INFECTION AND IMMUNITY, Mar. 1981, p. 1152-1160
0019-9567/81/031152-09$02.00/0

Vol. 31, No. 3

Specific Skin-Reactive Protein from Culture Filtrate of *Mycobacterium bovis* BCG

SADAMU NAGAI,[1]* JUNJIRO MATSUMOTO,[2] AND TOKUKO NAGASUGA[2]

Toneyama Institute for Tuberculosis Research, Osaka City University Medical School,[1] and the Toneyama National Hospital,[2] Toyonaka 560, Japan

A highly purified protein, named MPB70, was isolated from the culture filtrate of *Mycobacterium bovis* BCG. This protein accounted for more than 10% of the proteins secreted into the culture medium. MPB70 was purified by precipitation with ammonium sulfate, followed by treatment with diethylaminoethyl ion exchanger, with or without 3 M urea, and by gel filtration. The final MPB70 preparation was homogenous as judged by several analyses. The molecular weight was estimated to be 18,000 by electrophoresis or molecular sieve and 15,100 by sedimentation equilibrium. The preparation did not contain sugars. The amino acid composition did not include cysteine or tryptophan. In skin reaction, MPB70 was a strictly BCG-specific antigen and, among the guinea pigs sensitized with the heat-killed cells of the various species of mycobacteria—*Mycobacterium tuberculosis* strains H37Rv and Aoyama B, *Mycobacterium kansasii, Mycobacterium intracellulare, Mycobacterium phlei,* and BCG, it elicited a delayed cutaneous reaction only in the guinea pigs sensitized with BCG. The potency of MPB70 in the skin reaction was about one-twentieth of the standard purified protein derivative.

Purified protein derivative (PPD), which is widely used in the diagnosis of tuberculosis and in immunological studies as a specific antigen, is prepared from the heated culture filtrates of mycobacteria. The heating process used to kill virulent cells preparatory to the isolation procedure is a drastic treatment, which also affects the component proteins secreted in the culture medium, and many attempts at further purification of PPD to isolate the active component of tuberculin have faced considerable difficulties (10, 18, 28). We proposed in a previous paper (27) the use of urea to unfold the tangled proteins which were produced while autoclaving. It is preferable, however, to avoid sterilization by heating, if possible. The avirulent H37Ra strain of *Mycobacterium tuberculosis* is one suitable source of an elicitin prepared without heating, and some purified preparations from its culture filtrate have already been reported (6, 7, 9). In another species of mycobacteria, *Mycobacterium bovis* BCG, the heating step can also be omitted. All preparations from BCG so far reported have been closely cross-reactive with *M. tuberculosis* in delayed skin reaction (4, 5, 24); therefore, a preparation from BCG might be used as a skin-reactive agent in place of PPD from *M. tuberculosis*. With this point of view, some tuberculin active components were purified from the cell extract of living BCG (22, 23). We chose to isolate the active protein from culture filtrate of BCG because a special component made up one-tenth of the total proteins detected.

A good yield of the protein, named MPB70, was obtained in a highly pure form by relatively simple procedures. More remarkably, MPB70 was a unique antigen with regard to its strict species specificity: it elicited delayed skin reaction only in guinea pigs sensitized with the heat-killed cells of BCG and not in those sensitized with the heat-killed cells of any other species of mycobacteria tested. In this paper we describe the isolation procedures and the properties of MPB70.

MATERIALS AND METHODS

Growth of organisms. *M. bovis* strain BCG (Tokyo 172), *M. tuberculosis* strains H37Rv and Aoyama B, *Mycobacterium kansasii,* and *Mycobacterium intracellulare* were grown at 37°C for 4 to 5 weeks on Sauton medium as described previously (28). *Mycobacterium phlei* was grown for 10 days on the same medium.

Polyacrylamide gel electrophoresis. Proteins were identified by their electrophoretic mobility in 7.7% polyacrylamide gels [7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide (BIS)] at the running pH of 9.4 by the method of Davis (11) or at the running pH of 7.0 to 8.0 in the triethanolamine–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer system of Orr et al. (29). The gels, in tubes with an inside diameter of 6 mm, were subjected to electro-

phoresis at room temperature with a constant current of 3 mA/tube at pH 9.4 or 2 mA/tube at pH 7.0 to 8.0. The relative mobility of the protein bands stained by Coomassie brilliant blue at the running pH of 9.4 was used to identify the proteins. MPB70 means a mycobacterin (31) which is a protein fraction from BCG as a band with a relative mobility of 0.70 under this condition. In the purification procedures, we found electrophoresis in slab-type gels (15 by 15 by 0.1 cm) to be convenient for the identification of bands in fractions, applying 30 mA at the running pH of 9.4.

The electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) in sodium phosphate buffer (pH 7.0) was performed by the method of Weber and Osborn (34). The gels contained 12.5% acrylamide and 0.34% BIS with 0.1% SDS. Samples were heated at 95°C for 3 min with 1% SDS and a 1/10 volume of mercaptoethanol at pH 7.0 before loading.

