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# SDS-PAGE characterization of dialyzed protein fractions from mycobacterium tuberculosis isolates for tuberculosis biomarker screening

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**ABSTRACT**

Tuberculosis is an infectious disease caused by Mycobacterium tuberculosis, in which the identification of protein profiles serves as an important approach in diagnostic research and specific antigen development. This study aimed to analyze the protein profile of dialyzed fractions from Mycobacterium tuberculosis isolates using the SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) method. Protein isolation was carried out through sonication at 4 °C to lyse the cells and release intracellular proteins without causing denaturation. The resulting lysate supernatant was subjected to 30% ammonium sulfate precipitation to separate proteins based on their solubility, followed by dialysis to remove residual salts and unwanted small molecules. The total protein concentration was measured using a photometer with a total protein reagent, showing a relatively low value, indicating that the protein fraction was concentrated on specific molecules with higher purity. The SDS-PAGE analysis revealed the presence of a protein band with an estimated molecular weight of 77,6 kDa, suggesting the successful purification of protein fractions based on molecular weight. Based on these findings, it can be concluded that the combination of sonication, ammonium sulfate precipitation, and dialysis is effective as a preliminary purification method to obtain purer proteins from Mycobacterium tuberculosis isolates and enables more specific characterization of protein profiles through SDS-PAGE.



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# INTRODUCTION

Tuberculosis (TB) remains one of the most impactful infectious diseases worldwide, particularly in countries with a high TB burden such as Indonesia. This disease requires more advanced

diagnostic, therapeutic, and molecular monitoring strategies to be effectively controlled. Tubercolosis (TB) is one of the oldest known diseases and a leading cause of mortality among infectious diseases, despite the widespread use of attenuated vaccines and several antibiotics. According to the World Health Organization (WHO) in 2016, there were an estimated 10.4 million new TB cases globally, resulting in 1.8 million deaths, with more than 95% occurring in low- and middle-income countrie (WHO, 2016).

Globally, TB remains the leading infectious killer. In 2023, an estimated 10.8 million people suffered from TB, with approximately 1.25 million deaths, the majority occurring in low- and middle-income countries. The 2024 WH report highlighted the stagnation in Sprogress toward the 2025 targets and emphasized the urgent need for more effective diagnostic innovations and preventive measures (WHO, 2024). The increasing incidence of multidrug-resistant tuberculosis (MDR-TB) further complicates global control efforts. Therefore, scientific strategies are required to gain a deeper understanding of the biological characteristics of M. tuberculosis, including its protein profile Proteins not only play vital roles in bacterial metabolism but also act as antigens capable of eliciting host immune responses. The identification of dominant proteins from clinical isolates holds potential for use as specific biomarkers for diagnosis and vaccine development (Advani et al., 2019).

The bacterium *Mycobacterium tuberculosis* (M.tb), the causative agent of TB, exhibits complex adaptive mechanisms through the expression of proteins involved in metabolism, virulence, and interactions with the host immune system. For example, a study conducted in Indonesia demonstrated that antibodies against secretory proteins of *M. tuberculosis* such as Ag85B and Rv1860 could distinguish between active TB patients and healthy controls, confirming the crucial role of proteins in immune responses and their diagnostic potential (Dewi et al., 2023). However, most previous studies have primarily focused on selected secreted or recombinant antigens, serological responses, or single target proteins, whereas comprehensive characterization of the whole protein profile directly derived from local clinical isolates remains limited. This is an important gap because local clinical isolates may reflect regional biological variations and protein expression patterns that are not fully represented by laboratory strains or recombinant protein models. To identify and further characterize the proteins involved in the pathogenesis of *M. tuberculosis*, the isolation and purification of bacterial proteins are required using sonication and purification techniques.

