Original Article

Zahedan Journal of Research in Medical Sciences

Journal homepage: www.zjrms.ir

Isolation and Purification of Low Molecular Weight Proteins from Liquid Culture for Mycobacterium Tuberculosis by Chromatography

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Article information

Article history:
Received: 27 Nov 2011
Accepted: 22 Oct 2012
Available online: 18 Nov 2012
ZJRMS 2013; 15(5): 35-38

Keywords:
Mycobacterium tuberculosis
Secretory proteins
chromatography

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Abstract

***Background*:** Tuberculosis (TB) is a disease caused by a bacterium called *Mycobacterium tuberculosis*. *M. tuberculosis* has different molecular weight secreted antigens. Low molecular weight proteins secreted into the culture medium by *M. tuberculosis* are thought to play an important role in the development new TB diagnostic tests and new vaccines against tuberculosis. In this report, we describe isolation and purification of low-molecular-weight proteins secreted by *M. tuberculosis*.

***Materials and Methods*:** Initially by biphasic medium, bacteria from Lowenstein-Jensen solid medium transferred to a Dorset-Henley liquid medium and After 6 weeks of growth, the bacteria with a 0.22 micron filters of liquid medium containing secreted proteins were isolated and the secreted proteins was precipitated by ammonium sulfate. Protein concentrations were determined by using the lowry protein assay. Then low molecular weight proteins were purified by Sephadex-G75 gel chromatography and we studied purification of low molecular weight proteins by Coomassie-Blue stained SDS-PAGE.

***Results*:** The results showed that low molecular weight secreted proteins purified from *M. tuberculosis* strain DT. Also, low molecular weight proteins made up approximately 65.3% of total proteins.

***Conclusion*:** this study demonstrated that without break down of bacteria bodies can be purified low molecular weight secreted proteins from *M. tuberculosis* liquid medium by Sephadex-G75 gel chromatography.

Introduction

Tuberculosis disease has global distribution. This infectious disease is contagious and is transmitted easily from one person to another and it is evaluated that one third of world population is polluted to *Mycobacterium tuberculosis* [1]. The prevention and on time diagnosis of disease is the best method to fight this disease. BCG (Bacillus Calmette-Guerin) is only vaccinate can prevent the spread of tuberculosis disease and cannot product complete immunity in the people to prevent the tuberculosis disease [2].

Tuberculosis skin test is a standard test to determine tuberculosis latent infection [3] but the result of this test is liar in the people who received BCG vaccine [4, 5]. So far, different antigens have been identified in the proteins secreted from *M. tuberculosis* which can stimulate the cellular immunity system. The antigens with the molecular weight lesser than 15 kD can be mentioned which are strong stimulators of INF-γ secretion. The most important protein of this family is 6-kDa early secretory antigenic target with molecular weight of 6 kD and its encoding gen name is esxA [6-8]. This gen has been placed in genomic region of RD-1 (Region of Difference-1) and this region has been kept in all pathogenic Strains and does not include non pathogenic Strains and *M. bovis* BCG, so this antigen exists only in *M. tuberculosis* pathogenic type. The mentioned antigen is a suitable indicator to determine Mycobacterium infections and finally is a suitable antigen to use in the diagnosis methods of tuberculosis disease [9-11].

According to the immunity response to these proteins in all people infected to this disease and important role in the competition to the immunity system, it seems that these proteins can be suitable candidate to be used as vaccination effective against tuberculosis. The small size of these peptides caused their structure not be changed in different stages of processing in phagosome and finally they were introduced naturally to $CD4^+$ cells [12, 13]. According to the characteristics of above proteins, they can be used in the studies of producing modern diagnosis test or producing modern vaccinate against tuberculosis.

Materials and Methods

In this study, Mycobacterium tuberculosis standard strain DT with number ATCC 35810 was prepared from the Tuberculin and Mallein Production & Research Department in Razi Vaccine and Serum Research Institute

and was cultured on Lowenstein-Jensen solid medium within eight weeks. Then the bacterium was transmitted to the Dorset-Henley Liquid medium by biphasic medium of potato-Dorset-Henley. After 6 weeks bacterium growth on the liquid medium, fehrenbach dish containing medium and bacterium within 20 minutes in the temperature 80°C to kill bacterium incubation [14].

