Cloning and expression of *pab* gene of *M. tuberculosis* isolated from pulmonary TB patient in *E.coli* DH5α

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Abstrak

Latar belakang: *Antigen38 Mycobacterium tuberculosis merupakan agen serodiagnostik yang potensial karena mengandung dua epitop spesifik untuk sel B. Mahalnya agen diagnostik menyebabkan lambatnya realisasi diagnosis TB secara cepat di negara berkembang. Kami memproduksi antigen 38 rekombinan yang berasal dari galur lokal yang kemungkinan dapat digunakan untuk memproduksi alat serodiagnostik TB yang ekonomis.*

Metode: *Gen pab diisolasi dari pasien TB paru di Malang, diklon ke plasmid pGEM-Teasy menjadi pMB38. Klon E.coli DH5α yang membawa pMB38 diseleksi di medium yang ditambah dengan X-Gal. Ekspresi pab dilakukan menggunakan pMBhis yang berasal dari pPRoExHTc dibawah kontrol promoter Trc dengan inang E.coli DH5α*

Hasil: *Pencocokan sekuen gen pab dari klon E.coli DH5α berwarna putih dengan gen pab dari M. tuberculosisH37Rv memperlihatkan homologi sebesar 98%. Protein rekombinan yang sudah dihilangkan signal peptidanya ditemukan di sitoplasma.*

Kesimpulan: *Gen pab dari pasien TB dapat diekspresikan secara intraseluler dengan sistem heterolog.* ***(Med J Indones 2011; 20:247-55)***

Abstract

Background: *Mycobacterium tuberculosis* antigen38 is a potent serodiagnostic agent containing two *M. tuberculosis*-specific B-cell epitopes. The high price of imported diagnostic agents hinders realization of fast clinical TB diagnosis in developing countries. Therefore, we produced recombinant antigen38 (recAg38M) from *M. tuberculosis* local strain, which might be used to produce economical tuberculosis serodiagnostic kit.

Methods: *Pab* gene that was isolated from pulmonary TB patient in Malang was cloned into a plasmid vector (pGEM-Teasy) to construct pMB38. The E.coli DH5α clone carrying pMb38 was selected on X-gal medium. The expression of *pab* was mediated using pPRoExHTc under the control of Trc promoter and *E.coli* DH5α as host.

Results: Alignment of the pab sequence from the white E.coli DH5α clones with that of *M. tuberculosis* H37Rv showed 98% homology. The recombinant protein in which the signal peptide has been deleted to prevent the protein being secreted into medium was found in the cytoplasm.

Conclusion: *pab* gene of *M. tuberculosis* isolated from a TB patient could be expressed in heterologous system in *E. coli*DH5α. ***(Med J Indones 2011; 20:247-54)***

Key words: *Mycobacterium tuberculosis, Pab gene expression, recombinant antigen38*

Mycobacterium tuberculosis (*M. tuberculosis*) Antigen38, a phosphate-binding protein, has a function that is similar to periplasmic phosphate-binding protein of *Escherichia coli* (*E.coli*) i.e. as an initial receptor for active transport.[1] Among other antigens that were used as tuberculosis serodiganostic agents, Antigen38, that contains two *M. tuberculosis*-specific B-cell epitopes has been shown as the most potent immunogen due to its high specificity and sensitivity.[2] The high price of imported diagnostic agent hinders realization of fast clinical TB diagnosis in developing countries. One of the urgent goals of mycobacterial research in developing countries is to provide serodiagnostic agents. For such purpose, antigen need to be produced in a large scale. However, mass production of mycobacterial antigen using conventional system encountered several problems such as high cost laboratory facility, difficulty in the protein identification and reduction of immunogenicity after purification.[3] An alternative solution to these problems would be the production of recombinant antigen in *E.coli*.

Therefore, in this study we aim to express the recombinant Antigen38 from *M. tuberculosis* local strain (recAg38M), in *E.coli* DH5a, which might be used to produce economical tuberculosis serodiagnostic kit.

METHODS

The antigen38 coding *pab* gene was amplified from *M. tuberculosis* that was isolated from the sputum of pulmonary TB patient. The *pab* gene containing overexpression plasmid was constructed by inserting the amplified fragment into the corresponding vector. In order to avoid a loss of protein in the medium during purification process, the putative signal peptide of

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the *pab* gene was omitted using specific primer design during the amplification of the gene and construction of the expression vector, enabling the protein to be expressed in the cytoplasm of *E. coli* rather than secreted into medium.

Strain and plasmid

The *M. tuberculosis* which gene was used in this work was isolated from a patient who was suffering from pulmonary tuberculosis and hospitalized in Batu public hospital, Malang. Written consent was obtained from the patient. The study was approved by the Ethics Committee of Saiful Anwar Public Hospital. Plasmid pGEM-Teasy was purchased from Promega (USA). Plasmid pPRoExHTc was kindly given by Dr Rintis Noviyanti from Eijkman Institute.

