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Cloning and expression of MPT83 gene from *Mycobacterium tuberculosis* in *E. coli* BL21 as vaccine candidate of tuberculosis: A preliminary studyAhyar Ahmad^{a,b,*}, Rosana Agus^c, Muh. Nasrum Massi^d, Rosdiana Natzir^e, Radha Madhyastha^f, Harish Kumar Madhyastha^g, Masugi Maruyama^h^a Chemistry Department, Mathematics and Natural Science Faculty, Hasanuddin University, Perintis Kemerdekaan Street km. 10 Tamalaea, Makassar 90245, Indonesia^b Laboratory of Research Centre and Developing of Sciences, Faculty of Mathematic and Natural Sciences, Hasanuddin University, J. Perintis Kemerdekaan KM 10, Makassar 90245, Indonesia^c Biology Department, Mathematics and Natural Science Faculty, Hasanuddin University, Perintis Kemerdekaan Street km. 10 Tamalaea, Makassar 90245, Indonesia^d Microbiology Department, Faculty of Medicine, Hasanuddin University, Perintis Kemerdekaan Street km. 10 Tamalaea, Makassar 90245, Indonesia^e Biochemistry Department, Faculty of Medicine, Hasanuddin University, Perintis Kemerdekaan Street km. 10 Tamalaea, Makassar 90245, Indonesia^f Dept. of Applied Physiology, Faculty of Medicine, University of Miyazaki, Miyazaki 885-1692, Japan

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ABSTRACT

The appearance of *Mycobacterium tuberculosis* strains leading to drug resistance has caused new problems in TB treatment in various parts of the world and forces WHO to declare TB as a global emergency. With the increase of TB drug resistance, it is convinced that a more effective vaccine development will stop the epidemic of TB. Some *M. tuberculosis* antigens, one of which is MPT83, have been examined as TB vaccine candidate. MPT83 antigen, which is very immunogenic in lipoprotein micro bacteria, is identified as surface cell interrelated to antigen with cytometry circulation. Having TB resistance from BCG vaccine, MPT83 is considered TB vaccine candidate that can protect people against TB at adult age. The purpose of this research is to conduct amplification of MPT83 antigen cloning, and expression of its antigen on *E. coli* bacteria. From the result of the research, it is expected that raw material to produce TB vaccine as well as a high-quality antigen can be obtained. The band of DNA is PCR product is 660 bp, while the one in pGEMT-Easy-Mpt83 recombinant plasmid is 3678 bp. This is expressed in *E. coli* BL21 strain and produces 48 kDa protein as well as GST-MPT83 fusion protein.

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1. Introduction

Together with malaria and AIDS, tuberculosis (TB) is classified as the big three of main contagious diseases in the world. It causes 2 million deaths per year with 9 new cases per year. Indonesia reaches the third rank in the highest number of TB cases in the world, following India and China [1]. In Indonesia, 70% TB occur

in productive age, leading to big social and economy problems. Every year about 8 million infected people progress into an active disease, of which 2 million die. The reason why there are high numbers of TB cases in Indonesia is that a lot of Indonesians live in the optimum place of the development and spread of TB. Moreover, the increase of HIV cases and *M. tuberculosis* (MDR) multidrug resistance worsens the situation. Therefore, it is necessary to find out an effective and affordable vaccine as well as its quick and appropriate diagnosis method of development. The treatment of TB is getting difficult following the appearance of *M. tuberculosis* that is resistant to TB drugs (*M. tuberculosis* multi-drug resistant, MDR-TB). This strain is resistant to two main antibiotics commonly used, i.e. isoniazid and rifampicin [2]. Even more, it is found out that *M. tuberculosis* is resistant to other TB drugs (*M. tuberculosis* Extensively Drug Resistant, XDR-TB). Therefore, this strain is resistant to fluoroquinolone and injections such as kanamycin, capreomycin or amikacin [1,2]. The appearance of the above strains

Abbreviations: TB, tuberculosis; MDR-TB, *M. tuberculosis* multi-drug resistant; XDR-TB, *M. tuberculosis* extensively drug resistant; BCG, Bacillus Calmette-Guérin; IPTG, isopropyl β-D-1-thiogalactopyranoside; X-CaCl₂, 5-Bromo-6-Chloro-3-Indolyl-D-Galactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; G-A, glutathion-agarose; GST, glutathione S-transferase.

