HAYATI
Journal of Biosciences

Vol. 31 No. 2, March 2024 374-381
DOI:10.4308/hjb.31.2.374-381
ISSN: 1978-3019
EISSN: 2086-4094

Cloning and Production of Antigen 85A *Mycobacterium tuberculosis* for Diagnostic Latent Tuberculosis: a Preliminary Study

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ARTICLE INFO

Article history:
Received July 25, 2023
Received in revised form November 6, 2023
Accepted November 8, 2023

KEYWORDS:
antigen,
cloning,
expression,
latent,
recombinant,
vector

ABSTRACT

The main challenge in the management of Tuberculosis (TB) is diagnosing quickly and accurately, especially Latent Tuberculosis Infection (LTBI). LTBI detection was carried out using the Tuberculin Skin Test (TST) and Interferon Gamma Release Assay (IGRA). In TB endemic areas, these two examinations have limitations, so current research is directed at finding specific antigens for the diagnosis of LTBI. One of the potential proteins is Antigen 85A (Ag85A) *Mycobacterium tuberculosis* (Mtb) encoded by Fibronectin-binding protein A (FbpA). The Ag85 complex induces the proliferation of T-cells and interferon-gamma in most healthy individuals infected with *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and BCG-vaccinated mice, making it a potential antigen. This study aims to clone and produce recombinant protein Ag85A from Mtb in *Escherichia coli* BL21. The methods used were ligation to the pET-32a expression vector, transformation to *Escherichia coli* BL21, and production of protein by IPTG induction. Characterization of recombinant clones by colony PCR and sequencing. The results obtained were that the fbpA gene isolated from Mtb clinical isolate had been amplified, and the PCR product was 900 bp. The production of Antigen 85A has been successfully carried out and produces 44 kDa.

1. Introduction

Indonesia is one of the countries with the highest burden of Tuberculosis (TB) in the world, with a total of 824,000 cases and 93,000 deaths per year. TB in Indonesia is ranked third in the world, after India and China. It is estimated that out of 824,000 TB sufferers, only 49% are found and treated, so there are still 500,000 people who have not been treated and can become a source of transmission (Ministry of Health 2022). Indonesia is still having problems with TB because in 2016-2020 it was included in the 20 countries with high TB cases, including TB/HIV cases, and drug-resistant TB/MDR (WHO 2018).

Latent tuberculosis infection (LTBI) is a condition of persistent immune response to Mtb stimulation without clinical evidence of active TB, or radiographic and bacteriological abnormalities (CDC 2013). LTBI does not cause clinical symptoms and has a normal chest X-ray picture with positive results of immunological tests such as the Tuberculin Skin Test (TST) or Interferon Gamma Release Assay (IGRA). There are no clinical signs and symptoms of active pulmonary or extrapulmonary TB (Cohen *et al.* 2019). It is estimated that one-third of the world's population is infected with Mtb and most have no signs or symptoms of TB disease and are not contagious, but still have a risk of becoming active and infectious TB.

The risk of TB reactivation is estimated at 5-10%, with the majority becoming active TB within the first five years after infection with TB (WHO 2015). The number of LTBI sufferers in the United States is estimated at 13 million people, and around 650,000-1,300,000 people are at risk of becoming active TB (CDC 2013).

Currently, the diagnosis of latent TB is done by TST and IGRA tests. It is known that the TST has many limitations. This test can only determine that a person has been infected with TB, but cannot determine whether the TB

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infection is still active or not. In addition, the TST test cannot distinguish whether a positive result is due to TB infection or BCG immunization. This test requires 2 patient visits and requires staff skills in conducting tests and readings. The TST is a test for the diagnosis of LTBI, and is not recommended for the diagnosis of active disease–its sensitivity is only 70-85%. Additionally, a positive TST result may not indicate the presence of *Mycobacterium tuberculosis* in the body because this test relies on the activation of memory T cells, which can persist in the body for years (Kruse and William 2019).

IGRA is a blood test performed by measuring the immune response of individuals who have been exposed to active tuberculosis. The principle of IGRA is to detect interferon-gamma secreted by T cells in response to the re-stimulation of Mtb antigens. The specificity and sensitivity of IGRA are low for detecting active TB, especially in areas with a high TB incidence. Several studies have shown that IGRA is not recommended as a substitute for the tuberculin test to predict TB infection in countries with a high prevalence of TB because the results are not significantly different from the tuberculin test (WHO 2018).

