ORIGINAL ARTICLE

Comparison of simple and rapid extracting methods of free-tags *Mycobacterium tuberculosis* protein 64 Recombinant Protein from polyacrylamide gel: Electroelution and the optimized passive elution

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Abstract

In this study, the *Mycobacterium tuberculosis* protein 64 (MPT64) protein was constructed without any tags to facilitate the purification using column affinity chromatography, but the MPT64 must be obtained as a pure protein. This study was purpose to ensure the efficient extracting method to purify protein MPT64 directly from the polyacrylamide gel. The crude extract of extracellular protein containing MPT64 protein was separated into single protein band and the targeted protein which is located in the size of 24 kDa was excised. Each of the six bands was collected in a sterile microtube to be eluted using electroelution and the optimized of the passive-elution method. Both the elution methods demonstrated the purity level of the MPT64 protein by detecting a solely band on the gel at the 24 kDa. Among the variety of passive-elution time, the highest MPT64 protein concentration was 0.549 mg/ml after elution for 72 h. However, the electroelution result provided higher MPT64 protein concentration, i.e., 0.683 mg/mL. However, based on the recognition of the purified MPT64 protein on commercial detection kit of MPT64 protein, it showed that the positive result was only showed by the passive-elution extracting protein. Therefore, for purifying the protein MPT64 from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, the efficient method was passive elution.

Key words: MPT64, passive elution, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

INTRODUCTION

The *Mycobacterium tuberculosis* protein 64 (MPT64) is an extracellular protein which is specifically secreted by *M. tuberculosis* complex and used as a detected antigen for the tuberculosis diagnosis using a rapid test kit containing a specific antibody.[1-6] In this present study, MPT64 was produced as a recombinant protein that free of tag for protein purification and constructed to be fused with pelB as the signal peptide that directed the MPT64 protein to be secreted into the medium of *Escherichia coli* BL21 (DE3). Not only expressed our targeted protein, it should be noted

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that *E. coli* also produces other extracellular proteins which are naturally secreted into the medium. Therefore, the secreted MPT64 protein must be isolated to acquire pure antigens to be immunized into the animal as the antibody bioreactor. The antigen purity is the most important factor to be considered to allow a specific immune response.[7,8] Notably, the diagnostic kit used to detect a specific antigen as the parameter of a certain disease depends on the immunochemistry bind of specific antibody proteins to an antigen target.

There are several purification methods for preparing pure protein as the antigen. Due to the MPT64 protein that was constructed without any tags to facilitate the purification using column affinity chromatography, but the MPT64 must be obtained as a pure protein, then we conducted sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) to purify MPT64 protein into single band on the gel. The use of this protein electrophoresis contributes to advances in proteomic applications, particularly in biomedicine, biotechnology, or pharmaceuticals. This method is a profitable alternative when the purification of other proteins is ineffective or the target protein concentration is low.[9] In this way, the MPT64 protein can be separated from other proteins based on its molecular weight by extracting the protein from the polyacrylamide gel to obtain the identity of the protein. The methods commonly used are passive elution and electroelution.[10-12] Actually, the passive elution is relatively easy to be conducted and inexpensive, however, it is not as efficient as electroelution. Therefore, this study aimed to optimize the passive-elution method using simple modification and comparing it with the electroelution method by observing the purity and the final yield of MPT64 protein.

MATERIALS AND METHODS

Materials

The chemicals used were SDS, bis-acrylamide, tris-HCl, 10% Ammonium Persulfate (APS), Tetramethylethylenediamine (TEMED) SDS sample buffer, methanol, glacial acetic acid, 70% ethanol, unstained protein markers (PeqLab), phosphate-buffered saline (PBS) powder, Tris base, glycine, and distilled water.

Gel electrophoresis

The crude extract of extracellular protein containing MPT64 protein was separated into single protein band using SDS-PAGE. The gel electrophoresis was composed of a separating gel (12%) and stacking gel (4%). The gelling solution mixture of the separating gel was put into an electrophoresis device, followed by the addition of distilled water to the upper limit of the glass and it was allowed to be solidified. After that, the solution of stacking gel was put on the solid separating gel and a comb-like tool was placed in the stacking gel solution to form reservoirs to storage the protein samples. The stacking gel was allowed to solidify, then a comb-like tool was removed from the gel. The prepared gel was then soaked in SDS electrophoresis buffer. Before the protein extract was located in the hole, the protein was diluted in 5 µL of × 5 buffer sample, then it was heated for 15 min at 95°C. The protein sample and the marker were loaded in the reservoir in a volume of 10 µL. The electrophoresis was run for 90 min at 100 V and 400 A. The gel was then washed three times with running water and stained with Coomassie blue solution at room temperature and gently agitated for 1–2 h. Furthermore, the stained gel was rinsed with distilled water, then put into the destaining solution for 18–24 h until the background of the gel become colorless and the bands of separated proteins colored as blue bands. The separated protein was observed based on their molecular weight, and the single band MPT64 protein located in the size of 24 kDa was excised and each of the six bands was collected in a sterile microtube to be eluted.

