

Real-time monitoring of rhamnose induction effect on the expression of *mpt64* gene fused with *pelB* signal peptide in *Escherichia coli* BL21 (DE3)

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ABSTRACT

In this research, *Escherichia coli* BL21 (DE3) harboring an expression vector constructed with a rhamnose-inducible promoter and a *pelB* signal peptide was used as a host cell to produce MPT64 protein. The objective of this research was to figure out the optimum time of *mpt64* gene expression through real-time monitoring of MPT64 protein production and distribution in host compartments. The *mpt64* expression was regulated by the rhamnose presence at a concentration of 4 mM. The real-time isolated protein was monitored using polyacrylamide gel electrophoresis in denaturation condition. Based on real-time monitoring, the MPT64 protein (24 kDa) in the cytoplasm was optimum detected at 24 h after induction. For periplasmic fraction, the protein was detected at 4 h after induction but thinning at 15 h after induction. At 16 h after induction, the MPT64 protein band was found in the medium with increasing concentrations until 24 h. Thus, it can be concluded that the *mpt64* gene expression was regulated in the presence of rhamnose as an inducer, and the proteins were shown to be translocated throughout the host cell compartment with different levels of protein accumulation at different times, according to the role of *pelB* as a signal peptide.

Key words: *Escherichia coli* BL21 (DE3), MPT64, *pelB*, real time, rhamnose

INTRODUCTION

Tuberculosis is a contagious ailment generated by *Mycobacterium tuberculosis* and is reported to be one of the ten death cases worldwide.^[1] Thus, increasing number of tuberculosis patients has forced the need for rapid and accurate detection of *M. tuberculosis* infection. Rapid detection to differentiate the *M. tuberculosis* complex

and mycobacteria other than tuberculosis (MOTT) is important to diagnose early tuberculosis infection, which is needed for the effective treatment.^[2] MPT64 is one of the most widely released proteins (24 kDa) which secreted during the bacterial growth and has been reported as a specific differentiator between mycobacterium tuberculosis complex (MTBC) and MOTT species.^[3] Hence, MPT64 antibodies can be utilized as a detection tool for *M. tuberculosis* presence. Thus, to produce the antibodies, MPT64 protein is needed in a large amount. In this study, the *mpt64* gene used was a synthetic gene that was constructed in the expression vector, equipped with a rhamnose-inducible promoter and *pelB* signal peptide to produce MPT64 proteins at high

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level, mainly the extracellular protein in *Escherichia coli* BL21 (DE3). *E. coli* is the most chosen host cell to express and secrete the target protein.^[4] The L-rhamnose system has been used successfully to express various genes.^[5] The expression vector system using the L-rhamnose promoter is reported to provide better results than other vectors, especially if the target gene expression has the potential to form insoluble proteins. Expression vector capability is notably evaluated by its efficiency in production and induction response. Those characteristics can be affected by genetic modifications. The effects of determinants cannot simply be predicted by the treatment of singular effects. The combined aspects can inhibit mutually.^[6] Thus, the L-rhamnose expression system must be analyzed, and the expression conditions must be optimized. In this study, the effect of L-rhamnose induction of the gene expression was evaluated to determine the optimum expression time of *mpt64* gene through real-time monitoring of MPT64 protein production.

Usually, in comparison with the cytosolic protein, the secreted proteins are correctly folded and more stable because the periplasmic or the culture medium has lower protease contents.^[4] Therefore, in this research, the expression vector was inserted with the *pelB* sequence as the signal peptide to secrete the proteins across the cytoplasmic membrane.^[7] However, the signal peptide is not only simple marker to secrete the protein but also influences various steps of the whole process of secreting protein production. Therefore, the assistance of the signal peptide in the release of target protein is an important factor in producing high-yield extracellular protein.^[8] Thus, the real-time monitoring of protein translocation is also needed to analyze the optimum yield of MPT64 protein distribution in host compartments.