Due to the limitations of the TST and IGRA tests, it is advisable to perform serodiagnostic tests to obtain confirmatory results. Therefore, immunodiagnostic tests for latent TB urgently require specific antigens recognized by Peripheral Blood Mononuclear Cells (PBMC) cells to give a positive response to TB infection. In this case, it is the search for specific antigens for the development of TB diagnostic reagents, especially to identify new and latent infected individuals with a high risk of developing active TB.

M. tuberculosis produces many proteins in the extracellular environment, which can be recognized by the host immune system and induce protective immunity and immune responses with diagnostic value. The Ag85 complex is highly conserved in the TB cell epitope, indicating that it can be used for the diagnosis and vaccine for TB (Jiang *et al.* 2015). *M. tuberculosis* accumulates large amounts of triacylglycerol (TAG) as an energy and carbon storage compound. Mycobacterial triacylglycerol is stored in the form of intracellular lipid droplets for the survival of *M. tuberculosis* during the dormant state. The TAG synthesized by Ag85A plays a role in the formation of lipid storage bodies and the maintenance of persistent tuberculosis infection (Elamin *et al.* 2011).

The Ag85 complex is the major secretory antigen and has an important role in Mtb pathogenicity. The roles of this complex in virulence include binding to fibronectin, mycolyltransferases, and inhibition of phagosome maturation (Babaki *et al.* 2017). In literature, Ag85A known under different names: MtbAg85A, Rv3804c, P32 and FbpA (Huygen 2014). Maintenance of a highly hydrophobic cell wall is essential for the survival of Mtb in the host. Antigen 85 proteins (85A, 85B, and 85C) from Mtb help maintain cell wall integrity by catalyzing the transfer of mycolic acid to the cell wall of arabinogalactan and the synthesis of trehalose dimycolate (cord factor). This secreted protein makes it possible to rapidly invade alveolar macrophages through direct interactions between the host immune system and invading bacilli (Ronning *et al.* 2004).

Antigen complex 85 plays a role in mycobacterial pathogenesis and possesses mycolyltransferase enzymatic activity involved in cell wall synthesis. Ag85 functions as a virulence factor because its expression is essential for intracellular survival in macrophages, but it also contributes to mycobacterial attachment, invasion, and dissemination in host cells (Kuo *et al.* 2013). The Ag85 complex can be detected in bthe lood and sputum of pulmonary TB patients, cerebrospinal fluid of TB meningitis patients, and mycobacterial liquid culture media, so it can be a prominent marker for the diagnosis of TB (Yuk and Eun 2014).

These proteins are known to be potential candidates for TB vaccines and TB diagnostic kits. The antigen 85 complexes of *M. tuberculosis* have been shown to stimulate both humoral and cell-mediated immune responses. In addition, the large number of serum antibodies produced against Ag85 complexes in active TB patients proves that the Ag85 complex can serve as a promising diagnostic (Bekmurzayeva *et al.* 2013). Ag85A and Ag85B induce strong T-cell proliferation and IFN-γ production in most healthy individuals latently infected with Mtb and in BCG-vaccinated mice and humans but not in tuberculosis patients. Members of the Ag85 complex are highly conserved in other mycobacterial species (Huygen 2014).

Detection of Ag85 complexes in the serum of TB patients by ELISA provides a reliable diagnosis and does not give false results with other non-tuberculosis diseases. It can be used to develop immunodiagnostic assays with increased sensitivity and specificity. Such a test will be fast, sensitive, and cost-effective and can be performed easily in any standard pathology laboratory (Kashyap Rajpal *et al.* 2007). Based on the explanation, Ag85A has the potential as an antigen in diagnosing latent tuberculosis, so that the spread of active TB can be prevented.

Recombinant proteins can be produced by companies using bacteria and hosts as desired. The Novatein Biosciences can produce Ag85A recombinant protein using *M. tuberculosis* H37Rv and the expression host in Baculovirus. In this study, the recombinant protein Ag85A was obtained from clinical isolates of M tuberculosis from Indonesia and produced in *E. coli* BL21 host cells. It is hoped that this protein can function as an immunogenic antigen so that it has the potential to diagnose latent TB.