Electroelution

The gel sliced in microtube sterile was transferred into the dialysis membrane with the cutoff range of the protein molecular weight is >12.000 kda. Before used, the pretreatment of the membrane (10 cm) must be performed to activate the membrane. The activation was done by treating the membrane with 1 mM EDTA pH 8 and 2% sodium bicarbonate, then heated at 60°C for 10 min. The membrane was then rinsed using sterile distilled water, followed by heating in 100 mL 1 mM Ethylenediaminetetraacetic acid (EDTA) at 60°C for 10 min. The membrane was then re-washed with sterile distilled water, then the membrane was ready to be used for the electroelution process. The membrane dialysis tube was filled with 100 µl sterile PBS solution and the sliced gel was placed in the dialysis tube with the tied cap on both sides using sewing thread. The tubing was placed in electroelution apparatus containing elution buffer. The electroelution was performed at room temperature, 50 V, for 3 h. After the electroelution was completed, the sewing thread was removed and the solution contained eluted protein was stored in a sterile microtube.

Passive elution

The passive elution was performed at 4°C in different times of incubation, i.e., 24, 48, and 72 h, to optimize the protein release into the elution buffer solution. For passive elution, the excised gels (6 bands for each condition of time incubation) were crushed into small pieces using a sterile razor blade and incubated in 100 µL sterile PBS. The gel debris was centrifugated (3.000 g, 5 min) to isolate the supernatant.

Confirmation of the protein purity

A 10 µL of each elution yield was evaluated to detect the MPT64 presence by loading it to SDS-PAGE. The gel

electrophoresis procedure was performed as a previous mentioned method. The presence of a single band located at the position of 24 kDa on the gel indicated that the elution was successful.

Measurement of protein concentration

The soluble protein concentration was measured by the nanodrop 2000 spectrophotometer, as described in Desjardins (2009).[13]

RESULTS AND DISCUSSION

In this study, MPT64 protein was expressed as a recombinant protein that did not have a tag for protein purification in its gene construction. This protein was fused with the pelB signaling peptide which directs the MPT64 protein to be secreted into the medium. In our previous study, the MPT64 protein was successfully translocated across the host cell membrane due to using pelB as a signal peptide.[13] The efficiency of pelB to secrete protein into the medium was reported in another study that using the same signal peptide and cell host.[14] However, recovery of the MPT64 protein was low, thus, it needs a strategy to collect the target protein in high accumulation. However, *E. coli* not only expresses our target protein but also produces other extracellular proteins which are naturally secreted into the medium. Therefore, the secreted MPT64 protein must be isolated to obtain pure antigen to be immunized to animals as an antibody bioreactor.

Antigen purity is the most important factor that must be considered for generating a specific immune response.[7,8] In particular, the diagnostic kits used to detect specific antigens as a specific disease parameter are highly dependent on the immunochemical binding of specific antibody proteins to the target antigen. There are several purification methods for preparing pure protein as an antigen. Recombinant proteins are usually made by adding a "tag" to the target protein to facilitate the isolation of the target protein from the total protein extract of the host cell using affinity-based chromatography techniques.[15] MPT64 protein used in this study was not designed with His-tag fusion to purify the protein. As reported in the several studies that protein purification using nickel column affinity chromatography sometimes failed to avoid the impurities of targeted protein from other bands protein. This is because His-tag splits using of nickel column affinity chromatography are not 100% efficient. Thus, we suggest that MPT64 protein extraction can be purified using SDS PAGE as a simple method to provide highly purified His-tag free MPT64 protein.

