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Optimization of pGEX System to Express and Isolate *Mycobacterium tuberculosis* Inclusion Body Protein in Combining with Modified Refolding Method

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Abstract

Antigen sub units for vaccine studies are typically isolated from recombinant proteins in an expression system. However, not all protein expression systems are used to express the specific protein. In this study, we optimized the pGEX system combined with the modified protein refolding to express and isolate *M. tuberculosis* proteins, especially proteins that are expressed as an inclusion body. Resuscitation promoting factor B (RpfB) protein is one of the Resuscitation promoting factor (Rpf) family of proteins that has been studied for its ability to induce cellular immunity in animal tests. Silico analyses demonstrate how RpfB is included in cell wall and cell processes. The Rpf family proteins are promising antigens that can be used as a TB vaccine candidate. The polymerase chain reaction was briefly performed using specific primers to amplify the full length of the *rpfB*. PCR amplification products were then purified, cut by restriction endonucleases, and cloned in to pGEX 6-P1. Protein expression was done in the *Escherichia coli* BL21 strain, and expressed protein was isolated using the modified protein refolding and solubilization method. The complex protein expression that appeared as inclusion bodies were successfully isolated and can be detected as complex GST-RpfB through the western blotting process. Our study results indicate that this system and our modified method are suitable for *M. tuberculosis* inclusion body protein expression and isolation.

Abstrak

Optimasi System Ekspresi pGEX untuk Mengekpresikan dan Mengisolasi Protein RpfB *Mycobacterium tuberculosis* dalam Bentuk Badan Inklusi Menggunakan Metode Refolding yang Dimodifikasi. Antigen sub unit untuk studi vaksin umumnya diisolasi dari protein rekombinan menggunakan suatu sistem ekspresi. Sementara itu, tidak semua sistem ekspresi protein dapat digunakan untuk mengekspresikan protein tertentu. Dalam studi ini kami melakukan optimasi sistim pGEX untuk mengekspresikan dan mengisolasi protein *M. tuberculosis* yang diekspresikan sebagai badan inklusi. *Resuscitation promoting factor* B (RpfB) protein adalah salah satu protein keluarga *Resuscitation promoting factor* (Rpf) yang telah dipelajari kemampuannya dalam menginduksi imunitas seluler hewan uji. RpfB protein terlibat dalam pembentukan dinding sel dan proses-proses dalam sel. Protein keluarga Rpf berpotensi sebagai antigen yang dapat digunakan sebagai kandidat vaksin TB. Secara singkat, reaksi berantai polimerase menggunakan primer spesifik digunakan untuk mendapatkan panjang DNA sintetik *rpfB* yang dibutuhkan. Produk amplifikasi PCR kemudian dimurnikan, dipotong oleh ensim restriksi endonuclease yang dilanjutkan dengan pengklonaan kedalam pGEX 6-P1. Ekspresi protein dilakukan di bakteri non pathogen *Escherichia coli* strain BL21, selanjutnya protein terekspresi diisolasi menggunakan metode refolding dan solubilisasi yang dimodifikasi. Komplek protein yang terekpresi muncul sebagai badan inklusi dan terdeteksi sebagai komplek GST-RpfB melalui metode western blotting. Hasil studi menunjukkan sistem pGEX dan modifikasi metode solubilisasi dan *refolding* dapat mengisolasi protein *M. tuberculosis* yang diekspresikan dalam bentuk badan inklusi.

Keywords: Mycobacterium tuberculosis, Beijing strain, RpfB, recombinant protein

Introduction

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis* and is one of the most serious health problems in Indonesia. The World Health Organization (WHO) reported that the mortality rate of tuberculosis (TB) disease in Indonesia is about 67,000 cases/year and 2,100 cases/year for TB-HIV [1]. Increasing TB infections can be prevented by preventative means such as vaccination. However, the BCG vaccine used

broadly in the world has variable protective efficacy, ranging from 0 to 77% [2,3]. The weakness of the BCG vaccine has led to new vaccine studies with the aim of determining alternative vaccines to use as primary or booster vaccines.