2. Materials and Methods

2.1. Ethics Approval

This research was carried out with recommendations for ethical approval from the Hasanuddin University Research Ethics Commission with number: 189/UN4.6.4.5.31/PP36/2023.

2.2. Isolation of *M. tuberculosis*

TB patient sputum was decontaminated by adding NaOH, sodium citrate and N acetyl-L-cysteine, then centrifuged at 12,000 rpm. The precipitate is diluted with Phosphate Buffer Saline (PBS). Sputum ready for culture in Lowenstein Jensen's medium (LJ). In this study, Mtb isolates were obtained from positive TB cultures and Mtb isolates strain H37Rv as the positive control. The isolated DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). The Mtb DNA will be used as a template for the amplification of target genes by PCR.

2.3. Gene Amplification with Polymerase Chain Reaction (PCR)

Target gene amplification was carried out by PCR technique using specific primers. Forward:5'-AGGAT CCATGTTTTCCCGGCCGGGCTTG-3' and Reverse: 5'GG AATTCTGTTCGGAGCTAGGCGCCCTGGG-3' (Fan *et al.* 2007). The PCR conditions were pre-denatured at 95°C for 2 minutes, denatured at 95°C for 30 seconds, annealed at 60°C for 30 seconds and extended at 72°C. The PCR product of the FbpA was 900 bp. The PCR product was analyzed by electrophoresis on 2% agarose gel in Tris boric acid/EDTA buffer and observations were conducted in transilluminator UV light.

2.4. Cloning of FbpA to pET-32a Expression Vector

The pET-32a expression vector was cut with restriction enzymes *Bam*HI and *Eco*RI. The restriction compositions were 13 µl nuclease-free water, 3 µl NE buffer, 1 µl/µg pET-32a, restriction enzyme unit/µl *Eco*RI and 5 unit/µl *Bam*HI, put into an eppendorf tube and incubated overnight at 37°C. Insertion DNA purification using the QIAquick® PCR Purification Kit. 5 µl PB buffer and 25 µl PCR product were put into the spin column, homogenized and centrifuged at 13,000 rpm for 1 minute. The supernatant was removed, and 750 µl PE buffer was added and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and centrifuged at 13,000 rpm for 1 minute. EB buffer 50 µl was added and centrifuged at 13,000 rpm for 1 minute.

Ligation of the FbpA to the pET-32a expression vector with the composition of the ligation reaction consisted of 1 µl T4 DNA Ligase buffer, 50 ng/µl pET-32a expression vector, 18 ng inserted DNA, 1 µl T4 DNA ligase (3u/µl), 4 µl Nuclease Free Water and stored in the refrigerator for 24 hours. Transformation of Recombinant Plasmids to *Escherichia coli* BL21 with the method is heat shock based on Sambrook *et al.* 1989.

A solid Luria Bertani (LB) medium containing ampicillin and IPTG was prepared. 10 µl of the ligation product was added to 50 µl of *E. coli* BL21 competent cells, and then incubated on ice for 30 minutes. Furthermore, the heat shock process was carried out in a water bath with a temperature of 42°C for 1 minute and incubated on ice for 1 hour. Next, 100 µl of liquid LB media was added and homogenized. Samples were incubated using an incubator shaker at 37°C for 3 hours at 150 rpm and centrifuged for 1 minute at 10,000 rpm. The supernatant was discarded until 50 µl pellet remained. The transformation products were spread into a solid Luria Bertani medium containing ampicillin, X-Gal and IPTG, then incubated at 37°C for 16-18 hours.

2.5. Characterization of Recombinant Clone (FbpA-pET-32a)

2.5.1. Colony PCR

The white colonies growing on the Luria Bertani (LB) medium were recombinant clones (FbpA-pET-32a). White colonies were isolated in 20 µl of nuclease-free water. The PCR reagent composition consisted of Go Taq Green 12 µl, 1 µl of Forward and Reverse primers each, 3 µl of the sample, 8.5 µl of nuclease- free water, homogenized and PCR carried out as before.