MATERIALS AND METHODS

Materials

The *E. coli* BL21 (DE3) harboring synthetic plasmids (ATUM) of pD861-*pelB*-MPT64 with kanamycin resistance were used in this research.

Bacterial starter preparation

A volume of 100 μ L transformant from glycerol stock was cultured in 5 mL LB broth with kanamycin addition (100 μ g/mL). Then, the culture medium was put in a shaker incubator for 16–18 h at 37°C and set it up with a speed of 180 rpm.

Gene expression

A volume of 1 mL bacterial starter was suspended into 99 mL broth medium with kanamycin addition and then cultured at 37°C for 3 h on 180 rpm. The culture was incubated and induced with L-rhamnose when the cells grew to OD600 = 0.684. L-Rhamnose was put to

the cell culture until reached a 4 mM of the rhamnose concentration in the medium. Before induction, 1 mL of the cell culture was analyzed as a protein fraction at zero time (t_0). After L-rhamnose addition, the incubation was continued at 37°C and 180 rpm for 24 h. Then, 1 mL from the cell culture was taken in per hour during 24 h and analyzed as per hour protein (t_{1-24}). The protein isolation was conducted to isolate the MPT64 protein by cell fractionation included isolation of the cytoplasmic, periplasmic, and extracellular media.

Isolation of cytoplasmic protein

For protein extracted from the cell cytoplasm, about 1 mL of the cells harboring pD861-SR: 319895 was centrifuged to separate the cell pellets and supernatants with a speed of 6000 \times g, 4°C for 20 min. The pellets were then suspended in Tris-HCl ethylenediaminetetraacetic acid buffer (500 μ L) and then homogenized with a shearing rod. The cells were lysed using a sonicator afterward, with an amplitude of 20 kHz, setting the on-off every 2 s for 2 min with a break time of 4 min, and the cycle was running for three times. The sonication process resulted in the cell debris and the lysate. The MPT64 protein was contained in the lysate; thus, the suspension was centrifuged to isolate the lysate from the cell debris at 4°C on 10,000 \times g for 10 min. The obtained lysate was separated as a cytoplasmic fraction.

Isolation of periplasmic protein

The periplasmic protein was isolated by taking about 1 mL of the cells harboring pD861-SR: 319895 and then centrifuged to separate the cell pellets and supernatants with a speed of 6000 g at 4°C for 20 min. The cell pellets were then mixed in TSE buffer (100 μ L) at pH 8.0 which supplemented by 20% of sucrose and incubated at 4°C for 30 min. Then, the soluble protein was separated from the other cell components by centrifugation using a speed of 20,000 \times g at 4°C for 30 min. Then, the supernatant was separated and collected as a fraction of the periplasmic protein.

Isolation of extracellular protein

The supernatant obtained from separation in the protein cytoplasmic isolation was an extracellular medium containing MPT64 protein. Then, phenylmethylsulfonyl fluoride was put into the medium fraction until its concentration was 1 mM. The extracellular protein was then stored at -20°C.

Protein characterization

All the protein fractions were characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and run at 100 V, 400 A, for 90 min. Then, the bands' migration was analyzed to determine the protein size (kDa) by comparing with the molecular weight of protein markers.

RESULTS AND DISCUSSION

SDS-PAGE results showed that bands of recombinant MPT64 protein at 24 kDa were existed in all cell fractions. Meanwhile, at t_0 (before induction with L-rhamnose), no MPT64 protein was expressed. These results clearly demonstrated the important role of L-rhamnose as an inducer. The *mpt64* gene has been successfully expressed by the presence of the L-rhamnose inducer on the rhaBAD promoter system. The presence of L-rhamnose as an inducer activated promoter rhaBAD in the transcription step of the gene expression process and produced the desired protein through positive regulation.^[9] An hour after induction, the MPT64 protein began to express and showed a band with the thickness increased along with increasing incubation time after induction. In each cell fraction, the MPT64 protein appeared with different band thickness per hour monitoring of gene expression. The thicker the band indicated that the amount of secreted protein was increasing. These different thicknesses were the effects of pelB as a peptide signal, which played an important role in translocating MPT64 protein into the outer compartment of *E. coli* cells.