Isolation and amplification of *pab* gene from chromosomal DNA of *M. tuberculosis*

Bacterial DNA was isolated according to van der Zanden. [4] The *pab* gene was amplified using PCR with modification, i.e. without putative signal sequence (amino acid position 1-23) and Cys1 was replaced with Met residue, and was amplified directly from the chromosomal DNA of *M tuberculosis* using two oligonucleotides, as forward (sense) and reverse (antisense) primers (figure 1).[5]

Primer 1: pabF224 (5'-GGCGGCCATGGGCTC GAA ACCACCGAGCGG-3') carried an *Nco*I cleavage site and attached to a position 63 to 93 starting from initiation codon (ATG).

Primer 2: pab1342 3' (5'-CCAGCAGGATCC GCA AAGCAGCCCGATGGC-3') was located downstream from stop codon. It carries restriction site BamH1 and correspond to the sequence at the position +1.002 to +974 counted from initiation codon ATG.

Construction of plasmid pMB38

The *pab* gene was amplified using PCR without the putative signal sequence. The amplified *pab* gene was cloned into pGEM-Teasy vector (Promega, USA) according to a standard method.[6] The bacterial host for cloning and expression was *E.coli* DH5α (F-*recAI endAI gyr*A96 thi-1 hsdr17(rk-mk-) *sup*44 *relAI*l(Φ80*lac*ZDM15) Δ(lacZYA-*arg* F) U169). To maintain compatibility of the alignment with Antigen38, the deduced amino acid of the recombinant protein was renumbered so that Cystein that was replaced by Met became the first residue. The new construct was then named pMB38. (Figure 1).

Screening of *E.coli* DH5α/pMB38

Plasmid pMB38 was then used to transform *E.coli* DH5α using heat shock method, [6] and the *E.coli* was spread on LB (Luria broth) solid medium supplemented with IPTG and X-gal. [7] The white clones were selected and recultured in liquid medium. The presence of the *pab* gene was confirmed using PCR followed by sequencing.

Construction of pMBhis

Plasmid pMB38 was digested with *Nco*I, then electrophoresed on 12.5% agarose gel, and generated three fragments. The 1130 bps fragment that corresponded to *pab* gene was then purified using Qiagen kit (USA) and ligated with pPRoExHTc vector that previously was linearised using the same restriction enzyme, and both DNA segments were then ligated to produce pMBhis (Figure 2). The ligation product was used to transform *E.coli* DH5α. Selection of *E.coli* DH5α/pMBhis was conducted on ampicillin 100mg/mL containing LB. The correct clone was confirmed via PCR.

Expression of recAg38M in *E.coli* DH5a/pMBhis

E.coli DH5α/pMBhis was cultivated at 37°C in Ampicillin (100mg/ml) containing LB medium to an OD600 of 0.6. The expression of *pab* was induced by addition of 0.6 mM isopropyl-B-D-thiogalactopyranoside (IPTG). After 3 hours cells were harvested by centrifugation at 6000xg for 15 min. The pellet was resuspended in 2.5 volume of 50 mM potassium phosphate buffer (pH 6.9) that contained 1 mM protease inhibitor, i.e. phenyl methyl sulphonyl fluoride (PMSF), and the cells were broken using a sonicator at 40% for 20 seconds, 3 times. The cell extract was then centrifuged for 15 minutes at 8.000x g. The supernatant was removed and further centrifuged (Bench Top Ultracentrifuge) at 30.000xg for 30 minutes to separate the S30 extract. The S30 extract was taken and underwent another centrifugation at 100.000xg for 90 minutes, and the supernatant was called S100, while the pellet was called P100. All cell fractions were subjected to 12.5% SDS-PAGE.

Purification of recAg38M using batch system

In this study the protein recAg38M was fused with 6x histidin tag that was located on N terminus site. Partial purification was performed in Eppendorf-cup according to the procedure from the manufacturer (Qiagen Ltd, Sweden). 250 ml of nickel-Nitriloacetic acid agarose in Phosphate buffer (pH 6.9) was incubated with 1 ml of S30 extract of *E.coli* DH5α/pMBhis at 4°C for 1 hour with gently shaking. After that, the mixture was loaded into a tube filter and centrifuged for 1 min. The tube was washed twice with Phosphate buffer containing 10 mM imidazol. In order to elute the protein that bound to Nickel, Phosphat buffer containing 250 mM imidazole was applied. Protein elution was repeated 4 times. Finally all fractions were analysed using SDS-PAGE 10%. The presence of protein 38 was confirmed using Commassie blue serva.

