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Purification and Antigenic Specificity of Alpha Protein (Yoneda and Fukui) from *Mycobacterium tuberculosis* and *Mycobacterium intracellulare*[*)]

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ABSTRACT

Alpha protein antigen isolated from culture filtrate of *Mycobacterium tuberculosis* by Yoneda and Fukui (1961) was a cross-reacting material among mycobacteria. Purified alpha protein of *M. tuberculosis* obtained by a series of procedures with gel filtration, ion-exchange chromatographies, and chromatofocusing. Alpha protein of *Mycobacterium intracellulare* was purified by an affinity chromatography on anti *M. tuberculosis*-alpha serum conjugated sepharose. Alpha antigen of *M. tuberculosis* possessed specific antigenic determinants for both *M. tuberculosis* and *M. bovis*, and so did alpha antigen of *M. intracellulare* for both *M. intracellulare* and *M. avium*. Extracts from *M. kansasii*, *M. scrofulaceum*, *M. gordonae*, *M. xenopi* and *M. gastri* showed alpha antigenicity somewhat different from those of *M. tuberculosis* and *M. intracellulare*. Alpha antigen was not detected in extracts from *M. nonchromogenicum*, *M. terrae* and *M. triviale*.

INTRODUCTION

A lot of efforts have been made by many investigators for classifying or idenitifying mycobacteria serologically[1,2,6]. However, methods for general application in the identification of mycobacteria, particularly atypical mycobacteria, have not yet been developed. The reasons for this may be due to the use of unpurified antigens in general. Only a few purified antigens are now available.

One of them is alpha protein antigen isolated from the culture filtrate of *Mycobacterium tuberculosis* by Yoneda and Fukui[3,4,16-18]. Alpha protein is a cross-reacting material which is widely distributed over slowly growing mycobacteria[19]. It is considered that alpha protein antigen possesses common antigenic determinants and specific antigenic determinants.

The present communication describes the method for purification of alpha proteins from *M. tuberculosis* and *Mycobacterium intracellulare* and demonstration of specific antigenic determinants of respective alpha protein.

MATERIALS AND METHODS

Culture filtrate: *M. tuberculosis* strain H37 Rv and *M. intracellulare* strain ATCC 13950 were cultivated on a modified Sauton medium at 37°C for 3 to 6 weeks, and the unheated filtrates obtained were used for preparation of alpha antigens[16].

Purification of alpha antigen. All the procedures were carried out at 4°C and summarized in Fig. 1. Solid ammonium sulfate was slowly added to the unheated culture filtrate with constant stirring until 70% saturation was obtained. The resulting protein precipitate was collected by centrifuging at 8,000 rpm for 30 min. The precipitate was dissolved in 0.02 M Tris buffer (pH 8.0) and dialyzed against the same buffer. The solution was centrifuged at 40,000 rpm for 2 hr (Hitachi 65P, Tokyo, Japan), and the supernatant was saturated at 50% with ammonium sulfate. After centrifuging at 8,000 rpm for 30 min, the precipitate was redissolved in a minimal volume of the same buffer and 1,000 mg of protein (the protein concentration

*) 田坂博信，清谷克寛，松尾吉恭：*Mycobacterium tuberculosis* および *Mycobacterium intracellulare* の αタンパク質（米田・福井）の精製法ならびに抗原特異性について