2.5.2. Recombinant Plasmid Amplification

Recombinant plasmid amplification was performed on white colonies using the QIAprep Spin

Miniprep plasmid extraction kit. Furthermore, PCR amplification used primers and the same conditions at the beginning of the study. Recombinant plasmid PCR was confirmed using agarose gel electrophoresis.

2.5.3. Sequencing

Characterization of recombinant clones was carried out by sequencing. White colonies growing on Luria Bertani medium were diluted in nuclease-free water, and colony PCR was performed using primers and conditions as at the beginning of the study. The PCR products were sequenced and BLAST (Basic Local Alignment Search Tool) was used to analyze target gene homology with *Mycobacterium tuberculosis* H37Rv.

3. Results

3.1. PCR Product

PCR results on FbpA isolate H37Rv as a positive control and clinical isolates obtained a band size of 900 bp (Figure 1).

3.2. Cloning of FbpA to pET-32a Expression Vector

Transformation using the heat shock method in LB medium with the addition of ampicillin, IPTG and X-Gal. Transformation to *Escherichia coli* BL21 aims to amplify recombinant plasmid DNA. The results of the transformation can be seen in Figure 2.

Figure 1. PCR product of FbpA. 1 = 100 bp Marker, 2, 3 = clinical isolates, 4, 5 = H37Rv isolates

3.3. Characterization of Recombinant Clones (FbpA-pET-32a)

3.3.1. Colony PCR

The characterization was carried out to determine whether the correct gene was inserted into the plasmid. Characterization of recombinant plasmids was carried out by colony PCR using primers and the same conditions at the early stages of this study. The results obtained in Figure 3 show that the white colonies carry the FbpA measuring 900 bp.