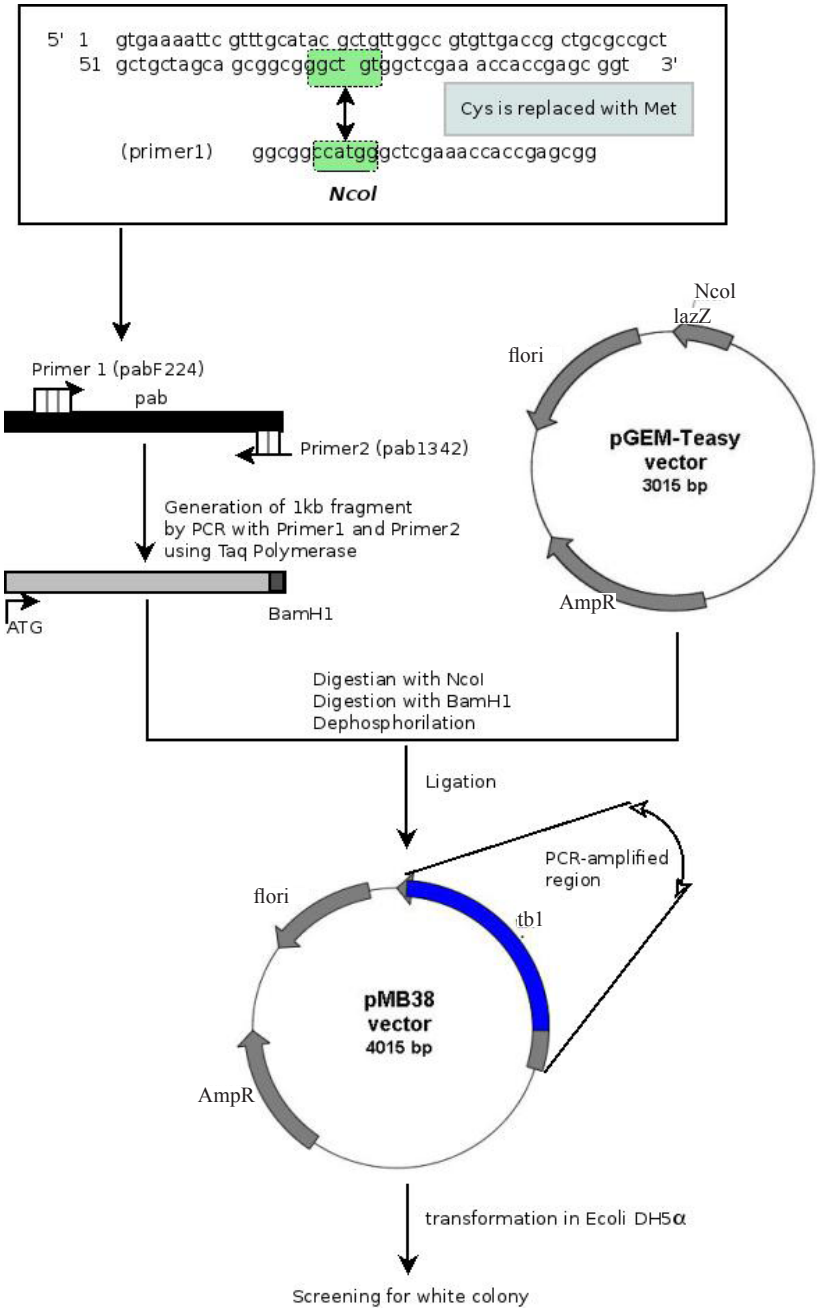

Figure 1. Construction of plasmid pMB38 (derivate of pGEM-Teasy). Plasmid pGEM-Teasy was digested with NcoI and ligated with PCR amplified product that was digested previously with NcoI and BamHI. The 3' overhang of insert (product of BamHI digestion) should ligate with 5' overhang of the plasmid (product of NcoI digestion) because there were only two different nucleotides within the cleavage site.

Growth of *E.coli* DH*5α*/pMB38

To examine whether the expression of pMB38 influence the growth of the host, growth tests were performed.

RESULTS

Construction of pMB38 and pMBhis

Primers were designed to modify *pab* gene by omitting the putative signal peptide. As a result, a band corresponded to 950 bp appeared after DNA amplification (Figure 3). This segment (*tb1*) was slightly shorter than that of the *pab* gene from *M. tuberculosis* 37Rv (1260bp). The segment *tb1* was then inserted into pGEM-Teasy to produce pMB38. Transformation of *E. coli* with pMB38 generated numerous white clones on plates containing X-Gal. We tested 12 clones and all carried the insert. Sequence analysis of *tb*1 showed a 98% homology to the sequence of *pab* gene of the *M. tuberculosis* R37v that was retrieved from published sequence in Gene Bank NCBI (Figure 4).

