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Cloning, Overexpression, and Purification of PhoR Cytoplasmic Domain Protein from *Mycobacterium tuberculosis* strain H37Rv

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Tuberculosis is still a major health problem in the world. This infectious disease is caused by *Mycobacterium tuberculosis* (Mtb). Novel anti-tubercular drug is urgently needed to counter multidrug resistant cases and Mtb's spread. The cytoplasmic domain of PhoR histidine kinase, a part of the two-component system PhoR-PhoP in Mtb, is one of the potential candidates for anti-tubercular drug target. Three dimensional structure (3D-structure) of the protein (drug target) is needed to screen potential drug candidate using rational drug design approaches. Previous studies have successfully characterized and isolated putative cytoplasmic domain of PhoR (CytoPhoR) from Mtb strain H37Rv. This study aimed to clone, overexpress, and purify CytoPhoR protein. CytoPhoR was fused with thioredoxin protein in pET32b expression vector and overexpressed in *Escherichia coli* (*E.coli*) BL21(DE3) as soluble fraction by induction with 1 mM IPTG. Purification of His-tagged CytoPhoR was carried out using IMAC Ni-NTA Agarose His-tag affinity column. SDS-PAGE analysis showed that another protein was co-purified (~35 kDa) along with the CytoPhoR protein. Subsequent protein purification using DEAE-ion exchange column generated a strong single band of 37 kDa on SDS–PAGE which was identified as CytoPhoR protein. The purified CytoPhoR protein was successfully obtained and could be used for further analysis to determine the 3D-structure of CytoPhoR protein.

Key words: *Mycobacterium tuberculosis,* rational drug design, tuberculosis*,* two-component system

Tuberkulosis (TB) masih menjadi masalah kesehatan utama di dunia. Penyakit menular ini disebabkan oleh bakteri *Mycobacterium tuberculosis* (Mtb). Obat anti-TB baru sangat dibutuhkan untuk menanggulangi kasus multi-resisten obat TB yang ada saat ini dan penyebaran Mtb. Domain sitoplasmik protein PhoR histidin kinase yang merupakan bagian dari *two component system* PhoR-PhoP di Mtb, adalah salah satu kandidat yang sangat potensial untuk dijadikan sebagai target baru obat anti-TB. Pada studi *rational drug design,* struktur tiga dimensi protein target obat dibutuhkan untuk menyeleksi kandidat obat yang potensial ini. Studi sebelumnya telah berhasil mengkarakterisasi dan mengisolasi domain sitoplasmik PhoR (CytoPhoR) dari Mtb strain H37Rv. Penelitian ini bertujuan untuk mengkloning, mengekspresikan, dan memurnikan protein CytoPhoR. CytoPhoR disubkloning dalam vektor ekspresi pET32b. Protein berhasil diekspresikan sebagai fraksi terlarut dalam *E. coli*. Pemurnian CytoPhoR dilakukan dengan menggunakan IMAC Ni - NTA agarosa kolom afinitas His-tag. Analisis SDS-PAGE pada hasil pemurnian menunjukkan bahwa protein kontaminan masih terbawa(~ 35 kDa). Selanjutnya, pemurnian protein dilakukan dengan menggunakan kolom penukar ion DEAE menghasilkan satu band berukuran 37 kDa pada analsis SDS–PAGE. Dengan demikian, protein CytoPhoR telah berhasil dimurnikan dan dapat digunakan untuk analisis lebih lanjut dalam menentukan struktur tiga dimensi protein CytoPhoR.

Kata kunci: *Mycobacterium tuberculosis*, *rational drug design,* tuberkulosis, *two-component system*

Tuberculosis (TB) is still one of the high prevalence diseases in the world. Latent infection of *Mycobaterium tuberculosis* (Mtb) is believed to infect almost one third of the world population (Koul *et al*. 2011). No existing anti-tubercular drugs can be used to against this latent infection. Recently, poor control of anti-tubercular drugs intake leads to the emergence of resistance (Araújo-filho *et al*. 2008; Lawn and Zumla 2011). PhoR-PhoP, a two-component system signal transduction in Mtb, is known to regulate 114 genes related to the virulence of Mtb (Walters *et al*. 2006; Gonzalo-Asensio *et al*. 2008). The currently available anti-tubercular drugs recognise only single targets. Using this system as target is very useful, since it will block and shut down multiple targets simultaneously. Previous studies showed that *in vivo* disruption of PhoR-PhoP drastically attenuated Mtb (Walters *et al*.

