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Original Research Article

Analysis of Secreted Proteins and Detergent Soluble Sonicates Of Mycobacterium Tuberculosis by Polyacrylamide Gel Electrophoresis

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ABSTRACT

Rapid and specific diagnostic methods and improved vaccines are considered a high priority to control tuberculosis. For advances in both these areas, the appropriate antigenic fractions of the bacterium are required. This study was done to compare and analyse the excretory-secretory proteins of Mycobacterium tuberculosis obtained from Sauton's liquid culture medium and detergent soluble sonicate (DSS) proteins from Lowenstein Jensen medium. The proteins were extracted from the standard strain, H37Rv of Mycobacterium tuberculosis and analysed using SDS-PAGE. The band patterns were similar with H37Rv DSS and H37Rv Sauton's proteins. However, sharper and thicker bands were seen with DSS protein, since the protein concentration was higher in DSS protein (2.227mg/ml) compared to Sauton's protein (2mg/ml). Therefore, Detergent soluble sonicate is a promising antigen source for diagnostic tests obviating the need for cumbersome and tedious procedures like filtration and dialysis for extracting the excretory-secretory proteins.

Key words: Mycobacterium tuberculosis, H37Rv, culture filtrate proteins, excretory-secretory proteins, detergent soluble sonicate

INTRODUCTION

Tuberculosis is a major global health problem and is the most frequent cause of death from a single infectious agent. BCG has been tried extensively as a vaccine for prevention of the disease, but with varying success. [1] The incidence of the disease has increased in recent years, mainly due to its association with human immunodeficiency virus and also due to the occurrence of multidrug resistance. [2]

The early diagnosis of tuberculosis plays an important role in disease control. Rapid and specific diagnostic methods should be available to initiate prompt treatment. Development of improved vaccines is considered a high priority in the effort to control tuberculosis. For advances in both these areas, the appropriate antigenic fractions of the bacterium are required. Further, study of proteins also helps in elucidating protein modifications and identifying drug resistance. [3]

Proteins secreted by M. tuberculosis are believed to mediate important biological functions by interacting with host cells, notably macrophages and are thus potentially important for virulence and pathogenesis. [4] Seibert and Munday were the first to provide evidence that the culture filtrate is a major repository of antigens involved in the protective immune response. [5] In comparison to the proteins of other sub cellular fractions of M. tuberculosis (cell

wall, cell membrane and cytoplasm), the culture filtrate proteins, as a family, are well defined in terms of function, immunogenicity and composition. [6] However, the slow growth rate of tubercle bacilli resulting in decreased yield of culture filtrate antigen was observed to be a major limitation in getting the required quantity of antigen for extensive diagnostic studies. Supplementation of suitable metabolic stimulants in culture medium of tubercle bacilli was explored to stimulate bacillary growth. [7-9] Thyroid hormone supplementation has been shown to be effective in accelerating various microbial growths including that of *Mycobacterium tuberculosis.* [10-12]

Secreted proteins are also considered to be key T-cell antigens of protective immune responses against M. tuberculosis. [13] Along with actively secreted proteins, the culture filtrate proteins possess somatic molecules that are released into the medium during replication or by autolysis. Advances in separating and probing techniques like immunoelectrophoresis, SDS-PAGE, and western blotting have provided useful tools to define several Mycobacterial antigens. [14]

This study describes protocols for extracting the excretory-secretory proteins and detergent soluble sonicate (DSS) proteins from the standard strain H37Rv of Mycobacterium tuberculosis and to compare and analyse these proteins by Polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

The standard strain of Mycobacterium tuberculosis, H37Rv was used for protein preparation. The strain was maintained on Lowenstein Jensen (LJ) slopes.

Two culture media were used for protein extraction, Sauton's medium, which is a chemically defined medium and thyroxine-supplemented Lowenstein Jensen (LJ) medium. For making thyroxine supplemented LJ medium, thyroxine tablets 100 µg each was powdered and added to LJ medium at a final concentration of 4µg/ ml.

For preparation of the Sauton's medium tap water was used instead of distilled water, since tap water contains trace amounts of different metal ions, which improves growth of Mycobacterium tuberculosis. The medium was allowed to remain in the autoclave after sterilization for cooling, after which the medium acquired a brown color which proved beneficial for the growth of M. tuberculosis. All procedures for protein extraction were done inside a Biosafety cabinet.

Isolation of culture filtrate (excretory-secretory/ES) protein from Sauton's culture [15]

Preparation of starter culture: 400ml Sauton's medium was prepared and 50 mL each was aliquoted into eight flasks. After autoclaving, the medium was cooled and 2-3 loopfuls of *H37Rv* grown on Lowenstein Jensen slope was inoculated to each of the above 50 mL flasks with Sauton's medium. The flasks were incubated with constant shaking in a shaking incubator at 37°C for 5 days.

Culturing of Mycobacterium tuberculosis for culture filtrate production: One litre each of Sauton's medium was prepared in four flasks and autoclaved. Two flasks each of the incubated starter cultures were added into each of the one litre of Sautons medium. 1 M phenyl methyl sulphonyl fluoride (PMSF) was made and 250 µL each was added to each of the bottles. The flasks were incubated with constant shaking at 37°C for 5 days.

Harvest of culture filtrate(Excretory secretory proteins): The culture medium was harvested after sterile filtration using Whatman No. 3 filter paper and membrane filters of porosity 0.44 µm, followed by 0.22 µm.