Sonication is a protein extraction technique that employs ultrasonic waves to disrupt cell membranes, thereby facilitating the release of proteins from biological matrices. This technique is effectively applied to various protein sources, including seeds, insects, and marine products. Sonication enhances the protein extraction yield and may alter functional properties such as solubility and biological activity (Wijatniko et al., 2023). Ammonium sulfate precipitation is one of the most widely used methods for protein purification, both for natural and recombinant proteins produced through genetic engineering. This method aims to isolate proteins from complex environments by reducing protein aggregation, thus yielding proteins in a more stable and easily separable form (Khosrobeygi et al., 2021).

After obtaining the purified protein fractions, the next step is dialysis, which aims to stabilize the proteins by removing small molecules that could interfere with protein stability. Dialysis serves to purify proteins in solution from other interfering particles. The principle of dialysis involves placing enzyme preparations inside a dialysis bag made of a semipermeable membrane that allows small molecules to diffuse through it. The semipermeable membrane retains protein molecules while allowing smaller molecules such as salts and water to pass through (Widowati et al., 2018).

Once the proteins have undergone purification and dialysis, the total protein concentration is determined to ensure sufficient yield. Basic proteomic methods such as SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) enable the separation of proteins based on

molecular weight and generate characteristic banding patterns. For instance, a local recombinant study on the MPT63 protein characterized by SDS-PAGE produced a ~16 kDa band (Agus et al., 2021). In addition, a local study on the expression of Ag85A and Ag85B proteins by *fbpA/fbpB* clinical isolates using SDS-PAGE and Western blot reported molecular weights of approximately 48 kDa and 46 kDa, respectively (Fihiruddin et al., 2020). To date, only a few studies have comprehensively examined the protein profiles of local *Mycobacterium tuberculosis* clinical isolates using SDS-PAGE. This limitation underscores the need for systematic studies to identify protein bands with consistent molecular weights across isolates, which could serve as a foundation for further analysis and the development of protein-based diagnostic tools (Dewi et al., 2023; Li & Zhang, 2017).

In this context, the novelty of the present study lies in its focus on characterizing protein fractions extracted directly from local clinical isolates of M. tuberculosis through a sequential workflow of sonication, ammonium sulfate precipitation, dialysis, protein quantification, and SDS-PAGE profiling. Rather than evaluating only one predefined antigen, this study seeks to identify reproducible dominant protein bands from local isolates as an initial step toward discovering context-relevant protein-based TB biomarkers that may better reflect circulating strains in Indonesia.

# RESEARCH METHOD

The clinical isolate of *Mycobacterium tuberculosis* used in this study, labeled as sample code 321, was obtained from the Laboratory of Rotinsulu Hospital, Bandung. The selection of one clinical isolate, coded 321, in this study was intended as an initial step to explore the protein profile of *Mycobacterium tuberculosis*. This isolate was used as a preliminary representative sample and was not intended to reflect the entire variation of protein profiles among different isolates. The chemicals used included a lysis buffer composed of EDTA, Phosphate-Buffered Saline (PBS), and a protease inhibitor to maintain protein stability (Ardiansyah et al., 2025). Ammonium sulfate was employed during the purification process, while dialysis utilized dialysis tubing with PBS at concentrations of 0.025 M and 0.05 M. The separating gel was prepared using bis-acrylamide, distilled water, TEMED, and APS, whereas the stacking gel was composed of bis-acrylamide, TEMED, and 10% APS. Electrophoresis was performed using a running buffer. Protein bands were visualized with a staining solution, and the background was removed using a destaining solution. All reagents were obtained from Ekolab (Agus et al., 2021; Alonso Villela et al., 2020).

The instruments included a sonic cleaner for cell lysis and an SDS-PAGE apparatus (BIO-Rad) consisting of a power supply, casting tray, and electrophoresis chamber. Lysis was carried out by sonication in pulse on–off mode at 4°C to prevent protein denaturation caused by heat generation during the process. This step aimed to disrupt the bacterial cell wall to release intracellular protein fractions optimally. The lysate was then centrifuged to remove cell debris, and the supernatant containing soluble proteins was collected as the test sample (Setyaningsih & Musdaniaty, 2019).