The *rpf* family consists of five genes, i.e. *rpfA*, *B*, *C*, *D*, and *E,* which play a role in inducing the bacteria to change from a stationary condition to an active growth condition [4,5]. The experiment results do by some researchers have shown the capability of this family to induce antibodies and the cellular immune system in animal tests [6]. Conversely, further studies reveal that the RpfB and RpfD proteins stimulated a higher response of cellular immunity compared to the other proteins in this family [7]. Both characteristics of the Rpf protein family, the ability to induce stationary bacterial cells and the ability to generate immune responses, are interesting study themes for further investigation. Nonetheless, exploring protein expression systems is necessary as it continues to be a challenge to find alternative systems that can be used to express *M. tuberculosis* proteins.

The aims of this study were to examine the manufacturing of the recombinant protein of *M. tuberculosis,* Beijing strain, from Indonesian patients collected by Pusat Biomedis dan Teknologi Kesehatan Kementrian Kesehatan Republik Indonesia. The focus was particularly RpfB, in the pGEX system, and to examine the protein expression system itself. Several studies demonstrate success in cloning and in the protein expression of *M. tuberculosis* proteins in vector systems, such as the pET system, the pColdII system, the pGEX system itself, and others [8-10]. Although the pGEX system has been used for protein expression of *M. tuberculosis*, information on the pGEX system is still limited, especially for proteins that have the potential to be expressed as inclusion bodies. Therefore, it is necessary for further testing of the pGEX system with other proteins of *M. tuberculosis*. The pGEX system has some advantages compared to other systems. One of these advantages is the availability of anti-GST, as the expression of GST fusion proteins can be easily analyzed using western blotting. Similarly, tools to isolate and purify the proteins based on GST affinity are available to simplify and shorten the workflow. The results of this study provide useful information on use of the pGEX system, along with useful information on the protocol to obtain recombinant *M. tuberculosis* proteins. The RpfB protein designed and produced in this study can be used in subsequent studies with the aim of discovering vaccine candidates that can be produced in Indonesia.

Materials and Methods

Strain. The *M. tuberculosis* H37Rv strain used in this study is a bacterial stock from the Department of Microbiology, Medical Faculty, Universitas Indonesia. The Beijing strain was kindly donated from the Center for Biomedical and Health Technology, Ministry of Health Republic of Indonesia (Pusat Biomedis dan Teknologi Kesehatan Kementrian Kesehatan Republik, Indonesia).

Genomic DNA extraction. *M. tuberculosis* genomic DNA was isolated from the cells using the boiling method [11]. The bacterial cells were scraped from Lowenstein-Jensen media and inserted into 1.5 mL tubes, containing 500 µL sterile dH_2O. They were then incubated at 100 °C in a heat block for 10 minutes. The tubes were centrifuged to separate genomic DNA from cell debris. The supernatant containing *M. tuberculosis* genomic DNA was isolated and stored at minus 30 °C until used.

Amplification of the *rpfB* gene. The *rpfB* gene was amplified using the polymerase chain reaction (PCR) method with specific primers annealing to the *rpfB* region (primers used in this study are not published for patent purposes). The PCR product size was confirmed by gel electrophoresis and then purified using a PCR purification kit (Qiagen). The purified PCR product was then used for cloning.

Cloning of the *rpfB* gene into the pGEX system and transformation. pGEX 6-1 and *rpfB* PCR products were digested by *Eco*RI and *Xho*I restriction enzymes (Fermentas) and then purified before ligation. The ligation process was done using T4 DNA ligase (Fermentas), resulting in plasmid harboring the inserted gene. Transformation of recombinant plasmid into *E. coli* DH5α was done using the heat shock method and resulted in the MRB1 and MRB3 strain [12]. The ligation product was mixed with bacterial competent cells in a 1.5 mL plastic tube and incubated on ice for 30 minutes, and then incubated under heat shock conditions at 42 °C for 1.5 minutes. The bacterial cells were sub cultured in LB medium and shaken for 1 hour at 37 °C. LB agar containing 100 µg/mL ampicillin was used to select bacterial cells carrying recombinant plasmid. Plasmids isolated from bacteria growing on the selective medium were examined for the correct orientation of the inserted gene by PCR, and for correct base composition by sequencing. The plasmid containing the *rpfB* gene was then transformed into *E. coli* strain BL21 as the expression host resulting in the MRB2 and MRB4 strain.

