KOR. JOUR. MICROBIOL. December, 1986 p. 377-384 Vol. 24, No. 4

Serological Activity of Fractions of Mycobacterial Antigens

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항산성균 항원분획의 혈청학적 활성

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Abstract: A study on the production of mycobacterial antigens has been made in order to improve immunological reactivity and specificity, which have long been explored for the better use of immunological diagnosis of mycobacterial infections. Instead of culture filtrate, cell extract was used as a starting material for the production of antigens in this study. Cell extract was fractionated though several steps such as salting out, gel filtration and ion exchange column chromatography and reactivity and specificity of the fractions so produced were evaluated by the various serological methods. The result showed that the species-specific antigenic components distributed mostly in the fractions, Tc of *M. tuberculosis*, Kc of *M. kansasii*, Sa of *M. scrofulaceum*, Aa of *M. avium* and Fa, Fb, Fc (FF1) of *M. fortuitum*, which were fractionated by ion exchange column prior to concentrating by salting out and molecular sieving.
Key Words: mycobacterial antrigens, *Mycobacterium tuberculosis*, *Mycobacterium fortuitum*.

Tuberculin has long been used to identify tuberculous infection in the community since it was introduced by Robert Koch. However tuberculin was suffered from the lack of specificity, in other words it comprised large amount of cross reactive substances, which present difficulty in the interpretation of immunological test results. In the 1930's to 1940's Fiorence Seibert had reported purified protein derivative (PPD) prepared from OT with ammomium sulfate or trichloroacetic acid (TCA) precipitation (Seibert *et. al.*, 1932, 1934, 1941, 1949). Her pioneering study still serves as the bench mark for all efforts to purify mycobacterial antigens. However, as they are autolytic products and subjected to continuous enzymatic alteration and degradation during incubation, the antigenic composition of culture filtrate can vary markedly, not only between strains but also identical cultures of the same strains. It is also known that the selection of heat-killed cultures as source of antigen influence to a great extent on the following purification study because considerable protein denaturation occurs when antigenic proteins are subjected to heating.

In other studies, bacillary extracts have been found to be suitable materials for immunoserological study because it may be less likely to incur denaturation or degradation than are

those in filtrates of cultures, although Daniel *et. al.* (1970) reported that there were no significant differences in antigen composition detectable between culture filtrates and cell extracts.

Some investigators reported that the total yield from bacillary extracts were considerably less than from filtrates. Yoneda (Yoneda *et. al.*, 1965), Daniel (Daniel *et. al.*, 1978), observed that the antigens produced from culture filtrates which had been highly purified to eliminate the cross-reactivity, was too small to use practically. Castelnuovo and his colleague (Castelnuovo *et. al.*, 1964) found more antigens in the cell extracts than in the culture filtrates.

Despite the great progress in mycobacterial studies owing to the improvement of biological and immunological analytic methods, still well standardized preparations possessing fairly well antigenicity and specificity are not available for general use of diagnostic purpose. Indeed, the results of many physicochemical studies suggest that still there are a considerable molecular heterogenicity in most of mycobacterial antigens developed and introduced recently. The purpose of the present study was to isolate fairly specific antigenic constituents of the various mycobacterial species which could be used as sensitin for skin tests or for other immunological studies.

MATERIALS AND METHODS

Mycobacterial cell culture and crude antigen preparation

Five reference strains of mycobacterial species (*M. tuberculosis, M. kansasii, M. scrofulaceum M. avium,* and *M. fortuitum)* were grown as a surface pellicle on sauton's medum at 37℃. After 1 week incubation for *M. fortuitum,* and 6 to 8 weeks for others, cultures were killed with 1% phenol solution for one day at 37℃. Then bacilli were harvested and washed three times with saline. Cell mass was resuspended on the two volume of Tris-buffer (50mM Tris-Cl, 5% glycerol 0.01% merthiolate, pH7.6) and disrupted by the ultrasonicator (Fisher Sonic Dismembrator, Model-300). First , cells were disrupted on the cup-shaped transmitter 3 times for 10 miniutes at the 80% of maximum output in the ice-chilled conditions. This partially disrupted bacillary homogenates were sonicated again using spindle-shaped transmitter at the 80% of maximum output. More than 99% of cells stained by Ziehl-Nelson's method were shown disrupted.