was determined by the method of Lowry et al.[8]) were placed on a Ultrogel AcA 54 (LKB-Produkter, Bromma, Sweden) column (5 by 100 cm). The column was previously equilibrated with 0.02 M Tris buffer (pH 8.0) containing 0.2 M sodium chloride and was eluted with the same buffer at a flow rate of 60 ml/hr (Fig. 2). Effluent fractions (10 ml) were collected and the antigenic reactivity was checked by agar-gel diffusion test[5]) with anti-alpha serum (kindly provided by late Professor Yoneda, Osaka University, Japan), anti-gamma serum[13]), and anti-H37 Rv extract serum. Fractions showing the highest reactivity against anti-alpha serum were pooled concentrated by Amicon diaflo membrane (UM 10, Amicon, Mass., U. S. A.) and dialyzed against 0.02 M Tris buffer (pH 8.0). One hundred mg of protein were placed on a DEAE sephacel (Pharmacia Fine Chemical, Uppsala, Sweden) column (2.6 by 45 cm) previously equilibrated with 0.02 M Tris buffer (pH 8.0), and chromatographed with a linear gradient (0 to 0.5 M sodium chloride with the same buffer: flow rate 30 ml/hr) (Fig. 3). Effluent fractions (5 ml) were collected. Fractions showing the highest reactivity against anti-alpha serum were pooled, concentrated and dialyzed against 0.025 M citrate buffer (pH 3.5). Fifty mg of protein were placed on a SP sephadex C 50 (Pharmacia) column (2.6 by 45 cm) previously equilibrated with the same buffer, and chromatographed with a linear gradient (0 to 0.5 M sodium chloride with the same buffer, at a flow rate of 30 ml/hr) (Fig. 4). Effluent fractions (5 ml) were collected. Fractions showing the highest reactivity against anti-alpha serum were pooled, concentrated and dialyzed against 0.025 M histidine-HCl buffer (pH 6.2). Fifty mg of protein were placed on a Polybuffer exchanger 94 (Pharmacia) column (1 by 40 cm) previously equilibrated with the same buffer, and chromatographed with Polybuffer 74 (1 : 8) (Pharmacia, pH 4.0) at a flow rate of 15 ml/hr (Fig. 5). Alpha antigen was eluted as a single peak.

On the other hand, an affinity chromatography was carried out on the partialy purified alpha antigen from *M. intracellulare* after DEAE sephacel chromatography. Ten mg of protein was placed on a CNBr-activated sepharose 4B (Pharmacia) column (1.5 by 20 cm) bound with anti-alpha (*M. tuberculosis*) serum. The column was previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride and chromatographed with 200 ml of the same buffer (flow rate 18 ml/hr). Subsequently, the buffer was changed to 2 M ammonium thiocyanate (pH 7.0) (flow rate 15 ml/hr). *M. intracellulare*-alpha antigen was eluted as a single peak.

Preparation of anti-alpha serum: An appropriate volume of alpha antigen solution (750 μg/ml) was emulsfied with an equal volume of Freund's incomplete adjuvant (Iatron, Tokyo, Japan). Each rabbit was given by intramuscular injection of the antigen emulsion containing 750 μg protein (2 ml). Three weeks after the first injection, the rabbit were boostered twice with the same antigen at two weeks interval. Ten to 14 days later, the rabbits were sacrified for total bleeding. The sera were separated, inactivated, and kept at $-80°$C.

Preparation of mycobacterial cell extract: Every mycobacterium cultured on 1% Ogawa egg medium for 2 to 3 weeks at 37°C was collected into a chilled mortar, and ground with an equivalent amount of glass beads by adding 0.02 M Tris buffer (pH 8.0) drop by drop. The supernatant was collected after centrifugation at 3,000 rpm for 30 min.

Absorption of test serum: The anti-serum (1.0 ml) was mixed with 10 mg of protein of partialy purified alpha antigen after DEAE sephacel chromatography. The mixture was incubated at 37°C for 1 hr and 4°C overnight, and centrifuged at 10,000 rpm for 30 min. The clear supernatant was referred to as absorbed anti-serum.

RESULTS

As shown in Fig. 1 to 5, purified alpha protein from *M. tuberculosis* was obtained by a series of procedures with gel filtration, ion exchange chromatographies, and chromatofocusing, and purified antigen of *M. intracellulare* was isolated by an affinity chromatography (Fig. 6).