Then the incubation medium was passed from 0.22 µm filter after passing the liquid from the EKS filter, few drops of filtered liquid was transported to Lowenstein-Jensen solid medium to assure complete omit of bacterium from the liquid medium. Precipitating method by ammonium sulfate was applied to isolate the proteins from the liquid medium. 561 g ammonium sulfate in lieu of every liter was added from the liquid medium to do this work and then the above solution was mixed during 24 h in the temperature 4°C. The solution was centrifuge in 2500 rpm and its supernatant was discarded. Then phosphate buffer (pH=7) was added to the residue precipitation. Dialysis method was used to omit the salts in the protein solution.

The protein solution was poured inside dialysis bag and dialysis bag was placed 72 h in the distilled water. To assure the exit of salt from the protein solution by conductometer, the conductivity of above solution was studied. The Concentration level of protein solution was determined by the Lowry protein assay. Before doing chromatography, at first protein solution was condensed by using polyethylene glycol. To do this the protein solution with 150 ml volume was poured in the dialysis bag and dialysis bag was placed in a dish containing polyethylene glycol. After 24 h, protein solution volume inside dialysis bag was reach to 15 ml and the residue of the water in the protein solution was absorbed by polyethylene glycol.

Chromatography column with the height 150 cm and diameter 2 cm in which the packed volume was 470 ml was used as stationary phase and ammonium acetate buffer 0.1 molar was used as mobile phase. The volume of protein solution was 5 ml and its Concentration was 15 mg/ml. After doing chromatography, the absorption spectrum of protein solutions was read by spectrophotometer in the wavelength 280 nm and its chromatograph was drawn and fraction isolation was done by chromatograph. SDS-PAGE method with 17% gel was used to confirm the proteins with low molecular weight. Multicolor low range protein markers of Fermentas company was used in this study.

Results

The bacterium transport was done from Lowenstein-Jensen solid medium to Dorset-Henley Liquid medium by biphasic medium of potato-Dorset-Henley. According to this fact that *M. tuberculosis* is aerobic and should grow on the liquid medium surface and the direct transport of bacterium colony from the solid medium to the liquid medium, cause the bacterium to be drown, so the bacterium was transported to Dorset-Henley Liquid medium by biphasic medium after habituating the bacterium to the liquid medium. The beginning of bacterium growth on the solid medium to the end of bacterium growth on the Liquid medium was 5 months.

After passing the Liquid medium from EKS filter and then filtering 0.22 µm and cultivating the filtered liquid on the solid medium, to assure the complete omit if bacterium incrustation from the liquid medium containing the proteins, filtration was confirmed and no colony was produce on the above culture.

After dialysis of protein solution, to assure the exit of salt from the protein solution, and after dialysis by conductometer, the above solution conductivity was studied. If the number conductometer indicates lesser than 1000 µs/cm, it means the salt has been come out from the solution and dialysis has been done correctly and here the system indicates 280 µs/cm. After isolation of bacterium mass from the liquid medium and precipitating the proteins in the medium, the concentration level of protein in the precipitated solution (1.48 mg/ml) was obtained by Lowry protein assay. After doing chromatography and drawn chromatograph, two picks were determined (Fig. 1)

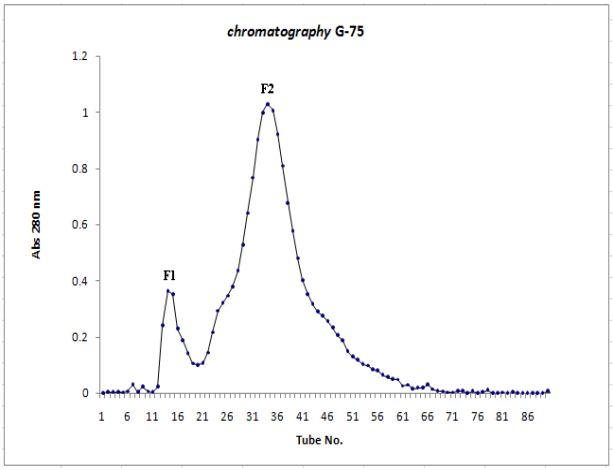

Figure1. Chromatograph of exit of proteins in the culture filtrate from Sephadex-G75 column