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2006; Gonzalo-Asensio *et al*. 2008). Homology study revealed that PhoR is different from other prokaryotic proteins (Suwanto and Giri-rachman 2012) Also, this signal transduction system is not found in human (Suwanto 2012). Therefore, the drug candidate hopefully has high selectivity and limited toxicity.

One potential drug target is the dimerization domain of PhoR. Dimerization plays a key role in the activation of the transduction system PhoR-PhoP. The dimerization domain is located in the cytoplasmic domain of PhoR (CytoPhoR) (Ryndak *et al*. 2008; Yamada and Shiro 2008). 3D-structure of CytoPhoR is needed to screen novel drugs using rational drug design method. Rational drug design is a method for developing new drugs based on structural simulation. Although the 3D-structure of the protein target can be used to screen hundreds of molecules that can potentially inhibit the protein targets, currently, the 3D-structure of PhoR in Mtb has not been determined. To determine the protein structure, large quantities of purified protein are required. Previous studies have successfully isolated and cloned CytoPhoR coding sequence from Mtb strain H37Rv into pRSET expression vector (Kurnia 2012). In this study, we subcloned, overexpressed, and purified CytoPhoR recombinant protein in *E. coli* BL21 (DE3). The focus of our research is to produce and purify CytoPhoR protein in soluble form. Thus, it will be useful for determination of CytoPhoR 3D-structure.

MATERIALS AND METHODS

Subcloning of PhoR Cytoplasmic Domain into Vector Expression pET32b. A DNA fragment containing the putative cytoplasmic domain of PhoR was previously isolated from genomic DNA of Mtb strain H37Rv (Kurnia 2012). The DNA fragment was subcloned into pET32b expression vector by PCR (pET32b-CytoPhoR). The forward primer used contained a *Kpn*I site upstream of the start codon: 5‟-GGTACCATGCGACAGTTCATCACC-3‟ (CytoPhoR-Forward primer). The reverse primer contained an *Eco*RI site and a stop codon downstream of CytoPhoR: 5'-GAATTCTCACAACCCCAGTC CGGT-3' (CytoPhoR-Reverse primer). The PCR amplified fragment was ligated into pET32b vector. The plasmids were introduced into *E. coli* BL21(DE3) by transformation using heat shock method (Sambrook and Russel, 2001). The ligation product (pET32b-CytoPhoR) was examined by 1% agarose gel electrophoresis and sequenced. The construct was validated by restriction digestion using *Kpn*I-*Eco*RI, PCR amplification, and DNA sequencing. DNA sequence and translation analyses were carried out using BioEdit© and CLC Genomic® programs.

Overexpression of Recombinant PhoR Cytoplasmic Domain. *E. coli* BL21(DE3) containing pET32b-CytoPhoR plasmid were grown in LB agar containing 100 ppm ampicillin. A single colony was inoculated into 5 mL LB broth containing ampicillin. The culture was incubated at 37 °C, with 200 rpm agitation overnight. Then, the culture was used to inoculate 1 liter LB broth and ampicillin. When the optical density (OD_{600nm}) reached 0.4-0.7, the culture was induced with 1 mM IPTG (Isopropyl-1-thio-β-galactopyranoside) for 4 hours. Cells were harvested by centrifugation at 7000x*g*, 4°C, for 30 minutes. The cell pellet was washed with 20 mM Tris Cl (pH 8.0), then resuspended in 2 mL lysis buffer (20 mM Tris Cl pH 8.0, 1 mM PMSF), followed with 10 minutes sonication . The insoluble material and cell debris (pellet) were separated by centrifugation at 12000x*g*, 4 °C, for 10 minutes. The cell lysate containing soluble fraction of His-tagged Cyto-PhoR was filtered using 0.22 μm Millipore filter and used for protein purification.