All data obtained were entered into an online calculator to determine the required amount of ammonium sulfate, a 30% ammonium sulfate concentration was used as an initial protein precipitation step because it provides mild salting-out conditions that help concentrate soluble proteins while minimizing excessive co-precipitation of contaminants and reducing the risk of protein denaturation. In this study, this concentration was selected to obtain a preliminary protein fraction from *Mycobacterium tuberculosis* isolate 321 for further characterization, as the main objective was to evaluate the protein isolation and characterization method rather than to perform complete protein fractionation. The calculated amount was weighed, and a magnetic stirrer was prepared in an ice-filled basin to maintain the temperature at approximately 4°C. The precipitate bottle was kept on ice, and ammonium sulfate was gradually added with continuous stirring. The precipitation process was carried out for 2 hours at 4°C. After precipitation, the mixture was

centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting pellet, containing both enzymatic and non-enzymatic proteins, was separated and subjected to dialysis for further purification (Widowati et al., 2018).

Dialysis is an essential step in protein purification, particularly after ammonium sulfate precipitation. First, a magnetic stirrer and an ice-filled basin were prepared to maintain the temperature at around 4°C. The pellet obtained from the ammonium sulfate precipitation was transferred into dialysis tubing, and both ends were tightly sealed with thread to ensure that proteins remained inside. The dialysis bag was then immersed in a beaker containing 0.05 M Tris-HCl buffer at pH 8. The beaker was placed in an ice bath to maintain low temperature during dialysis. The magnetic stirrer was turned on, and dialysis was allowed to proceed for 2 hours. Afterward, the buffer was replaced with 0.025 M Tris-HCl pH 8, and dialysis continued for another 2 hours. Buffer replacement was repeated periodically until no residual ammonium sulfate remained inside the dialysis bag, indicating that small molecules such as salts and impurities had been successfully removed while larger protein molecules were retained for subsequent purification steps (Nunu Prihantini et al., 2013; Widowati et al., 2018).

The total protein concentration was determined using a photometric method based on the Biuret reaction principle. In this method, copper ions ($Cu^{2+}$) under alkaline conditions react with peptide bonds in protein molecules, forming a purple complex whose intensity is proportional to the protein concentration in the sample. Measurements were performed using a Microlab 300 photometer at wavelengths of 540–546 nm with ready-to-use AIM reagents. Absorbance was read against a reagent blank, and the total protein concentration was calculated by comparing the sample absorbance with that of a protein standard (Tangkelangi et al., 2023).

Protein analysis was conducted using the Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique with a BIO-Rad SDS-PAGE apparatus. The polyacrylamide gel system consisted of a separating gel (containing bis-acrylamide, TEMED, and APS) and a stacking gel (bis-acrylamide, TEMED, and 10% APS), with running buffer serving as the migration medium (Emawati et al., 2019). Protein samples obtained from the lysis process were loaded into the gel wells in three volume variations—4 µL, 8 µL, and 16 µL—to evaluate the effect of loading volume on protein band sharpness and intensity. Electrophoresis was conducted at a constant voltage of 95 V until the tracking dye reached the bottom of the gel, ensuring optimal separation of proteins based on molecular weight.

# RESULTS AND DISCUSSIONS

As part of the effort to obtain specific antigens or proteins from Mycobacterium tuberculosis (M.tb), a series of processing steps was carried out to ensure optimal protein isolation and purification. This process involves several main stages, including cell lysis, protein precipitation, dialysis, and the analysis of protein purity and characterization using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Each stage plays an important role in maintaining the integrity and stability of the resulting proteins. Cell lysis functions to release proteins from the bacterium's thick cell wall, while precipitation and dialysis are used to separate the target proteins from contaminants and remove substances that may interfere with subsequent analyses. Successful purification is indicated by the presence of clear protein band profiles on SDS-PAGE and a measurable total protein concentration determined by photometric analysis (Alonso Villela et al., 2020; Heidari et al., 2015).