RpfB protein expression. Strain MRB2 and MRB4 was grown on selective media until the optical density (OD_{600}) reached 0.4, and then IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 1 mM. Incubation was continued for 4 hours before bacterial cells were harvested. Ten milliliters of 1x phosphate-buffered saline (PBS) was added to the bacterial pellet after centrifugation and the re-suspended pellet was subjected to sonication using a Memert sonicator. Lysed bacterial cells after sonication was analyzed by electrophoresis on a 15% SDS-

polyacrylamide gel (SDS-PAGE) to detect the expression of RpfB protein. RpfB protein expression was confirmed by western blotting with anti GST antibody.

Extraction, solubilization, and refolding of recombinant RpfB protein. The pellet, from centrifugation after sonication, was re-suspended in the lysis buffer containing lysozyme. Sonication was performed again for 30 minutes to break down cell membranes and then subjected for centrifugation at 5000 rpm. This was done for 10 minutes at 4 °C. Subsequently, the pellet was re-suspended in buffer TNMFX — 2M urea containing 50 mM Tris-Base, 150 mM NaCl, 1 mM EDTA, and 2 M urea — and then subjected to 10 minutes sonication, followed by centrifugation at 5,000 rpm for 10 minutes at 4 °C. Pellets were then re-suspended in buffer TNMFX-0.1% Triton-X100 contained 50 mM Tris, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton-X100. The suspension was sonicated for 10 minutes, incubated for 30 minutes at 4 °C, and then centrifuged at 5000 rpm for 10 minutes. The pellet was re-suspended in the solubilization buffer, containing 8M urea, then centrifuged at 1000 rpm for 10 minutes at 4 °C. The supernatant was collected and stored at -80 °C until refolding. For refolding, the supernatant was diluted fivefold in the refolding buffer containing 50 mM Tris - HCl, 100 Mm NaCl, 1 mM reduced glutathione, 1 mM DTT, and 1 mM PMSF. The diluted solution was incubated overnight at 4 °C with stirring.

Isolation of recombinant protein RpfB. Refolded recombinant RpfB protein was isolated using the GST affinity column; this was based on the manufacturer's instructions. Prior to this, the affinity column was equilibrated with the equilibration buffer containing 50 mM Tris-HCl, 100 mM NaCl, 1 mM reduced glutathione, 1 mM DTT, and 1 mM PMSF in a volume four times the volume of the resin. The column was incubated, with shaking, for one hour and then the equilibration buffer was removed from the column. A volume of refolding protein solution, three times the volume of the resin, was poured into the column and incubated on a shaker for 1 hour. The liquid in the column was collected in a sterile tube as the flow through. The column was then washed 2–3 times with a wash buffer. Recombinant protein bound to the resin was eluted with an elution buffer, collected in a sterile tube, and stored at -80° until use.

Results and Discussion

In order to construct the AR1 and AR2 plasmids, we inserted the *rpfB* gene downstream of the GST sequence, between the multi cloning site (MCS) and the terminal sequence (Figure 1). Gene insertion in this area is advantageous because the addition of a terminal sequence to the PCR product is not necessary. With this construct, expression of a protein in accordance with the expected length is more likely.

