Expression and Purification of PhoR Sensor-Domain Histidine Kinase of Mycobacterium tuberculosis in Escherichia coli

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Globally, tuberculosis (TB) remains a leading cause of death. The emergence of multidrug-resistant strains (MDR-TB) and extensively drug-resistant strains (XDR-TB) has fuelled the discovery for novel drugs and drug targets for its successful and better treatment. One of the potential candidates for drug target is PhoR sensory protein histidine kinase, a part of the Two Component System (TCS) PhoP/PhoR in Mycobacterium tuberculosis (Mtbc). This protein system was known for its role on regulating hundred of Mtbc virulence factors, from genes for cell wall and lipid synthesis to genes for adaptation in human leukocyte and hypoxia response. Previous studies have successfully characterized, isolated, and cloned the putative sensory domain of PhoR protein gene into pRSET vector expression system. In this study, Escherichia coli was transformed with pRSET-SensPhoR and cultivated at 37 £°C under IPTG induction to express PhoR sensor-domain protein. Most of the proteins were overexpressed in the form of inclusion bodies. Subsequent protein purification in Ni-NTA system under refolding condition on urea gradient was performed to isolate PhoR sensor-domain protein in soluble form. Arginine was supplemented in purified protein solution to prevent aggregation during long term storage. While highly purified protein was acquired, small angle X-ray scattering (SAXS) analysis was conducted to obtain 3-dimensional (3D) protein structures in solution.

Key words: multi-drug resistance, rational drug design, tuberculosis, two-component signal transduction system of histidine kinase

PhoP/PhoR histidine kinase two-component system in Mycobacterium tuberculosis has been studied as potential target for new antitubercular drugs. The system is not found in mammals and the M. tuberculosis PhoP/PhoR amino acid sequence shows little homology with other prokaryotic proteins (Suwanto 2012). These findings implied that PhoP/PhoR have low non-targetting potential, either on humans or other organisms, a required characteristic...
for new drug target. Previous studies also demonstrated that interrupting PhoP/PhoR two-component-system drastically decreased \textit{M. tuberculosis} virulence in vivo (Walters et al. 2006, Gonzalo-Asensio et al. 2008). PhoP/PhoR two-component-system also induces the expression of 114 virulence-related genes (Pathak et al. 2011), including those involved in lipoarabinomannan (a cell wall component) metabolism and \textit{M. tuberculosis} resistance inside macrophage (Walters et al. 2006, Gonzalo-Asensio et al. 2008). Inhibition of PhoR sensor-domain will block the first step in the signal transduction pathway and shut down the expression of the virulence-related genes. Since many genes are affected by the inhibition, anti-tubercular drug which attack the PhoR sensor-domain should be more effective in treating \textit{M. tuberculosis} infection and have shorter duration of treatment than existing drugs.

Previous studies by Suwanto et al (2012) have successfully characterized, isolated, and cloned PhoR sensor-domain coding sequence from \textit{M. tuberculosis} strain H37Rv into pRSET expression vector. In this study, we over expressed recombinant PhoR sensor-domain protein in \textit{E. coli} BL21 (DE3) and conducted subsequent purification process to isolate the protein. Protein in pure condition is required to be proceed for structural study. The resulting PhoR sensor-domain 3D structure could be used for further characterization of protein function (Shumilin et al. 2012). Moreover, structural information will provide basis for in silico screening of new anti-tubercular drug using rational drug design, primarily from natural chemical products found in Indonesia. Instead of determining the 3D structure of protein crystal, solution scattering, a new promising method for studying conformational changes of protein in physiological conditions has been established (Svergun et al. 2003, Jacquelet al. 2010) and was proposed in this work. Overall 3D structure of protein in low-resolution can be provided from the small-angle scattering (X-ray, SAXS or neutron, SANS) of protein solution as an important complementary method of protein crystallography due to the limitation in preparing the protein single crystal. An isotropic scattering function \( I(q) \) which is proportional to the averaged of over all structure orientations from a single protein molecule has to be attained in order to reconstruct the protein structure from solution. This constraint is only achieved from a monodisperse system as non-interacting of identical particles (scatterers) in dilute solutions.

