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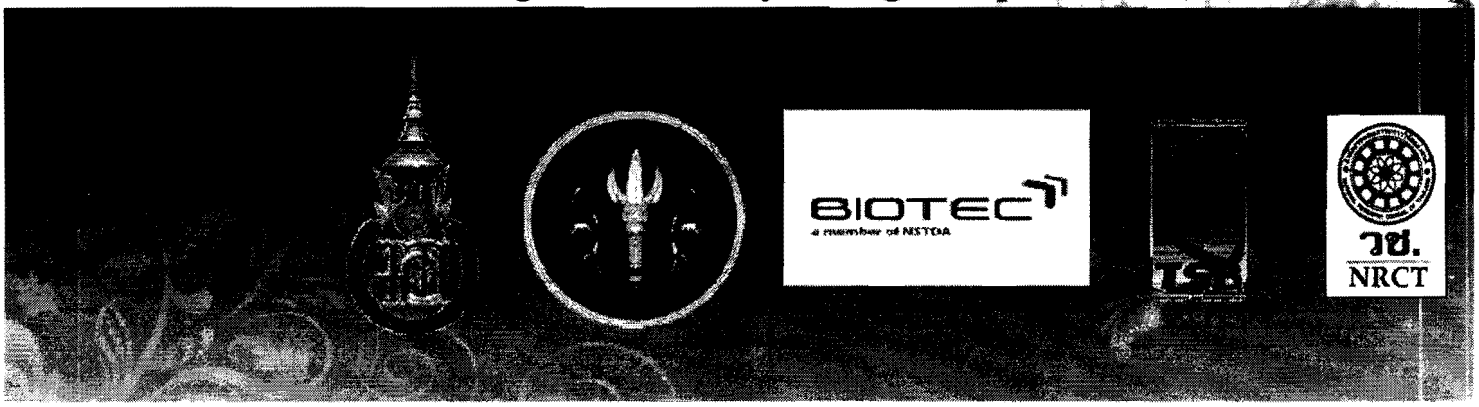

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cDNA cloning, expression and preliminary biochemical characterization of adenine phosphoribosyltransferase from *Brugia malayi*

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Abstract

Brugia malayi is the etiologic cause of lymphatic filariasis that is prominently confined to Southeast Asia region including Thailand. Moreover, current drug therapy has several disadvantages. Drug resistance in *B. malayi* has not yet been reported, however, drug resistance in other nematodes have been reported. Analysis of *B. malayi* genome suggests that the nematode relies on purine salvage pathway to meet their purine nucleotide requirement; therefore enzymes in this pathway might be potential drug targets for antifilarial drugs. Adenine phosphoribosyltransferase (APRT: E.C 2.4.2.7) of *B. malayi* is a key enzyme that has not been characterized biochemically. The catalytically active, recombinant *B. malayi* APRT, as a *N*-terminal His-tagged fusion protein was successfully expressed in *Escherichia coli* strain Rosetta2™ (DE3) and purified to near homogeneity using Ni-IDA resin. Preliminary steady-state kinetic analysis with adenine and 5-phospho-α-D-ribose-1-diphosphate (PRPP) yielded K_m values of 1.0 μM and 2.7 μM, respectively. Analysis of a 3D model of *B. malayi* APRT compared with human APRT crystal structure revealed unique difference of the non-conserved residues in the purine binding site. Functional contribution of these residues in *B. malayi* APRT together with their homologs in human APRT will be further investigated by site-directed mutagenesis.

Keywords: *Brugia malayi*; Adenine phosphoribosyltransferase; biochemical characterization; 3D-model

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Introduction

Brugia malayi is the pathogen that causes lymphatic filariasis (LF) in human. It is prominently confined to South-East Asia including Thailand (Hertz-Fowler and Pain 2008). Currently, anthelminthic drugs that are used for treatment of LF have several disadvantages such as being restricted to use in some patients (Briggs, et al. 2001; Molyneux, et al. 2003) and not fully active against adult filarial worms.

Even though resistance to these drugs in *B. malayi* has not yet been reported, drug resistance in other nematodes has been documented (Geerts and Gryseels 2000; Hoti,

et al. 2003). Therefore, the study of new molecular targets is essential to provide foundation for effective antifilarial drug design.

For modern drug design, identification and characterization of new molecular targets are required for design and development of new anthelminthic drugs that act selectively against etiologic parasites without causing harmful effects to human. Adenine phosphoribosyltransferase (APRT; E.C. 2.4.2.7) is an enzyme involved in transfer of phosphoribosyl moiety from 5-phospho-α-D-ribose-1-diphosphate (PRPP) to adenine to form adenosine monophosphate (AMP). The nucleoside monophosphate is further phosphorylated and finally incorporated into the nucleic acids. APRT is essential to *B. malayi* because it lacks 9 of 10 key enzymes in *de novo* purine biosynthesis (Ghedin, et al. 2007). Therefore, inhibition of APRT potentially leads to growth retardation of the organisms. For this reason, APRT could be a potential target of antifilarial drugs. A molecular property of *B. malayi* APRT which is essential for the design of selective inhibitors has not been studied. Hence, the cDNA cloning and biochemical characterization of APRT from *B. malayi* are the primary aims of this research.