M. tuberculosis alpha protein (alpha-T) and *M. intracellulare* alpha protein (alpha-I) were tested against their unabsorbed antisera by gel diffusion technique. Photo. 1 shows that alpha-T and alpha-I each formed a single precipitation band and these bands partially fused with each other. Several mycobacterial cell ex-

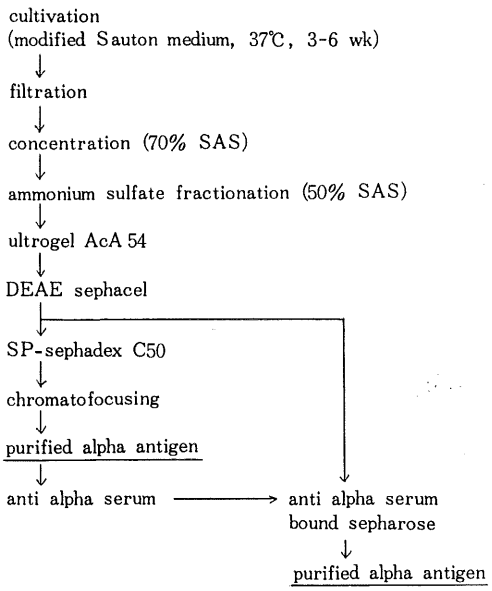

Fig. 1. Flow chart for purification of alpha protein from mycobacterial culture filtrate

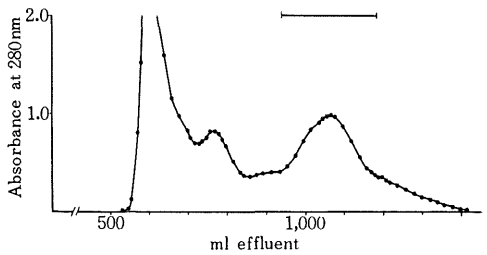

Fig. 2. Gel filtration pattern of 50% SAS fraction of culture filtrate on Ultrogel AcA 54. The bar represents the fractions that contain alpha protein.

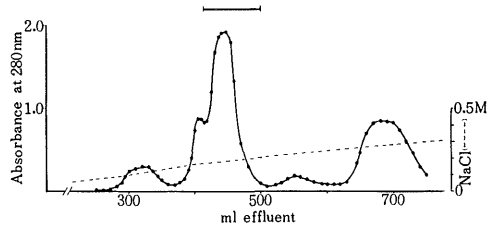

Fig. 3. Elution pattern of alpha protein contained in fractions by Ultrogel on DEAE sephacel. The bar represents the fractions that contain alpha protein.

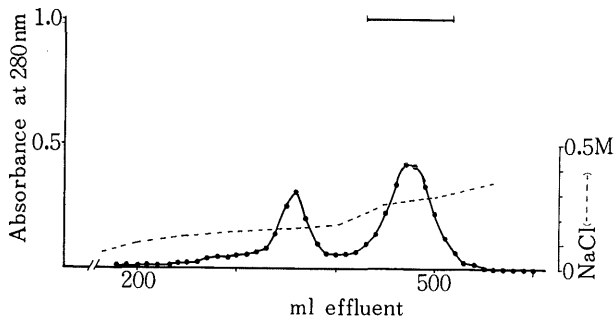

Fig. 4. Elution pattern of alpha protein contained in fractions by DEAE sephacel on SP sephadex C50. The bar represents the fractions that contain alpha protein.

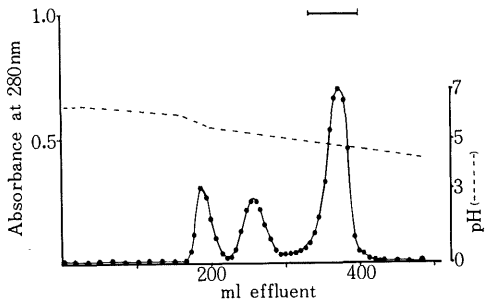

Fig. 5. Elution pattern of chromatofocusing through a Polybuffer exchanger 94, after SP sephadex C50 chromatography. The bar represents the fractions that contain purified alpha protein.