**MATERIALS AND METHODS**

**Cloning Summary and Overexpression of Recombinant PhoR Sensor-Domain Protein.** Previous study by Suwanto et al (2012) has amplified DNA segment encoding sensor-domain of PhoR protein (GenBank, NC_000962.3, NP_215272.1) from \textit{Mycobacterium tuberculosis} H37Rv genome using PCR technique with forward (5’-GAATTCCGTACCT CGATGTTCGACGA-3’) and reverse (5’-CCATGGTT AGACGTCGGCCAGATCAATG-3’) primers. The amplified DNA then was cloned primarily into pGEM-T easy vector before transferred into pRSET expression vector. The expressed protein will be observed around 17 kDa according to in silico predicted product (MRGSHHHHHHMASMTGGQMGDLDDDKDRWGSEVTSMQLHRSLTRVLELIEQI WAQITLPLAPDPYPGHNPDRPSRFYVRVISPDMQSYTALNDNTAIPAVANVDVGHRHTTLPSIG GSKTLWRAVSVRASDGYLTVAIDLADV).

pRSET-SensPhoR vector was transformed into \textit{E. coli} BL21(DE3) using heat shock method (Sambrook and Russel, 2001). A single transformant colony was grown in LB medium containing 0.1 mg mL\(^{-1}\) ampicillin at 37 °C and agitated at 200 rpm until the optical density at 600 nm reached 0.4-0.8. The overexpression was induced with 1 mM IPTG for 4-6 h. Cells were harvested by centrifugation at 2000 g, 4 °C, for 30 min.

**Protein Purification.** The cell pellet from overexpression cultures was resuspended in lysis buffer containing 20 mM Tris-HCl pH 8.0, 1 mM PMSF, disrupted by sonication, and then centrifuged at 12,000 g, 4 °C, for 10 min to separate soluble proteins (supernatant) from insoluble proteins and cell debris (pellet). The pellet was washed twice with Triton X-100 -Urea (2% triton X-100, 2M urea, 0.5 M NaCl, 20 mM Tris-HCl pH 8.0) and once with Tris-HCl 20 mM pH 8.0 followed by sonication and centrifugation at 12,000 g, 4 °C, for 10 min. The pellet containing inclusion bodies was dissolved in binding buffer containing 8 M urea, 0.5 M NaCl, 5 mM imidazole, 2.5 mM β-mercaptoethanol, and 20 mM Tris-HCl pH 8.0 stirred for 30-60 min, and then centrifuged at 12000 g, 4 °C, for 15 min. The resulting supernatant was filtered using millipore membrane of 0.22 μm. The filtrate was prepared for purification.

Protein purification was carried out using nickel Ni-NTA-agarose system on 50 mL of Econo column (Biorad) in the refolding state at flow rate of 0.5-1 mL
The column was equilibrated with solution containing 0.5 M NaCl, 5 mM imidazole, 8 M urea, 2.5 mM β-mercaptoethanol, 20 mM Tris-HCl pH 8.0 (GE Life Sciences). The protein was refolded using a urea linear gradient of 8-0 M and then eluted with 0.5 M NaCl, 250 mM imidazole, 2.5 mM β-mercaptoethanol, 20 mM Tris-HCl pH 8 added with 200 mM arginine to prevent precipitation formation during storage. Results from protein overexpression and purification were profiled by SDS-PAGE (Fig 1). To confirm the identity of recombinant PhoR sensor-domain protein, slices of acrylamide gel containing a desired band were sent to Center for Mass Spectrometry & Proteomics University of Minnesota for LC-MS analysis.

Small Angle X-ray Scattering (SAXS) for Determining Protein Structure in Solution. The purified protein was dialyzed using servapor membrane (molecular weight cut off /MWCO = 12-14 kDa, Serva) with protein sample/dialysis buffer volume ratio of 1:22 to dispose imidazole and β-mercaptoethanol and to reduce the NaCl concentration in the purified sample to 200 mM. The sample was concentrated using Nanosep Ultrafiltrator (MWCO/Molecular Weight Cut-off = 10 kDa, Pall). After filtration by millipore membrane of 0.22 µm, acquired protein in final concentration of 1.5 – 5 mg mL\(^{-1}\) then was subjected for SAXS of protein in solution. Sample equilibration was conducted prior to data collection by dialysing protein sample in its solution buffer. Both of equilibrated protein sample and the background solution from its dialysed buffer were exposed to X-ray beam for 10 h and collected at every hour, at 4 °C. The collected scattering data of protein samples was subtracted by the background solution data in obtaining corrected scattering data of proteins. The corrected 2-dimensional scattering data was then radially averaged to obtain the scattering intensity \(I(q)\) as a function of the momentum transfer \(q\) in Å\(^{-1}\) using data reduction software of Bruker NANOSTAR.