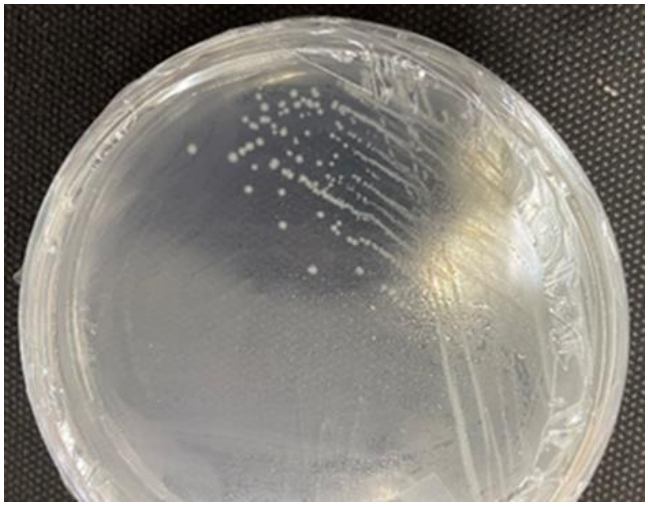

Figure 2. Recombinant clone in *E. coli* BL21

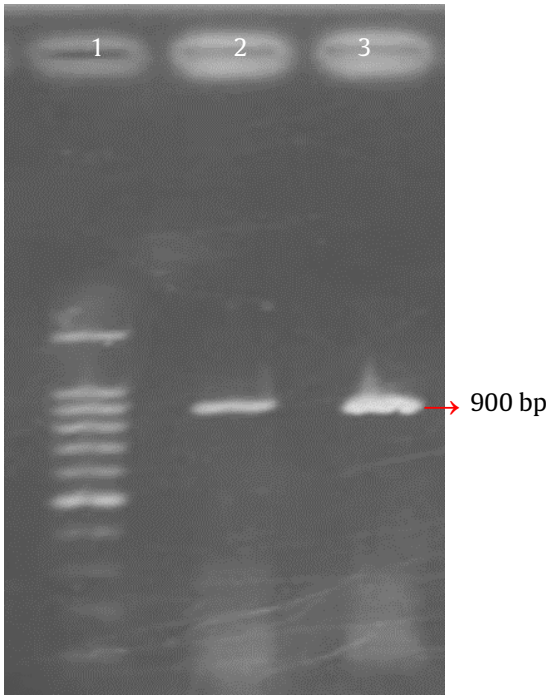

Figure 3. Colony PCR. 1 = 100 bp marker, 2, 3 = white colonies

3.3.2. Sequencing

Sequencing was performed to ensure that the inserted DNA isolated from the white colonies was FbpA, which encodes the Ag85A protein based on the confirmed gene sequence from NCBI. The results of the sequences producing significant alignments are shown in Figure 4. The alignment results show high sequence similarity with FbpA in *M. tuberculosis* H37Rv was 99%.

```
>Mycobacterium tuberculosis H37Rv complete genome
Sequence ID: AL123456.3 Length: 4411532
Range 1: 4265640 to 4266454

Score:1435 bits(777), Expect:0.0,
Identities:803/815(99%),  Gaps:7/815(0%), Strand: Plus/Minus

Query  64       TTCCAAAGTGGTGGTGCCAACTCGCCCGCCCTGTACCTGCACGACGGCCTGCGCGCGCAG  123
                 |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  4266454  TTCCAAAGTGGTGGTGCCAACTCGCCCGCCCTGTACCTGCTCGACGGCCTGCGCGCGCAG  4266395

Query  124      GACGACTTCAGCGGCTGGGACATCAACACCCCGGCGTTCGAGTGGTACGACCAGTCGGGC  183
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266394  GACGACTTCAGCGGCTGGGACATCAACACCCCGGCGTTCGAGTGGTACGACCAGTCGGGC  4266335

Query  184      CTGTCGGTGGTCATGCCGGTGGGTGGCCAGTCAAGCTTCTACTCCGACTGGTACCAGCCC  243
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266334  CTGTCGGTGGTCATGCCGGTGGGTGGCCAGTCAAGCTTCTACTCCGACTGGTACCAGCCC  4266275

Query  244      GCCTGCGGCAAGGCCGGTTGCCAGACTTACAAGTGGGAGACCTTCCTGACCAGCGAGCTG  303
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266274  GCCTGCGGCAAGGCCGGTTGCCAGACTTACAAGTGGGAGACCTTCCTGACCAGCGAGCTG  4266215

Query  304      CCGGGGTGGCTGCAGGCCAACAGGCACGTCAAGCCCACCGGAAGCGCCGTCGTCGGTCTT  363
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266214  CCGGGGTGGCTGCAGGCCAACAGGCACGTCAAGCCCACCGGAAGCGCCGTCGTCGGTCTT  4266155

Query  364      TCGATGGCTGCTTCTTCGGCGCTGACGCTGGCGATCTATCACCCCCAGCAGTTCGTCTAC  423
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266154  TCGATGGCTGCTTCTTCGGCGCTGACGCTGGCGATCTATCACCCCCAGCAGTTCGTCTAC  4266095

Query  424      GCGGGAGCGATGTCGGGCCTGTTGGACCCCTCCCAGGCGATGGGTCCCACCCTGATCGGC  483
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266094  GCGGGAGCGATGTCGGGCCTGTTGGACCCCTCCCAGGCGATGGGTCCCACCCTGATCGGC  4266035

Query  484      CTGGCGATGGGTGACGCTGGCGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGGAGGAC  543
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4266034  CTGGCGATGGGTGACGCTGGCGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGGAGGAC  4265975

Query  544      CCGGCGTGGCAGCGCAACGACCCGCTGTTGAACGTCGGGAAGCTGATCGCCAACAACACC  603
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4265974  CCGGCGTGGCAGCGCAACGACCCGCTGTTGAACGTCGGGAAGCTGATCGCCAACAACACC  4265915

Query  604      CGCGTCTGGGTGTACTGCGGCAACGGCAAGCCGTCGGATCTGGGTGGCAACAACCTGCCG  663
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4265914  CGCGTCTGGGTGTACTGCGGCAACGGCAAGCCGTCGGATCTGGGTGGCAACAACCTGCCG  4265855

Query  664      GCCA-GTTCCTCGAGGGCTTCGTGCGGACCAGCA-CATCAAGTTCCAAGACGCCTACAAC  721
                 |||| |||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  4265854  GCCAAGTTCCTCGAGGGCTTCGTGCGGACCAGCAACATCAAGTTCCAAGACGCCTACAAC  4265795

Query  722      GCCGGTGGCGGCCACAACGGCGTGTTCGACTTCCCGGACAGCGGTACGCACAGCTGGGAG  781
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  782      TACTGGGGCGCGCAGCTCAACGCTATGAAGCCCGAC-TGCAAC-GGCACTGGGTGCCACG  839
                 |||||||||||||||||||||||||||||||||||| |||||| ||||||||||||||||
Sbjct  4265734  TACTGGGGCGCGCAGCTCAACGCTATGAAGCCCGACCTGCAACGGGCACTGGGTGCCACG  4265675

Query  840      CCCAACACCGGTCC-GSSCCCAAGG-CGC-TAGCT  871
                 |||||||||||| || |  ||| ||| ||| |||||
Sbjct  4265674  CCCAACACCGGGCCCGCGCCCCAGGGCGCCTAGCT  4265640
```