Purification of MPB70. The source of MPB70 was the culture filtrate of BCG strain Tokyo 172 (BCG vaccine, Nihon BCG Seizo Co., Tokyo, Japan). After 4 to 5 weeks of cultivation on Sauton medium, cells were removed by filtration through layered gauze. The pH of the filtrate was about 7.5, and 11.3 liters of it were obtained from one batch of 50 culture bottles. After every 10 continuous inoculations onto Sauton medium, the inoculum was renewed from the original vaccine.

(i) Ammonium sulfate precipitation. The proteins in the filtrate were concentrated by solid ammonium sulfate at 80% saturation (561 g/liter) as previously described (27), and the concentrate was dialyzed against 30 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer (Tris buffer, pH 8.7) and then was passed through a membrane filter of pore size 0.45 μm to remove any BCG cells. The electrophoretic pattern of this preparation is shown in Fig. 1.

(ii) DEAE-Sephadex chromatography. The following isolation procedures were carried out at 4°C. The concentrate, 8 g of protein, estimated by the method of Lowry et al. (25), was applied to a column (5.5 by 44 cm) of diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden), which was equilibrated with 30 mM Tris buffer, pH 8.7. The gel was washed with 500 ml of the same buffer, and most of the carbohydrate in the original sample was removed from the column. The next eluate was collected with 1,500 ml of 30 mM Tris buffer (pH 8.7) containing 0.2 M sodium chloride, and the protein was precipitated with ammonium sulfate at 70% saturation (472 g/liter). The precipitate was collected by centrifugation at 7,000 × *g* for 10 min, dissolved in water, and dialyzed against 30 mM Tris buffer, pH 8.7. A total of 5.3 g of protein was obtained.

(iii) DEAE-Sepharose CL-6B chromatography (first). The dialyzed protein solution was applied to a column of DEAE-Sepharose CL-6B (Pharmacia), 3.6 by 49 cm, which was equilibrated with 30 mM Tris buffer (pH 8.7), and then chromatographed with 3,000 ml of 30 mM Tris buffer (pH 8.7) containing a linear concentration gradient of sodium chloride from 0 to 0.2 M, at a rate of 90 ml/h (Fig. 2). Every fraction of the eluate was analyzed by electrophoresis at the running pH of 9.4. The fractions containing MPB70, which appeared as the large, sharp peak in Fig. 2, were pooled. Another protein with a relative mobility of 0.70 in the gel analysis also appeared in the fraction as the small plateau following the peak (fraction number of around 90 in Fig. 2). This protein, named MPB70a, was collected separately. Several other proteins, which were also some of the main components of the original preparation, followed in the elution (Fig. 2), and MPB80 was found in the fraction between 98 and 104; MPBs 64, 59, and 83 were in the fraction between 105 and 121. The proteins were precipitated with ammonium sulfate at 70% saturation, collected by centrifugation at 12,000 × *g* for 10 min, dissolved in water, and dialyzed against 30 mM Tris buffer, pH 7.5. The amount of protein in the MPB70 preparation was 1.5 g.

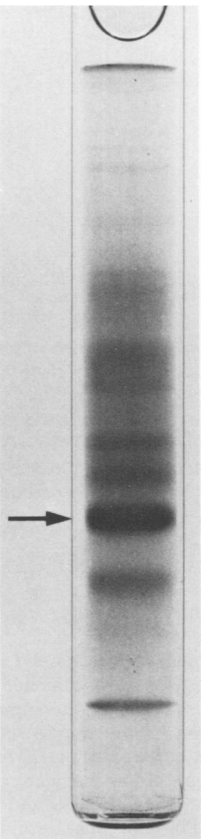

FIG. 1. *Gel electrophoresis of the culture filtrate of BCG. The concentrated culture filtrate, 130 μg of protein, made with ammonium sulfate at 80% saturation [see the text, purification of MPB70 (i)], was applied to a 7.7% polyacrylamide (7.5% acrylamide + 0.2% BIS) gel and was electrophoresed at a running pH of 9.4. The arrow indicates the position of the band of MPB70.*

(iv) DEAE-Sepharose CL-6B chromatography (second). A DEAE-Sepharose CL-6B column of the same size as that in (iii) was equilibrated with 30 mM Tris buffer (pH 7.5) containing 3 M urea. Any insoluble

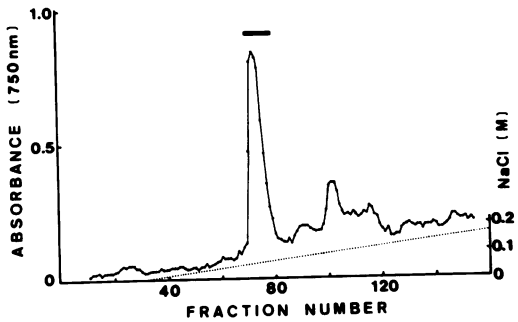

FIG. 2. *Elution pattern through a DEAE-Sepharose CL-6B column with an elution gradient of sodium chloride in 30 mM Tris-hydrochloride buffer, pH 8.7 [see the text, purification of MPB70 (iii)]. The bed volume of the column was 500 ml. The protein content was analyzed by the method of Lowry et al. (25) using a 0.03-ml sample of each fraction (20-g fractionation). The bar indicates the fractions that contained MPB70.*