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causes new problems in treating TB in various parts of the world and forces WHO to declare TB as a global emergency. With the increase of TB drug resistance, it is convinced that a more effective vaccine development will stop or decrease the epidemic of TB. Some criteria that should be fulfilled by TB vaccine are the abilities to provide better resistance than BCG, to give a quick response toward TB protection, to decrease the pain, and to prevent TB reactivation [3].

Currently, *Bacillus Calmette-Guérin* (BCG) is the only vaccine available for TB prophylaxis. The advantage of this vaccine is that it is quite safe and contains efficacy in order to prevent severe TB in children [4]. In some clinical experiments, BCG efficacy to prevent active TB in adults is found very low, and the lowest protection is found in countries with high TB cases, like China, India and Indonesia. When given to someone with immunologic system defiance, lack of BCG can lead to infection [5]. As a consequence, a more effective vaccine development is needed so that it can provide protection towards active TB on adults. Previously study revealed that the antigen MPT64 can be isolated from nontoxic MTB, H37Ra and a large quantity of MPT64 was obtained when the expression in *E. coli* BL21 and purified using a Ni²⁺ affinity column, resulting the pure MPT64 with the molecular weight was 23,497 Da [6]. A recent study showed the Rv1096 gene from the *M. tuberculosis* H37Rv strain could be expressed in both *Escherichia coli* and *M. smegmatis* is a soluble protein and this protein showed peptidoglycan deacetylase activity [7]. A very recent study also report the HspX/ExcS and MPT83 gene from the *M. tuberculosis* H37Rv strain can be cloning and expression in *E. coli* BL21 as a soluble fusion protein [8,9].

In connection to this, research on the production of MPT83 antigen is conducted using DNA recombinant technology. At first, the production of antigen is carried out by cloning the gene encoding MPT83 recombinant antigen. This gene expression is conducted *in vivo* in bacteria, followed by quality and quantity tests on the antigen gained. Purification has to be done in the last phase to ensure that MPT83 antigen can be used as vaccine candidate. The production of the antigen can, therefore, reduce the production cost of TB vaccine, provide protection towards active TB on adults, and finally, reduce the rate of morbidity and mortality caused by TB. In the present study, an antigen MPT83 from the *M. tuberculosis* local strain in Makassar Indonesia was cloning and expressed in *E. coli* BL21 as a soluble fusion protein might be an vaccine candidate of tuberculosis in future clinical trial studies, especially in the developing countries.

2. Materials and methods

2.1. Materials

Blood sample as source local strain of *M. tuberculosis* was collected from the pulmonary tuberculosis patient from the Wahidin Sudirohusodo Hospital, Makassar, Indonesia. Signed written informed consent was obtained according to Ethics Committee from Hasanuddin University Hospital, Makassar, Indonesia. pGEX-2TK vector and *E. coli* BL21 component cells were purchased from Amersham Pharmacia Biotech, pGEM-T Easy vector and *E. coli* JM109 cells was purchased from Promega, USA. Glutathione-agarose beads was purchased from GE Healthcare Hong Kong. Ampicillin, IPTG, X-gal, phenylbenzothioyl fluoride, dithiothreitol, Sarkosyl, and lysozyme were purchased from Sigma.

2.2. Genomic DNA isolation and PCR

Genomic DNA was isolated from *M. tuberculosis* using the phenol, chloroform, and isoamyl alcohol (25:24:1) extraction method.