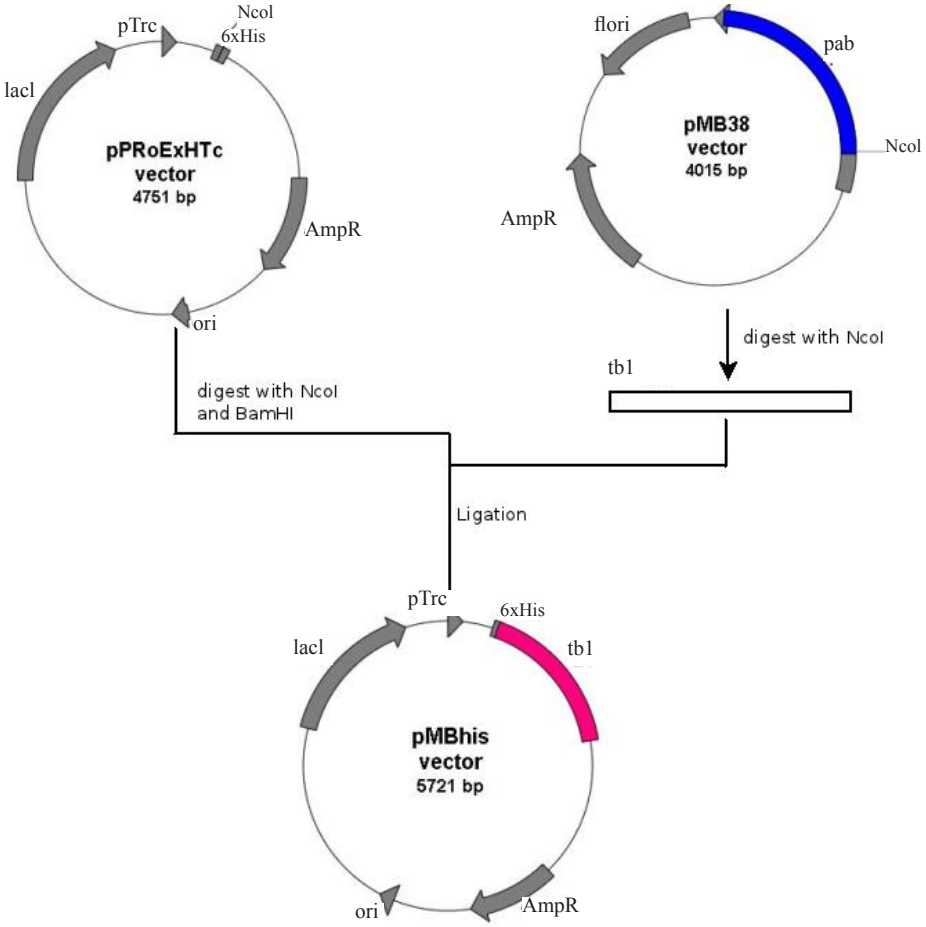

Figure 2. Construction of plasmid pMBhis (derivate of pPRoEXHTc). Plasmid pMB38 was digested with NcoI producing insert (tb1) while pPRoExHTc was double digested with NcoI and BamHI. The fragment tb1 was then ligated into pPRoExHTc. To ensure that the insert (tb1) bound to the plasmid in the correct direction, restriction test was perfomed.

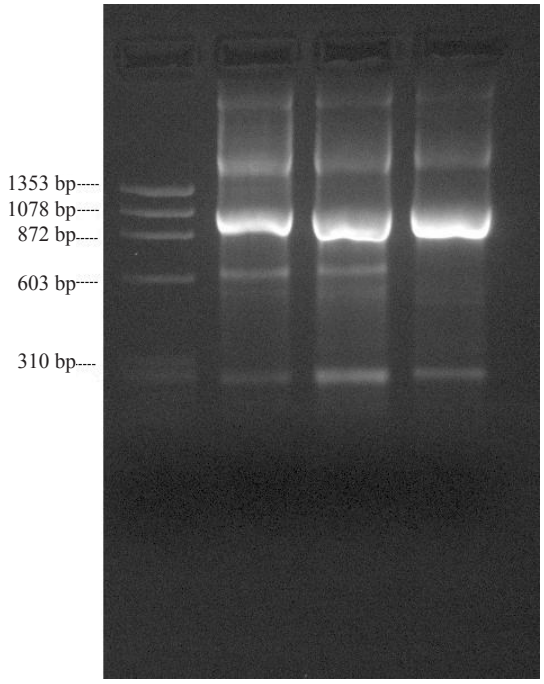

Figure 3. Amplified pab fragment from chromosomal DNA of M. tuberculosis. Lanes: 1= DNA marker, 2-4= amplified pab gene correspond to 970 bp.