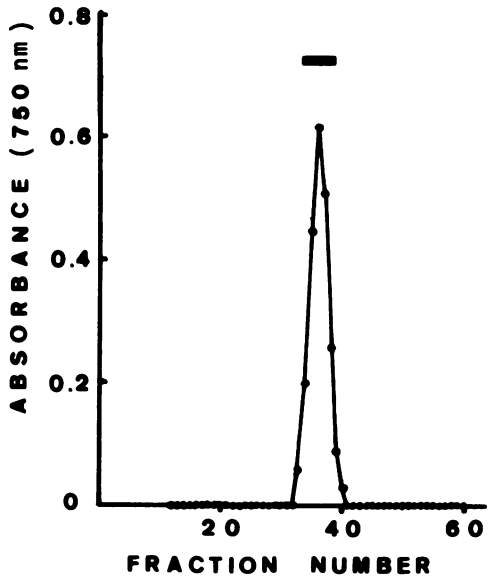

FIG. 3. *Gel filtration through a Sephadex G-50 column [see the text, purification of MPB70 (vi)]. The bed volume of the gel was 530 ml, and the volume of the applied sample was 7.6 ml. A 30 mM Tris-hydrochloride buffer (pH 7.5) containing 0.1 M sodium chloride was used for the chromatography. The protein content was analyzed using a 0.03-ml sample of each fraction (7.4-ml fractionation). The bar indicates the fractions collected.*

substances in the urea-containing Tris buffer were removed beforehand by passing the buffer through a membrane filter of pore size 0.45 μm. Urea was dissolved in the preparation obtained in (iii) to a final concentration of 3 M. The addition of the urea increased the final volume of the sample to 1.16 times the original volume. The sample was applied to the column and then eluted with 3,000 ml of 30 mM Tris buffer (pH 7.5) containing a linear concentration gradient of sodium chloride from 0 to 0.08 M in addition to the 3 M urea. The fractions containing MPB70 were pooled, and the protein was precipitated with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation and suspended in water. The suspension was gradually dissolved in 30 mM Tris buffer (pH 8.7) containing 0.04 M sodium chloride during overnight dialysis. The amount of protein in the preparation was 1.09 g.

(v) DEAE-Sepharose CL-6B chromatography (third). A DEAE-Sepharose CL-6B column of the same size as that in (iii) was washed with 30 mM Tris buffer (pH 8.7) containing 0.04 M sodium chloride. The sample was applied to the column and then eluted with 3,000 ml of 30 mM Tris buffer (pH 8.7) containing a linear concentration gradient of sodium chloride from 0.04 M to 0.09 M. The fractions containing MPB70 were pooled, and the protein was collected as described in (iii). The concentrated protein solution was dialyzed against 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride. The amount of protein in the preparation was 730 mg.

(vi) Sephadex G-50 filtration. The preparation obtained from (v) was divided into a few portions of less than 8 ml, and each of them was applied separately to a column of Sephadex G-50 (Pharmacia), 2.6 by 100 cm, and chromatographed with 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride, at a flow rate of 18 ml/h. The fractions around the peak (Fig. 3) were combined, and the protein was collected with ammonium sulfate at 60% saturation (390 g/liter). The concentrated MPB70 was dialyzed against water to completely remove salts. The dialyzed solution, which had 708 mg of protein estimated by the method of Lowry et al. (25), was passed through a membrane filter of pore size 0.22 μm and then was lyophilized. The MPB70, as a white powder (430 mg in dry weight), was stored in a desiccator over silica gel in a refrigerator.

Estimation of molecular weight. The molecular weight of MPB70 was estimated by three methods. For the electrophoresis in polyacrylamide gel containing SDS (34) and for the molecular sieve (1) with the Sephadex G-50 column (2.5 by 100 cm) in 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride, the following protein standards were used: ovalbumin (Sigma Chemical Co.), molecular weight 27,000; α-chymotrypsinogen A from porcine pancreas (Sigma), 25,700; myoglobin from whale skeletal muscle (Sigma), 17,200; and cytochrome *c* from horse heart (Sigma), 11,740. For the method of sedimentation equilibrium, the partial specific volume of MPB70 was assumed to be 0.74 in the calculation of the molecular weight.

Amino-terminal amino acid analysis. The amino-terminal amino acid was analyzed by the dansylation method (17).

Amino acid analysis. The lyophilized preparation of MPB70 was hydrolyzed with 6 N hydrochloric acid, and the amino acids were analyzed with an amino acid analyzer. The amount of tryptophan was estimated by measuring the absorption of ultraviolet light (16).