The initial stage of the isolation process was performed through sonication to disrupt M.tb cells and release intracellular proteins. Sonication was carried out in pulse on–off mode at 4°C to prevent denaturation due to temperature increase during the process. After sonication, the sample was centrifuged to separate cell debris, and the supernatant containing proteins was collected for the next step. In the sonication method, the shear force and pressure generated are able to rupture the cell membrane so that cytoplasmic components, including proteins, can be released into the

solution. Lysis efficiency directly affects both protein yield and protein composition, because more effective cell disruption increases the release of intracellular proteins, whereas incomplete or excessive sonication may reduce recovery or alter the extracted protein profile through selective loss or degradation (Saputra et al., 2024; Setyaningsih & Musdaniaty, 2019).

After cell lysis, protein purification was performed using ammonium sulfate precipitation (salting-out) at a concentration of 30%. This method works based on the principle of decreasing protein solubility due to increased ionic strength, so that the target protein precipitates and can be separated from other components. Precipitation with 30% $(NH_4)_2SO_4$ has been reported to effectively achieve up to 95% purity after the dialysis process (Khosrobeygi et al., 2021). However, the use of ammonium sulfate may leave residual salts and other small molecules that can interfere with analysis, therefore dialysis is required as a subsequent step. Dialysis was performed using a semipermeable membrane with a molecular weight cut-off (MWCO) of 10 kDa at 4 °C for 12–24 hours to remove residual salts, urea, and other small molecules (Heidari et al., 2015). Dialysis also plays a role in maintaining protein stability and preventing aggregation, thereby preparing the sample for SDS-PAGE analysis. The purity of the resulting protein fraction is strongly influenced by precipitation conditions, including ammonium sulfate saturation, rate of salt addition, and temperature control, because appropriate conditions improve selective precipitation of target proteins while minimizing co-precipitation of contaminants.

After precipitation and dialysis, total protein concentration was measured using a photometric method with the Biuret reagent at a wavelength of 546 nm. Measurements were performed in triplicate to ensure consistency of the results. The absorbance values obtained were 0.023, 0.021, and 0.023, with total protein concentrations of 1.3 g/L, 1.4 g/L, and 1.3 g/L, respectively.

**Table 1.** Results of total protein concentration analysis

| Repetition of the Test | ABS | Total Protein Concentration (g/L) |
|---|---|---|
| 1 | 0,041 | 1,3 |
| 2 | 0,039 | 1,4 |
| 3 | 0,041 | 1,3 |

The relatively consistent results across repetitions indicate that the sample was homogeneous and that the mixing and pipetting procedures were performed properly. In addition, the color change of the reagent to purple indicates that the Biuret reaction occurred well, namely the interaction between $Cu^{2+}$ ions and peptide bonds in proteins proceeded optimally (Seely & Gagnon, 2022).

Although the total protein concentration obtained was relatively low, this condition may be influenced by several factors. The low protein concentration may indicate that some proteins were lost during the precipitation and dialysis processes, considering that the precipitation method does not always cause all protein molecules to precipitate completely (Ardiansyah et al., 2025). In addition, the biological characteristics of M.tb also contribute to the low protein yield. M.tb has a thick and lipid-rich cell wall containing components such as mycolic acids, arabinogalactan, and peptidoglycan, which function to protect the cell, but at the same time can limit the release of intracellular proteins and secreted proteins. This causes the amount of protein successfully extracted to be relatively lower compared to other microorganisms such as E. coli. (Khosrobeygi et al., 2021) reported that the total protein concentration in M.tb culture filtrates ranged only from 0.20 to 0.30 mg/mL and remained low even after precipitation and dialysis. These findings support that low protein content is an inherent characteristic of M.tb, rather than merely the result of technical errors (Alonso Villela et al., 2020).

Protein characterization was performed using SDS-PAGE to observe the protein profile and the purification level of the isolated product. Prior to molecular weight estimation of the protein bands, a semi-logarithmic curve of the SDS-PAGE protein marker was generated.