Forward sequence:

```
>gb|M30046.1|MSGPABA M.tuberculosis protein antigen b (Pab) gene, complete
cds
Length=1993

 Score = 1072 bits (580),  Expect = 0.0
 Identities = 586/592 (98%), Gaps = 0/592 (0%)
 Strand=Plus/Plus

Query  2    TGTCGCGNNTACCCCCGCGTCGTCGCCGGTGACGTTGNNGGANACCGGTAGCACGCTGCT  61
            |||||||  ||||||||||||||||||||||||||||  ||| ||||||||||||||||||
Sbjct  274  TGTCGCGACTACCCCCGCGTCGTCGCCGGTGACGTTGGCGGAGACCGGTAGCACGCTGCT  333

Query  62   CTACCCGCTGTTCAACCTGTGGGGTCCGGCCTTTCACGAGAGGTATCCGAACGTCACGAT  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  334  CTACCCGCTGTTCAACCTGTGGGGTCCGGCCTTTCACGAGAGGTATCCGAACGTCACGAT  393

Query  122  CACCGCTCAGGGCACCGGTTCTGGTGCCGGGATCGCGCAGGCCGCCGCCGGGACGGTCAA  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  CACCGCTCAGGGCACCGGTTCTGGTGCCGGGATCGCGCAGGCCGCCGCCGGGACGGTCAA  453

Query  182  CATTGGGGCCTCCGACGCCTATCTGTCGGAAGGTGATATGGCCGCGCACAAGGGGCTGAT  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454  CATTGGGGCCTCCGACGCCTATCTGTCGGAAGGTGATATGGCCGCGCACAAGGGGCTGAT  513

Query  242  GAACATCGCGCTAGCCATCTCCGCTCAGCAGGTCAACTACAACCTGCCCGGAGTGAGCGA  301
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Sbjct  514  GAACATCGCGCTAGCCATCTCCGCTCAGCAGGTCAACTACAACCTGCCCGGAGTGAGCGA  573

Query  302  GCACCTCAAGCTGAACGGAAAAGTCCTGGCGGCCATGTACCAGGGCACCATCAAAACCTG  361
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Sbjct  574  GCACCTCAAGCTGAACGGAAAAGTCCTGGCGGCCATGTACCAGGGCACCATCAAAACCTG  633

Query  362  GGACGACCCGCAGATCGCTGCGCTCAACCCCGGCGTGAACCTGCCCGGCACCGCGGTAGT  421
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Query  422  TCCGCTGCACCGCTCCGACGGGTCCGGTGACACCTTCTTGTTCACCCAGTACCTGTCCAA  481
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Sbjct  694  TCCGCTGCACCGCTCCGACGGGTCCGGTGACACCTTCTTGTTCACCCAGTACCTGTCCAA  753

Query  482  GCAAGATCCCGAGGGCTGGGGCAAGTCGCCCGGCTTCGGCACCACCGTCGACTTCCCGGC  541
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Sbjct  754  GCAAGATCCCGAGGGCTGGGGCAAGTCGCCCGGCTTCGGCACCACCGTCGACTTCCCGGC  813

Query  542  GGTGCCGGGTGCGCTGGGTGAGAACGGCAACGGCGGCNTGGTGACCGGTTGC  593
            |||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  814  GGTGCCGGGTGCGCTGGGTGAGAACGGCAACGGCGGCATGGTGACCGGTTGC  865
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Reverse sequence:

```
>gb|M30046.1|MSGPABA M.tuberculosis protein antigen b (Pab) gene, complete
cds
Length=1993

 Score = 1077 bits (583),  Expect = 0.0
 Identities = 591/597 (98%), Gaps = 1/597 (0%)
 Strand=Plus/Minus

Query  7     GGTC-ACGAGGCTAGCTGGAAATCGTCGCGATCAACGCGTCANACAACTTCACCACCGCG  65
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Query  66    GGCGGCAGCGGCTGGAAATGAACCTGGTCGAGGAACGAGGCCTTGTTGCCGTCGGTGATC  125
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Sbjct  1227  GGCGGCAGCGGCTGGAAATGAACCTGGTCGAGGAACGAGGCCTTGTTGCCGTCGGTGATC  1168

Query  126   GCCCAGTGCANAAATGCCTGCAAGGTCTGCGCGGTGGCGGCGTCCTTTTGCCGGTTGTTG  185
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Query  186   ACGATGGCGTACTCGTAGTTGATGATCGGGTAGCCGTCCGGGGGCGGGCCCGTCGATCATC  245
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Sbjct  1107  ACGATGGCGTACTCGTAGTTGATGATCGGGTAGCCGTCCGGGGCGGGCCCGTCGATCATC  1048

Query  246   GAAATCGCCTGGTTCGCCGGGGTTTTCGATGCGAAGCCAGCCGCCGCGGCCTGAATGCTT  305
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1047  GAAATCGCCTGGTTCGCCGGGGTTTTCGATGCGAAGCCAGCCGCCGCGGCCTGAATGCTT  988

Query  306   TGCGCGTCGGGCAACAAGAAATTGCCAGAGCTATTGCCTAGTTGGGCCTCGCCGAGTCCC  365
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  987   TGCGCGTCGGGCAACAAGAAATTGCCAGAGCTATTGCCTAGTTGGGCCTCGCCGAGTCCC  928

Query  366   CGTTGACTGGCCTGNTCNAGGAAGCTGATGCCGATATAGGCCACGCAGCCCGGTGTCTCG  425
             |||||||||||||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  927   CGTTGACTGGCCTGGTCGAGGAAGCTGATGCCGATATAGGCCACGCAGCCCGGTGTCTCG  868