Protein Purification Using Ni-NTA Affinity Chromatography (IMAC). Protein was purified using Ni-NTA-agarose system at 4 °C. Ni-NTA agarose was first equilibrated using buffer containing 20 mM TrisCl pH 8.0, 20 mM imidazole, and 300 mM NaCl. The cell lysate was applied on Ni-NTA agarose for 1 hour at 60 rpm, 4 °C. Purification was carried out in the native state at 4 °C with a linear flow rate of 0.5-1 mL min^{-1}, using 50 mL of Econo column (Biorad). The column was washed with binding buffer (20 mM TrisCl pH 8.0, 300 mM NaCl, 50 mM imidazole) several times to remove unbound proteins. CytoPhoR protein was eluted with elution buffer containing 250-500 mM imidazole. The base-buffer used in this purification contained 20 mM TrisCl pH 8.0 and 300 mM NaCl. Eluted fractions were pooled and concentrated to about 5 mL using Microsep™ advanced centrifugal filter with 10 kDa MW cut off (Pall). The sample was dialyzed against buffer containing 20 mM TrisCl pH 8.0, and 200 mM Arginin, at 4 °C overnight.

Protein Purification Using Ion Exchange Chromatography. Final purification was achieved by ion exchange chromatography using DEAE (diethylaminoethyl) resin. The partially purified His-tagged Cyto-PhoR protein was applied to DEAE on 50 mL Econo column (Biorad). The column was eluted

with 0-800 mM NaCl gradient using 20 mM Tris-Cl buffer pH 8.5 flow rate of 0.5-1 mL at 4 °C. The eluted fraction were collected and pooled in one falcon before being dialyzed against buffer containing 20 mM Tris-Cl pH 8.0, 200 mM NaCl, and 200 mM Arginin, and concentrated by ultrafiltration using Microsep™ advanced centrifugal filter with 10 kDa MW cut off (Pall). CytoPhoR concentration was determined using Bradford assay (Bradford 1976), with bovine serum albumin (BSA) as protein standard. CytoPhoR protein was then run on 12% SDS-PAGE gel according to Laemmli method (Laemmli 1970). Protein bands were visualized by Coomassie Brilliant Blue staining and silver staining.

RESULTS

Subcloning of the Cytoplasmic Domain of PhoR into pET32b Expression Vector. The specific primers (CytoPhoR-forward and reverse) were designed to amplify CytoPhoR gene fragment. The PCR amplified fragment was inserted via its unique KpnI and EcoRI restriction sites into the corresponding sites in pET32b vector. CytoPhoR was fused with another protein in this expression vector. A genetic map of pET32B-CytoPhoR (Fig 1) shows thioredoxin, His-tag, and S-tag located upstream from CytoPhoR. PCR analysis using CytoPhoR-forward and reverse primer was performed to verify CytoPhoR in pET32b. The specific amplicon (~561 bp) in the electropherogram confirmed the presence of CytoPhoR in the pET32b (Fig 2). Sequencing analysis was performed by BioEdit© and CLC Genomic® programs, indicating the insertion of CytoPhoR in the correct direction without shift in the reading frame. This result indicated that CytoPhoR had been successfully inserted in frame within pET32b.

Overexpression of the Cytoplasmic Domain of PhoR Recombinant Protein. The expression system in pET32b was driven by T7 promoter and *lac* operator upon induction with IPTG. No expression of the protein of interest was observed in the non-induced sample. Calculation of total molecular weight using MW prediction program (http://www.expasy.org/compute_pi/Mw) revealed that this fusion protein has molecular weight of 36.8 kDa. SDS-PAGE analysis of the CytoPhoR fusion protein showed the presence of a band approximately ~37 kDa (Fig 3). This result was consistent with the prediction of its molecular weight.

