 required 30 mM KCl and at higher concentration (200 mM) the activity was completely inhibited.

The optimum concentration of  $MgCl_2$  was 1 mM and at 8 mM the unwinding activity was completely inhibited. In the presence of  $Mn^{2+}$  the enzyme had almost the same activity as in the presence of  $Mg^{2+}$

(Table 1). Other divalent cations such as  $Ag^{2+}$ ,  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ ,  $Ni^{2+}$  and  $Zn^{2+}$  were unable to support any unwinding activity (Fig. 3c) (Table 1). DNA helicase activity of PfdDH60 was totally dependent on the hydrolysis of ATP, with an optimum concentration requirement of 1.0 mM. At 8.0 mM ATP concentration the helicase activity was completely inhibited. Only dATP supported approximately similar activity while other NTPs or dNTPs could not support any unwinding activity (Fig. 3d) (Table 1). The unwinding activity of PfdDH60 required the hydrolysis of ATP since the poorly hydrolysable analog ATP $\gamma$ S was inactive as a cofactor (Table 1).

The results of ATPase assay showed that there was negligible hydrolysis without ssDNA and  $Mg^{2+}$ . ssDNA and synthetic RNA were able to stimulate ATP hydrolysis several folds. The ATPase activity also followed the same kinetics as helicase activity (Figs. 4a and b). The results of ATP-binding activity of PfdDH60 showed that a polypeptide of 60 kDa is radiolabeled after the reaction. As a positive control, *Escherichia coli* DNA polymerase I (approximately 100 kDa) was also affinity-labeled to provide a 100 kDa band as a marker on SDS-PAGE (Figs. 4c and d).

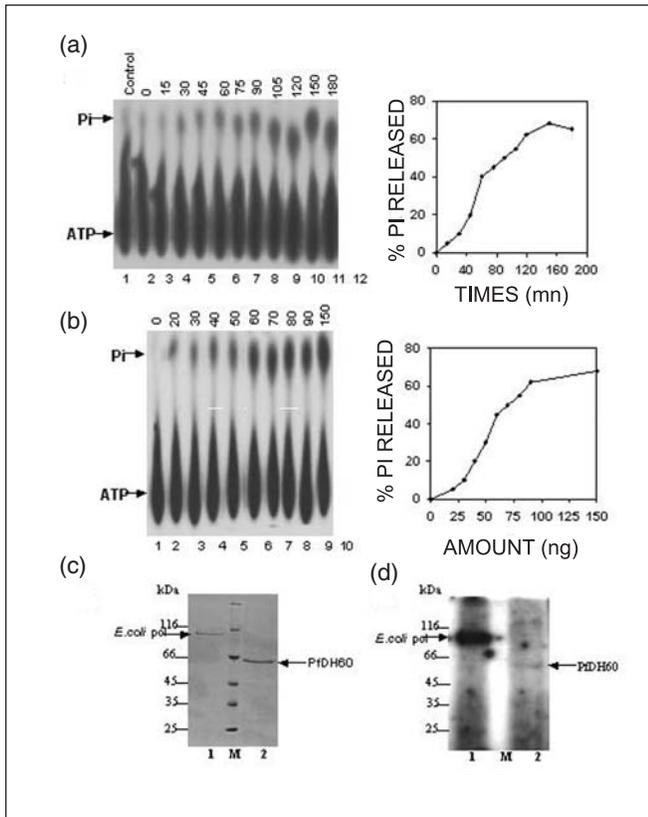


**Fig. 3:** Effect of different pH on helicase activity. (a) – The partially duplex DNA substrate was incubated for 1 h using buffers of different pH. Control is the substrate at pH 8.0 and enzyme is the reaction in the presence of pure PfdDH60. pH is written on the top of each lane in both the panels; (b) – The enzyme reaction was carried out for 1 h using buffers of different pH. Control is the substrate alone at pH 8.0 and lanes 2–9 are the reactions in the presence of pure PfdDH60 at different pH. Lane 10 is reaction with heat-denatured substrate; (c) – Effect of different divalent cations and nucleoside triphosphates; and (d) – on helicase activity of PfdDH60. Lanes marked ‘Control’ and ‘Boiled’ are the reactions without the enzyme and with heat-denatured substrates, respectively

*Localisation of PfdDH60 by immunofluorescence assay:* It is interesting to note that there was no expression of PfdDH60 in the ring and trophozoite stages and the expression was mainly in the schizont stages of the parasite (Figs. 5a–d). The results indicate that PfdDH60 is expressed in a cell cycle-dependent manner with peak expression in the schizont stages.

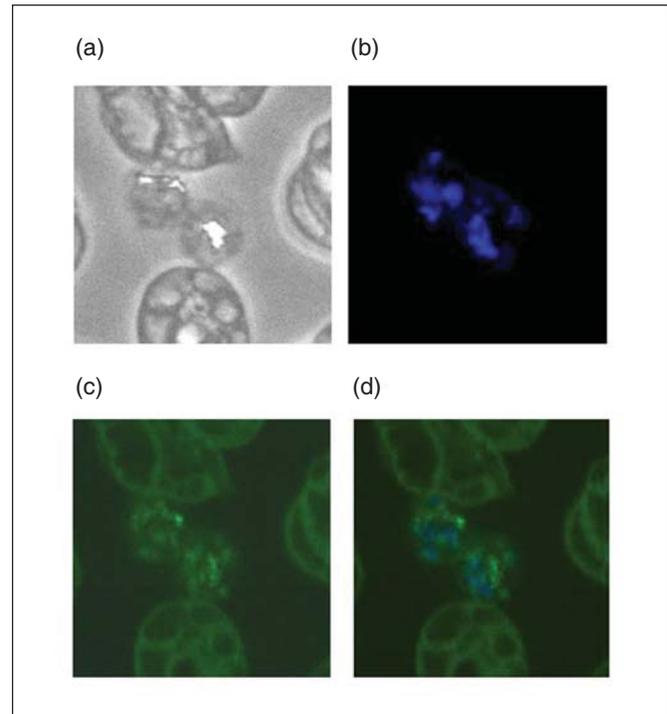
## Discussion

The DNA helicases are very important enzymes since they are known to play essential role in unwinding of duplex DNA or RNA strands in every aspect of nucleic acid metabolism. Although a number of DNA helicases have been characterised from a variety of sources such as bacteriophage, bacteria, fungal, viral, plant and animal systems<sup>4,5,33,34</sup>, little is known about