Materials and Methods

Total RNA isolation

Total RNA was extracted from microfilaria of *B. malayi*. The microfilariae were preserved in Trizol® reagent and subjected to phase separation technique using chloroform, isopropanol and 70% ethanol. The purified total RNA was dissolved in DNase-RNase free water.

cDNA pool synthesis

The cDNA pool of *B. malayi* was prepared from the total RNA and poly(T) primer (RACE42: 5'-GACTACGTTAGCATCTAGAATTCTCGACTTTTTTTTT TTTTTT-3') by reverse transcription using reverse transcriptase. Total RNA was heated at 65 °C for 5 minutes and chilled on ice immediately prior to reverse transcription. The resulting cDNA pool was purified and collected in DNase-RNase free water and stored at -20 °C until use.

PCR-based cloning of cDNA encoding *B. malayi* APRT (*Bmaprt*)

I. Primer design for amplification of Bmaprt open reading frame (ORF)

The design of primers annealing to 5'-end and 3'-end of *Bmaprt* cDNAs was based on the available sequence of *B. malayi* adenine phosphoribosyltransferase partial mRNA (NCBI accession: XM_001901344), *B. malayi* adult male DNA (NCBI accession: AI771039) and *B. malayi* infective larva DNA (NCBI accession: AA273141).

II. PCR amplification of Bmaprt ORF

The ORF for *Bmaprt* was amplified with a nested PCR approach using the aforementioned forward and reverse primers. The *Kpn*I site and *Hind*III site were incorporated into the forward and reverse primers, respectively.

III. Construction of recombinant pQE-30Xa harboring cDNA encoding (pQE-30Xa-Bmaprt)

The obtained *Bmaprt* cDNA with corresponding restriction recognition sites was digested with *Kpn*I and *Hind* III simultaneously and subsequently ligated into the corresponding sites of pQE-30Xa (Qiagen, Hilden, Germany) yielding pQE-30Xa-*Bmaprt.* The pQE-30Xa plasmid conferred a 6 His-tagged coding sequence to 5'-end of *Bmaprt* ORF. The desired pQE-30Xa-*Bmaprt* was cloned in *E. coli* strain JM109.

Expression and purification of recombinant *B. malayi* APRT (*Bm*APRT)

The recombinant *Bm*APRT was expressed as a *N*-terminal His-tagged fusion protein in *E. coli* strain Rosetta 2™ (DE3) (Merck KGaA, Darmstadt, Germany). The strain harbours pRARE2 which supplies tRNAs for seven rare codons for efficient expression of eukaryotic proteins. The transformants were cultured in LB medium supplemented with ampicillin and chloramphenicol and subsequently induced with IPTG. The fusion protein was purified by affinity chromatography using Ni-IDA resin (Macherey-Nagel, Dürea, Germany) in a gradient elution manner with increasing imidazole concentrations.

Preliminary biochemical characterization of recombinant *Bm*APRT

Initial rates were determined spectrophotometrically in an assay buffer containing 100 mM Tris-HCl, pH 7.5 and 12 mM $MgCl_2$ at 27°C. The reaction was initiated by adding *Bm*APRT into the mixture of adenine, PRPP and assay buffer to a final volume of 1 mL. Initial rates for the production of AMP were measured at 260 nm using UV-Visible Spectrophotometry (Shimadzu UV-1601). For steady-state kinetic determination, initial velocities were determined in the presence of varying substrate concentrations (either adenine or PRPP) at a saturating concentration of the other substrate. Initial velocities were fitted to Michaelis-Menten equation by non-linear least square fit using GraphPad Prism®5 for Windows (GraphPad Software, Inc. CA, USA).

Results and Discussion

cDNA cloning, expression and purification of the recombinant *Bm*APRT

The full-length cDNA encoding *B. malayi* adenine phosphoribosyltransferase (*Bmaprt*) was successfully amplified from a nested-PCR approach. The cloned cDNA was verified to be consistent with the reported sequence. The catalytically active recombinant *Bm*APRT, as a *N*-terminal His-tagged fusion protein was successfully expressed in *E. coli* strain Rosetta 2™ (DE3). The recombinant *Bm*APRT was purified to near homogeneity, as assessed by SDS-PAGE (**Fig. 1**). Application of a gradient elution with increasing imidazole concentrations conferred an efficient, single-step

purification process. The purified protein was consistent with the recombinant *N*-terminal His-tagged fusion *Bm*APRT with a calculated molecular mass of 24.98 kDa (Fig. 1).