Query  426   GCGCAACCGGTCACCATGCCGCCGTTGCCGTTCTCACCCAGCGCACCCGGCACCGCCGGG  485
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  867   GCGCAACCGGTCACCATGCCGCCGTTGCCGTTCTCACCCAGCGCACCCGGCACCGCCGGG  808

Query  486   AAGTCGACGGTGGTGCCGAAGCCGGGCGACTTGCCCCAGCCCTCGGGATCTTGCTTGGAC  545
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Sbjct  807   AAGTCGACGGTGGTGCCGAAGCCGGGCGACTTGCCCCAGCCCTCGGGATCTTGCTTGGAC  748

Query  546   AGGTACTGGGTGAACAAGAAGGTGTCACCGGACCCGTCGGANCGGTGCAGCGGAACT  602
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  747   AGGTACTGGGTGAACAAGAAGGTGTCACCGGACCCGTCGGAGCGGTGCAGCGGAACT  691
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Figure 4. Alignment of amplified pab gene of M. tuberculosis from Malang (query) and pab gene from M. tuberculosis H37Rv (sbjct) using two primers.

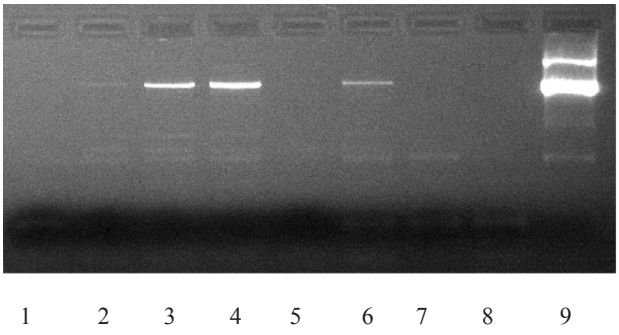

Figure 5. PCR-results of the amplification of pab gene on several clones of E.coliDH5a/pMBhis

Lanes: 1= Negative control, 2-8= Colonies 1-7, 9= tb1. Lane 3,4 and ,6 were positive showing the presence of fragment tb1.

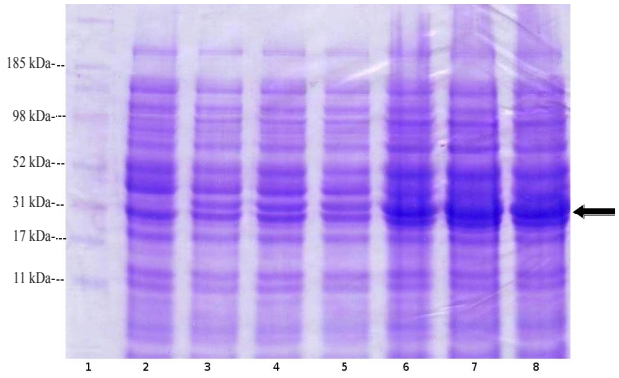

Figure 6. SDS-PAGE of extracts from cells that expressed the Ag38-His fusion protein

Lanes: 1= molecular mass standard, 2= cell extract of E.coliDH5a/pMBhis before induction, 3, 6= 1and 2 hours after induction with 200mM IPTG, 4, 7= 1 and 2hours after induction with 400mM IPTG, 5, 8= 1and 2hours after induction with 600mM IPTG.

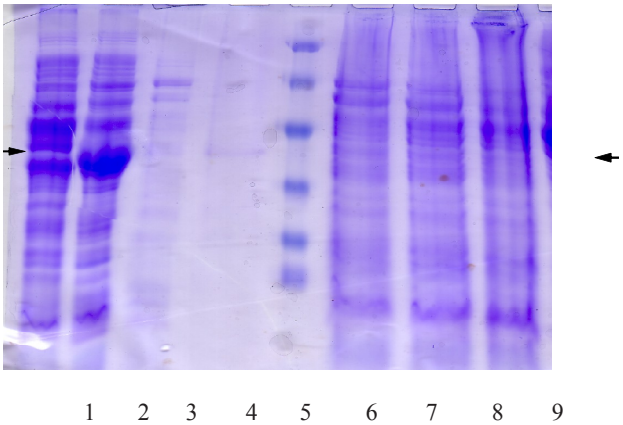

Figure 7. SDS-PAGE of extract of cells producing the Ag38M fusion protein

Lanes: 1= cell extract of E.coliDH5α/pMBhis without IPTG induction, 2= after IPTG induction, 3= the 8,000 x g supernatant, 4= the 8,000 x g precipitate, 5= Molecular mass standard (from top to bottom): galactosidase(118 kD), bovine serum albumin(90 kD), ovalbumin (50 kD), carbonic anhydrase (34 kD), b-lactoglobulin (26 kD), lysozyme (19 kD), 6= the 30,000 x g supernatant (S30), 7= the 30,000 x g precipitate (P30), 8= the 100,000 x g supernatant (S100), 9= the 100,000 x g precipitate (P100)