In order to amplify the specific gene of our interest antigen MPT83 product, PCR was performed using the following conditions: complete denaturation: 94 °C for 3 min; Annealing: 55 °C for 30 s; Extension: 72 °C for 1 min, followed by 25 cycles of amplification and the final elongation step (72 °C for 7 min) using Forward Primer: (5' CGAAGCTAGCATGATCAACGTTTCAGGCCAA 3') and Reverse Primer: (5' GCGCAAGCTTTTACTGTGCCGGGGGCATCA 3'). PCR products were separated and analyzed on 1% agarose gel electrophoresis essentially as previously described earlier [10,11].

2.3. Cloning of MPT83 gene

The PCR product of antigen MPT83 gene was eluted from the gel and cloned into pGEM-T Easy vector (Promega, USA) as per manufacturer's instructions to yield recombinant plasmid pGEM-T Easy-Mpt83. The ligated mix was then transformed into competent *E. coli* JM109 cells, by CaCl₂ transfection method [10]. The transformants were plated on Luria broth (LB) agar supplemented with Ampicillin (100 µg/mL), in addition with isopropyl β-D-1-thiogalactopyranoside, IPTG (40 µg/mL), and X-gal (20 µg/mL). Cells were incubated at 37 °C overnight. Blue-White screening colony selection method was performed to choose the white colored recombinant clone and followed by colony PCR amplification for confirmation of cloning of our gene of interest (MPT83 gene).

2.4. Plasmid isolation

The plasmid was isolated from positive clones by alkali-lysis method described by Sambrook et al. [10]. Briefly, 2 mL of overnight culture was centrifuged. The cell pellet was resuspended in 200 µL ice cold lysis solution 1 (15% glucose, 25 mM Tris, 10 mM EDTA) and followed by vortexing gently. Then 400 µL of freshly prepared solution 2 (0.2 N NaOH, 1% SDS) and 50 µL solution 3 (3 M Sodium acetate) were added and centrifuged at 10,000 rpm for 10 min. Equal volume of iso-propanol was added to the supernatant and incubated at room temperature for 15 min. Following centrifugation at 10,000 rpm for 10 min, the pellet was washed with 70% ethanol, dried, and dissolved in 50 µL TE buffer for further use.

2.5. Confirmation of recombinant clone by plasmid isolation and restriction digestion

The recombinant white colonies were isolated from the LB-Ampicillin agar plates and inoculated in LB Ampicillin containing broth and incubated at 37 °C overnight. Plasmid DNA was isolated by Alkaline-lysis method. The purified plasmid was subjected to restriction digestion using *NheI* and *HindIII* restriction enzymes. The release of the gene product was checked on 1% agarose gel.

2.6. Expression and purification of GST-MPT83 fusion proteins

The MPT83 gene of pGEM-T Easy-Mpt83 recombinant plasmid was then sub cloned into pGEX-2TK expression vector, resulting in pGEX-2TK-Mpt83 plasmid. *E. coli* BL-21 cells were transformed with pGEX-2TK-Mpt83, harboring the GST-Mpt83 cDNAs and grown to A₆₀₀ nm = 0.2 in 400 mL of LB medium supplemented with 200 µg/mL ampicillin. Upon induction with 50 µM IPTG overnight at 20 °C, the cells were collected by centrifugation and suspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 0.1% (v/v) phenylbenzothioyl fluoride and 1 µg/mL lysozyme. Following a 15-min incubation on ice, dithiothreitol and Sarkosyl were added to 5 mM and 1.5% final volumes, respectively. The sample was sonicated for 2 min on ice in a water bath sonicator, centrifuged, and Triton-X 100 (2% final

volume) was added to the supernatant. Continuously, the GST fusion proteins (GST-MPT83) were purified with glutathione-agarose beads essentially as described earlier [11; GE Healthcare Life Sciences].

2.7. Data analysis

The data obtained from the reading of the two methods were used for a comparison study. Data analysis from SDS-PAGE and DNA sequencing were later analyzed using v.7.0.9 Bioedit application. The result of data analysis is presented in tables or figures, as well as described.