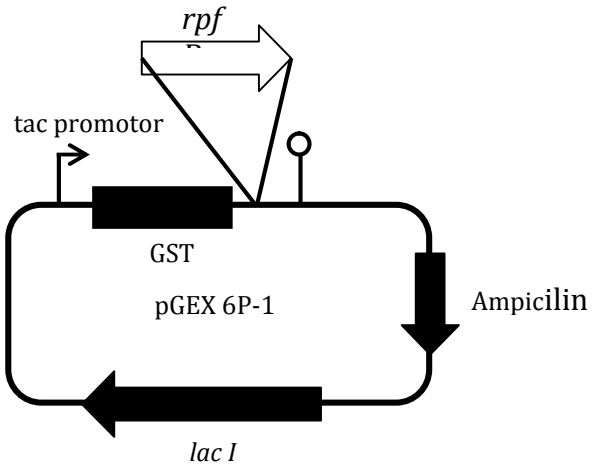

Figure 1. Plasmid Construction to Find AR1 Plasmid

The transformation result produced by colony PCR displayed some positive colonies carrying the *rpfB* gene on a plasmid. Successful DNA transformation of AR1 and AR2 plasmid into *E. coli* BL21 was demonstrated, and elicited the MRB2 and MRB4 strains. Success of the transformation was determined by colony PCR results, which exhibited bands with length as expected (data not shown). All strains and plasmids used in this study are listed in Table 1 and Table 2.

Analysis results for the gene sequencing of *rpfB* gene *M. tuberculosis* strains Beijing, from recombinant plasmids, elicited the presence of mutations outside of the epitopes region when compared to strain H37Rv (Figure 2). RpfB protein have two epitops for $CD4^+$ cell T (codon 146-160, codon 251-256) and one epitope for $CD8^+$ cell T (codon 66-80) Based on analysis using Genetyx ver.4 software. Even though mutations occurred in cloning, we believe that these mutations will not affect the ability of the protein to stimulate the immune response, since the mutations are out of epitope regions.

To observe RpfB protein expression in the host, we cultured MRB2 and MRB4 in a Luria Bertani (LB) medium. Addition of 1 mM IPTG as an inducer at OD_{600} 0.4 succeeded in inducing RpfB expression, as detected in SDS PAGE (Figure 3). The length of the RpfB protein band was as expected based on analysis in silico [13]. Unexpectedly, the expression of RpfB strain from strain was higher than the H37Rv strain. We speculate that the appearance of this gene mutation may have resulted from changes in the protein conformational, a protein that is normally accepted and overexpressed by *E. coli* but was instead rejected. It will be advantageous to isolate the protein in a large scale. However, this experimental step revealed that the expression of the Rpf protein family of *M. tuberculosis* can be performed in the pGEX system with *E. coli* as a host.

Tabel 1. Bacterial Strain List

Strain	Genotype	Source
M. tuberculosis strain H37Rv		Bacterial stock of Microbiology Department Medical Faculty Universitas Indonesia
M. tuberculosis strain Beijing		Bacterial stock of Pusat Biomedis dan Teknologi Kesehatan Kementrian Kesehatan Republik Indonesia
E. coli DH5α	FΦ80*lac*ZΔM15Δ(*lac*ZYA*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Bacterial stock of Microbiology Department Medical Faculty Universitas Indonesia
E. coli BL21pLys	F–ompT hsdS(rB–mB–) gal dcm λ(DE3) pLysS (Camr) (λ(DE3):lacI, lacUV5-T7 gene 1, ind1, sam7, nin5)	Bacterial stock of Microbiology Department Medical Faculty Universitas Indonesia
MRB1 (H37Rv)	*E. coli* DH5αpGEX6P-1 *rpfB*	This work
MRB2 (H37Rv)	*E.coli* BL21pGEX6P-1 *rpfB*	This work
MRB3 (Beijing)	*E. coli* DH5αpGEX6P-1 *rpfB*	This work
MRB4 (Beijing)	*E.coli* BL21pGEX6P-1 *rpfB*	This work

Table 2. Plasmid List

Plasmid	Genotype	Source
pGEX 6P-1		Amersham
R1 (H37Rv)	pGEX 6P-1Δ*rpfB*	This work
R2 (Beijing)	pGEX 6P-1Δ*rpfB*	This work