Sugar analysis. The content of carbohydrate in

the fractions of the gel filtration was determined by the use of anthrone (32). With 20 mg of the lyophilized preparation of the purified MPB70 at each analysis, its neutral sugar content was determined with phenol-sulfuric acid (13), and the amino sugar content was analyzed by the method of Elson and Morgan (14) after hydrolysis by 2 N hydrochloric acid (3).

Ouchterlony gel diffusion. Antibody against MPB70 was prepared by intramuscular injection into rabbit of the final purified MPB70 (5 mg), homogenized in 1 ml of incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). A booster was given 2 weeks after the last injection. The antibody was repeatedly precipitated with ammonium sulfate at 33% saturation and finally dissolved in saline at one-fifth of the volume of the original serum. Immunodiffusion tests on microscope slides were performed essentially as outlined by Ouchterlony (30).

Sensitization of guinea pigs. A 0.1-ml suspension in liquid paraffin containing 0.1 mg of dried mycobacterial cells (28) was injected intramuscularly into the hind limbs of female guinea pigs. These animals were able to be skin tested 4 weeks after injection.

Skin test. Guinea pigs were skin tested on a shaved flank by intradermal injection of 0.1 ml of antigen, MPB70 or PPD, dissolved in physiological saline without addition of Tween 80. The protein content of MPB70 was calculated from the nitrogen content, which was determined by the method of Kjeldahl. The final dilution with saline was performed within 1 h before injection. The mean diameter of induration was measured at 24 h after injection. Commercial PPD was used as the control for the skin tests. This product, obtained from Nihon BCG Seizo Co., Japan, was prepared from the heated culture filtrate of *M. tuberculosis* Aoyama B, and its potency was approved by the National Institute of Health, Japan. The reactivity of the PPD preparation was expressed as the diameter of induration against the amount of potency of international standard PPD.

RESULTS

MPB70 as the main component in the culture filtrate. As clearly shown in Fig. 1, the band of MPB70 was outstandingly stained among the bands of the proteins which were concentrated with ammonium sulfate at 80% saturation from the culture filtrate of BCG. Estimated from the final yield of 8.9%, the MPB70 in the culture medium originally accounted for more than 10% of the amount of proteins secreted into the medium. This massive amount of MPB70 in the culture filtrate was one of the important reasons which made it possible for us to get an intensely purified preparation.

Analyses on MPB70. There was evidence of the homogeneity of the final MPB70 preparation from all the various analyses: the single band in electrophoresis under five different conditions (Fig. 4); the one precipitin line against anti-MPB70 serum in the Ouchterlony gel diffusion (Fig. 5); and the single, symmetrical schlieren

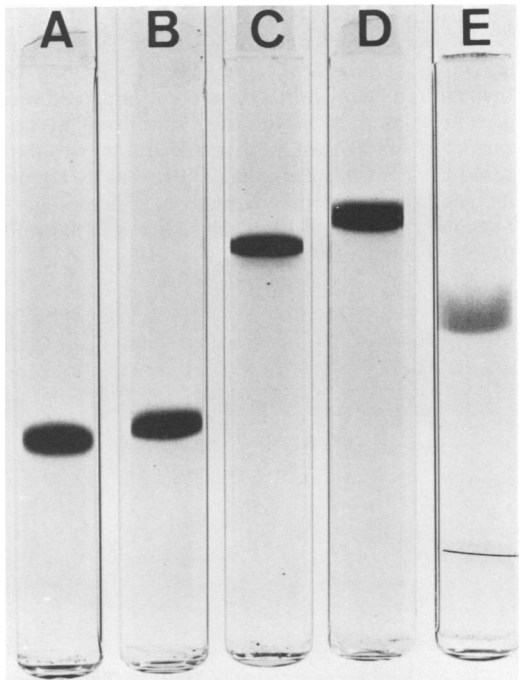

FIG. 4. *Homogeneity of MPB70 in gel electrophoresis. MPB70 (100 μg) was applied to each gel. (A) 7.7% polyacrylamide gel at a running pH of 9.4, and (B) at a running pH of 7.0 to 8.0; (C) 6.16% (6% acrylamide + 0.16% BIS) gel with 8 M urea at a running pH of 9.4, and (D) at a running pH of 7.0 to 8.0; (E) 12.84% (12.5% acrylamide + 0.34% BIS) gel with 0.1% SDS at pH 7.0.*

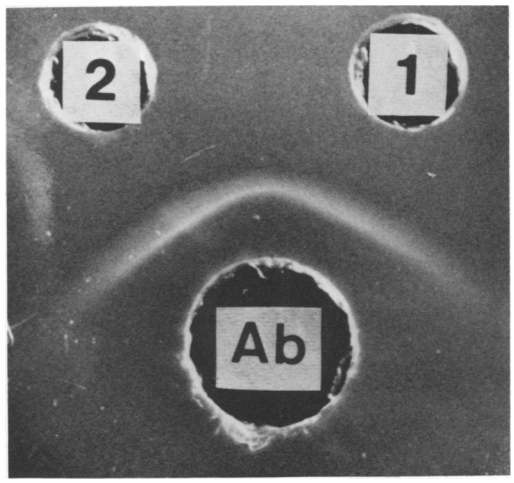

FIG. 5. *Ouchterlony gel diffusion with MPB70. Well contents: Ab, anti-MPB70 serum; 1, purified MPB70 (0.1 mg per ml); 2, concentrated culture filtrate of (i) (see the text, purification of MPB70) (1 mg per ml).*

pattern in the ultracentrifugation (Fig. 6).