3. Results

3.1. Genomic DNA isolation and quantification

The blood sample was incubated in Lowenstein-Jensen (LJ) medium for 2–3 weeks. Small colonies developed in the plate. Based on the colony morphology six bacteria were transferred into LJ broth and used for genomic DNA isolation. *M. tuberculosis* was cultured in the LJ broth media and genomic DNA was isolated by modified CTAB method [10]. The isolated DNA was electrophorized in 1% agarose gel (Fig. 1). The quantity and quality of DNA was analyzed by UV visible Spectrophotometer (Shimadzu, Japan). The A260/280 value of genomic DNA samples showed the purity of

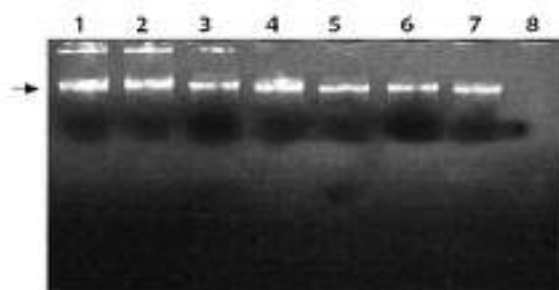


Fig. 1. Genomic DNA isolated from H37Rv strain as positive control (1), clinical samples from local strains (2–7) of *M. tuberculosis*, and negative control (8).

the isolated DNA, where the value from 1.8 to 2.0 was considered as high purity.

3.2. Optimization of PCR condition and cloning of Mpt83 gene

The initial stage of this research, i.e. optimizing PCR products, was to identify the range of the optimum temperature of PCR machine in order to get a high density DNA amplification result. The optimum condition gained was on primary concentration of 40 μ mol and 58.1 °C on annealing phase, and the result depicted in Fig. 2.

After purification, the formed DNA band was ligated into pGEM-T Easy plasmid vector in order to produce pGEM-T Easy-MPT83 recombinant vector. The ligation between plasmid and PCR product was very carefully carried out in an uncontaminated and sterile place, so that the fusion between plasmid vector and the product purified using T4 DNA Ligase enzyme was reached with a low contamination. This was then incubated overnight at 4 °C and was ready to be transformed into *E. coli* JM109 competent cell. Finally, a growth pattern of recombinant bacteria colony was obtained, as seen in Fig. 3.

Seven white colonies of the recombinant plasmid isolation from the bacteria growing in a LB media plate in Fig. 2 was picked up and cultured in a liquid Luria Broth (LB) media (Fig. 4). After using mini prep purification, the isolation resultant plasmid isolation encoding MPT83 antigen from *M. tuberculosis* was successfully done towards six colonies. Meanwhile, the third colony did not grow (Line 1 and 3 in Fig. 4). The result of recombinant DNA of the six colonies was tested using agarose gel on electrophoresis and result are shown in Fig. 5.

pGEM-T Easy Mpt83 recombinant plasmid was successfully reconstructed. This was shown by the existence of 3678 bp band which was the result of insertion of MPT83 gene into pGEM-T Easy gene. Next, pGEM-T Easy-Mpt83 was sequenced and the result is shown in Fig. 6. As shown in Fig. 6, the open reading frame for the cDNA encoded for a native protein MPT83 of 219 amino acid residues with the calculated by Bioedit 7.9 software was molecular weight of 21,992 Da (result not shown).

MPT83 gene of pGEM-T Easy-Mpt83 recombinant plasmid was then sub cloned into pGEX-2TK expression vector, resulting in pGEX-2TK-Mpt83 plasmid. The above expression vector was expressed into *E. coli* BL-21 bacteria as GST-MPT83 fusion protein and purified using glutathione agarose matrix (GE Healthcare Life