Protein Purification Using Ni-NTA Affinity Chromatography (IMAC). In this study, we used IMAC Ni-NTA to purify the overexpressed CytoPhoR fusion protein, making use of the His-tag present in this recombinant protein. Purification process was performed under native condition, since CytoPhoR fusion protein was overexpressed as soluble fraction. SDS-PAGE analysis showed that several impurities

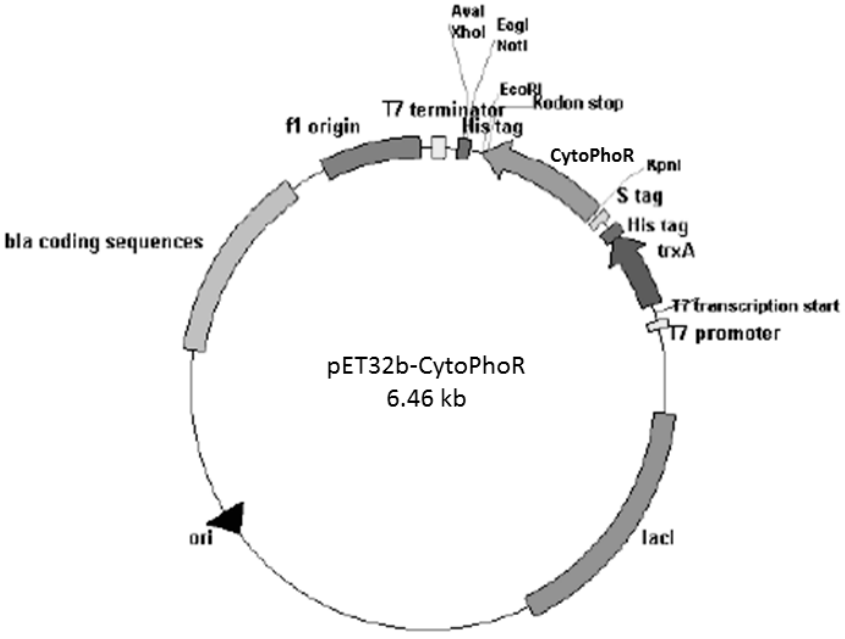

Fig 1 Genetic map of pET32B-CytoPhoR vector. Location and transcriptional orientation of *bla* coding sequence, β-lactamase for ampicillin resistance; *ori* (origin of replication); *lacI*, *lac*I repressor; T7 promoter (upstream) and T7 terminator (downstream); TrxA (thioredoxin); 6xHis-tag; S-tag; and f1 origin of replication are shown. The amplified DNA fragment of CytoPhoR gene (561 bp) was ligated into *Kpn*I/*EcoR*I site of pET32b expression vector under control of T7 promoter. CytoPhoR was fused with thioredoxin protein, 6xHis-tag and S-tag.

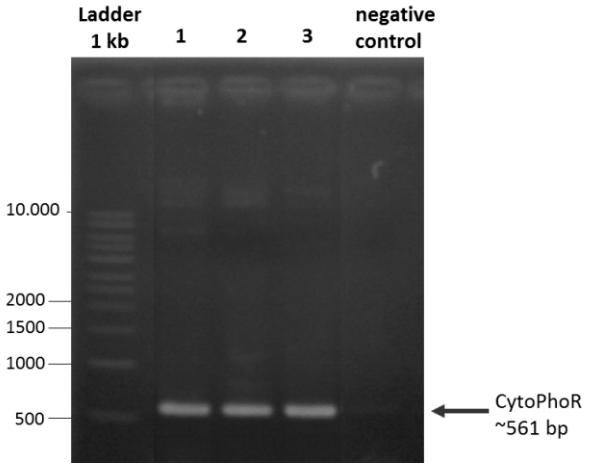

Fig 2 CytoPhoR gene amplification using specific primer CytoPhoR-forward and primer CytoPhoR-reverse. The PCR produced ~561 bp bands on agarose gel (1% w/v) representing the CytoPhoR gene. Left lane: DNA ladder 1 kb (Thermo Scientific); middle lane: 1, 2, 3 CytoPhoR gene ~561 bp; right lane: negative control.

were co-eluted with CytoPhoR (Fig 4A). Other non-tagged but histidine rich proteins might also interact with the Ni-NTA resin. This non-specific binding was reduced by adding low concentration of imidazole in wash buffer and during pre-treatment of protein.