The amino acid composition of MPB70 is shown in Table 1. There was no cysteine or tryptophan. The amino-terminal amino acid was glycine. Using 20 mg of the lyophilized preparation of MPB70 in each experiment, we analyzed the content of sugars. There was no significant amount of either neutral or amino sugars because their contents were less than 0.03 and 0.02%, respectively.

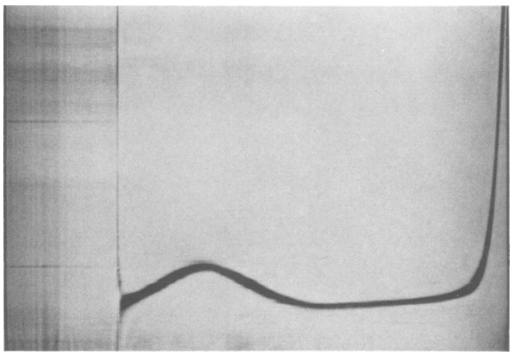

FIG. 6. *Schlieren pattern of MPB70. The protein concentration was 5.8 mg per ml in 0.1 M sodium acetate buffer, pH 6.0. The photograph was taken at 100 min after the centrifuge had reached 56,000 rpm at 25°C with a phase plate angle of 60°.*

TABLE 1. *Amino acid composition of MPB70*

Amino acid	No. of residues[a]/molecule of MPB70
Lysine	2.84
Histidine	0.94
Arginine	0.95
Aspartic acid	17.7
Threonine	17.9[b]
Serine	13.2[b]
Glutamic acid	13.0
Proline	9.91
Glycine	14.1
Alanine	18.4
Half-cystine	0
Valine	16.3
Methionine	1.69
Isoleucine	2.60
Leucine	12.9
Tyrosine	3.80[b]
Phenylalanine	1.72
Tryptophan	0[c]

[a] The values were calculated by assuming that the molecular weight of MPB70 was 15,100. Except for threonine, serine, tyrosine, and tryptophan, each value is the average of values obtained by hydrolysis for 24, 40, and 72 h.

[b] Values extrapolated to zero time of hydrolysis.

[c] Determined spectrophotometrically (16).

The molecular weight of MPB70 was found to be 18,000 by SDS-gel electrophoresis and gel filtration with Sephadex G-50 (Fig. 7) and 15,100 by sedimentation equilibrium analysis. We used the latter value for the calculation of the amino acid composition of MPB70. The sedimentation constant was 1.9S.

MPB70 could be crystallized by slow addition of ammonium sulfate even from the crude preparation. The crystals, however, were not in a regular shape and were usually rocky or round (Fig. 8). The electrophoresis analysis showed that repeating of crystallization did not bring about any further purification of MPB70.

Skin reactivity of MPB70. To compare it with the PPD preparation from *M. tuberculosis*

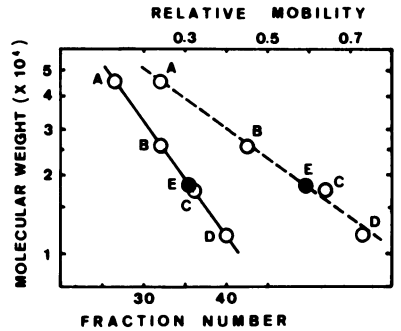

FIG. 7. *Estimation of the molecular weight of MPB70 by electrophoresis (O-----O) in SDS-containing gels (12.84% polyacrylamide with 0.1% SDS) and by gel filtration (O——O) through a Sephadex G-50 column (2.6 by 100 cm, 7.4-ml fractionation). A, Ovalbumin; B, α-chymotrypsinogen A; C, myoglobin; D, cytochrome c; E, MPB70.*