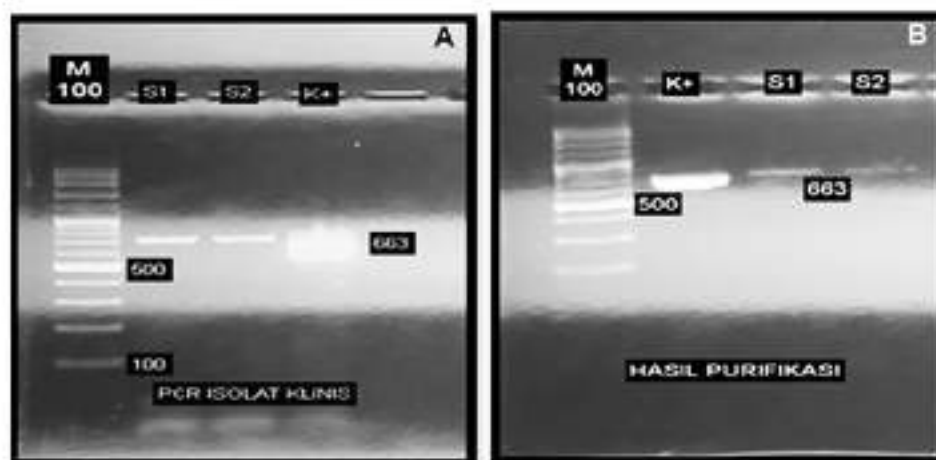


Fig. 2. A. PCR result of positive control isolate (H37Rv) and its clinical samples from local strains (S1 and S2). B. Purification result of DNA positive control and its clinical samples (S1 and S2) from panel A.



Fig. 3. *E. coli* growth pattern in LB-Amp plus IPTG and X-Gal Plate containing pGEM-T Easy-Mpt83 recombinant plasmid.

Sciences). As shown in Fig. 7, the result analysis of SDS-PAGE was GST protein band of 26 kDa, while the GST-MPT83 fusion protein molecule was 48 kDa. As a result, the size of MPT83 alone was 22 kDa.

As shown in Fig. 7, Column 1, protein marker; column 2, total protein whole extract from *E. coli* containing pGEX-2TK Mpt83 cDNA with IPTG induction; column 3, total protein rough extract from *E. coli* containing pGEX-2TK Mpt83 cDNA without IPTG induction; column 4, GST recombinant protein complex prior to elution from glutathion-agarose (G-A) matrix; column 5, GST recombinant protein complex prior to elution from G-A matrix; column 6, GST recombinant protein complex subsequent to elution from G-A matrix; column 7, GST recombinant protein complex subsequent to elution from G-A matrix. Based on SDS-PAGE (12%) in Fig. 7, the molecular mass of the GST-MPT83 fusion protein product was ~48 kDa were dramatically accumulated in *E. coli* BL 21 cell containing the pGEX-2TK Mpt83 recombinant plasmid with induction by 50 μ M IPTG and could be purified by single step process to more than 97% homogeneity, using glutathione agarose beads (see lane 7 in Fig. 7).



Fig. 4. Bacteria recombinant cultures in several liquid LB medium supplemented with 200 μ g/mL ampicillin.

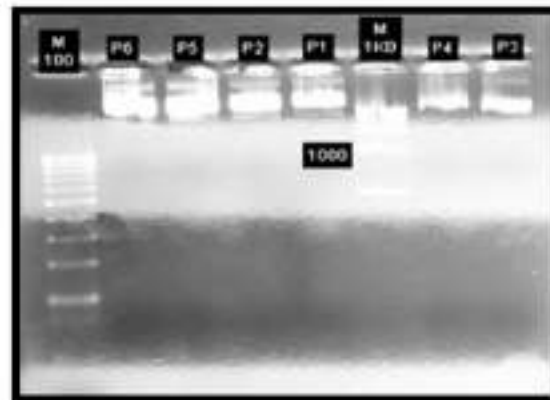


Fig. 5. Electrophoresis result of recombinant plasmid pGEM-T Easy-Mpt83 (P1-P6).