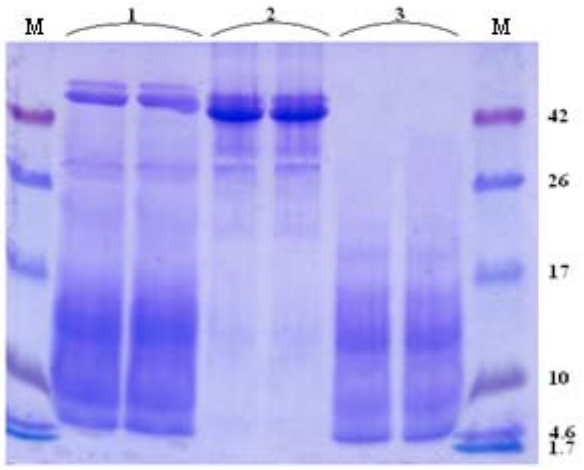

Figure2. The profile of the exited different proteins of Sephadex-G75 column using Coomassie- Blue stained SDS-PAGE

and studying the protein concentration in everyone of fractions determined that out of total 75 mg protein, 14 mg protein in fraction 1 and 49 mg protein in fraction 2 and 12 mg protein were omitted. So, 65.3% out of total secreted proteins were the proteins with low molecular weight.

Conducting Coomassie-Blue stained SDS-PAGE, purification of proteins with low molecular weight was confirmed, and the bands indicated that fraction 2 in the limit of 6-15 kD contains the proteins with low molecular weight and also the high concentration of protein in limit of 14 kD is ESAT6/CFP10 protein complex which is one of the most important *M. tube*rclosis secreted proteins (Fig. 2).

Discussion

During the recent years the antigens in the *M. tuberclosis* culture filtrate were studied widely. Among these, the antigen with molecular weight lesser than 15 kD can be mentioned which are strong stimulator for secretion of INF-γ and their most famous one is ESAT6 [6-8] and purifying these proteins plays important role in the studies for prevention and diagnosis of tuberculosis disease.

Anderson and Marien purified ESAT6 from *M. tuberclosis* [15, 16]. They used *M. tuberclosis* strain H37RV to purify ESAT6 family. Also, they used Sauton liquid medium to grow bacterium and then purified the protein. Sauton medium needs Albumin to grow *M. tuberclosis*, otherwise, the bacterium will not grow in that medium. The existence of albumin in the culture increased purification stages to omit albumin from culture containing the proteins secreted from *M. tuberclosis*. Dorset-Henley liquid medium was used in the present study to prepare tuberculin that it is a medium without added protein. So, passing from several stages to omit albumin was not needed and this caused the purification stages to be reduced [15, 16].

Also bacterium mass was destroyed by physical and chemical methods such as Sonication and heat to purify one protein from the bacterium. These processes cause the bacterium to be released in different parts of bacterium mass to the culture and damage the structure and performance of considered protein. According to the lack of secretion signal in encoding gen of these proteins [17], these proteins can be secreted in the culture without need to the special factor, so the proteins secreted in the culture were purified in this study without destroying the bacterium mass. Also, according to this fact that the bacterium incrustation was not destroyed, EKS filter and 0.22 µm filter were used to omit the bacterium from the culture before precipitation (that to assure complete omit of bacterium from the liquid medium, few drops of filtered liquid was transported to Lowenstein-Jensen solid medium).

The important point about these proteins is low expression of these proteins under in vitro conditions [12]. To obtain these proteins, cloning methods and gene expression were used [18] but the proteins which are recombinant do not have natural proteins abilities [16]. The bacteria were cultured in high volume of liquid culture medium in this study to purify the natural proteins according to low induction of these proteins in the culture medium in vitro condition.

In a study conducted by Mohammadi et al., the volume of primary solution in both precipitating methods was equal and the protein concentration obtained from the precipitating with ammonium sulfate is greater than precipitating methods with TCA, so ammonium sulfate was used in the present study to precipitate the proteins in the culture [19].

Lowry protein assay was used to determine the protein concentration. Due to the high protein concentration in the solutions, Lowry protein assay was more suitable than Bradford protein assay because Bradford protein assay is suitable to measure the biological amounts in microgram and nanogram/milliliter and is not used to measure in high amounts. Also, due to this fact that kjeldahl protein assay is a time-consuming and old method, Lowry protein assay is a suitable methods to measure the protein [19].