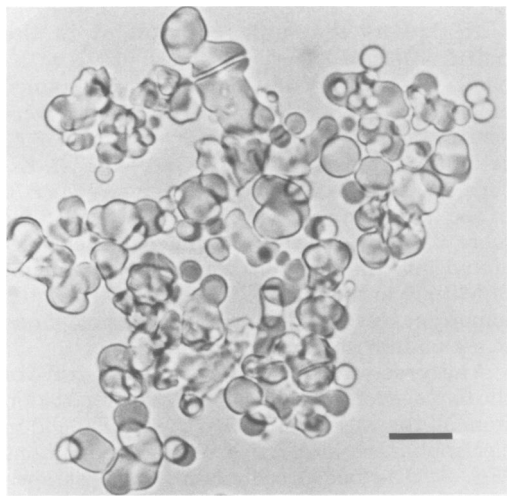

FIG. 8. *Micrograph of crystallized MPB70. The bar represents 10 μm.*

Aoyama B, we tested the skin reactivity of MPB70 in guinea pigs sensitized with heat-killed cells of six different mycobacteria. As shown in Table 2, MPB70 was unique as a mycobacterial antigen in the delayed skin reaction, in that it reacted only in the guinea pigs sensitized with BCG. In the tests of the guinea pigs sensitized with any of the other mycobacteria, there was essentially no skin reaction, even after the injection of 16 μg of MPB70 in 0.1 ml. The potency of MPB70, however, in the skin reaction was not so strong and was approximately one-twentieth of that of the approved PPD. The time course of the reaction to MPB70 was similar to that of the reaction to PPD, and no cutaneous reaction appeared within 3 h after injection.

Skin reactivity of fractions of crude culture filtrate in gel filtration. The specific skin reactivity which we found in MPB70 has not been reported, and we tested the specificity again with the original preparation of the culture filtrate of BCG with which we started to check the results of the broad specificity so far reported. A concentrated solution (5 ml), obtained with ammonium sulfate at 80% saturation from culture filtrate, was applied to a Sephadex G-75 column (2.5 by 100 cm) and was chromatographed with 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride as shown in Fig. 9. The contents of protein and carbohydrate in the fractions were plotted in the upper part of the figure. The scale for molecular weight was obtained with a run of standard proteins under the same conditions. Most of the carbohydrate appeared just after the void, as reported (4), and the protein was distributed over a wide range with two peaks. Six samples at intervals of six fractions were taken, and 0.4 μg of protein from each sample, estimated by the Kjeldahl method, was tested for skin reactivity in guinea pigs sensitized with heat-killed cells of either *M. tuberculosis* H37Rv or Aoyama B, or BCG, and the results are shown in the lower part of the figure. All six samples tested were skin reactive to an almost similar potency, although in the samples containing the smaller size proteins around 10,000, the reaction was rather weaker than in the others. MPB70 was distributed in the sample of fraction number 52 out of these six samples, but this sample did not indicate so strict a specificity against guinea pigs sensitized with BCG. The broad specificity in skin reaction observed with these crude preparations coincided with the results in earlier reports that any preparations from BCG were reactive on animals sensitized with *M. tuberculosis* (4, 5, 24).

DISCUSSION

MPB70 is a unique protein for several reasons: the extremely large amount of it in the culture medium, its BCG-specific skin reactivity among guinea pigs sensitized with heat-killed cells of mycobacteria, the fact that it is a highly purified skin-reactive protein which can be reproduced by rather simple procedures, and the fact that it is clearly definable both chemically and physi-

TABLE 2. *Skin test reactivity of MPB70 and PPD[a] in guinea pigs sensitized with six species of mycobacteria*

Antigen	Protein concn (μg/0.1 ml)	Diam of induration (mm) 24 h after injection in guinea pigs sensitized with heat-killed cells of:						
		BCG (10)[b]	*M. tuberculosis* H37Rv (8)	*M. tuberculosis* Aoyama B (8)	*M. kansasii* (8)	*M. intracellulare* (10)	*M. phlei* (8)	None (4)
MPB70	0.2[c]	11.8 ± 3.5	—	—	—	—	—	—
	0.4	12.7 ± 3.8	—	—	—	—	—	—
	0.8	14.3 ± 3.3	—	—	—	—	—	—
	1.6	15.1 ± 3.1	—	—	—	—	—	—
	2.0	—	2.9 ± 1.6	3.5 ± 0.9	2.8 ± 1.5	3.4 ± 1.6	3.3 ± 1.6	—
	3.2	16.8 ± 3.0	—	—	—	—	—	—
	4.0	—	2.9 ± 1.4	3.4 ± 0.7	3.0 ± 0.9	3.1 ± 1.6	3.3 ± 1.2	—
	8.0	—	3.4 ± 1.1	3.4 ± 0.7	2.5 ± 1.1	3.1 ± 1.4	3.6 ± 1.3	—
	16.0	—	3.1 ± 1.4	3.0 ± 0.8	2.6 ± 0.5	3.0 ± 1.5	3.5 ± 1.2	2.0 ± 0.0
PPD	0.025[d]	13.0 ± 1.2	12.6 ± 1.9	12.1 ± 1.1	—	—	—	—
	0.05	14.8 ± 1.0	13.5 ± 1.9	13.3 ± 1.0	10.9 ± 1.7	8.1 ± 1.5	6.0 ± 1.6	—
	0.1	15.4 ± 1.8	15.1 ± 2.5	15.3 ± 1.3	12.3 ± 1.0	10.3 ± 1.3	6.5 ± 2.0	—
	0.2	—	—	—	13.8 ± 1.3	11.9 ± 1.5	7.5 ± 2.1	2.0 ± 1.2

[a] Approved PPD from *M. tuberculosis* Aoyama B. See the text.
[b] Number of guinea pigs used.
[c] Calculated from the amount of nitrogen determined by the method of Kjeldahl.
[d] Calculated from the amount corresponding to the standard potency.