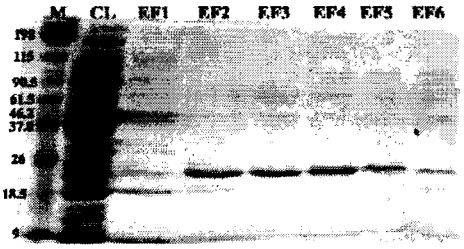

Figure 1. 12% SDS-PAGE representing the Ni-IDA purification profiles of the recombinant *Bm*APRT expressed in *E. coli* strain Rosetta 2™ (DE3). M represented standard proteins whose sizes were in kDa. CL and EF represented crude soluble lysate, and consecutive eluted fractions, respectively. A gradient elution with increasing imidazole concentrations was applied. EF1 and EF2 were obtained with 50 mM imidazole. EF3, EF4, EF5 and EF6 were obtained with 100 mM, 150 mM, 200 mM and 250 mM imidazole, respectively.

Preliminary biochemical characterization of *Bm*APRT

The desalted, purified recombinant *Bm*APRT (combined EF2-EF4) was catalytically active as demonstrated by a spectrophotometric measurement of the reaction initial velocity (**Fig. 2**). The result further verified the activity of the purified recombinant *Bm*APRT.

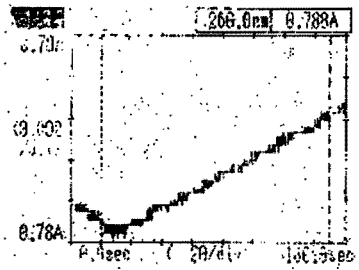

Figure 2. A spectrophotometric measurement of the increase in absorbance at 260 nm of a reaction catalyzed by the purified recombinant *Bm*APRT. The absorbance increment coincided with the formation of AMP, one of the two products yielded in an APRT-catalyzed reaction.

Steady-state kinetic parameters of the recombinant *Bm*APRT and the reported values of the recombinant human APRT (*Hs*APRT) were shown in (**Table 1**). The result revealed apparent difference in the K_m values of both substrates between the species, reflecting unique difference in dynamics of substrate binding sites.

Table 1. Summary of steady-state kinetic parameters

Enzyme	K_m for adenine	K_m for PRPP
recombinant *Bm*APRT	1 μM	2.7 μM
Recombinant Human APRT *	4 μM	8.9 μM

* reported by Silva, et al. 2004

Comparison of a 3D-homology model of *Bm*APRT, constructed by SWISS-MODEL, an automated protein homology modeling server (Schwede, et al. 2003), with an experimental 3D structure of *Hs*APRT (1ZN9.pdb) facilitates the identification of the unique difference that could be exploited in the design of selective antifilarial drugs. The 3D structure alignment reveals difference constituting two non-conserved residues, K37 and V39, in adenine binding pocket of *Bm*APRT. These two residues correspond to their homologs, namely V25 and R27, in *Hs*APRT, respectively. Currently functional contribution of these two homologous residues to unique biochemical properties of the APRTs are under investigation.

Acknowledgements

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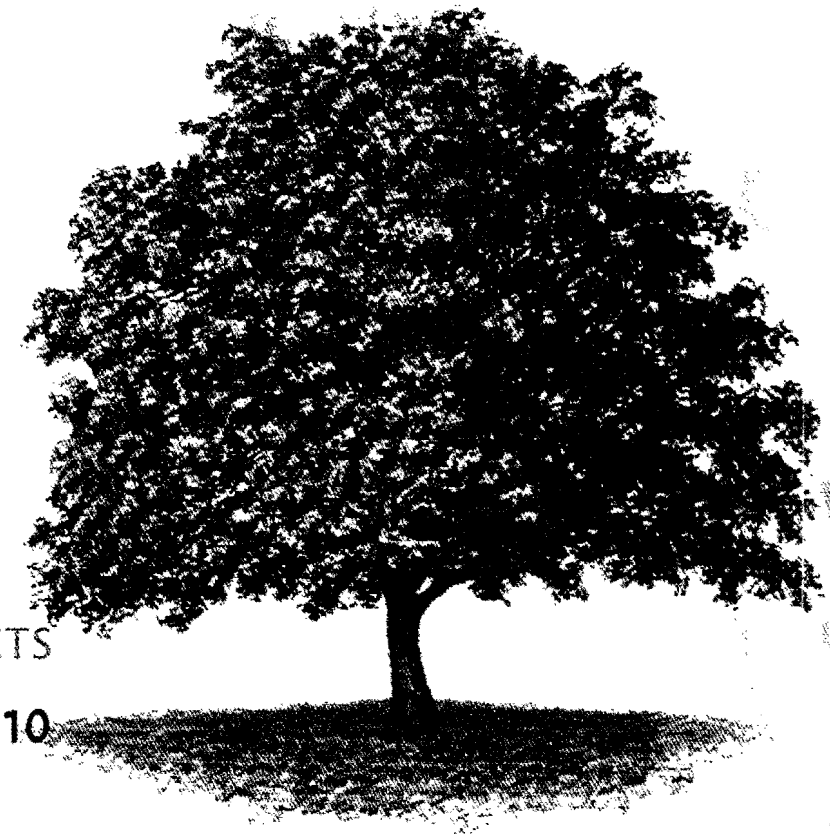

PROGRAM AND ABSTRACTS

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