Figure 4. FbpA gene alignment with NCBI BLAST

3.4. Production of the Recombinant Protein Ag85A

The *E. coli* transformants were grown in liquid LB and ampicillin media (100 µg/ml) and induced with IPTG (Isopropyl-β-D-thiogalactopyranoside) with a concentration of 1.0 mM and incubated for 18 hours at 37°C. The *E. coli* transformants were lysed using a sonicator. The supernatant containing recombinant protein Ag85A in the cytoplasm was analyzed by Sodium Dodecyl Sulfate – Polyacrylamide Electrophoresis (SDS-PAGE) and Coomassie Blue staining. The recombinant protein Ag85A to be produced is 44 kDa can shown in Figure 5.

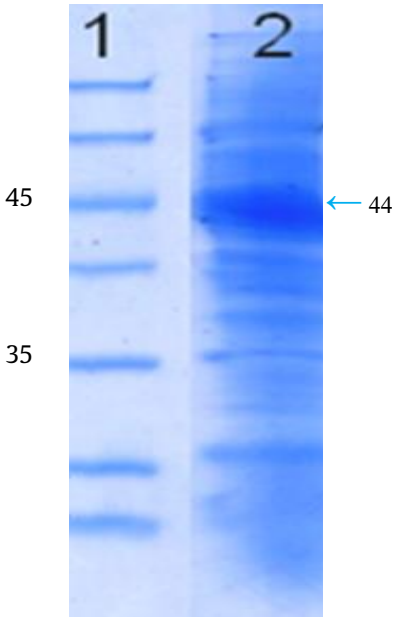

Figure 5. Recombinant protein of Ag85A. 1 = protein marker 2 = Ag85A

4. Discussion

4.1. PCR Product

Isolation of the FbpA gene encoding Ag85A was obtained from clinical isolates and H37Rv isolates. The Reverse primer sequence is 5'-AGGATCCATGTTTTCCCGGCCGGGCTTG-3' and the Forward primer sequence is 5'-GGAATTCGTTCGGAGCTAGGCGCCCTG GG-3'. The yellow color is the *Bam*HI restriction site and the red color is the EcoRI restriction site. PCR results in H37Rv isolate as a positive control and clinical isolates obtained a band size of 900 bp (Figure 1).

4.2. Cloning of FbpA to pET-32a Expression Vector

The pET-32a vector was designed for the expression of peptide sequences coupled to the thioredoxin 109 aa Trx•Tag™ protein. It belongs to a kind of expression system that can produce the desired protein after being introduced into prokaryotic host cells. Prokaryotic cells such as *Escherichia coli* are preferred hosts for protein expression because of their low cost, rapid accumulation of biomass and simple process enhancement. Fusion protein expressed by recombinant pET-32a vector containing His-Tag which is often used for affinity purification and for identification of fusion protein after purification (Liu and Yang 2012).

Transformation using the heat shock method. Transformation to *Escherichia coli* BL21 aims to amplify recombinant plasmid DNA. *Escherichia coli* is an important experimental, medical and industrial cell factory for the production of recombinant proteins. The inducible lac promoter is one of the most commonly used promoters for heterologous protein expression in *E. coli.* Isopropyl-β-D-thiogalactoside (IPTG) is currently the most efficient molecular inducer to regulate the transcriptional activity of this promoter (Briand *et al.* 2016).

X-Gal is a widely used chromogenic substrate for β-galactosidase. This results in a dark blue precipitate at the site of the enzymatic activity. X-Gal is useful for a variety of histochemical and molecular biology applications, including the detection of lacZ activity in cells and tissues (Thermo Fisher Scientific 2022).