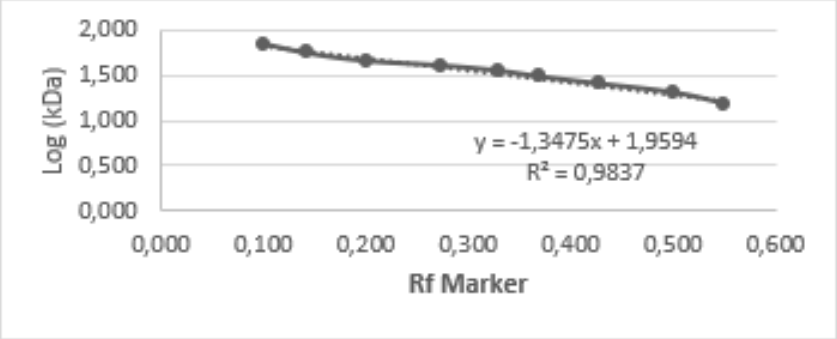


**Figure 1.** Semi-logarithmic curve of SDS-Page protein marker for molecular weight determination

The analysis results showed a linear relationship between the retardation factor (Rf) values and the logarithm of protein molecular weight [log(MW/kDa)]. The regression equation obtained was log(MW) = −1.3475 × Rf + 1.9594 with an $R^2$ value of 0.9837. The negative slope indicates an inverse relationship between migration distance and molecular weight, where smaller proteins migrate further. The high $R^2$ value indicates that the calibration model is sufficiently accurate to estimate molecular weight based on protein migration patterns on the SDS-PAGE gel (Tucci et al., 2020).

The SDS-PAGE visualization results showed protein separation across four lanes, consisting of one molecular weight marker lane (M) and three sample lanes with different loading volumes (S 4 µL, S 8 µL, and S 16 µL).

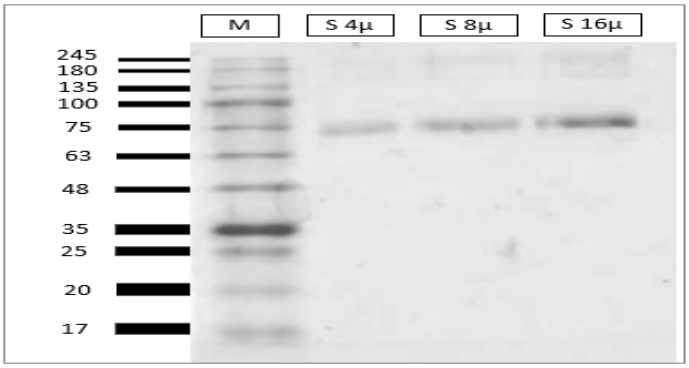


**Figure 2.** Visualization of protein bands of M.tb protein samples obtained from dialysis

In the sample lanes, a clear and consistent band was observed at approximately 77.6 kDa. Band intensity increased with increasing sample volume from 4 µL to 16 µL, indicating a proportional correlation between protein amount and staining intensity. The minimal presence of non-specific bands or contaminants suggests that precipitation and dialysis successfully enriched the dominant protein fraction at approximately 77.6 kDa. The minimal non-specific bands on SDS-PAGE suggest that the purification steps were sufficiently selective, so the dominant 77.6 kDa band likely represents an enriched major protein fraction rather than a highly heterogeneous crude lysate.