One simple purification method is to isolate MPT64 protein directly from SDS PAGE gel. The use of SDS PAGE has been known to contribute to the advancement of proteomics applications, particularly in the biomedical, biotechnology, or pharmaceutical fields. This method is especially applicable when purification of target proteins is impractical or the target protein concentration is low.[9] In this study, purification was carried out by isolating the MPT64 protein band from SDS PAGE gel at a size of 24 kDa, as shown in Figure 1. In this way, MPT64 protein can be separated from other proteins based on its molecular weight, then extracted from the polyacrylamide gel, and then characterized to obtain the correct protein identity. The extraction method that can be used is by eluting the protein from the gel into the liquid phase. The advantage of this extraction method is it avoids chemical modification or denaturation of the eluted target protein. The methods commonly used are passive elution and electroelution.[10,11] In fact, passive elution is relatively easy to perform and cheap, but the efficiency of protein recovery is lower than the electroelution method. Based on the optimization results presented in Table 1, it can be seen that longer the passive-elution time was used, the higher MPT64 protein concentration was obtained. Among the passive-elution variations, the highest MPT64 protein concentration was 0.549 mg/ml after elution for 72 h. The effectiveness of this passive elution can be seen by comparing the level of protein extraction from SDS PAGE gel without the passive elution method, which was 0.021 mg/ml. However, the highest concentration of MPT64 protein was achieved by the electroelution method. Both the elution methods demonstrated the purity level of the MPT64 protein, as presented in Figure 2. Confirmation of the MPT64 protein identity is proven by the presence of positive recognition of anti-MPT64 antibodies found in commercial MPT64 detection kits, as shown in Figure 3. It found that the positive

Table 1: The results of protein *Mycobacterium tuberculosis* protein 64 elution

Treatment	Concentration (mg/mL)
Without elution	0.021±0.001
Passive elution for 24 h	0.488±0.001
Passive elution for 48 h	0.498±0.003
Passive elution for 72 h	0.549±0.001
Electroelution	0.683 ± 0.001

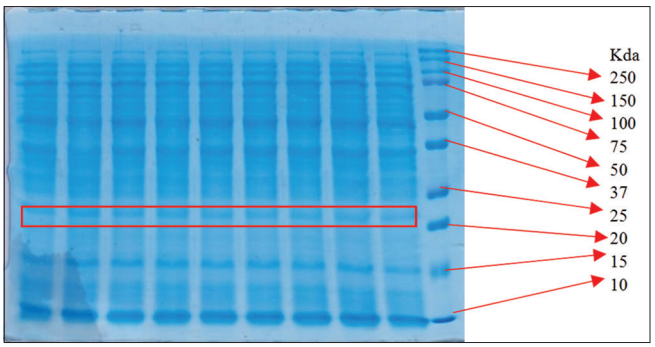

Figure 1: Characterization of extracellular *Mycobacterium tuberculosis* protein 64 protein using sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

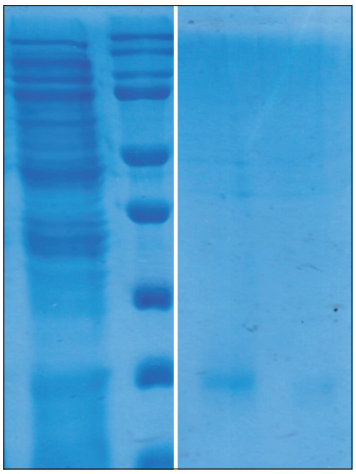

Figure 2: *Mycobacterium tuberculosis* protein 64 protein purity using different elution method. Notes: 1 = Extracellular protein extract; 2 = Protein marker; 3 = Electroelution; 4 = Passive elution (72 h)

result was only showed by the passive-elution extracting protein. The reading of the kit might be disturbed by the presence of Coomassie blue in the protein solution of the electroelution method. Furthermore, although this method has a high efficiency of protein recovery, it has a number of disadvantages, as follows: (1) the isolation of samples in a small number is relatively slow, (2) the eluted protein might be contaminated by SDS and other impurities, (3) cleavage of the peptide chain during elution due to the longer elution times, and (4) N-terminal blockage can be occurred due to chemical modification during elution. Those problems were hypothesized as the reason for the failed recognition of the MPT64 commercial kit against the MPT64 protein extracted using the electroelution method. However, the isolated MPT64 protein identity by the electroelution method can still be confirmed through the SDS PAGE results which showed one clear band according to the size of the MPT64 protein.

Purification of proteins using the electroelution method enables rapid and quantitative elution of the gel.[16] The only prerequisite for protein elution using this method is that the desired protein molecule can migrate in an electric field.[17] This electroelution method is an efficient and reproducible method of eluting multiple protein bands, thus saving a lot of time. This technique can be applied for soluble and membrane-bound proteins.[18] The success of protein purification using electroelution has been reported in several studies.[19,20] For the small obtain volume, this method makes the sample is not difficult to handle. By eluting several samples simultaneously then, a large amount of protein can be produced.[18] However, not all proteins can be transferred by this electrotransfer method.[10]

CONCLUSION

It can be concluded that for purifying the protein MPT64 directly from the SDS PAGE gels, the efficient method was passive elution after elution for 72 h.

Figure 3: Confirmation of the *Mycobacterium tuberculosis* protein 64 protein identity. Note: (a) Electroelution; (b) Passive-elution

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Conflicts of interest

There are no conflicts of interest.

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