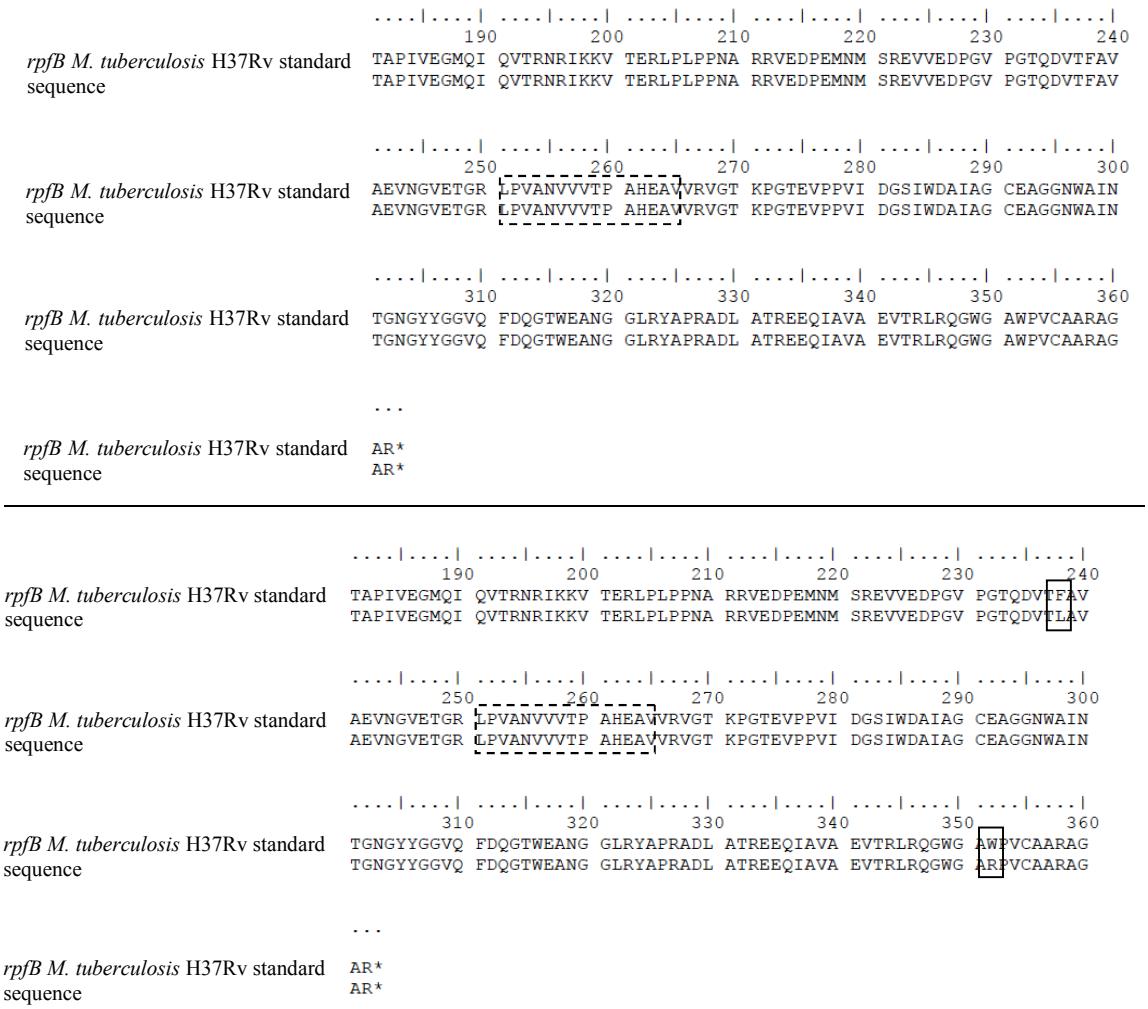

Figure 2. Analysis of *rpfB* Recombinant Sequences Comparing to *M. tuberculosis* H37Rv Standard Sequence

To examine the expression of RpfB, we performed western blotting with an anti-GST antibody as reporter. The results support previous data that the expressed protein is a fusion between RpfB and GST (Figure 4). The result of this experiment, using pellet and supernatant fractions, demonstrated that the protein was expressed mostly in the pellet. This indicates that the *rpfB* gene may have been translated as inclusion bodies in *E. coli*. To confirm that the protein expression of RpfB was not in inclusion bodies as a result of the high culture incubation temperature (37 °C), we repeated the culture step with incubation temperature variations up to 20 °C and examined the protein expression (data not shown). The result reveals that the RpfB protein consistently translates as inclusion bodies.