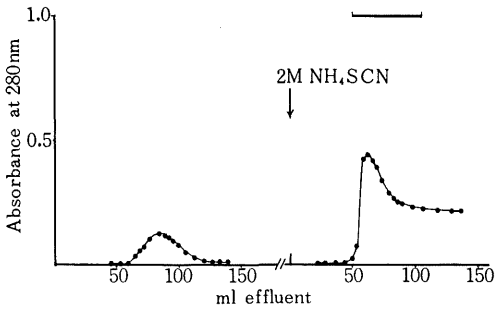

Fig. 6. Elution pattern of on anti alpha-T bound sepharose, after DEAE sephacel chromatography. The bar represents fractions that contain purified alpha protein.

tracts were tested against unabsorbed anti-alpha-T or anti-alpha-I serum. A single precipitation band formed by alpha-T fused completely with that by *M. bovis* extract (Photo. 2, left) and a similar immunodiffusion pattern was obtained between alpha-I and *M. avium* extract (Photo. 2, right). Precipitation bands of both alpha-T and alpha-I fused with the band of each of other mycobacterial extracts with clear spur formation (Photo. 3). These results are summarized in Table 1. Each protein obtained from *M. kansasii*, *M. scrofulaceum*, *M. gordonae*, *M. xenopi* and *M. gastri* developed a single precipitation band fused partially with that formed by alpha-T or alpha-I. Proteins from *M. nonchromogenicum*, *M. terrae* and *M. triviale* failed to produce precipitation band

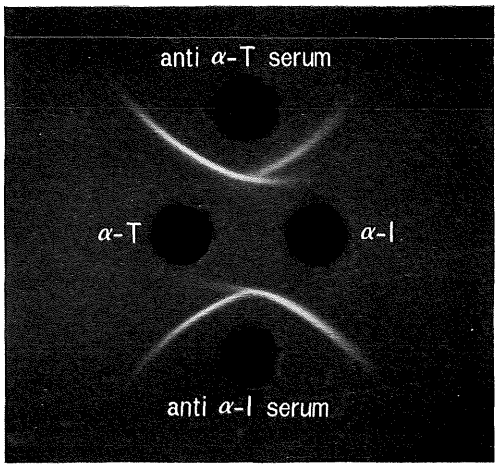

Photo. 1. Antigen analysis of alpha-T and alpha-I with respective unabsorbed anti-serum.

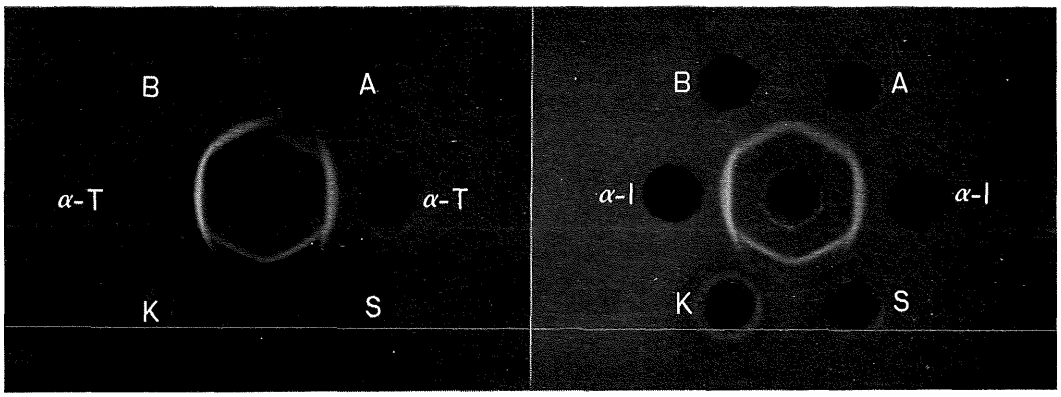

Photo. 2. Agar gel precipitation patterns demonstrating the interrelationships among alpha antigens in cell extracts from some mycobacterial species. The left central well containrd anti-alpha-T and the right anti-alpha-I sera. Extract: B; *M. bovis*, A; *M. avium*, S; *M. scrofulaceum*, and K; *M. kansasii*.