Protein Purification Using Ion Exchange Chromatography. Partially purified CytoPhoR was applied to DEAE ion exchange chromatography column and eluted using NaCl gradient (0-800 mM). Peak fraction containing the highest levels of protein was eluted at 400 mM NaCl. SDS-PAGE analysis showed that CytoPhoR had been purified to near homogeneity (Fig 4B)

DISCUSSION

CytoPhoR contains several conserved motif from histidine kinase: H-box (HELR), N-box (NLVANA), G1-box (DDGPG), F-box (FERF) dan G2-box (GTGLGL) (Kurnia 2012; Sundari 2010). Histidine kinase PhoR from Mtb strain H37Rv was categorized as Class I of histidine kinase (Yamada and Shiro 2008). H-box in this histidine kinase is located in the dimerization domain (Jung *et al*. 2012; Yamada and Shiro 2008). This domain plays a key role in the activation of signal transduction. Blockade of the dimerization domain will block the signal transduction pathway and subsequently stop the expression of virulent genes.

In this study, thioredoxin was used as fusion partner to increase protein solubility. Recombinant proteins that were produced as fusion with thioredoxin had been proven to be soluble (La Vallie 1994). *In silico* analysis using protein-solubilization prediction software (http://www.biotech.ou.edu/#r) indicated that CytoPhoR fusion protein is produced in soluble form in *E.coli* . Soluble protein is usually easier to purify than protein that is expressed in inclusion bodies.

Several *E.coli* proteins rich of histidine residue had been reported to co-purify in Ni-NTA agarose affinity chromatography system, for examples peptidyl prolyl cis-trans isomerase (SlyD) 27 kDa; class carbonic anhydrase (Can) 25 kDa; UDP-L-Ara4N formyltransferase (ArnA) 74.3 kDa; subunit E1 multienzyme complex pyruvate dehydrogenase (AceE) 99.7 kDa; and L-glutamine D- fructose-6-phosphate aminotransferase (GlmS) 67 kDa) (Öberg *et al*. 2004; Robichon *et al.* 2011). These proteins bound non-specifically to Ni-NTA resin and hardly removed. Therefore, another purification system must be performed in order to remove all contaminant proteins. In this experiment, the purification step had successfully removed almost all impurities. However, we found that CytoPhoR fusion protein easily formed aggregate at high protein concentration. This suggests that protein storage and analysis must be performed carefully. Addition of 200 mM arginine was aimed to prevent rapid aggregation. Amino acids sequence analysis and 3D prediction revealed that this protein had three cysteine residues. The presence of these cysteines may interfere with protein solubility, since non-reducing SDS-PAGE analysis showed that this protein formed a trimer (data not shown.).

In conclusion, the cytoplasmic domain of PhoR has successfully been cloned and expressed in *E. coli* BL21(DE3) as soluble form. The protein has also been successfully purified using chromatography affinity (IMAC) Ni-NTA, followed by ion exchange