**Fig. 4:** ATPase and ATP-binding activities of PfdH60. The quantitative enzyme activity data from the autoradiogram are shown on the right side of the TLC plate in (a) and (b). (a) – The standard ATPase reaction was carried out with 90 ng of pure PfdH60; (b) – An increasing amount of PfdH60 was used in the standard ATPase assay. The positions of the Pi and ATP spots are indicated by arrows. The activity is shown as percent Pi released; (c) – Coomassie- blue stained SDS-PAGE gel after photoaffinity labeling and UV cross-linking reaction. Lane M is the molecular weight marker and the size in kDa is written on the left in both the panels; and (d) – Autoradiogram of the gel shown in panel (c). Lane 1 is labeled *E. coli* DNA polymerase; and lane 2, is labeled PfdH60

DNA helicases from malaria parasite. Recently, we have reported an eIF-4A homologue PcDDH45 from *P. cynomolgi* and characterised it<sup>15,16</sup>. In this study we have cloned, expressed, purified and characterised a novel functionally active ‘DEAD-box’ DNA helicase named PfdH60 from *P. falciparum* and have shown that it is a homologue of p68 from various sources. Previously p68 has been reported as RNA



**Fig. 5:** Localisation of PfdH60 in schizont stages of the development of *P. falciparum* by immunofluorescence staining and confocal microscopy. In each panel single confocal image is shown. (a) – Phase contrast image; (b) – Image of cell stained with DAPI (blue); (c) – Immunofluorescently stained cell (green); and (d) – Super-imposed image

helicase<sup>30,32</sup>, but its DNA helicase activity has not been reported so far. Biochemical analysis with purified PfdH60 confirmed that it functions as a DNA helicase and it also contains ssDNA-dependent ATPase and ATP-binding activities. This is the first report to show that a homologue of p68 from *P. falciparum* encompasses the DNA helicase activity.

The PfdH60 protein sequence contained all the nine conserved helicase domains including ‘Q’ motif and therefore it belongs to the ‘DEAD-box’ protein family<sup>32</sup>. It is clear from the alignment results that PfdH60 contains a similar core region encompassing the conserved domains but it has different N- and C-terminal extensions, which endow the protein with specialised function<sup>35</sup>. The alignment results also show that PfdH60 is a homologue of p68 from vari-

ous sources and phylogenetic analysis shows that it is closer to plant p68. Most of the members of the 'DEAD-box' protein family have been identified as 'putative computer predicted helicases'. Only a few members have been shown to contain DNA or RNA unwinding activity. The main examples of DNA helicases from this family are the *E. coli* RecQ gene product, a 74 kDa protein<sup>36</sup>, yeast Rad3<sup>37</sup>, a product of excision repair gene *ERCC3*<sup>38</sup>, a 72 kDa protein encoded by the human *REQL* gene<sup>39</sup>, the 172 kDa protein encoded by *DNA2* gene in yeast<sup>40</sup>, PDH45 from pea<sup>41</sup> and eIF-4A from *P. cynomolgi*<sup>16</sup>. On the other hand some other members of 'DEAD-box' protein family such as *Arabidopsis thaliana* DRH1, *Drosophila* VASA, pea PDH45, mouse eIF-4A, Xenopus-an3 and Xp54, human p68, *E. coli* CsdA, RhlE and SrmB and hepatitis C virus NS3 helicase<sup>32,35,41-44</sup> have been shown to contain RNA unwinding activity. PfdH60 is homologous to p68 and various studies have shown that p68 is required for normal growth and development and it has been shown to be associated with organ differentiation/maturation in the foetus<sup>45</sup>.

Biochemical characterisation of PfdH60 showed that it could unwind DNA duplex almost equally well at all the pH ranging from 5.0–10.0. PfdH60 required ATP or dATP as a cofactor for optimal activity, although dATP showed ~95% of the activity, no other NTPs or dNTPs could be utilised as a cofactor. It is evident that ATP hydrolysis is required for the DNA unwinding activity of PfdH60, as the non-hydrolysable analogue of ATP (ATP $\gamma$ S) could not support any unwinding activity.

PfdH60 contains ATPase activity, which was maximally stimulated by ssDNA. ssDNA-dependent ATPase activity has been reported to be the intrinsic property of all the helicases and the energy released is also required for translocation of the helicase protein on the DNA<sup>4,33</sup>. Although a number of helicases have been characterised biochemically, it is generally difficult to determine the *in vivo* role of a specific

helicase. However, till to date the biological role of a few selected helicases have been determined<sup>5</sup>. PfdH60 could be a novel homologue of p68 from malaria parasite with DNA helicase activity. The findings demonstrate that p68 might also have a role in DNA metabolism. Using immunofluorescent assays we have shown that PfdH60 is present at low levels in the trophozoites and accumulates to peak levels in the schizont stages of the development of *P. falciparum*, which is in agreement with the expression of other *P. falciparum* DNA replication genes such as topoisomerase I. The isolation and characterisation of the first DNA helicase from the malaria parasite is the first step towards elucidating the DNA transaction mechanism in the parasite.

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