RpfB protein isolation, using the solubilization and refolding solution containing urea, transferred the protein originally in the pellet fraction to be in supernatant. Modifying this fraction means that the proteins can be isolated using an affinity column (Figure 5). Although the refolding was successful, the amount of isolated protein was nonetheless not optimal, as RpfB cit can be seen from the amount of target protein observed in the flow through. We speculate that the content of urea in the solubilization and refolding solution possibly blocked the binding of the GST protein to the slurry.

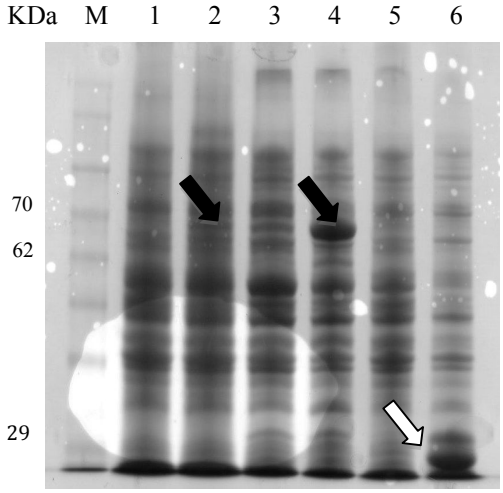

Figure 3. RpfB Expression on SDS PAGE. M = Marker; 1. *E. coli* BL21+pGEX-rpfB (H37Rv) without Induction, 2. *E. coli* BL21+pGEX-rpfB (H37Rv) was Inducted by 1 mM IPTG, 3. *E. coli* BL21+pGEX-rpfB (Beijing) without Induction, 4. *E. coli* BL21+pGEX-rpfB (Beijing) was Inducted by 1 mM IPTG, 5. *E. coli* BL21+pGEX without Induction, 6. *E. coli* BL21+pGEX was Inducted by 1 mM IPTG. Black Arrow Show Expression of GST+RpfB, White Arrow Show Expression of GST. Molecular Weight GST = 26 KDa, GST+RpfB = 64 KDa.

