EFFICIENT HETEROLOGOUS EXPRESSION AND ONE-STEP PURIFICATION OF FULLY ACTIVE C-TERMINAL HISTIDINE-TAGGED URIDINE MONOPHOSPHATE KINASE FROM *MYCOBACTERIUM TUBERCULOSIS*

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Abstract. Tuberculosis has long been recognized as one of the most significant public health problems. Finding novel antituberculous drugs is always a necessary approach for controlling the disease. *Mycobacterium tuberculosis pyrH* gene (Rv2883c) encodes for uridine monophosphate kinase (UMK), which is a key enzyme in the uridine nucleotide interconversion pathway. The enzyme is essential for *M. tuberculosis* to sustain growth and hence is a potential drug target. In this study, we have developed a rapid protocol for production and purification of *M. tuberculosis* UMK by cloning *pyrH* (Rv2883c) of *M. tuberculosis* H37Rv with the addition of 6-histidine residues to the C-terminus of the protein, and expressing in *E. coli* BL21-CodonPlus®(DE3)-RIPL using an auto-induction medium. The enzyme was efficiently purified by a single-step TALON® cobalt affinity chromatography with about 8 fold increase in specific activity, which was determined by a coupled assay with the pyruvate kinase and lactate dehydrogenase. The molecular mass of monomeric UMK was 28.2 kDa and that of the native enzyme was 217 kDa. The enzyme uses UMP as a substrate but not CMP and TMP and activity was enhanced by GTP. Measurements of enzyme kinetics revealed the k_{cat} value of 7.6 ± 0.4 U mg^{-1} or 0.127 ± 0.006 sec^{-1}.The protocol reported here can be used for expression of *M. tuberculosis* UMK in large quantity for formulating a high throughput target-based assay for screening anti-tuberculosis UMK compounds.

Keywords: *Mycobacterium tuberculosis*, uridine monophosphate kinase, antituberculous drugs, pyrimidine, protein purification

INTRODUCTION

The widespread of tuberculosis, caused by *Mycobacterium tuberculosis*, has become more worrisome than in the past mainly due to the emergence of multi-drug resistant strains (Dheda *et al*, 2010). The cases are often untreatable, signifying an urgent need for new antituberculous agents.

Current antituberculous drugs are known to act on a limited number of drug targets, while recent investigations have shown hundreds of potential drug targets

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(Duncan, 2004). It is therefore necessary to exploit these new drug targets for the development of new antituberculous compounds.

Nucleotides are essential substrates for bacterial growth, making interruption of their metabolism being a viable strategy in developing antituberculous drugs. Most bacteria obtain their nucleotides by both *de novo* biosynthesis and salvage pathways. The pyrimidine *de novo* biosynthesis pathway appears to be nearly identical in all bacteria (Evrin *et al*, 2007). The six-membered pyrimidine is synthesized first from the reaction between carbamoyl phosphate and aspartate, catalyzed by aspartate transcarbamoylase. Dihydroorotase then catalyzes the closure of the pyrimidine ring, which is subsequently oxidized to orotate by dihydroorotate dehydrogenase using NAD^+ as an electron acceptor. Phosphoribosyl pyrophosphate (PRPP) is attached by the enzyme orotate phosphorybosyl transferase, resulting in orotidylate, which is decarboxylated to yield uridine 5′-monophosphate (UMP), which is subsequently phosphorylated by uridine monophosphate kinase (UMK) to yield uridine 5′-diphosphate (UDP). UDP is converted to uridine 5′-triphosphate (UTP) by a non-specific nucleoside diphosphate kinase, and subsequently forms cytidine 5′-triphosphate (CTP) by cytidylate synthetase. In the salvage pathway, pyrimidine bases are converted to uracil and subsequently to UMP by UMK. To our knowledge, no viable bacterial deletion mutant of UMK has ever been reported. Mushegian and Koonin (1996) identify *pyrH* as a member of the minimal gene set a highly conserved set of genes supposed to provide minimal essential functions of bacterial life. Its essentiality for *M. tuberculosis* has been experimentally confirmed (Robertson *et al*, 2007).