RESULTS

Overexpression of Recombinant PhoR Sensor-Domain Protein. Since pRSET containing T7 promoter, IPTG induction was conducted to increase the expression of the recombinant protein in E. coli BL21 (DE3). SDS PAGE result showed that recombinant PhoR sensor-domain protein was overexpressed and found dominantly in pellet fraction of cell lysate to supernatant fraction (Fig 1). Subsequent process to isolate and characterize inclusion bodies protein from pellet fraction confirmed that the estimated molecular weight of 17 kDa protein existed in this form (Fig 1).

Protein Purification. PhoR sensor-domain protein was constructed in pRSET vector which contains hexahistidine as fusion tag. This tag facilitates rapid purification of recombinant protein by immobilized metal ion affinity chromatography (IMAC). Purification was performed alongside the refolding process and highly purified protein was obtained from the single Ni-NTA column running (Fig 2A). Post-purification observation showed that the purified protein was unstable and aggregated after storage at -80 °C (Fig 2B). Arginine in final concentration of 0.2 M should be added into the protein solution immediately after elution to prevent further aggregation.

LC-MS analysis was conducted to determine the peptide sequences of ~17 kDa band of purified protein. Several chemical modifications on the protein residues (Fig 3) were performed prior to trypsin digestion to increase peptide fragments generation and readability. All spectra of detected fragments were searched against sequence reference of recombinant PhoR sensor-domain using peptide de novo sequence software PEAK 7.5 (Ma et al. 2003). The result then confirmed the identity of recombinant PhoR sensor domain protein with 79% sequence coverage (Fig 3).

Small Angle X-ray Scattering for Determining Protein Structure in Solution. The scattering intensity \(I(q)\) vs. momentum transfer \(q\) of recombinant PhoR sensor-domain protein in two different concentrations, (A) 3.9 mg mL\(^{-1}\) (B) 1.56 mg mL\(^{-1}\) was showed (Fig 4). The scattering intensity still increased in low q-range indicating that this protein has a naturally propensity to aggregate under the buffer conditions used in the preparation whereas the protein solution was diluted two times. This aggregated form was also confirmed using dynamic light scattering (DLS) measurements which dominant peak referred to particle with size 11 nm and molecular weight 1052 kDa, significantly different with 3 nm and 17 kDa of PhoR sensor-domain actual features.

DISCUSSION

The presence of recombinant PhoR sensor-domain in the inclusion bodies confirmed in silico analysis using protein-solubilization prediction software from
Fig 1 SDS PAGE of PhoR sensor-domain recombinant protein expressed in E. coli. Lane M shows molecular mass marker (in kDa). Lane P contains pellet-cell lysate; Lane S contains supernatant-cell lysate; lane Incbod the inclusion bodies. Sample was run on 20% acrylamide and visualized using Coomassie Brilliant Blue staining.

Fig 2 SDS-PAGE of purified recombinant PhoR sensor-domain protein purification in 20% acrylamide (A). Lane M shows molecular mass marker (in kDa); Lane E the elution fraction. The gel was stained with Coomassie Brilliant Blue. Precipitation of purified protein was observed in the sample without additives (B).

University of Oklahoma. The software had developed on basis of logistic regression model with interaction of parameters that accurately predicted 96% insoluble protein from large set of data base (Diaz et al. 2010). Results from the analysis estimated that PhoR sensor-domain protein was expressed as insoluble protein inside E. coli. The intrinsic factor of protein might affect the inclusion bodies formation of PhoR sensor domain in E.coli rather than overexpression factor since significant amount of protein was acquired in insoluble fraction (Fig 1) (Diaz et al. 2010). On different approach, The higher numbers of β-sheet structure than α helices in a PDC fold-like characteristic prediction (Geneious) of PhoR sensor domain (Suwanto 2012) was considered to be responsible for the insolubility character of this protein (Vilasi et al. 2006).