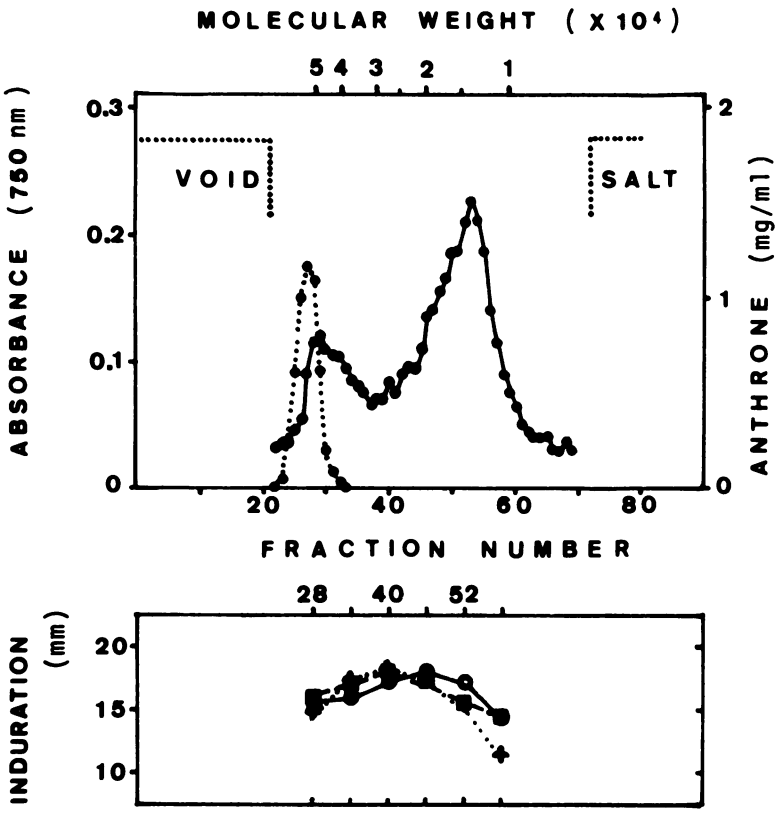

FIG. 9. *Gel filtration of the concentrated culture filtrate through a Sephadex G-75 column and the skin reactivity of the fractions. Five milliliters of the concentrated culture filtrate, prepared with ammonium sulfate at 80% saturation [see the text, purification of MPB70 (i)], was applied to a Sephadex G-75 column (2.5 by 100 cm) and chromatographed with 30 mM Tris-hydrochloride buffer (pH 7.5) containing 0.1 M sodium chloride. The protein content (●——●) was analyzed by the method of Lowry et al. (25), using an 0.05-ml sample of each fraction (6.3 ml). The carbohydrate content (● · · · · · ●) was analyzed by the anthrone method (32). The molecular weight scale was calibrated with the standard proteins (see the text). The skin reactivity was expressed by plotting the mean values of the induration diameter at 24 h after the injection of 0.4 μg of protein, determined by the method of Kjeldahl, of the fractions indicated, in guinea pigs sensitized with BCG (○; 12 guinea pigs were used) or M. tuberculosis strain H37Rv (□; 10 guinea pigs were used) or Aoyama B (+, 8 guinea pigs were used).*

cally. Of these, its large amount in the culture filtrate is most important, and this has not been reported. This high content of MPB70, however, does not seem to be the special property of the BCG strain Tokyo 172 which we used, because similar antigenic properties were observed in 11 other strains of BCG (5). Estimating from the final yield (8.9% by the Lowry method), it can be supposed that MPB70 existed originally at a content of more than 10% of the protein in the culture filtrate. The relative amount of MPB70 was high even in a young culture medium of 3 weeks, and MPB70 is probably not an autolyzed protein but one secreted into the medium from cells as a natural product when the cells are growing actively.

The amount of protein in BCG cells and the culture filtrate of Sauton medium, in relation to the cultivation term, has already been reported, and after 5 weeks of cultivation the amount of protein in the medium did not change (33). Our observations agreed with these results, and we recommend that a culture filtrate of less than 5 weeks of cultivation be used for MPB70 preparation to avoid contamination by autolyzed materials. The physiological aspects of MPB70 in the cell growth of BCG are not known, and it will be very interesting to study the mechanism for the massive secretion of a single such specific protein into the culture medium.

MPB70 was purified essentially by treatment with DEAE ion exchangers. Changes in pH or salt concentration in the elution buffers, however, did not improve the purification of MPB70.

