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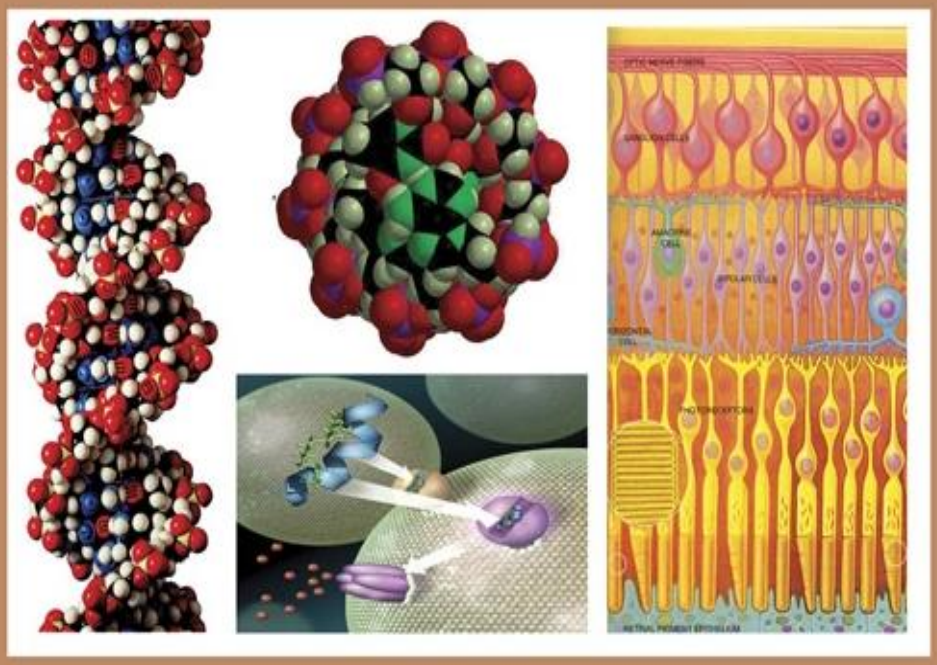

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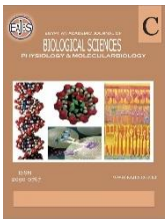

Isolation, Purification and Identification of CFP29 from *Mycobacterium tuberculosis* H37Rv culture filtrate proteins

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ABSTRACT

Tuberculosis (TB) continues to be a serious worldwide health issue. Human tuberculosis (TB) is the commonest cause of mortality from one infectious agent, with eight million new cases and two million fatalities each year. In many animal models of TB, proteins isolated from the culture filtrate of Mycobacterium tuberculosis promote protective immunity. The extracellular proteins of *Mycobacterium tuberculosis* were isolated, purified and identified in the current study. *M. tuberculosis* H_{37}Rv was cultured in Souton's medium and the extracellular proteins were isolated employing an ion-exchange column chromatography before their purification and characterization using SDS- PAGE, western blotting and N- terminal sequencing. CFP29 was identified and purified in M. tuberculosis H37Rv culture filtrate. However, further characterization of this protein is needed to be utilized as an effective T-cell antigen produced from culture filtrates.

INTRODUCTION

The World Health Organization named the tuberculosis epidemic a worldwide emergency situation many decades back still Tuberculosis (TB) continues to be a serious worldwide health issue (Organization 1992). Given the inconsistency of the Mycobacterium Bovis bacillus Calmette-Gue'rin (BCG) vaccine's performance (Fine 1989), the discovery of a better TB vaccine is of paramount importance.

Immunity against tuberculosis is derived through the immune system's cellular branch. Early studies show that culture filtrate proteins of Mycobacterium tuberculosis are efficiently identified by T cells engaged in tuberculosis protection (Andersen 1994; Orme *et al.,* 1992). Culture filtrate proteins, when used as experimental subunit vaccinations, produce effective acquired cellular resistance to the infection (Andersen 1994; Pal and Horwitz 1992; Roberts *et al.,* 1995). M. tuberculosis culture media contains several important secretory proteins emerging as culture filtrate proteins (CFPs). M. tuberculosis CFP contains around 200 proteins (Bahk *et al.,* 2004; Sable *et al.,* 2005; Sonnenberg and Belisle 1997). Because several of these proteins are linked to cells, the CFP characterization is fundamental to any application diagnostic or preventative application.

Individual protective antigens might be incorporated for vaccine development as a subunit vaccine, or as recombinant Mycobacterium bovis- bacillus Calmette-Gue'rin including key CFP proteins.

However, so far there is relatively little information available on specific antigens identified by T cells.