Supernatents after centrifugation (Sorvall GA, 10,000 rpm for 30 minutes) were used as a crude cell extract, and parts of them were stored at −70℃ in 50% glycerol, which can be used after dialysis.

Antisera production

About 5g of cell mass suspended in saline were disrupted by the ultrasonicator. Small amounts of them were taken and freeze-dried in order to measure dry-weight.

Freeze dried bacillary homogenate prepared by ultrasonifier was suspended to 80mg/m*l* in saline and then mixed with the same volume of Freund's incomplete adjuvant. The 20mg of bacillary homogenate was injected subcutaneously in each site of inguinal areas of two rabbits weighing approximately 3kg. After 3 weeks from immunization, the booster injections were followed at one week intervals until no more increase of antimycobacterial antibodies was observed. Animals were bled and serum separated. Hyperimmune sera so produced were pooled, distributed in a small aliquot per vial, and stored at −20℃. Sera were thawed at 4℃ prior to use.

DEAE-Cellulose Column Chromatography and Ammonium Sulfate salting-out.

Column (2.6×90cm) was filled with DEAE-cellulose resin suspended in Tris-buffer (pH7.6) and washed three times of column volume with initial buffer (0.01M NaCl in Tris-buffer). Cell extracts were applied to column with 2-4mg per m*l* of resin. After washing with initial buffer, bound proteins were eluted with 0.01M to 2.0M NaCl linear gradiented Tris-buffer and absorbance was monitored at 276nm (LKB: uv-detector and recorder). Sev-

eral peaks were noticed and their fractions were pooled and then further fractionated by ammonium sulfate method. At the less than 30%, 30-50% and 50-90% saturation of ammonium sulfate, the precipitates formed were separated and resuspended in small volume of Tris-buffer and dialyzed against the same buffer.

Serolgical Analysis of the Fractions

Immunoelectrophoresis and immunodiffusion

Molten 1.2% agarose in Tricine buffer (25 mM Tricine, 80mM Tris-Cl, 0.5mM calcium lactate, 0.01% merthiolate) was poured on the agar-coated plate (10×10cm) by 1.5-2m*l*/cm². Antigen and antisera were applied in the wells made by punchers or knives, according to the proper geometry and then plates were electrophoresed (10V/cm) and incubated for 2-3 days at room temperature in a moist chamber. The plate was soaked in 5% sodium citrate in order to dissolve CRP-produced precipitin bands if any. Precipitin bands were stained with amido black.

Rocket immunoelectrophresis

The method was described elsewhere (Kim *et. al.*, 1978). The 5m*l* of molten 1.2% agarose in Tricine buffer was poured 10×10 cm glass by 10×3cm and then the wells were made according to the proper geometry after the gel was solidified. The gel above 2cm from the bottom was removed and replaced with 15m*l* of molten agarose containing 10% (v/v) antiserum before harden. The plate was electrophoresed for 3-4 hrs at 100V in order to separate antigenic components of the fractions according to their isoelectric points. After electrophoresis, plate was left in a moist chamber for 1 day. Then the plate was washed with 5% sodium citrate and saline and dried prior to staining with amido black.

Gel filtration

Further concentration of species-specific antigen in the fractions has been made by sephacryl-S 300 (Farmacia Fine Chemical) gel filtration.

Species-specific antigen containing fractions were concentrated to a 1-2m*l* volume by vaccum dialysis using micro collodion bag (Saltorius). This concentrated antigen were applied to the sephacryl-S 300 column (1.6×90cm) and eluted. Elutents of peaks were pooled and concentrated to 3-5m*l* by 80% ammonium sulfate precipitation. Serological reactivity and cross-activity were analysed after dialysis.

RESULT AND DISCUSSION

Antibody production

It has been found that maximum production of antiantibody was obstained 3 weeks after immunization of *M. kansasii*, *M. scrofulaceum*, and *M. avium*, but in case of *M. tuberculosis*, after 5 weeks, as seen in figure 1.