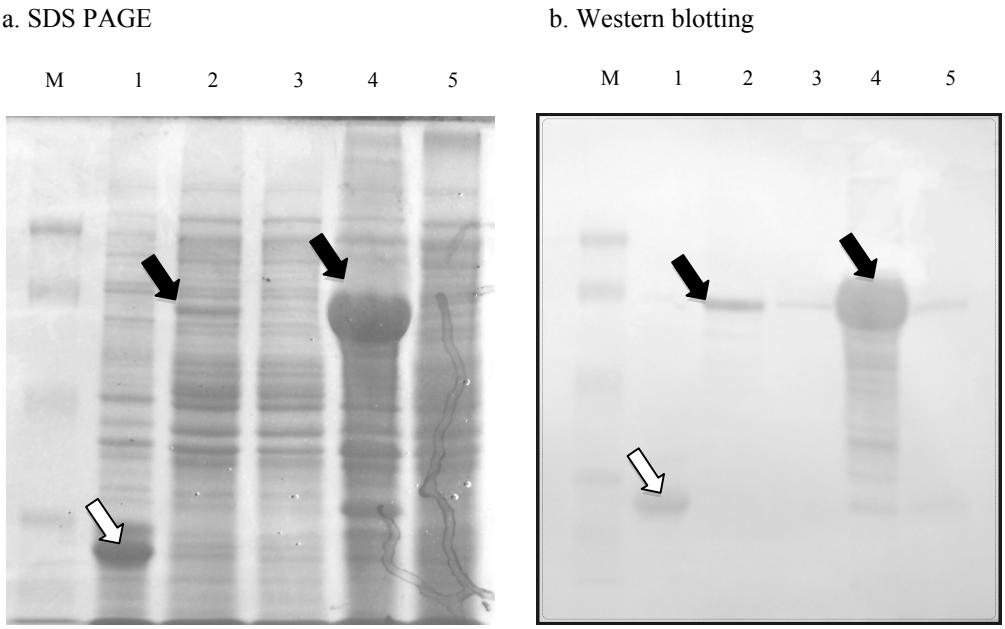

Figure 4. Confirmation of RpfB Protein Expression. a) SDS PAGE: Lane 1, BL21+pGEX 6P-1; Lane 2 BL21+pGEX-rpfB (H37Rv) Pellet, Lane 3 BL21+pGEX-rpfB (H37Rv) Supernatant; Lane 4 BL21+pGEX-rpfB (Beijing) Pellet, Lane 5 BL21+pGEX-rpfB (Beijing) Supernatant. White Arrow Show Expression of GST Protein, Black Arrow Show Expression of GST+RpfB. b). Western Blotting with Antibody-Anti GST: Lane 1, BL21+pGEX 6P-1; Lane 2 BL21+pGEX-rpfB (H37Rv) Pellet, Lane 3 BL21+pGEX-rpfB (H37Rv) Supernatant; Lane 4 BL21+pGEX-rpfB (Beijing) Pellet, Lane 5 BL21+pGEX-rpfB (Beijing) Supernatant. White Arrow Show Expression of GST Protein, Black Arrow Show Expression of GST+RpfB.

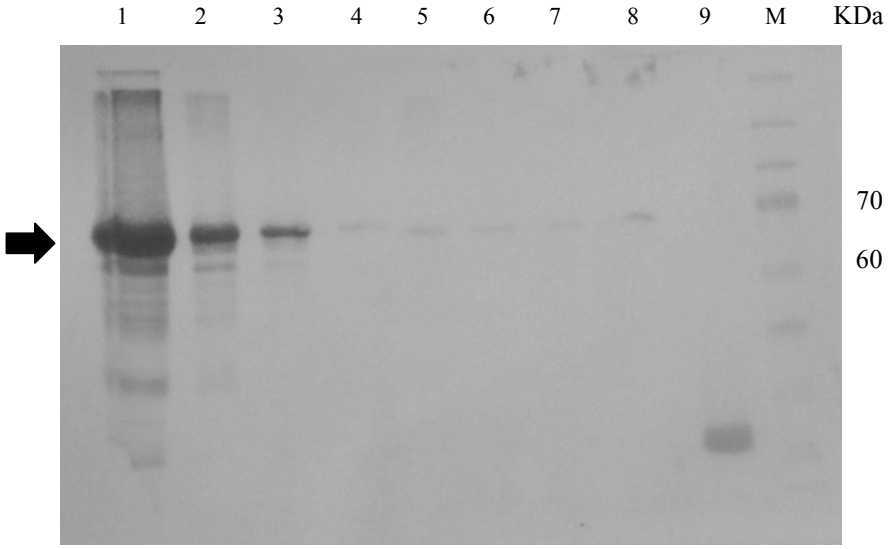

Figure 5. Isolation of RpfB Protein After Solubilizing and Refolding. Lane 1, Supernatant in Urea 8 M; Lane 2, Flow Trough; Lane, 3-4, wash; Lane 5-8, Elute; Lane 9, GST Protein; M = Marker, Black Arrow Show Expression of GST+RpfB.

Further research in protein expression system testing is needed to determine an efficient system in protein expression. In this study, we succeeded in cloning and expressing one gene from the *rpf* family, *rpfB*, into the pGEX system. However, analysis of the sequencing results exhibited two point mutations in the gene clone area. These mutations may alter the structure of the RpfB protein strain from Beijing. Analysis of the epitopes of CD4+ and CD8+ T cells demonstrated that the amino acid change occurred outside the epitope recognition of CD4+ and CD8+ T cells. Recognition of CD4+ and CD8+ epitopes to be important on TB vaccine study caused by protection against tuberculosis infection is generally mediated by IFγ and IL-12 that are secreted by CD4+ and CD8+ [14]. All of these mutations likely have no effect on the RpfB antigenicity properties. Changes in two amino acids, as a result of gene mutations that still allow RpfB protein, can be expressed in *E. coli* BL21. Results of the protein expression were detected by SDS PAGE, and demonstrated that the efficiency of protein expression RpfB in the mutant clone is better than the control strain (H37Rv). We suspect that these two amino acid changes supports the efficiency of the expression of the protein. Further testing is required to better understand the involvement of these mutations in RpfB expression. Nevertheless, we think it is this unexpected finding that will foster protein production for our next study, one in further exploring antigenicity of RpfB protein.