Precipitation of culture filtrate proteins: By combining ultrafiltration and ammonium sulfate precipitation, the filtrate was conveniently concentrated 100-200 times. Excretory- Secretory (ES) antigens were obtained after overnight dialysis. These proteins were labelled as Sauton's proteins. Sauton's proteins obtained were stored in

aliquots at -20°C with sodium azide (0.1 %). The concentration of protein in the preparation was determined by the Lowry method of protein estimation.

Extraction of M. tuberculosis *H37Rv*/clinical isolate antigens from LJ slopes [16]

2-week old cultures of *Mycobacterium tuberculosis H37Rv* grown on thyroxine-supplemented LJ slant was used. Ten loopfuls of the growth were scraped from the LJ slopes and inactivated using 5 mL of 5 % phenol for 1 hour at 4 °C to render them non-infectious. The cells were washed twice in normal saline and then suspended in 4 mL of 0.05 mol/L PBS (pH 7.2), containing a cocktail of protease inhibitors. The bacilli were sonicated with 30 second burst at 1 minute intervals for 30 min. The sonicate was incubated with 2 ml of sodium dodecyl sulphate (SDS) extraction buffer in boiling water bath for 5 minutes, followed by incubation at 4 °C for 24 hours. After centrifugation at 16,000 g at 4 °C for 20 minutes, the supernatant was separated. The supernatant was dialyzed against 0.01 mol/L PBS (pH 7.2) for 48 hours and the protein obtained was labelled as Detergent soluble sonicate (DSS) antigen. The concentration of protein in the preparation was determined by the Lowry method of protein estimation.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed using 12% gel. 30µl of 1: 10 diluted antigen preparation was loaded into each well. A low molecular weight protein marker in the range 14.3 kDa to 97.2 kDa from Takara was also loaded into one of the wells. Before loading the samples, a pre-run at 50V was made for 10 minutes. After loading the samples, the voltage was set at 75V and after the migration of the sample in the stacking buffer; the voltage was raised to 100V for separation.

RESULTS

The standard strain H37Rv was used for protein extraction. H37Rv was grown in 4 litres of Sauton's medium for 11days and then the filtrate was used for protein extraction. The quantity of protein obtained was 4mg each. Protein concentration in the isolate as determined by Lowry method of protein estimation was 2mg/mL.

Approximately 4mg DSS protein was obtained from H37Rv grown on five LJ slopes. Protein concentration in the isolate as determined by Lowry method of protein estimation was 2.227mg/mL, slightly more than in Sauton's protein.

The secretory proteins from Sautons and DSS proteins obtained from growth on LJ medium of H37Rv were subjected to Polyacrylamide gel electrophoresis.

Results of PAGE of Sauton's and DSS proteins

- Most of the predominant bands were visible in 66- 97kDa regions.
- The band patterns were similar with H37Rv DSS and H37Rv Sauton's proteins as seen in Figure 1.
- Sharper and thicker bands were seen with DSS protein. This may be because the protein concentration is higher in DSS protein (2.227mg/ml) compared to H37Rv
- (2mg/ml).

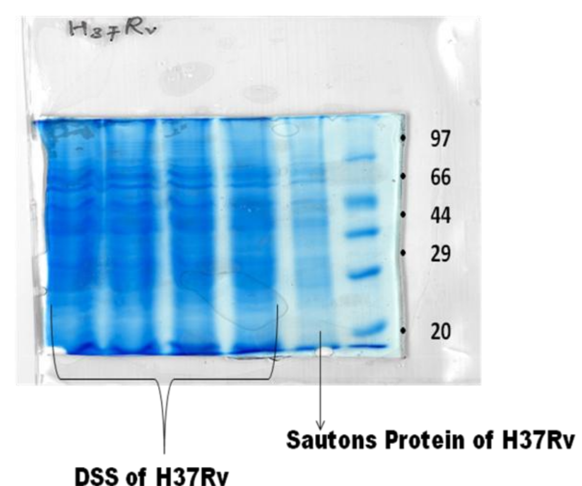

Figure 1: PAGE on H37Rv DSS proteins and H37RV Sauton's protein

DISCUSSION

The study of proteins of Mycobacterium tuberculosis has been of special interest in the field of tuberculosis research since certain secreted proteins interact with the host to promote virulence, while others may serve as biomarkers of infection. Mycobacterial proteins secreted in liquid media are known as important targets for early diagnosis of tuberculosis as well as candidates for protective immunity. [17,18]

M. tuberculosis secretes active culture filtrate proteins, which are missing in non-tuberculous Mycobacteria which serve as diagnostic markers. The availability of better diagnostic reagents from culture filtrates of *M. tuberculosis* such as more species–specific skin test antigens and serological markers has been a driving force in the efforts devoted to diagnosis of *M. tuberculosis*.

A crucial factor in protein extraction is complete solubilisation and denaturation of sample proteins. In an ideal procedure of sample preparation time should be kept as short as possible to avoid protein modifications and losses.

In this study, excretory-secretory proteins were extracted after growing the bacterium in a Sauton's liquid culture medium followed by procedures like centrifugation, filtration and ammonium sulphate precipitation. Detergent soluble sonicates were prepared from Lowenstein Jensen medium following sonication. Polyacrylamide gel electrophoresis of DSS proteins shows greater resolution in comparison to Sauton's protein. Hence, detergent soluble sonicate is a promising antigen source for diagnostic tests obviating the need for cumbersome and tedious procedures like filtration and dialysis involved in extraction of Mycobacterium tuberculosis excretory- secretory proteins.

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