Serological reactivity of the fractions

The profiles of DEAE-cellulose column chromatography of study strains were shown in Figure 2. Though salt concentrations at which peaks were eluted were little different from each strain. Crude extracts of all strains

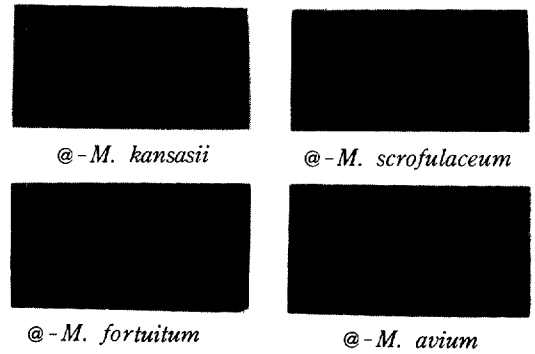
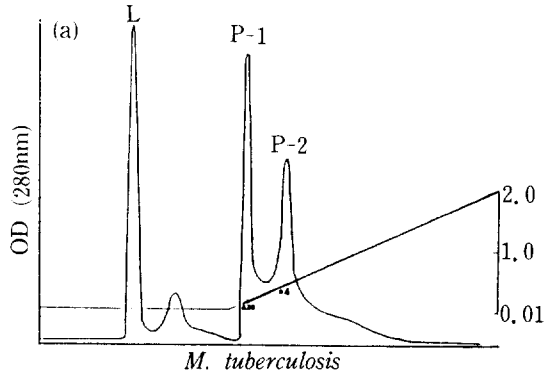
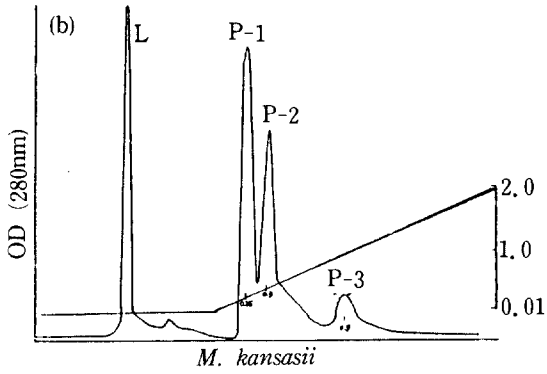
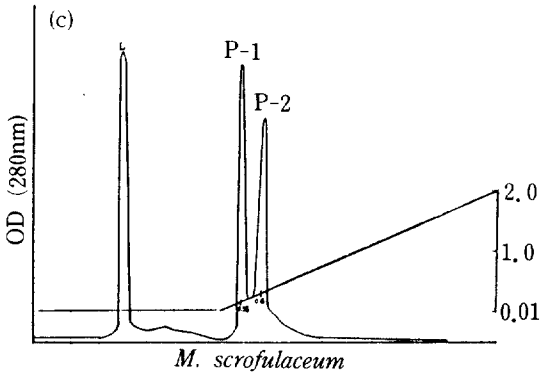


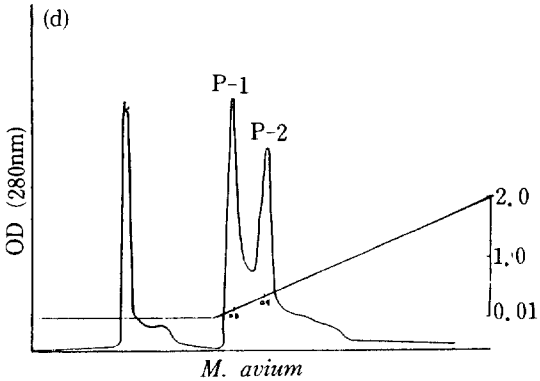
Fig. 1. *Precipitating antibodies of serial rabbit hyperimmunesera.*

Rabbit antisera to each strain were raised in two adult rabbits per strain. Each rabbit was immunized subcutaneously with 1ml of Freund's complete adjuvant containing 0.5ml of cell homogenate (40mg/ml, protein) freshly emulsified in equal volumn of Freund's incomplete adjuvant. After 3 weeks from immunization, booster injections were followed at 1 week intervals. Antibody titre from the sera were measured as described in Materials and Methods.