Blue-white screening is a fast and efficient technique for the identification of recombinant bacteria. This depends on the activity of β-galactosidase, an enzyme present in *E. coli*, which cleaves lactose into glucose and galactose (Merck 2022). In Figure 2, the presence of white colonies indicates that the FbpA insertion DNA was successfully inserted into the vector and successfully cloned. The lacZ gene will code for an enzyme β-galactosidase which functions to break down lactose into glucose and galactose. The presence of the β-galactosidase enzyme can be detected by the breakdown of the colorless X-gal substrate into galactose and blue 5-bromo-4-chloroindigo. The presence of an active β-galactosidase enzyme will produce blue bacterial cell colonies. This shows that there is no inserted DNA in the vector plasmid. Cells that do not have β-galactosidase enzyme activity will produce white bacterial cell colonies (GoldBio 2018).

4.3. Characterization of Recombinant Plasmid

Methods of selection and screening of recombinant transformants can be carried out with blue-white screening, insertional inactivation, antibiotic sensitivity and auxotrophic yeast strain (Indurkar 2017). In this study, clone characterization was carried out by colony PCR and sequencing. The characterization was carried out to determine whether the correct gene was inserted into the plasmid. Characterization of recombinant plasmids was carried out by colony PCR using primers and the same conditions at the early stages of this study. The results obtained in Figure 3 show that the white colonies carry the FbpA gene measuring 900 bp.

Sequencing was carried out to ensure that the inserted DNA isolated from the white colonies was FbpA which encodes the Ag85A protein based on the confirmed gene sequence from NCBI. The results of the alignment of FbpA with NCBI can be seen in Figure 4. The results of the sequencing showed that there was 99% homology of the FbpA gene with NCBI.

The same results were obtained by Fihiruddin *et al.* 2020 that the homology of FbpA with *Mycobacterium tuberculosis* H37Rv was 99%. The presence of 1% of mutations could be caused by SNPs (Single Nucleotide Polymorphisms). *Mycobacterium tuberculosis* sequences undergoing SNPs were reported by Chitale *et al.* 2022. They published and identified 109 SNPs, 35 insertion-deletion, and 10 large regions of difference in M.tuberculosis H37Rv.

Cloning of FbpA into *Escherichia coli* BL21 host cells has been successful. Characterization of the recombinant clones by colony PCR and sequencing showed that the DNA inserted into the pET-32a vector was FbpA with a size of 900 bp.

4.4. Production of the Recombinant Protein Ag85A

Production of recombinant protein of Ag85A was carried out in *Escherichia coli* Bl 21. *Escherichia coli* is a commonly used cell for the production of recombinant proteins in experimental, medical and industrial applications. The induced lac promoter is one of the most commonly used promoters for heterologous protein expression. Isopropyl-β-D-thiogalactoside (IPTG) is an inducer that regulates the transcription activity of this promoter (Huygen 2014). IPTG has a negative effect on the growth of Escherichia coli due to IPTG, which is an analog of lactose and can inactivate the lac repressor (Law *et al.* 2002). Cell clones were lysed by sonication and characterization of Ag85A with SDS-PAGE.

The pET-32a(+) vector was designed for cloning and high-level expression of peptides fused to the thioredoxin 109aa Trx•Tag™ protein. This protein is grafted onto the recombinant protein to help the protein fold properly and prevent it from precipitating. Thioredoxin protein can be removed by adding enterokinase (Liu and Yang 2012). The molecular weight of Ag85A is 32 kDa, but there is an increase in molecular weight caused by the addition of a protein tag from pET 32a, which is 12 kDa so the molecular weight of the recombinant protein Ag85A is 44 kDa (Figure 5).

Cloning of FbpA into *Escherichia coli* BL21 and production of Ag85A has been successfully carried out with a size of 44 kDa. It is necessary to produce recombinant Ag85A protein from clinical isolates from other countries and isolates recorded in the Gene Bank so that Ag85A can be used as an antigen to complete the latent TB diagnostic test.

Acknowledgements

The authors would like to thank the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia and Hasanuddin University for funding this research through an Internal Research Grant in 2022.

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