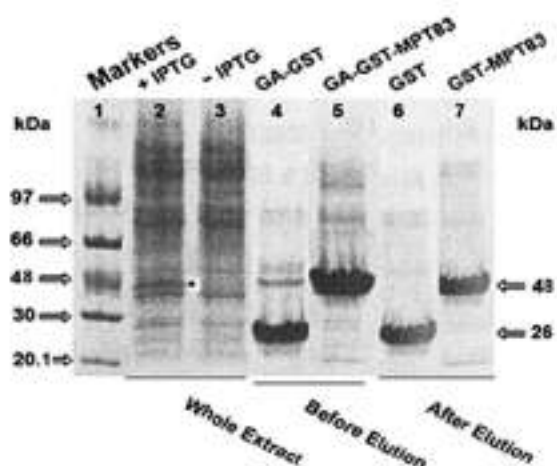
4. Discussion

The optimization phase of PCR product was carried out to get a high density DNA amplification result. By doing PCR gradient on product result of Positive Control (H37Rv) and considering product concentration, the result gained on 40 μ mol and temperature of 58.1 $^{\circ}$ C at annealing phase showed a thick band. Next was to amplify the DNA on positive sample (H37Rv) and clinical sample by using PCR and to analyze them using electrophoresis method. After electrophoresis, positive control (H37Rv) showed higher intensity clinical sample (S1 and S2) with 663 bp. This shows the higher the cloning phase possibility.

During the purification phase, positive control (H37Rv) showed higher intensity than the clinical samples (S1) and (S2). Final step we ligation between pGEM-T Easy plasmid/vector and purified DNA product. This was then incubated overnight at 4 $^{\circ}$ C and was ready to be transformed into *E. coli* JM109 competent cell. The transformation on the competent cell was done by creating lysis on the cell wall of *E. coli* JM109. By doing so, the result of recombinant plasmid ligation could be inserted into the bacteria cell without killing it. Next, the recombinant bacteria was bred on a selective breeding media, i.e. solid LB medium, in order to multiply the recombinant bacteria as well as to learn whether the recombinant bacteria had been inserted by pGEM-T-Easy-Mpt83 recombinant plasmid or not. For this reason, IPTG and X-Gal were added to the agar LB media so as to obtain blue and white colonies. The bacteria colony inserted by recombinant plasmid would showed white color, while the one without recombinant plasmid (self ligation

1	ATG ATC AAC GTT CAG GCC AAA CCG GCC GCA GCA GCG AGC CTC GCA	45
1	Met Ile Asn Val Gln Ala Lys Pro Ala Ala Ala Ala Ser Leu Ala	15
46	GCC ATC GTG ATT GCG TTC ITA GCG GGT TGT TCG AGC TCC AAA CCC	90
14	Ala Ile Val Ile Ala Phe Leu Ala Gly Cys Ser Ser Ser Lys Pro	30
91	GTG TCG CAA GAC ACC ASA CCG AAA CCG GCG ACC AGC CCG GCG GCG	135
21	Val Ser Gln Asp Thr Arg Pro Lys Pro Ala Thr Ser Pro Ala Ala	45
136	CCC GTT ACC ACG GCG GCA ATG GCT GAC CCC GCA GCG GAC CTG ATT	180
46	Pro Val Thr Thr Ala Ala Met Ala Asp Pro Ala Ala Asp Leu Ile	60
181	GGT CGT GGG TGC GCG CAA TAC GCG GCG CAA AAT CCC ACC GGT CCC	225
61	Gly Arg Gly Cys Ala Gln Tyr Ala Ala Gln Asn Pro Thr Gly Pro	75
226	GGA TCG GTG GCC GGA ATG GCG CAA GAC CCG GTC GCT ACC GCG GCT	270
76	Gly Ser Val Ala Gly Met Ala Gln Asp Pro Val Ala Thr Ala Ala	90
271	TCC AAC AAC CCG ATG CTC AGT ACC CTG ACC TCG GCT CTG TCG GGC	315
91	Ser Asn Asn Pro Met Leu Ser Thr Leu Thr Ser Ala Leu Ser Gly	105
316	AAG CTG AAC CCG GAT GTG AAT CTG GTC GAC ACC CTC AAC GGC GGC	360
106	Lys Leu Asn Pro Asp Val Asn Leu Val Asp Thr Leu Asn Gly Gly	120
361	GAG TAC ACC GTT TTC GCC CCC ACC AAC GCC GCA TTC GAC AAG CTG	405
121	Glu Tyr Thr Val Phe Ala Pro Thr Asn Ala Ala Phe Asp Lys Leu	135
406	CCG GCG GCC ACT ATC GAT CAA CTC AAG ACT GAC GCC AAG CTG CTC	450
126	Pro Ala Ala Thr Ile Asp Gln Leu Lys Thr Asp Ala Lys Leu Leu	150
451	AGC AGC ATC CTG ACC TAC CAC GTG ATA GCC GGC CAG GCG AGT CCG	495
151	Ser Ser Ile Leu Thr Tyr His Val Ile Ala Gly Gln Ala Ser Pro	165
496	AGC AGG ATC GAC GGC ACC CAT CAG ACC CTG CAA GGT GCC GAC CTG	540
166	Ser Arg Ile Asp Gly Thr His Gln Thr Leu Gln Gly Ala Asp Leu	180
541	ACG GTG ATA GCC GCC CGC GAC GAC CTC ATG GTC AAC AAC GCC GGT	585
161	Thr Val Ile Gly Ala Arg Asp Asp Leu Met Val Asn Asn Ala Gly	195
586	TTG GTA TGT GCC GGA GTT CAC ACC GCC AAC GCG ACG GTG TAC ATT	630
196	Leu Val Cys Gly Gly Val His Thr Ala Asn Ala Thr Val Tyr Ile	210
631	ATT GAT ACG GTG CTG ATG CCC CCG CAG TAA	660
211	Ile Asp Thr Val Leu Met Pro Pro Gln End	219