Pyrimidine monophosphate kinases found in bacteria are different from those of eukaryotes (Jones, 1980). There are three distinct prokaryotic pyrimidine monophosphate kinases, UMK, CMK and TMK, each specific for an individual pyrimidylate. In contrast, phosphorylation of UMP and CMP in eukaryotic organisms is carried out by a bifunctional UMP/CMP kinase, which phosphorylates both UMP and CMP with comparable efficiencies. The amino acid sequences of bacterial CMK and eukaryotic UMP/CMP kinases are similar to those of adenylate kinases, while bacterial UMK is not homologous to any eukaryotic enzymes (Gagyi *et al*, 2003). This suggests that UMK can serve as a potential anti-tuberculous drug target as a chemical compound inhibiting *M. tuberculosis* enzyme should not affect the human enzymes. Biochemical and catalytic properties of the enzyme has recently been reported and the data are useful for developing specific inhibitors against the enzyme (Rostirolla *et al*, 2011), but the production yield is low (20 mg/2g of wet cell paste and 21% enzyme yield). In order to improve the yield, which would be beneficial for high-throughput inhibitor screen assays, we report the development of an expression and purification protocol for production of fully active C-terminal histidine-tagged *M. tuberculosis* UMP ($MtUMK_{His6}$) in high yield. The purified enzyme had the expected biochemical properties of *M. tuberculosis* UMK, indicating that the heterologously expressed enzyme can be used in high throughput drug screening assays.

MATERIALS AND METHODS

Strains and plasmids

M. tuberculosis H37Rv was used for genomic DNA extraction. *E. coli* XL-1

blue (Invitrogen, Carlsbad, CA) was used for production of recombinant plasmid and *E. coli* BL21-CodonPlus®(DE3)-RIPL (Invitrogen, Carlsbad, Germany) for over-expression of *pyrH*.

Cloning of *M. tuberculosis pyrH* coding sequence

A 786 bp fragment containing the entire coding region of *pyrH* of *M. tuberculosis* H37Rv was amplified by PCR using *pfu* DNA polymerase with two primers, containing *Nde*I and *Xho*I restriction sites (underlined), (pyrHF: 5'- CGTTAAC-CATATGACAGAGCCCGAT -3', pyrHR: 5'-TTTCTCGAGGGTGGTGACCAGCGT -3'). PCR was carried out in a 50 µl final volume containing 5 µl of 10x buffer (Fermentas AB, Vilnius, Lituania), 25 µM $MgSO_4$, 100 µM each dNTP, 0.5 µM each primer, 2.0 U *pfu* DNA polymerase (Fermentas) and 1 µg of *M. tuberculosis* H37Rv DNA, using the following thermocycling parameters: 5 minutes at 95ºC, 30 cycles of 95ºC for 60 seconds, 69.6ºC for 60 seconds and 72ºC for 90 seconds with the final step of 5 minutes at 72ºC. PCR amplicons were analyzed by electrophoresis in 1.5% (w/v) agarose gel and stained with ethidium bromide. The amplicon was inserted and ligated into pET-24b plasmid (EMB bioscience, Madison, WI) at *Nde*I and *Xho*I sites using T4 DNA polymerase (Fermentas), resulting in the plasmid pMtUMK, which was used for expression of the recombinant protein fused to His_6-tag at the C-terminus. The whole sequence of *pyrH* in the pMtUMK plasmid was sequenced (Macrogen, Seoul, Korea) to verify the absence of any mutation.