We expected that fusion of RpfB with GST proteins would increase the solubility RpfB. In fact the expression of this protein, always in pellet fraction, indicates that the protein was in inclusion body form. To confirm RpfB protein expression was not in inclusion bodies due to the high incubation temperature (37 °C) or IPTG, we repeated the culture step with variation of IPTG concentration and incubation temperature variations below 37 °C (data not shown). Variation of IPTG concentration and temperature did not appear to solubilize these proteins, and the results reveal that the RpfB protein is translated as inclusion bodies. We do not have an argument to explain this phenomena, but it is possible that the *tac* promoter in this vector might induce overexpression of proteins. Several other factors may result in the formation of inclusion bodies, such as pH level, osmolarity, cofactors, or folding mechanisms. Conversely, high expression levels also induce polypeptide production in higher concentrations and would interact with a similar region causing an inclusion body [15]. Although it is possible to isolate the target proteins from the purify-native antigen of *M. tuberculosis*, expression of the proteins on the host, such as *E. coli,* will be more efficient and safe [16,17]. More than that, through this method the target protein can be re-designed or re-assembled with other target proteins [18].

Some expression vectors have also been developed and used to obtain the target proteins. The pET system, containing the T7 promoter, has been used for the expression and isolation of *M. tuberculosis* proteins [18]. This system is very popular, with more than 50% of cases of successful protein expression with other bacteria [19]. Nevertheless, our other studies demonstrated that protein isolation using this vector is not able to distinguish between the target and non-target protein on a western blot (unpublished data). We suspect an anti-His antibody was cross-reacted with non-target proteins of *E. coli* containing His residue that could not be separated with an affinity column. Research conducted to study the pET system expressed *M. tuberculosis* proteins and show this system has successfully performed expression

and purification of target proteins. When the detection of protein recombinants were observed using mass spectrometry, it is a more sensitive and expensive process that requires more trained people compared to the conventional western blot method that we used [18]. However, there is a successful study that expressed *M. tuberculosis* protein in pDE22 plasmid, inserted with sequences that encode His's-tag protein upstream of the target gene [20]. This study is important information for His use as there is a tag protein where the results differ from our study. Several plasmids are also generally used to express the protein, but have some weaknesses that can be covered by the pGEX system. For example, under the control of the promoter *cspA,* the expression of proteins requires lowering incubation temperature of the cell culture [21], while the expression of the protein under the control of the promoter λcI^{857} need a temperature up to 42 °C [22,23].

Finally, the pGEX system has proven useful for cloning and expression of *M. tuberculosis* proteins. This system appears suitable for isolating *M. tuberculosis* proteins that form inclusion bodies, and is easily refolded and resolubilized. Our study provides necessary information for the expression and isolation of *M. tuberculosis* proteins. The successful application of the pGEX system and the modified method for protein isolation in this study can be used by other researchers to assess the expression and isolation of other *M. tuberculosis* target proteins. Moreover, isolated protein from this study can then be used for cellular immunogenicity assay to determine the necessary immunogenic properties and can aid in the discovery of new tuberculosis vaccine candidates.

Conclusion

RpfB protein of *M. tuberculosis* was successfully cloned, expressed, and isolated in pGEX system. The expression of RpfB as membrane protein, this target protein was successfully isolated using the urea method.

Ethical clearance. This study is part of a study under ethical clearence from the Ethics Committee of the Faculty of Medicine Universitas Indonesia No. 906/UN2.F1/ETHICS/2014.

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