Fig. 6. Predicted nucleotide and amino acid sequences of MPT83 gen.

Fig. 7. Analysis pattern of SDS-PAGE (12%) from expression of MPT83 recombinant protein in *E. coli* strain BL21.

plasmid) would showed blue color. Next, the recombinant plasmid is isolated from white colony.

The purpose of isolating recombinant plasmid using Miniprep Kit Purification was to obtain a pure recombinant plasmid, so that the DNA needed was really pure. By adding IPTG and X-Gal, pGEM-T Easy-Mpt83 recombinant plasmid was found in five white colonies chosen from the breeding of recombinant bacteria. The five white colonies were bred in a liquid LB media. After isolation using Miniprep Kit Purification, a product of plasmid isolation encoding MPT83 antigen from *M. tuberculosis* was obtained, and then furthered tested using agarose gel on electrophoresis.

The results gained, in PCR cloning and subsequent PCR, showed that the length of the band is 3678 bp. With this length, cloning of pGEM-T Easy-Mpt83 recombinant plasmid gene was considered successfully carried out. Also, having 3015 bp pGEM-T plasmid size and 663 MPT83 antigen size, it was concluded that pGEM-T Easy plasmid and MPT83 antigen were already fused. Therefore, the product of recombinant plasmid was ready for sequencing. The sequencing process of pGEM-T Easy-Mpt83 plasmid was carried out using Applied ABI PRISM 310 Biosystem, while data analysis was done using Bioedit v.7.0.9. The nucleotide order data gained were compiled and translated into ordered amino acid, i.e. 219

amino acid, starting with 660 bp including ATG start codon and TAA stop codon, and 220 amino acid for *M. tuberculosis* H37Rv wild type. After pGEM-T Easy-Mpt83 plasmid had been cloned, protein was expressed to form fusion protein, i.e. GST-MPT83 fused protein as vaccine candidate of tuberculosis.

5. Conclusions

In this paper, a novel antigen as candidate TB vaccine, MPT83 antigen from local strain *M. tuberculosis* bacteria has been successfully amplified using PCR technique, resulting in 660 bp band. The cloning of MPT83 gene into pGEM-T Easy vector results in recombinant plasmid with the size of 3678 bp. Based on the order of amino acid encoding protein in recombinant plasmid, pGEM-T Easy-Mpt83 was subcloned and expressed into *E. coli* BL-21 cell in order to produce protein recombinant using 48 kDa molecules as GST-MPT83 fusion protein. In the future, MPT83 is considered as TB vaccine candidate that can protect people against TB at adult and productive age.

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