Division of secretory M. tuberculosis proteins according to low molecular mass has led to the identification of two areas that binds strongly to gamma interferon (IFN-g) and in turn upregulate IFN-G production in T cells especially during the early phase of MTB infection (Andersen *et al.*, 1995; Andersen and Heron 1993; Boesen *et al.*, 1995; Pollock and Andersen 1997).

ESAT-6 (6-kDa secretory antigen), member of 5 to 12 kDa low-molecular-mass fraction is a key antigenic target (Andersen et al., 1995; Jha *et al.*, 2020), while MPT44, MPT45, and MPT59, members of antigen 85 complexes found in 24 to 36 kDa molecular mass fraction, are other important T-cell antigens. Accumulating evidence suggest that there are other key proteins including T-cell antigens present in CFP (Dhar *et al.*, 2016; Nagai *et al.*, 1991; Rambukkana *et al.*, 1993).

The goal of this work was to identify and characterize a 29-kDa T-cell antigen of M. tuberculosis protein that is an antigenic target of memory effector cells of the immune system. Presently, CFP29 was successfully identified and purified as a result of this research.

MATERIALS AND METHODS

Bacterial Strains and Media:

Mycobacterium tuberculosis H37Rv (ATCC-27294) ($2x10^9$ cfu/ml) was cultivated in a modified Sauton medium till the late log phase. The culture filtrate proteins (CFP), containing high extracellular growth of *M. tuberculosis* were collected by centrifugation at 4°C for 20 minutes at 10,000g, and the bacterial pellet was washed in 100 ml of PBS (pH 7.2). The bacterial growth, as well as the protein content in the supernatant, was periodically determined spectrophotometrically at A_{580} after inactivating an aliquot of supernatant with formaldehyde.

Culture filtrate protein (CFP) isolation M. tuberculosis H37Rv was utilized to isolate proteins secreted in the culture filtrate after a 6-week growth period. The whole growth was centrifuged at 15,000 g for 30 minutes at 4°C in a Beckman centrifuge to extract the Mycobacterial cell mass. The supernatant was collected and kept in 50 mL sterile containers at –20°C until needed. The filtrate was then treated to ammonium sulphate precipitation after a significant volume of culture filtrate was collected. Protein was recovered from various batches and harvested at the same time point. The culture filtrate supernatant was precipitated with three concentrations of ammonium sulphate, namely 50, 75 and 80% saturated ammonium sulphate, in order to optimize the concentration of ammonium sulphate giving the highest protein yield. Subsequently, the precipitant samples were centrifuged at 18,000g to remove the supernatant and collect the precipitated protein as pellets. Bradford method and SDS-PAGE were used to determine the yield from various precipitation methods, and to examine the results, respectively. The culture supernatant was eventually precipitated with 80 percent saturated ammonium sulphate at 4°C overnight after optimizing harvesting and precipitation conditions.

Dialysis of CFP Precipitant:

In an ultracentrifuge, the precipitate was centrifuged at 12,000 g, 18,000 g, 28000 g, and 90,000 g for 30 minutes at 4°C. The pellet was recovered and dissolved in Phosphate Buffer (10 mM) (pH-7.2). The substance was dialyzed against the PB (10 KDa membrane) (10mM, pH 7.2). Every four hours, the buffer was replaced, and six to seven changes of 10mM PB were administered to guarantee full dialysis. Meanwhile, the dialysis buffer's conductivity was measured using a conductivity meter until it equaled that of 10mM PB. The total

protein, transferred to a new sterilized glass tube and maintained at -20^{o}C once the membrane was taken from the dialysis buffer.

Purification of Individual:

Proteins were isolated from the total culture filtrate protein using the column chromatographic method as follows (42). DEAE–Sepharose CL-6B (anion exchange) packed gel was equilibrated using 10 mM Tris HCl buffer containing 3% methylcellose. Approximately 100 mg of the concentrated culture filtrate proteins already dialyzed against Tris buffer was placed onto the column, at 4°C for 30 minutes to allow the proteins to bind to the gel matrix. The column was rinsed three times with equilibrating buffer to achieve maximal protein binding to the column and to eliminate unbound protein from the gel, which was finally washed with the equilibrating buffer. In the equilibrating buffer, a linear gradient of 50- 300mM NaCl was used to elute the bound protein from the column. On a spectrophotometer, the absorbance was measured at 280nm using equilibrating buffer as a blank. The pure protein was subsequently concentrated using an Amicon unit with a 5kDa cut-off filter, and the Tris salt was removed by dialysis against PBS overnight at 4°C. The Bradford technique was used to determine the protein concentration of each pooled fraction, and SDS-PAGE was used to examine the protein profile (43).