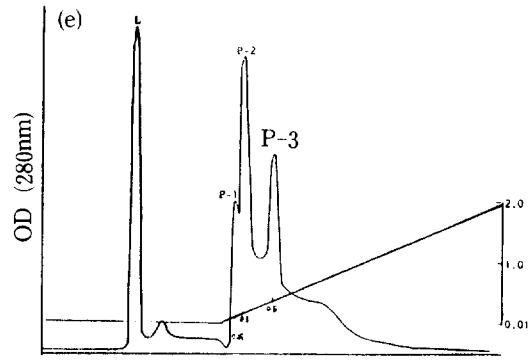


Fig. 2. *DEAE-cellulose column chromatography profiles of tuberculous and nontuberculous Mycobacteria. L is nonbinding materials to the resin and P-1 and P-2 are peak part fractions.*

showed a three major peaks (PL, P-1, P-2). L seems to be neutral material, such as lipid, polysaccharide, or basic protein. P-1 and P-2 seems to be composed of proteins, lipoproteins or glycoproteins, eluted upon increase of the salt concentration. P-1(P-1+P-2 in *M. fortuitum)* was further fractionated at 0-30%, 30-50% and 50-90% saturation of ammonium sulfate, and P-2 (P-3 in *M. fortuitum)* was precipitated by 0-90% of ammonium sulfate, thus designated P-1 0-30% as F1, P-1 30-50% as F2, P-1 50-90% as F3 and P-2 0-90% as F4. In case of *M. scrofulaceum.* F3 was a last fraction. Reactivity and specificity of each fraction (TF1, TF2, TF3, TF4, KF1, KF2, KF3, KF4, SF2, SF3, AF1, AF2, AF3, AF4, FF1, FF2, FF4) were analysed and the results were shown in Figure 3.

The species-specific antigens were found in TF3 fraction of *M. tuberculosis,* KF2 of *M. kansasii,* SF3 of *M. scrofulaceum,* AF2 of *M. avium,* and FF1 of *M. fortuitum.* These fractions were sieved through sephacryl S-300 gel filtration.

As shown in Figure 4, each fraction was separated further into 3 to 4 fractions, and reactivity and specificity of them were compared as seen in figure 5.