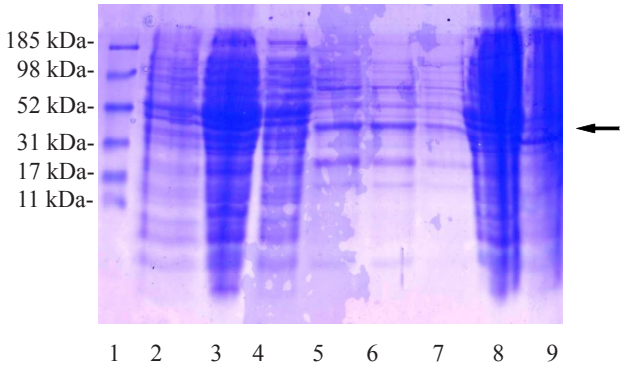

Figure 8. Purification of protein rec-Ag38M

S30 was mixed with matrix Ni-NTA, shaked gently for 1 hour and apllied onto filter.

Lanes : 1= Protein marker, 2= Flow through (FL), 3= Wash I (W1), 4= wash II (W2),5= Elution I (E1), 6= Elution II (EII), 7= Elution III (EIII), 8=S30, 9= P30.

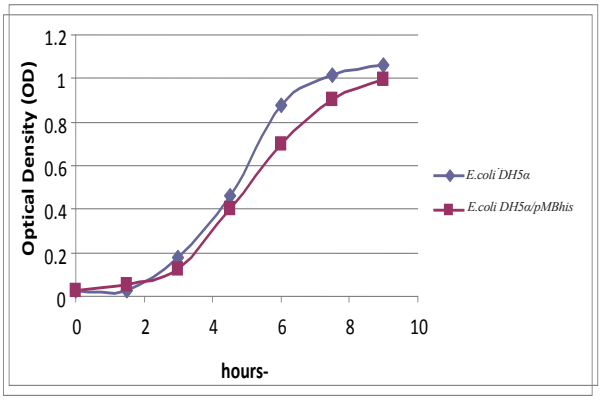

Figure 9. Growth curve of wild type E.coli DH5α and E.coli DH5α/pMBhis

For the expression of *tb*1, plasmid pMBhis was used and *E.coli* DH5α served as the host. Transformation of *E.coli*/pMBhis resulted in several white clones only. Seven white colonies were picked and confirmation with PCR showed that four colonies carried the *tb*1 gene (Figure 5).

Expression of *pab* gene of *M. tuberculosis* in *E.coli*

In this study *pab* gene could be successfully expressed in *E.coli* DH5α. For this purpose *E.coli* DH5α/pMBhis was grown on LB liquid medium suplemented with Ampicillin (100 mg/mL). Expression was conducted by inducing the transcription process with addition of various concentration of IPTG (isopropyl-D-thiogalactopyranose). We used several concentration of IPTG (100 mM – 600 mM), and the cells were harvested 3 hours after induction with IPTG. The results of SDS-PAGE of the various expression with and without induction can be seen in Figure 6. Furthermore, SDS-PAGE of the supernatants and pellets after various centrifugation can be seen in Figure 7. The expression of *pab* was confirmed using western Blot against recAg38 from *M tuberculosis* H37Rv (data not shown).

Purification of recAg38M

Loss of protein during purification was minimized by inclusion of a histidine tag at N-terminus, allowing purification using a Ni-matrix to capture histidine tag. The band of the protein recAg38M did not appear in the flowthrough (Figure 8 lane 2) as well as during wash steps (Figure 8 lanes 3 and 4), in contrast, the bands that coressponded to recAg38M were found during elution steps (Figure 8 lanes 5-7). However, there were still many other protein that were copurified so further washing may be warranted.

Growth of *E.coli* DH5α/pMB38

It was found that *E.coli* DH5α/pMB38 exhibited growth with slightly longer generation time (tD> 2 hours) compared to the host (tD= 1.5 hours) (Figure 9).

DISCUSSION

The present study aimed to express *pab* gene from a local strain of *M. tuberculosis* in heterologous system (*E.coli*) to achieve high antigen yields at potentially low cost. The construct was designed by omitting the putative signal peptide, so that the recombinant protein remained in the cytoplasm. Alignment of the amplified PCR product of *pab* gene isolated from malang showed 98% homology with sequence of *pab* gene from *M. tuberculosis*H37Rv. Hence, it was expected that the recombinant protein recAg38M from Malang could have a similar immunological properties to recAg38 synthesized from *M. tuberculosis*H37Rv when they are in the future used as serodiagnostic agent. However, we did not conduct immunological experiment yet.[5]

The *pab* gene from Malang was successfully expressed in *E.coli* DH5α, as the band did not present without IPTG induction. Therefore, the band that appeared after induction with IPTG must be the recAg38M protein. These was also confirmed using anti Ag38 (data was not shown). Nevertheless the amount of protein recAg38M was less than protein recAg38 originated from *M. tuberculosis* H37Rv, where Ag38 may account for 10% of the total protein.