Overexpression and purification of recombinant $MtUMK_{His6}$

Overexpression of MtUMK in cells grown in yeast extract tryptone (2xYT) and in auto-induction medium (ZYP-5052) was compared. *E. coli* BL21-CodonPlus®(DE3)-RIPL cells harboring pMtUMK were grown at 37ºC in 2xYT broth (16 g/l bacto-tryptone, 10 g/l yeast extract, and 0.5 g/l NaCl) supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. ZYP-5052 medium is composed of 10 g/l soluble enzymatic digest of casein, 5 g/l yeast extract, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM$(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.5 g/l glucose and 2 g/l lactose and 0.2x trace metals (Studier, 2005) and supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. For isopropyl-β-d-1-thiogalactopyranoside (IPTG) induction, cells were grown until the absorbance at 600 nm (OD_{600}) reached about 1.0. Then, induction was performed by adding IPTG (Fermentas) to a final concentration of 1 mM and was shaken at 20ºC, 250 rpm for 24 hours. For over-expression in auto-induction medium, *E. coli* BL21-CodonPlus®(DE3)-RIPL cells harboring pMtUMK were grown at 37ºC in ZYP-5052. Cells were grown until OD_{600} reached about 1.0 and then, without adding IPTG, the culture was shaken at 20ºC, 250 rpm for 24 hours. Bacteria were harvested by centrifugation at 4,000*g* for 10 minutes at 4ºC and stored at -80ºC until used. Cell pastes from both methods of expression were suspended in 50 mM sodium phosphate buffer pH 7.4 containing 1 mg/ml lysozyme and 1 mM phenyl methyl sulfonyl fluoride. Cells were lysed by sonication (6 x 10 s pulses) on ice. Clarified cell lysate was obtained by centrifugation at 20,800*g* for 30 minutes at 4ºC. The recombinant protein was purified from the supernatant by affinity chromatography using 15 ml TALON® Metal affinity column in ÄKTA™ automated liquid chromatography system (GE Healthcare, Uppsala, Sweden) employing a linear gradient of 5 to 400 mM imidazole.

Fractions containing $MtUMK_{His6}$ were pooled, concentrated and desalted using Sephadex G-25 gel filtration column previously equilibrated with 50 mM Tris-Cl pH 7.4, 50 mM KCl and 2 mM $MgCl_2$. Protein concentration was measured by Bradford Assay (Bradford, 1976).

Molecular mass determination

The subunit molecular mass of $MtUMK_{His6}$ was estimated by 12 % SDS-PAGE. Estimation of the native molecular mass was based on the elution volume of $MtUMK_{His6}$ from a Superdex™ 200 analytical gel filtration column in 50 mM Tris pH 7.4, 150 mM NaCl, operated by ÄKTA™ automated liquid chromatography system (GE Healthcare, Uppsala, Sweden). The following proteins with known molecular mass were used as standard markers: ribonuclease A, 15.6 kDa; oval albumin, 48.9 kDa; bovine serum albumin, 67 kDa; aldolase, 158 kDa; and ferrin 440 kDa (GE Healthcare, Uppsala, Sweden).

Enzyme activity measurement

$MtUMK_{His6}$ activity was measured with pyruvate kinase (PK)/ lactate dehydrogenase (LDH) coupling reaction as previously described (Blondin, 1994). Formation of ADP and UDP from ATP and UMP catalyzed by UMK is coupled to the formation of lactate and NAD^+ catalyzed by the reactions of PK and LDH in the presence of phosphenolpyruvate (PEP) and NADH.

Enzyme activity was measured in a total volume of 500 µl at 25°C using a Shimadzu UV-2501PC spectrophotometer. The assay reaction contained 0.3-0.45 nM $MtUMK_{His6}$, 50 mM Tris HCl (pH7.5), 50 mM KCl, 2 mM $MgCl_2$, 3 mM PEP, 0.2 mM NADH, 3 mM ATP, 3 mM UMP, and 28 and 16 units of PK and LDH, respectively. Initial rate was calculated from the decrease in absorbance at 340 nm (ε_{NADH} = 6.22×10^{-3} M^{-1} cm^{-1}), compared to the control in which UMK was absent. One unit of UMK is defined as the amount of enzyme that converts 1 µmol of UMP to UDP per minute. Values of k_{cat} and K_m were obtained by fitting the initial rates at various concentrations of UMP and ATP to the Michaelis-Menten equation. For investigating the effects of GTP, assays of $MtUMK_{His6}$ at the GTP concentration of 0, 0.1, 0.2, and 0.5 mM were conducted.