Our methods in this study is an applied methods to purify the natural proteins from Dorset-Henley liquid medium and decrease the purification stages which reducing the purification stages cause the structure and natural performance of protein to be damaged during different stages of purification and time and cost to be decreased.

Acknowledgements

All personals of Razi Vaccine and Serum Research Institute especially Tuberculin and Mallein Production & Research Department are acknowledged.

Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

Funding/Support

Razi Vaccine & Serum Research Institute, Karaj

References

1. Baumann S, Nasser-Eddine A, Kaufmann SH. Progress in tuberculosis vaccine development. Curr Opin Immunol 2006; 18(4): 438-48.
2. Barreto ML, Pereira SM, Ferreira AA. BCG vaccine: Efficacy and indications for vaccination and revaccination. J Pediatr (Rio J) 2006; 82(3 Suppl): S45-54.
3. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, et al. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Res Vet Sci 2006; 81(2): 190-210.
4. Ravn P, Munk ME, Andersen AB, et al. Prospective evaluation of a whole-blood test using Mycobacterium

tuberculosis-specific antigens ESAT-6 and CFP-10 for diagnosis of active tuberculosis. Clin Diagn Lab Immunol 2005; 12(4): 491-6.
5. Targeted tuberculin testing and treatment of latent tuberculosis infection. American Thoracic Society. MMWR Recomm Rep 2000 9; 49(RR-6): 1-51.
6. Brodin P, Rosenkrands I, Andersen P, Cole ST, Brosch R. ESAT-6 proteins: Protective antigens and virulence factors? Trends Microbiol 2004; 12(11): 500-8.
7. Yin Y, Li H, Wu S, et al. Hepatitis B virus core particles displaying Mycobacterium tuberculosis antigen ESAT-6 enhance ESAT-6-specific immune responses. Vaccine 2011; 29(34): 5645-51.
8. Farshadzadeh Z, Sankian M, Yousefi F, et al. Cloning, expression and purification of early secretory antigenic target 6kDa protein (ESAT-6) of Mycobacterium tuberculosis. Sci Med J Ahwaz Jundishapur Univ Med 2010; 3(2): 53-60.
9. Abdallah AM, Gey van Pittius NC, Champion PA, et al. Type VII secretion—mycobacteria show the way. Nat Rev Microbiol 2007; 5(11): 883-91.
10. Andersen P, Munk ME, Pollock JM and Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet 2000; 356(9235): 1099-1104.
11. Harboe M, Oettinger T, Wiker HG, et al. Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. Infect Immun. 1996; 64(1): 16-22.
12. Talaat AM, Lyons R, Howard ST and Johanston SA. The temporal expression profile of Mycobacterium tuberculosis infection in mice. Proc Natal Acad Sci USA 2004; 101(13): 4602-4607.
13. Lewinshon DM, Grotzke JE, Heinzel AS, et al. Secreted proteins from Mycobacterium tuberculosis gain access to the cytosolic MHC class-1 antigen-processing pathway. J Immunol 2006; 177(1): 437-442.
14. Alito A, Mcnair J, Cirrin RM, et al. Identification of Mycobacterium bovis antigens by analysis of bovine T-cell responses after infection with a virulent strain. Braz J Med Biol Res 2003; 36(11): 1523-1531.
15. de Jonge MI, Pehau-Arnaudet G, Fretz MM, et al. ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. J Bacteriol 2007; 189(16): 6028-6034.
16. Sorensen AL, Nagai S, Houen G, et al. Purification and characterization of a low-molecular-mass T-cell antigen secreted by Mycobacterium tuberculosis. Infec Immun 1995; 63(5): 1710-1717.
17. Pym AS, Brodin P, Majlessi L, et al. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med 2003; 9(5): 533-539.
18. Syhre M, Chambers ST. The scent of Mycobacterium tuberculosis. Tuberculosis 2008; 88(4): 317-23.
19. Mohamadi A. Research project of evaluation and preparation different methods PPD at different stages. Department of Education and Research Publication of Agricultural 1378.

Please cite this article as: Sadeghi D, Mosavari N, Rafiee B, Mohamad-Taheri M, Dashtipour S, Zare A, Tebyanian M, Rabii H. Isolation and purification of low molecular weight proteins from liquid culture for Mycobacterium tuberculosis by chromatography. Zahedan J Res Med Sci (ZJRMS) 2013; 15(5): 35-38.