The recombinant protein was dominated by the insoluble form, and only 25% of the protein was found in a soluble form. The failure to obtain recAg38M in a soluble form was the consequence of the fact that the expression of *pab* always lead to the formation of inclusion bodies that contain the insoluble form of the recombinant protein.[2,5] It might be worthwhile to try coexpression of *pab* with fusion partners that are known to aid in increasing the solubility such as maltose-binding protein MalE. [10]

During the course of the expression experiment, the plasmid containing host showed a different behavior. The low rate of transformation of pMBhis into *E.coli* DH5α suggested that a large amount of the recAg38M within the cell somehow disturb the homeostasis of host cell, so that the growth of *E.coli* DH5α/pMBhis in the liquid medium was slightly slower than *E.coli* DH5α alone with a generation time of 2 hours compared to 1 hour (figure 9). Previous study has noted that the expression of recombinant protein retarded the growth of the host, such as in the case of expression of *cymH* gene of *Klebsiella oxytoca* in *E.coli* DH5α. [11]

During the course of expression experiment, the pMBhis containing host has shown a different behaviour from the wild type. Comparison of growth curve of wild type *E.coli* DH5α and *E.coli* DH5α/pMBhis can be seen in Figure 9.

Subcellular localization showed that recAg38M was partly located in the cytoplasm as the band corresponding to 37 kDa that was found in supernatant (S100) after centrifugation at 100.000xg (figure 7 lane 8) was faint, whereas most of protein was found in the precipitate (P100) (figure 7 lane 9). The question whether recAg38 is only temporarily attached to periplasmic membrane or becomes a trans-membrane protein is still open. Attempts to release the protein from the membrane using Triton X-100 failed (data not shown). This finding was in contrasts with the recAg38 from *M. tuberculosis* H37Rv, which was found 90% in the cytoplasm. [2]

One crusial problem in producing recombinant protein is during purification process. To obtain pure protein, several purification steps are needed, so that at the end of the process the yield is low, and the proteins loss part of their immunogenicity. Hence there is a need to conduct a short and efficient purification process so that most of the protein could be purified with a high immunogenicity. This might be mediated through protein fusion with Histidin tag at the N-terminus that was purified through Ni-NTA matrix. During the course of purification, the Ni-NTA matrix was used to capture the Histidine tag to increase the protein resolution accordingly. The purification of recAg38M using Ni-NTA affinity batch system showed that there were still contaminations of several proteins. Therefore, it is sugessted to repeat the purification procedure with optimizations such as changing the buffer that could increase the purity of recombinant protein.

In conclusion, *pab* gene of *M. tuberculosis* isolated from TB patient could be expressed in heterologous system in *E.coli* DH5α. Although the *pab* gene in our study genetically showed high homology to *pab* gene from *M. tuberculosis* H37Rv, further study was required to purify the protein, and to identify its biological function.

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REFERENCES

1. Chang Z, Choudary A, Lathigra R, Quiocho FA. The immunodominant 38-kDa lipoprotein antigen of *Mycobacterium tuberculosis* is a Phosphate-binding protein. J Biol Chem. 1994;269;1956-68.
2. Wilkilson RJ, Haslov K, Rappuoli R, Giovannoni F, Narayanan PR, Desai CR, et al. Evaluation of the recombinant 38-kilodalton antigen of *Mycobacterium tuberculosis* as a potential immunodiagnostic reagent. J Clin Microbiol. 1997;35:553-7.
3. Cheng VCC, Yew WW, Yuen KY. Molecular diagnostics in tuberculosis. Eur J Clin Microbiol Infect Dis. 2005; 24:711-20.
4. Van der Zanden AGM. Spoligotyping, a tool in epidemiology, diagnosis and control of tuberculosis. Wageningen: Ponsen & Looijen BV; 2002.
5. Singh M, Andersen AB, McCarthy JEG, Rohde M, Schutte H, Sanders E, et al. The *Mycobacterium tuberculosis* 38-kD antigen: overproduction in *Escherichia coli*, purification and characterization. Gene. 1992;117:53-60.
6. Sambrook J, Fritsch E F,Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbour; 2001.
7. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidmann JG, Sminth JA, et al. Current protocol in molecular biology. New York : Wiley and Sons; 1997.
8. Young DB, Garbe TR. Heat shock protein and antigen of *Mycobacterium tuberculosis*. J Infec Immun. 2001;59: 3086-93.
9. Yasukawa C, Kanei-Ishii T, Maekawa J, Fujimoto T, Yamamoto, Ishii S. Increase of solubility of foreign protein in *E. coli* by overproduction of bacterial thioredoxin. J Biol Chem. 1995;270:25328-31.
10. Gubernator B, Seidler A, Rögner M, Szczepaniak A. Overexpression and reconstitution of a Rieske iron-sulfur protein from the higher plant. Protein Expr Purif. 2003;29:8-14.
11. Mardining Raras TY. Study on the cyclodextrine metabolism in *Klebsiella oxytoca* M5a1. A molecular Approach. Saarbrucken: Dr Mueller Publisher; 2010.