RESULTS

***E. coli* expression of $MtUMK_{His6}$**

M. tuberculosis pyrH was successfully cloned into *E. coli* BL21 CodonPlus®(DE3)-RIPL. The recombinant plasmid, pMtUMK, was isolated and sequenced. The plasmid contained the intact *pyrH* gene including 6 histidine codons as expected (data not shown).

Typically in our expression conditions, the 2YT medium yielded about 5-10 g of wet cell pellet per 1 liter while the auto-induction medium yielded about 20-25 g of wet cell pellet per 1 liter. The amount of UMK per gram of wet cell paste purified from the IPTG-induction method in this study (7.2 mg) was slightly less than a previous report (10 mg) (Rostirolla *et al*, 2011). As shown in the protein purification table (Table 1), the auto-induction medium produced 4-5 times more wet cell paste and total protein than did the IPTG induction medium from the same amount of culture medium while the specific enzymatic activity of the total proteins was also slightly higher. The His-tagged enzyme from both preparations could be purified to the same final specific activity of about 7.6 U/mg, which is very similar to the value recently reported for the recombinant UMK without a tag (Rostirolla *et al*, 2011).

Table 1
Purification table of $MtUMK_{His6}$ from IPTG induction and auto-induction.

Step	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
IPTG induction[a]					
Crude extract	421	370	0.88	100	1.00
IMAC column	32	243	7.59	66	8.65
Auto-induction[b]					
Crude extract	1,250	1,168	0.93	100	1.00
IMAC column	142	1,087	7.66	93	8.20

[a]From wet cell pellet 4.4 g (1 liter culture); [b]From wet cell pellet 10.2 g (0.5 liter culture)

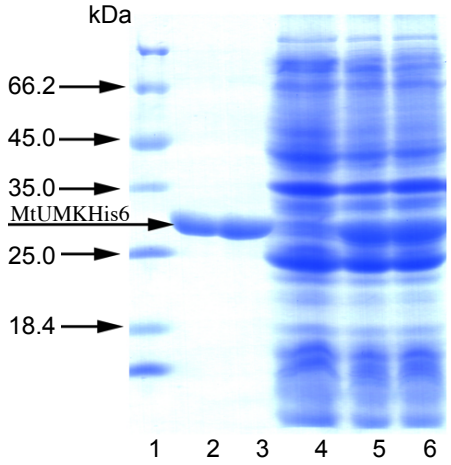

Fig 1–Expression of $MtUMK_{His6}$ in *E. coli* BL21(DE3)-RIPL using IPTG-induction and auto-induction analyzed by 12 % SDS-PAGE. The bacteria were grown in 2xYT or ZYP-5052 media, respectively until OD 600 mm reached 1.0 and then at 20°C for 24 hours with or without IPTG. Lane 1, molecular weight markers (kDa); lane 2, purified $MtUMK_{His6}$ from IPTG induction (1 µg); lane 3, purified $MtUMK_{His6}$ from auto-induction (1 µg); lane 4, clarified lysate of the control, *E. coli* BL21(DE3)-RIPL harboring pET24b without MtUMK insert (17 µg); lane 5, clarified lysate of *E. coli* BL21(DE3)-RIPL harboring pMtUMK induced by IPTG (15 µg); lane 6, clarified lysate of *E. coli* BL21(DE3)-RIPL harboring pMtUMK in auto-induction medium (15 µg).

The purified enzyme contained a single 28-kDa protein band (Fig 1) as expected from the sequence analysis, confirming the purity of the enzyme. After removing imidazole from the purified UMK by exchanging buffer in a desalting column and kept at -80°C, the frozen enzyme has maintained its original activity for at least 6 months.

Molecular mass determination

A subunit molecular mass of $MtUMK_{His6}$ was calculated based on its sequence as 28.2 kDa, consistent with that of the purified protein estimated by SDS-PAGE (Fig 1). The native molecular mass of $MtUMK_{His6}$ was estimated to be about 217 kDa based in Superdex S-200 gel filtration. Therefore, the oligomeric state of $MtUMK_{His6}$ should be around 8.