All extracellular proteins in *E. coli* are first produced in the cytoplasm and then enter a secretory pathway which targeting the protein based on their final cellular destination.^[10] Protein secretion is one of the most important problems of protein expression in the basic process of living cells. However, protein secretion requires effective translocation process to across the plasma membrane. Thus, the protein intended for secretion is targeted to the destination membrane using the signal peptide.^[11] The signal peptide at N-terminal has functioned as a target and identification signal.^[12] The type of signal peptide determines the secretion-type pathway to be used. Selection of suitable peptide signals must be considered because the use of signal sequences can reduce thermodynamic stabilization and its activity. In addition, the peptide signals may affect the forming of protein aggregation.^[13] Although certain proteins can be secreted naturally by the host cell, the recovery of target proteins is often very low or may not secrete at all.^[14,15] However, the stages in the pathway of protein secretion can directly determine the efficiency and kinetics of protein translocation. Likewise, if there is a merging of different signal peptides in the different target proteins, then different mRNA will be produced, which can develop various secondary structures that can affect the stability of the target protein. Thus, signal peptides can significantly affect the amount of protein of each precursor which is synthesized.^[15,16] Due to this fact, it is important to monitor the results of the *mpt64* gene expression and its protein translocation from the secretory expression system to determine the effective secretion of extracellular protein in *E. coli* as the host cell.

Based on real-time monitoring, the MPT64 protein (24 kDa) in the cytoplasm was most detected at 24 h after

induction [Figure 1a]. Normally, when the protein is translocated across the membrane pore, then the signal peptide will be cut by the signal peptidase at certain sequences during translocation.^[17] In this study, MPT64 protein was successfully translocated to periplasm at 4 h after induction, and the bands were thicker until the 14th h of induction [Figure 1b]. The distribution is important because the proteins accumulative in periplasm can increase the cell osmotic pressure. Recombinant protein production can cause membrane disruption, thereby increasing selective permeability which can facilitate leakage. Thus, periplasmic leak becomes important in extracellular secretions, and it can encourage protein secretion across the outer membrane. Therefore, 15 h after induction, the MPT64 protein bands were getting thinner. These showed that the MPT64 protein that accumulated in the periplasmic space was secreted into the extracellular media. As previously reported that protein harbored in the periplasm usually translocates into the medium.^[4] Thus, it was estimated that the MPT64 protein has been translocated to the medium after the 15 h induction. This can be seen from the extracellular MPT64 which was increasingly detected in 16–24 h after induction. Therefore, we monitored if the MPT64 protein can be obtained as extracellular protein in the culture medium.

The optimum recovery of extracellular MPT64 protein is expected; however, it is not easy to obtain because *E. coli* has inner and outer membrane that restricted the pulling out of the target proteins from the cytoplasm into the medium. Even though MPT64 protein has been successfully secreted into the periplasm, the outer membrane can still prevent the release of extracellular protein.^[18] Based on the protein monitoring, MPT64 protein in the medium fraction exhibited that recombinant MPT64 proteins were secreted into the medium. This was indicated by the presence of a protein band at the size of 24 kDa, and it was getting thicker until the 24th h after L-rhamnose induction. As seen in Figure 1c, the concentration of MPT64 protein that accumulated in the medium fraction was lower compared to MPT64 protein content in the periplasmic and cytoplasmic fractions. The impaired translocation of MPT64 protein was suspected because of the damaged signal peptides or slow protein export process; thus, precursors keep piling up in the cytoplasm.^[10] However, until now, the standard rule in determining an effective signal peptide for a certain protein to ensure the success of the secretion process is still unknown. A study reported that the cell host, the type of signal peptide, and secreted protein may affect the success of protein translocation.^[12] Another prediction is that the accumulation of target proteins in periplasmic is a consequence of protein misfolding in the cytoplasm, thus resulting in the impaired translocation and incorrect periplasmic folds.^[18] Thus, the translocation control of MPT64 protein should be considered to be further studied.