In Figure 5, Tc fraction shows a high

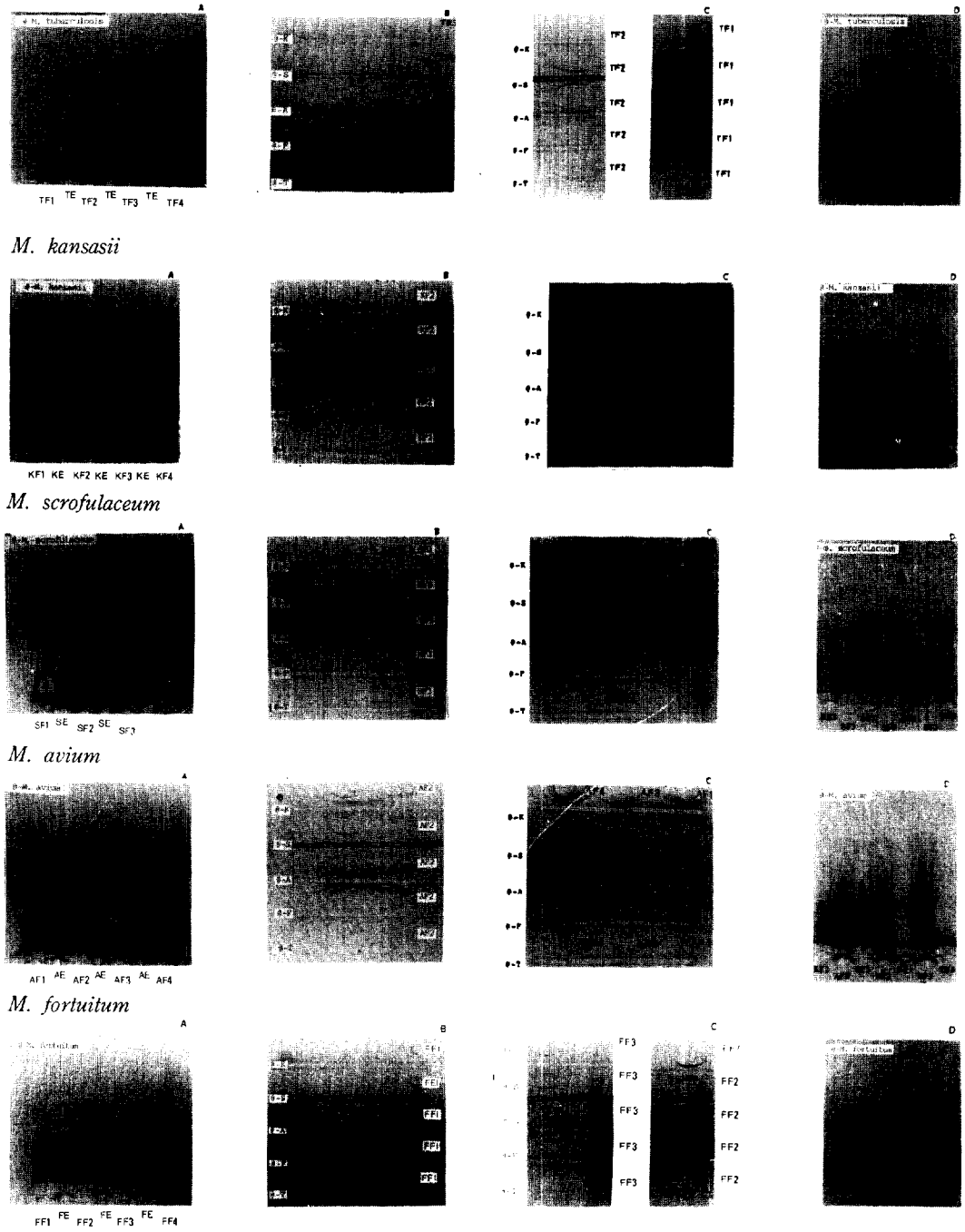


Fig. 3. *Antigenicity and specificity of the fractions.*

TF1 means fraction 1 of *M. tuberculosis*, KF1 fraction 1 of *M. kansaii*, and so on. (a); rocket immunoelectrophoresis of the fractions and cell extract using homologous antisera.(b) and (c); immunoelectrophoresis and immundiffusion using the homologous and heterologous antisera. (d); rocket immunoelectrophoresis the fractions containg the species-specific antigens in the homologours antisera.

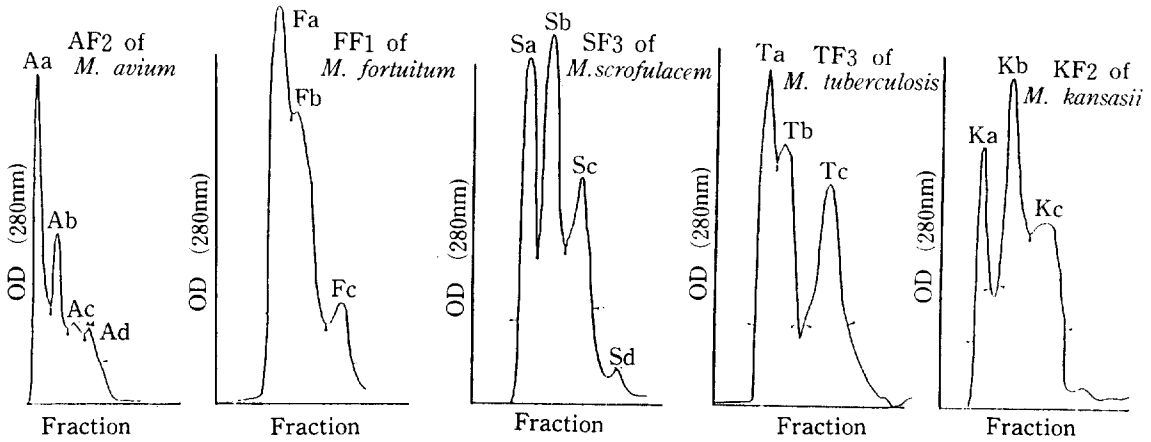


Fig. 4. *Gel (Sephacryl S-300) filtration column (90 × 1.6cm) chromatography profiles of species -specific antigen containing fractions (TF3, KF3, SF4, AF2, FF1).*