$MtUMK_{His6}$ kinetics and specificity

$MtUMK_{His6}$ activity was determined by coupling to the reactions catalyzed by PK and LDH in the presence of PEP and NADH. Steady-state kinetics of $MtUMK_{His6}$ with varying UMP or ATP concentrations at a saturating concentration of ATP or UMP (5 mM) revealed K_m for UMP of 1.9 mM ± 0.9 mM, K_m for ATP of 2.0 mM ± 1.1 mM, and k_{cat} of 7.6 ± 0.4 U mg^{-1}.

The recombinant enzyme could not use CMP or TMP as substrates, even at very high concentration of CMP and TMP of 10-50 mM (data not shown). We examined the activity of the enzyme in the presence of 0.1, 0.2 and 0.5 mM GTP and found that the UMK activity reached maximum at 0.2 mM GTP with activity being about twice the value in the absence of GTP (Table 2).

Table 2
$MtUMK_{His6}$ activity in the presence of GTP.

GTP (mM)	Specific activity (U/mg)	Relative activity
No GTP	7.54	1.00
0.1 mM	12.35	1.64
0.2 mM	14.23	1.89
0.5 mM	14.14	1.87

DISCUSSION

Pyrimidines are essential components of DNA and RNA, making interrupting their metabolic pathway being an interesting strategy for developing antituberculous drugs. Many works have been done on *M. tuberculosis* thymidylate kinase (Gasse *et al*, 2008; Familiar *et al*, 2010), resulting in the discovery of several interesting compounds. *M. tuberculosis* CMK (Thum *et al*, 2009) has been recently cloned and could be further used for drug discovery.

The availability of the purified MtUMK would certainly facilitate the development of inhibitors by making it possible to perform a high-throughput target-based assay. It is also a preliminary step to acquire high-resolution structural information of the protein, so that various approaches for structure-based drug designs can be applied.

In this study, MtUMK has been cloned and expressed as a C-his-tagged protein and has been shown that it retains the highly specific kinase activity for UMP but not for CMP and TMP, emphasizing its difference from UMP/CMP eukaryotic kinases. It can also be activated by GTP indicating that the $MtUMK_{His6}$ is allosterically controlled by GTP, similar to UMK from *E. coli* and other bacteria (Gagyi *et al*, 2003; Evrin *et al*, 2007; Tu *et al*, 2009).

While this work is ongoing, *pyrH* of *M. tuberculosis* has been cloned and expressed in its native form but the purification is more tedious, requiring multiple steps of chromatography. Unlike all known prokaryotic UMK, the heterologously expressed native MtUMK was a tetramer (Rostirolla *et al*, 2011), which was different from most native bacterial and archaeal UMK, which are generally hexamers, such as *E. coli* UMK, which is 56.4% identical to $MtUMK_{His6}$ (Briozzo *et al*, 2005), *Ureaplasma parvum* (Egeblad-

Welin *et al*, 2007), *Bacillus anthracis* (Meier *et al*, 2008), *Pyrococcus furiosus* (Marco-Marin *et al*, 2005) and *Sulfolobus solfataricus* (Jensen, 2007).

In contrast, C-His-MtUMK was found to be an octamer in this study, which was different from a report of a hexameric structure of recombinant $MtUMK_{His6}$ expressed in *E. coli* (Labesse *et al*, 2010). The cause of the discrepancy is unknown. It may be possible that the enzyme can adopt different quaternary structures depending on the environment. This warrants further study.

In summary, we have developed a method for production and purification of MtUMK with higher yield than the previously reported protocol while attaining the same level of purity and specific activity. The purified enzyme exhibited the functional characteristics expected for bacterial UMK, such as, being specific to UMP but not catalyzing the reaction of CMP or TMP, and being enhanced by GTP. The preparation of the histidine-tagged enzyme significantly simplifies the purification and also minimizes the possibility of contamination by *E. coli* UMK. The auto-induction protocol results in a high yield and also eliminates the need for IPTG, which significantly reduces the cost of the enzyme preparation. This should be beneficial for preparation of MtUMK for high-throughput drug-screening assays in the future.

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