Purification of Individual Protein from the Pooled Fraction:

Low molecular mass fraction of CFP was further isolated in pure form by multielution method using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, after electrophoresis, the band of interest was identified and excised using scalpel to excise the gel strip that included the molecular weight marker. One lane of the protein sample was silver-stained for the reference lane, and the remainder of the gel was placed on a moist glass plate at 4^{0}C. To find and clip off a part of the unstained gel that matches with the protein band of interest in the reference strip, the stained strip was aligned with the unstained gel. 1 mL of elution buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) was added to the excised gel fragments in microcentrifuge tubes. The excised gel pieces were crushed using a clean pestle and incubated on a rotary shaker at 30°C overnight before centrifuging at 10,000 x g for 10 min. The supernatant was carefully collected into a new microcentrifuge tube, a portion of which was tested for the presence of protein using SDS-PAGE.

N-terminal Sequencing:

Purified protein was blotted to PVDF membranes after SDS- PAGE and subjected to N-terminal sequence analysis by Edman degradation using Abi 494 Procise Protein Sequencer following the manufacturer's protocol.

RESULTS

Profile of Proteins Released in The Culture Medium During Growth:

A microbe releases a variety of chemicals into the medium during several growth phases. Metabolic by-products, lipids, carbohydrates, and proteins are examples of these compounds. The yield of total protein in the culture filtrate was determined using SDS-PAGE over the whole growth period to investigate proteins released by actively growing cells and those released following the lysis of bacterial cells into the culture medium during different growth phases. SDS-PAGE was used to assess the molecular weight of culture filtrate protein (CFP) by comparing relative mobility of standard molecular weight markers, yielding molecular weights of 6kDa, 13 kDa, 22kDa and 29kDa respectively (Fig. 1).

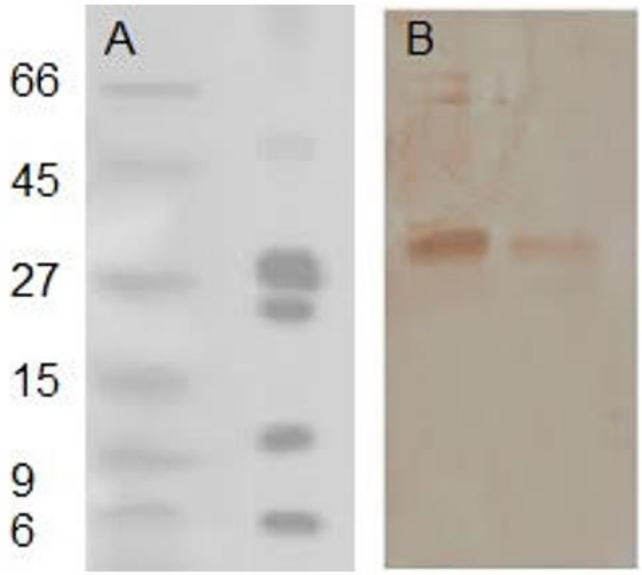

Fig. 1: Culture filtrate protein (CFP) analysed by SDS PAGE (A) and purified CFP29 product (B).

Purification of 29-kDa Antigen:

Culture filtrate proteins were loaded on DEAE–Sepharose CL-6B gel for anion exchange chromatography before the 29- Kda protein was multieluted and checked for purity using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

N-terminal Sequencing:

The microsequencing of 29- Kda band excised from PVDF membrane confirmed the presence of CFP29 antigen with N- terminal sequence MNNLYRDLAPVTEAAWAEIELEAAR.

DISCUSSION

Tuberculosis is a disease that affects people all around the world. MTB bacterium infected 1.7 billion people in 2018, accounting for around 23% of the world's population. TB is the world's greatest infectious disease killer, killing 1.5 million people each year. M. tuberculosis is carried latently by over a third of the global population, with 5% developing active illness during the first year of infection (Dye *et al.,* 1999; Kaufmann and McMichael 2005; Organization 2004; Reid *et al.,* 2019).

Furthermore, underlying diseases, immunosuppressive medication, malnutrition and most importantly, coinfection with the human immunodeficiency virus (HIV) significantly enhance the likelihood of reactivation (Bezerra *et al.,* 2009; Philips and Ernst 2012). Because treatment for latent TB infection can delay the development of active illness, a protein's capacity to identify antibodies present during subclinical disease is just as significant as its sensitivity in detecting antibodies generated during active tuberculosis.

Several M. tuberculosis antigens have been discovered to be beneficial in the serodiagnosis of clinical illness (Lange and Mori 2010; Pottumarthy *et al.,* 2000; Silva *et al.,* 2016; Spigelman 2007). Traditional techniques (smear and culture) are easier and less costly than modern molecular diagnostic procedures based on nucleic acid amplification, such as PCR. Many Mycobacterial antigens, such as cellular extracts, proteins (Tiwari *et al.,* 2005),(Zacharia *et al.,* 2010) polysaccharides (Yu *et al.,* 2012), DNA (Labugger *et al.,* 2017), RNA (Huang *et al.,* 2018), glycolipids (Nabeshima *et al.,* 2005; Tiwari et al., 2005), and other biomolecules, have been investigated using serological techniques (Donoghue 2017; Khan *et al.,* 2018).