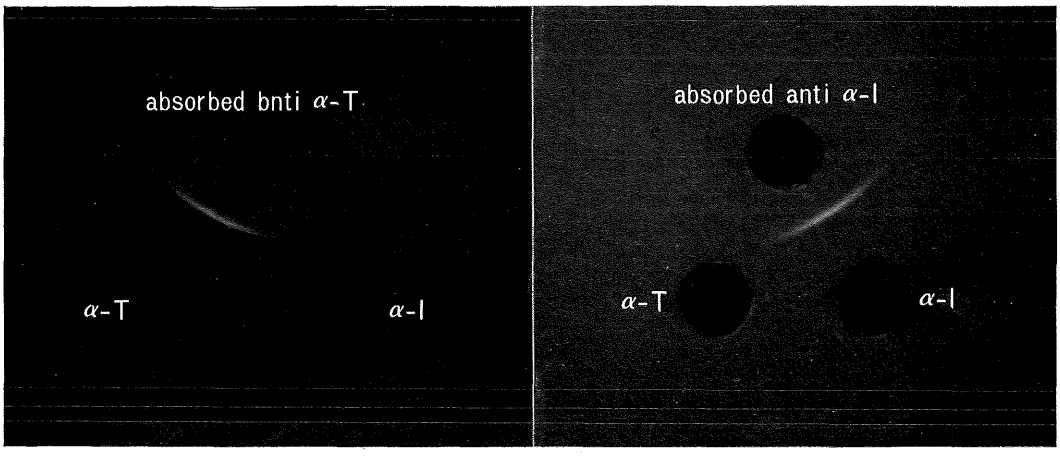

Photo. 3. Agar gel precipitation patterns of alpha-T and alpha-I antigens using absorbed anti-sera.

Table 1. Distribution of alpha antigen among slowly growing mycobacteria using agar gel diffusion technique

	anti alpha-T serum			anti alpha-I serum		
	positive fuse	negative spur		positive fuse	negative spur	
M. tuberculosis (H37 Ra)	○				○	
M. bovis (BCG)	○				○	
M. kansasii (ATCC 12478)		○			○	
M. scrofulaceum (ATCC 19981)		○			○	
M. gordonae (ATCC 14470)		○			○	
M. avium (B 92)		○		○		
M. intracellulare (ATCC 13950)		○		○		
M. xenopi (ATCC 19156)		○			○	
M. gastri (ATCC 15754)		○			○	
M. nonchromogenicum (ATCC 19530)			○			○
M. terrae (ATCC 15755)			○			○
M. triviale (ATCC 23292)			○			○

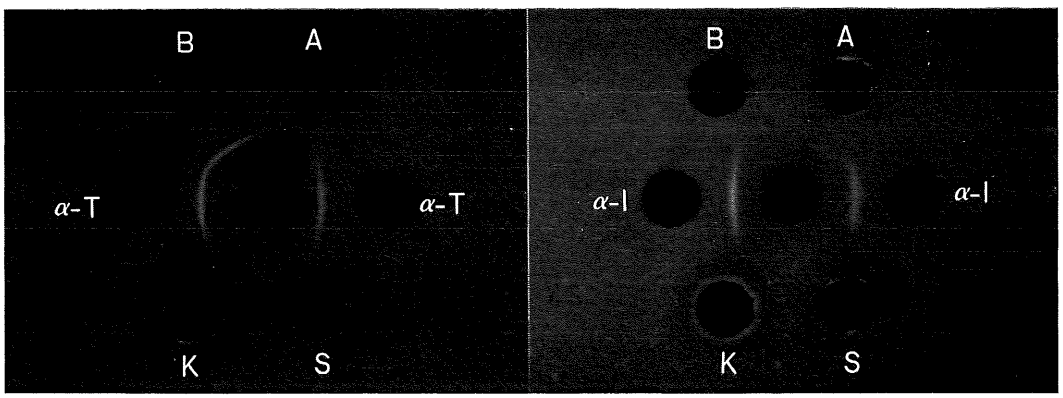

Photo. 4. Agar gel precipitation patterns demonstrating the interrelationships among alpha antigens of some mycobacterial species using absorbed anti alpha-T and alpha-I sera. The left central well contained anti alpha-T and right anti alpha-I sera.