The dominant protein band with a molecular weight of approximately 77.6 kDa in M.tb is generally associated with highly expressed cytoplasmic proteins, such as Elongation Factor G (EF-G) or Heat Shock Protein 70 (Hsp70/DnaK). EF-G is an important GTPase enzyme involved in translation, particularly facilitating ribosomal translocation during polypeptide chain elongation, with a molecular weight ranging from 76–78 kDa (Gao et al., 2021). Meanwhile, DnaK functions as

a molecular chaperone that assists protein folding and protects cells from environmental stress, with a molecular weight of approximately 70–72 kDa (Xiao et al., 2024). Both proteins often appear as dominant bands in SDS-PAGE analysis of M.tb lysates due to their high expression levels and essential roles in cellular metabolism. Under crude lysate conditions, these proteins may exist in large complexes due to associations with ribosomes or co-chaperones such as GrpE and DnaJ. However, SDS-PAGE alone cannot specifically identify the protein, because proteins with similar molecular weights may co-migrate in the same band; therefore, the identity of the 77.6 kDa protein must be confirmed using more specific methods such as LC-MS/MS or immunoblotting.

The presence of a sharper single band after purification and dialysis indicates an increased level of purity of the protein fraction. Dialysis plays a role in removing residual salts or small molecules that may affect protein stability and electrophoresis results. However, dialysis without stabilizing agents such as glycerol, $Mg^{2+}$, or nucleotides may lead to protein aggregation or reduced enzymatic activity, particularly for proteins such as EF-G that require GTP for structural stability (Xiao et al., 2024). Therefore, in further studies, buffer selection and the addition of stabilizing agents may be considered to maintain protein stability during purification.

The limited protein yield observed in this study may also be influenced by lysis efficiency and the composition of the extraction buffer. Recent studies have shown that combining mechanical methods such as sonication or bead-beating with buffers containing mild detergents and chaotropic agents (e.g., SDS or urea) can improve protein extraction, particularly in bacteria with thick cell walls such as M.tb (Machnik et al., 2024). Therefore, optimizing sonication parameters, including energy intensity, cycle duration, and maintaining stable low temperatures, may reduce protein loss during lysis.

Ammonium sulfate precipitation is an economical and efficient method for initial purification, but its effectiveness is strongly influenced by experimental conditions such as saturation percentage, salt addition rate, and temperature control. Fractional precipitation with proper temperature control can improve precipitation selectivity and reduce protein loss. In addition, combining this method with advanced techniques such as affinity chromatography or gel filtration may improve purity without significantly decreasing the amount of target protein. Stepwise dialysis using fresh buffer is also important to ensure complete removal of residual salts that may interfere with quantification and electrophoresis results (Koteshwara et al., 2021).

Overall, total protein measurement and SDS-PAGE results should be interpreted complementarily. Photometric analysis provides a quantitative estimate of total protein but is less sensitive at low concentrations. In contrast, SDS-PAGE provides visual evidence of specific proteins and demonstrates purification success even when total protein concentration is low. Combining both methods allows a more accurate interpretation of protein isolation and purification success (Alonso Villela et al., 2020).

The practical implication of this study is that the dominant protein at approximately 77.6 kDa identified through SDS-PAGE has the potential to be developed as an antigen candidate for immunodiagnostic assays or to be further analyzed using mass spectrometry (LC-MS/MS). Recent proteomic studies have shown that prefractionation techniques and label-free LC-MS analysis can increase the number of proteins identified from M.tb even when the initial yield is low (Yari et al., 2023). Therefore, these findings can serve as a basis for further exploration of specific M.tb proteins that play key roles in pathogenic mechanisms and the development of diagnostic biomarkers.

# CONCLUSION

Proteins from Mycobacterium tuberculosis were successfully isolated through sonication, ammonium sulfate precipitation, and dialysis. Although the total protein concentrations obtained were relatively low (1.3–1.4 g/L), SDS-PAGE analysis consistently revealed a distinct protein band at 77.6 kDa, indicating enrichment of a major protein fraction. These findings provide an initial basis for the identification of protein candidates from local clinical isolates that may contribute to

the development of protein-based TB biomarkers. In practical terms, the study demonstrates that a relatively simple extraction and purification workflow can yield protein fractions suitable for preliminary immunodiagnostic development. Nevertheless, further investigations are required to confirm the identity of the detected protein, particularly through LC-MS/MS analysis, and to evaluate its diagnostic relevance across a broader set of clinical isolates.

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