specificity and reactivity. While the fractions of atypical mycobacteria showed intensive cross -reactivity, which was often shared by many others. Small amount of species-specific antigen was detected in Ka fraction of *M. kansasii* and Sa fraction of *M. scrofulaceum*. Aa fraction of *M. avium* cross reacted with *M. kansasii* antiserum. The b fractions of *M. scrofulaceum* and *M. avium* showed a considerable cross-reactivity with heterologous antisera. All fractions of *M. fortuitum* (Fa, Fb, Fc) showed a fairly high specificity and little cross-reactivity. This result suggests that the rapid grower shared least antigens with the slowly growing mycobacteria in the present serological study.

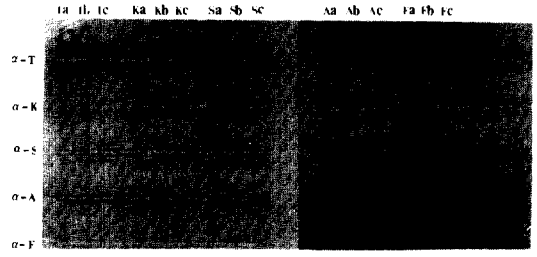


Fig. 5. *Antigenicity and cross-reactivity of the fractions purified from gel filtration column. 20 μl from each antigen fractions and 400 μl of antisera loaded on the well in the immunodiffusion technique as described in Materials and Methods.*

A crude cell extract prepared by low speed centrifugation showed a turbidity because of the lipids. Turbidity could be removed by DEAE -cellulose column, but a considerable amount of protein was also washed out simultaneously. As a matter of course this substances (L fraction on DEAE-cellulose column) had so poor reactivity that it was excluded in this study. Irreversable binding of proteins to DEAE-cellulose resin was also unavoidable so that approximately 85-90% of proteins of crude cell extract were recovered from the DEAE-cellulose ion exchange column. From the rest, the proportion of specific antigen-containing fraction was merely 5-7% of initial total proteins, and after final fractionation the specific fraction was at most 1% of initial total proteins. This situation make a difficulty produce a large lot of specific fractions. Molecular weights of all species-specific antigens were less than 140,000 in polyacrylamide gel electrophoresis. Of the immunogens used such as live cells, dead cell and cell homogenate of *M. tuberculosis*, cell homogenate turned out to be best for the production of antibody.

적 요

Phenol 처리로 사멸시킨 *M. tuberculosis, M. Kansasii, M. scrofulaceum, M. avium, M. fortuitum* 등 5개 균주를 초음파 분쇄기로 파쇄후 원심분리하여 세포추출항원을 얻었다. 이 세포추출항원을 DEAE-cellulose ion-exchange column chromatography에 적용시켜 0.01-2.0 N NaCl 1 차농도구배 Tris-buffer로 용출하고 peak 부분은 각각 황산암모니움 0-30%, 30-50%, 50-90%에 염석하였고, 이 항원분획은 다시 Sephacryl S-300 column을 통한 용출 및 황산암모니움 80%에 의한 재염석으로 더욱 정제하였다. 이때 얻어진 각각의 항원성분들은 파쇄전 각균체에 대한 가토 항혈청을 이용, 전기영동, 면역확산, Rocket 전기영동을 통해 항원성분들을 분석하였던 바 *M. tuberculosis*의 경우는 Tc. *M. Kansasii*는 Kc, *M. scrofulaceum*은 Sa, *M. avium*은 Aa, 그리고 *M. fortuitum*은 Fa, Fb, Fc에 각각 항원성과 특이성이 높은 항원성분들이 많이 함유되어 있음이 밝혀졌다.

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(Received Sep. 8, 1986)