Table 2. Distribution of specific antigenic determinants in alpha antigen among slowly growing mycobacteria using agar gel deffusion technique

	absorbed anti alpha-T serum		absorbed anti alpha-I serum	
	positive	negative fuse spur	positive	negative fuse spur
M. tuberculosis (H37 Ra)	○			○
M. bovis (BCG)	○			○
M. kansasii (ATCC 12478)		○		○
M. scrofulaceum (ATCC 19981)		○		○
M. gordonae (ATCC 14470)		○		○
M. avium (B 92)		○	○	
M. intracellulare (ATCC 13950)		○	○	
M. xenopi (ATCC 19156)		○		○
M. gastri (ATCC 15754)		○		○
M. nonchromogenicum (ATCC 19530)		○		○
M. terrae (ATCC 15755)		○		○
M. triviale (ATCC 23292)		○		○

against both the anti-sera.

When used absorbed anti-serum, alpha-T and alpha-I each formed a single precipitation band against only homologous antiserum (Photo. 3). Further, the band formed by alpha-T fused completely with that by *M. bovis* protein, and the band by alpha-I did with that by *M. avium* protein. However, these bands did not form any band of each of other mycobacterial proteins (Photo. 4 and Table 2).

DISCUSSION

It has been not easy to isolate species specific antigens from various mycobacteria. Recently, Hirai et al.[7] reported that eta protein isolated from *M. tuberculosis* strain H37 Rv was a species specific antigen and Nagai et al.[9] reported that MPB 70 isolated from *M. bovis* strain BCG was a species specific antigen. The alpha antigen (Yoneda and Fukui) is known to have cross reactivity with proteins from some of mycobacterial species, and Daniel and Janicki[2] have pointed out that the alpha antigen seems to be in fact antigen 6. Antigen 6 contains at least two antigenic determinants, one of which appears to be specific for *M. tuberculosis* and the other is present in several other mycobacteria. Therefore, we attempted to isolate purified alpha antigen. The alpha antigen from *M. tuberculosis* (alpha-T) was purified as a single peak following successive purification procedures. In addition, the alpha antigen from *M. inracellulare* (alpha-I) was successfully isolated by an affinity chromatography on a CNBr-activated sepharose column bound with anti-alpha-T serum.

Alpha-T and the extract from *M. bovis* formed a completely fused precipitation line

against unabsorbed as well as absorbed anti-alpha-T sera by gel diffusion analysis, while extracts from 7 species of atypical mycobacteria reacted only against unabsorbed anti-alpha-T serum with clear spur formation and did not react against absorbed anti-alpha-T serum. These results suggest that alpha-T and alpha-*M. bovis* are serologically identical, and that alpha-T possesses common antigenic determinants among proteins of some species of atypical mycobacteria.

Similarly, specific antigenic determinants exist between *M. intracellulare* and *M. avium*, but an interest is aroused that the determinants are not present in the extract from *M. scrofulaceum* in so-called *M. avium* complex[11,12,14,15]. It was reported by Yoneda et al.[19] that alpha protein was found in *M. kansasii*, and Norlin et al.[10] detected the presence of alpha protein in *M. gastri*. Although extracts from *M. kansasii*, *M. scrofulaceum*, *M. gordonae*, *M. xenopi* and *M. gastri* reacted partially against unabsorbed anti-alpha-T and anti-alpha-I sera. Extracts from *M. nonchromogenicum*, *M. terrae* and *M. triviale* did not. These 3 species of atypical mycobacteria seem not to have alpha protein, and are of interest in view of the proposal by Tsukamura[14] that these 3 species should be combined as *M. nonchromogenicum* complex because of their close relatedness.

From the results, absorbed anti-alpha-T and anti-alpha-I sera are adequate enough to identify *M. tuberculosis* complex[14] and *M. avium* complex. Further investigation is under way to obtain specific antisera for identifying other mycobacterial species.

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