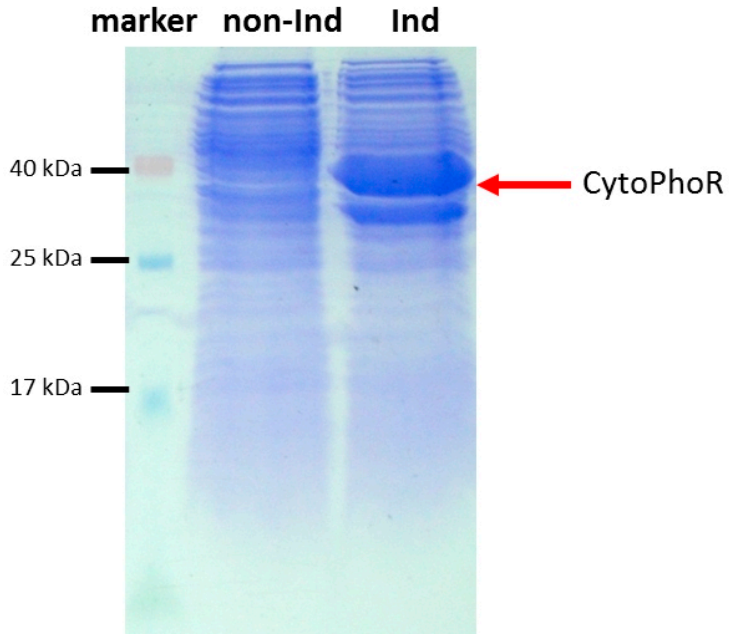

Fig 3 Overexpression of CytoPhoR protein in *E. coli* BL21(DE3) was analyzed by 12% SDS-PAGE gel. The recombinant *E. coli* was grown overnight then induced with 1 mM IPTG for 4 h. Left lane: low range protein markers (Thermo Scientific). non-Ind: cell lysate without IPTG induction. Ind: cell lysate with IPTG induction containing His-tagged CytoPhoR.

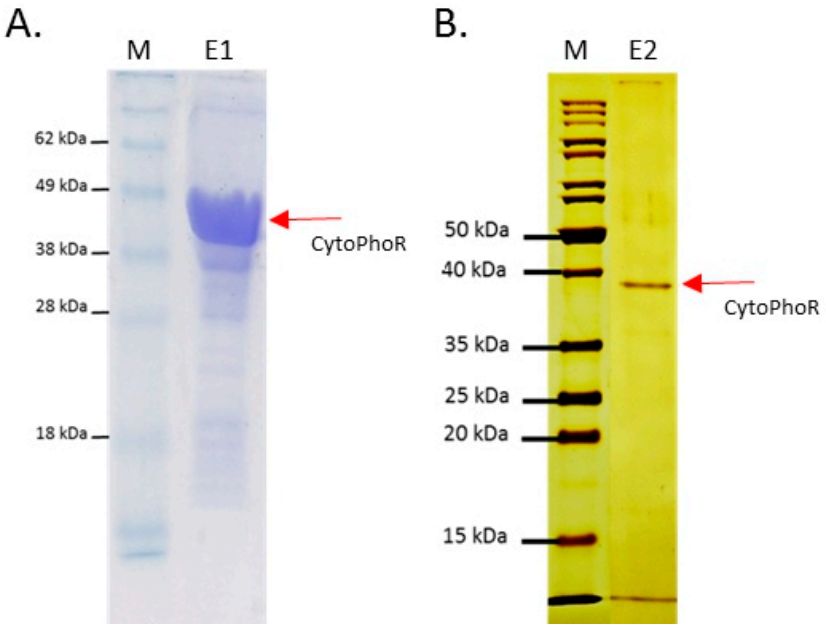

Fig 4 The purification of CytoPhoR recombinant proteins extracted from *E. coli* BL21(DE3) using Ni-NTA affinity chromatography (IMAC) followed by ion exchange chromatography. Protein samples were separated in 12% SDS-PAGE gels. (A) SDS–PAGE analysis of partially purified CytoPhoR protein stained with Coomassie brilliant blue. 6xHis-tagged CytoPhoR proteins from *E. coli* BL21(DE3) were purified under native conditions using affinity chromatography (IMAC) Ni-NTA. Lane M : broad range protein markers, EI : the eluent collected from affinity chromatography columns. (B) SDS–PAGE analysis of purified CytoPhoR protein stained with silver-stain. Protein was eluted using NaCl gradient (0-800 mM) on DEAE-ion exchange column. Eluted protein of DEAE-ion exchange chromatography produced specific band at ~37 kDa. SDS-PAGE analysis showed that purification process successfully remove almost all impurities proteins. Lane M : broad range protein markers (Thermo Scientific), E2 : the eluent collected from ion-exchange columns.

chromatography. The purified protein would be used for further crystallization studies as a target for structure-based discovery of novel anti-tubercular drugs.

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