Secretory protein antigens of Mycobacterium tuberculosis, which are generated by the actively developing M. tuberculosis culture and induce the desired immunological response, have recently received a lot of attention (Andersen *et al.,* 1991; Bekmurzayeva *et al.,* 2013; Delogu and Brennan 2001; Mustafa *et al.,* 2006). These proteins, also known as culture filtrate proteins (CFP), have been shown to induce robust immune responses in humans and animals infected with Mycobacterium TB / Mycobacterium bovis (Maue *et al.,* 2005; Samten *et al.,* 2009; Wedlock *et al.,* 2002). Secretory proteins, on the other hand, are well identified early in the course of M. tuberculosis infection in several species (Dorhoi *et al.,* 2011; Ganguly *et al.,* 2008a; Ganguly *et al.,* 2008b; Kassa *et al.,* 2012).

They have been recommended as a potential for in vitro TB diagnosis since they have distinguished TB patients from both BCG vaccinated and M. avium patients (Frigui *et al.,* 2008; Lien *et al.,* 1999; Ulrichs *et al.,* 1998). A combination of secretory protein (CFP) antigens now being researched may give information that may help researchers better understand the host immune response.

Current efforts were focused on the discovery of secretory protein antigens and the goal of this work was to identify and characterize CFP29 T-cell antigen, a 29-kDa M. tuberculosis protein, robustly recognized by mouse memory effector cells. Presently, the characterization of Mycobacterium tuberculosis H37Rv CFP29 secreted during the logarithmic phase was done. The 29 kDa protein was obtained in pure form using column chromatography, characterized by SDS PAGE (low molecular weight proteins) and the individual protein band eluted from the gel before N- terminal sequencing.

T cells implicated in tuberculosis protection have been shown to detect Mycobacterium tuberculosis culture filtrate proteins, indicating that immunity against TB is mediated through the immune system's cellular branch (Andersen 1994; Weinreich Olsen *et al.,* 2000). Culture filtrate proteins produce effective acquired cellular resistance to infection when used as experimental subunit vaccines (Pal and Horwitz 1992; Tundup *et al.,* 2008). Individual protective antigens might be utilized in the future for the development of a subunit or recombinant vaccine like BCG expressing important CFP proteins.

Extracellular M. tuberculosis secretory proteins further subdivided into two parts according to their molecular weights namely low and moderate includes key T cells antigenic targets which bind and induce string proliferation of gamma interferon (IFN-g) especially in the early phase of infection (Andersen et al., 1995; Jasenosky *et al.,* 2015).

Culture filtrate proteins include a few T-cell antigens namely ESAT-6, MPT44, MPT45, and MPT59 which are part of the antigen 85 complex (Cunha 2005). CFP29 is a component of M. tuberculosis CFP's extraordinarily stimulatory 24- to 36-kDa area. In many animal models of tuberculosis disease, memory effector cells detect antigens in this area (Cunha 2005; Hasløv *et al.,* 1995). Furthermore, Th1 cells in human TB patients with mild illness highly detect this area (Boesen et al., 1995; Hussain *et al.,* 1995). Enough native CFP29 for a partial biochemical characterization was extracted, despite the fact that CFP29 is present in CFP in extremely small levels. CFP29, as a T-cell antigen, stimulates the release of substantial amounts of IFN-g from memory effector cells during the recall of protective immunity in a mouse model of tuberculosis infection (Rosenkrands *et al.,* 1998). It's been proposed that the extracellular proteins most important for developing new vaccines are those found in large numbers in culture filtrates (Harth *et al.,* 1997).

M. tuberculosis intracellular

development in macrophages results in a considerable shift in protein expression when compared to extracellular growth in culture media (Kahnert *et al.,* 2006; Lee and Horwitz 1995; Yuan *et al.,* 1996). The discovery of the highly reactive T-cell antigens CFP29 and ESAT-6 (Lim *et al.,* 2004; Nayak *et al.,* 2015; Rosenkrands et al., 1998), which are both present in tiny levels in culture filtrates, shows that key antigens can be extracted in sufficiently enough quantities for further diagnostic or preventative application.

Conclusion

Several investigations have found that Mycobacterium TB culture filtrate proteins increase protective immunity. The extracellular CFP29 of Mycobacterium TB, which was isolated, purified, and identified in this work, has the potential to be used as a T-cell antigen.

Conflict of Interest: The Author declares that there is no conflict of interest.

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