Protein purification and refolding was performed to obtain PhoR sensor-domain protein in pure and native form. Refolding protein is an elaborate process and achieving correct folded protein is sometimes tricky. In other cases, expressing the sensor domain of histidine kinase in particular vector that contain soluble promoting tag such as GST and MBP is preferable (Cheung et al. 2012; Tan et al. 2014). Nevertheless, inclusion bodies production has several advantages for
isolating and purifying particular protein such as high number of protein production, resistance from cellular protease, and shorter purifying step. The point that is stated later proven well in this experiment while recombinant PhoR sensor-domain protein could be purified merely in single column process using Ni-NTA agarose with high level of protein purity acquired. The heterogeneity of protein sample might be decreased significantly during isolation of inclusion bodies step including removal of common co-purified contaminant of *E. coli* protein in nickle column (Bolanos-Garcia and Thomas 2006), simplifying the subsequent process of protein purification to separate protein target from trace amount of other proteins. Importantly, the lack of cysteine in primary sequence of PhoR sensor-domain protein trimming the elaborate process of protein refolding by finding out the suitable condition for arrangement of correct disulfide bridge. Nevertheless, limitation was found in further analysis of PhoR sensor-domain recovery after refolding process due to the
protein absence in enzymatic activity and lack of information about the protein ligand.

Post-purification observation showed that the purified and refolded protein was precipitated after storage in -80 °C. Supplementation with additive such as arginine significantly reduced progression of protein aggregates. Arginine might effectively prevent protein aggregation by several ways. Ho et al. (2003) showed that addition of arginine in the refolding solution of Lysozimes could shift the viral coefficient value into positive and accordingly increased repulsive interactions between proteins. In another mechanism, Tsumoto et al. (2004) proposed that interaction of arginine with exposed aromatic amino acid in protein could increased its solubility that led to repress protein aggregation. It was later strongly indicated by Das et al. (2007) experiment that cluster of arginine in aqueous solution displaying methyl groups that might interact with hydrophobic surface in protein and then prevented the aggregation.

Purified protein was confirmed accurate to recombinant PhoR sensor domain since mass spectral analysis shown high peptide coverage score (≥ 50%) and two and more unique peptides were identified (60 unique peptides) (Link and LaBaer 2009).

Crystallization trial demands on high concentration of protein target. Several attempts to produce final solution of PhoR sensor-domain protein at ≥ 10 mg mL⁻¹ led to sample aggregation (data not shown). Alternative method for analysing protein structure in lower concentration, such SAXS, was preferable since it was able to give useful signal at concentration of protein sample 2.5 mg mL⁻¹ (14 kDa of molecular weight) and even at 0.5 mg mL⁻¹ (66 kDa of molecular weight) (Skou et al. 2014).

It is not possible to have confidence in the structural models derived in obtaining useful biostuctural information from small-angle scattering data if the samples aggregated. It should be monodisperse, identical particles, and the data are measured to low enough q to reliably characterize their largest dimensions as the plot will have a discernible flat region in the lowest q regime (independent on q) — dashed lines. Moreover, it confirmed that subaggregate particles was still continually developed in the sample even after protein filtration and buffer supplementation with arginine. The highly sensitive technique was required to detect protein homogeneity preceding to SAXS analysis or other related methods for protein structural study. Nonetheless, from those initial SAXS experimental works, it can be concluded that the sample preparation protocols for solution scattering experiment, experimental setup, data reduction and analysis are well established for protein solution.

In conclusion, inclusion bodies of PhoR sensor-domain protein was produced in E. coli by expressing pRSET-Sensor PhoR system under cultivation temperature of 37 °C and IPTG induction. While purification and refolding process dominantly isolated the protein in soluble form, protein homogeneity still an issue before proceeding the protein onto structural study. Some improvements in protein production are required for providing a pure monodisperse protein which has a high stability. We propose to optimize this protein expression on alternative condition, host or vector system to obtain the soluble protein at the first time and to prevent elaborate process of protein refolding and storage optimization.

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