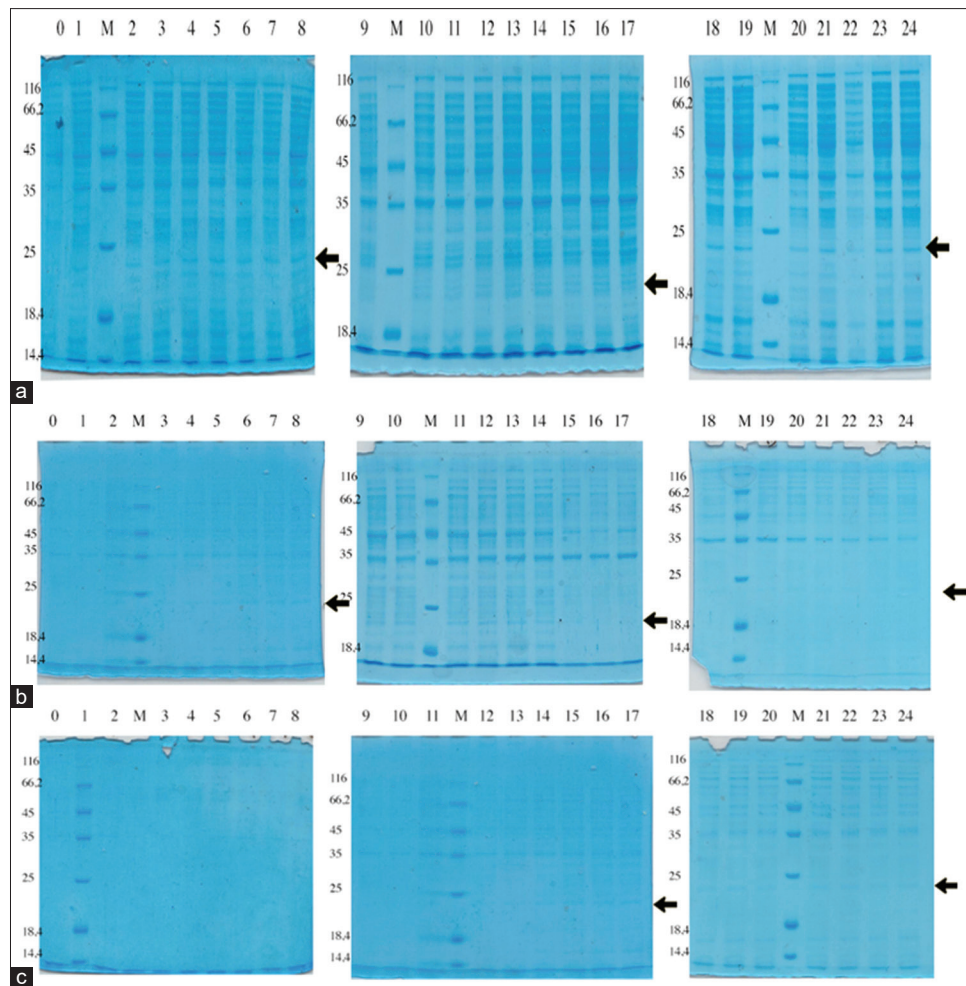


Figure 1: Characterization of recombinant MPT64 protein in the fraction: (a) cytoplasmic, (b) periplasmic, (c) extracellular. M: Marker, 0: Protein fraction before L-rhamnose induction, 1–24: Protein fraction after L-rhamnose induction in every hour monitoring, Arrow: Recombinant MPT64 protein (~24 kDa)

The harvesting time of MPT64 protein that obtained from this study can be used as a reference to obtain MPT64 protein in high yield from each of *E. coli* cell compartment.

CONCLUSION

It can be concluded that the presence of L-rhamnose as an inducer in improving the recovery yield of MPT64 protein at a certain time after induction in the different cell compartments, was correlated with the role of *pelB* as a signal peptide.

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Conflicts of interest

There are no conflicts of interest.

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