We found the additional use of urea in the buffer to be quite effective. In the preparation from a virulent strain, *M. tuberculosis* H37Rv, it is necessary to use a high concentration of urea to unfold the tangled proteins which are produced by autoclaving, as previously reported (27). The culture filtrate of BCG does not need to be sterilized by heating; therefore, the preparation method for BCG differs from that for *M. tuberculosis* H37Rv. The use of urea, however, was an important process in the preparation of MPB70 also. The critical concentration of urea for changing the molecular conformation of MPB70 was 2 M, checked with electrophoresis at a running pH of 7.0 to 8.0; in the 3 M urea contained in the elution buffer of DEAE-Sepharose CL-6B chromatography (iv), MPB70 changed its conformation completely. The detailed mechanism affecting MPB70 and other substances in the sample is not known, but this urea treatment was very effective and improved the purification of MPB70. The change in conformation of MPB70 by the 3 M urea was reversible and, by removing urea with dialysis, MPB70 gradually recovered its solubility and original properties.

The identification of protein in our preparation procedure was performed by analysis with 7.7% polyacrylamide gel at a running pH of 9.4. A protein with a relative mobility of 0.70, which is the same as that of MPB70 in gel electrophoresis, was eluted just after the peak of MPB70 in the DEAE-Sepharose CL-6B chromatography without urea. We named this protein fraction MPB70a because its nature was slightly more acidic than that of MPB70 in the DEAE ion exchanger. Other than this, we observed no difference from MPB70 in the electrophoresis with SDS-gel or in the Ouchterlony gel diffusion. There is a possibility that some acidic substances of small molecular weights bound to MPB70. In the experiments reported here, MPB70a was collected separately and was not used.

The molecular weights estimated with the SDS-gel electrophoresis or with the gel filtration through Sephadex G-50 were similar, which means that the MPB70 preparation did not contain subunits. The value of 15,100 obtained by the sedimentation equilibrium method was used as the molecular weight of MPB70 because this method is supported by a theoretical background.

MPB70 is a stable protein, but when it was kept at 95°C for 30 min at pH 5.0, 7.5, or 8.7, some degraded products appeared in the subsequent gel analysis. With regard to skin reactivity, however, the heated MPB70 at neutral pH did not show any different features from those of the unheated MPB70.

The anti-MPB70 serum did not form a precipitin line with PPD preparations from *M. tuberculosis* H37Rv or Aoyama B, *M. kansasii*, or *M. intracellulare*, but only with the PPD from BCG. This might be interpreted to mean that MPB70 is a protein constituent peculiar to BCG and not included in the other mycobacteria tested. This is a very strange observation because no report has been published on such a unique protein in BCG, and antigenic similarities and cross-reactivities were observed on preparations from either BCG or *M. tuberculosis* (2, 4, 5, 8, 15, 18, 19, 24, 27, 35).

The species specificity of MPB70 in skin reaction can be attributed to some unique units in its configuration. In the preliminary experiments (unpublished data) on some other proteins isolated, such as MPBs 80, 64, 59, and 83, from the culture filtrate of BCG, the species specificity in the skin reaction which was observed for MPB70 was also found for MPB80 to a slightly weaker extent, but all the other MPBs were cross-reactive with *M. tuberculosis*. The potency in skin reactivity of these MPBs was very different for each one, as observed with the isolated proteins from *M. phlei* (26).

Some other preparations from BCG cells or culture filtrate have been reported (12, 20–23), but they are different from MPB70 with their larger molecular weights or glycoprotein nature. From *M. tuberculosis* H37Ra, common antigens of both *M. tuberculosis* and BCG, especially antigen 5 (7–9), were isolated. These products are also differentiated from MPB70 for the same reasons as described above. It should be noted that MPB70 did not contain either neutral or amino sugars; this was determined with the use of a large amount of the sample (20 mg for each test).

We obtained at least 10 mg of the final lyophilized MPB70 preparation from 1 liter of the original culture filtrate of BCG. This high yield is a very important property of MPB70 with its unique specificity in skin reaction, and more detailed studies can be expected by the use of highly purified MPB70, for example, in the physiology of BCG cells, in the mechanism of delayed hypersensitivity in place of PPD, and possibly in the hygiene and epidemiology of tuberculosis, where MPB70 might be able to check sensitization by BCG vaccination.

ACKNOWLEDGMENT

This work was supported by a National Sanatorium Division grant from the Ministry of Health and Welfare, Japan.

LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochem. J. **91:** 222–233.

2. **Baker, R. E., W. E. Hill, and C. L. Larson.** 1973. Ribosomes of acid-fast bacilli: immunogenicity, serology, and in vitro correlates of delayed hypersensitivity. Infect. Immun. **8**:236–244.
3. **Boas, N. F.** 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204**:553–563.
4. **Chaparas, S. D., and H. Baer.** 1964. The immunology and chemistry of tuberculin. II. Chromatography with Sephadex of the nondialyzable tuberculin-active constituents of BCG culture filtrate. Am. Rev. Respir. Dis. **89**:41–48.
5. **Chaparas, S. D., and S. R. Hedrick.** 1973. Comparison of strains of BCG. I. Antigenic analysis and tuberculin reactivity. Infect. Immun. **7**:777–780.
6. **Daniel, T. M.** 1976. Tuberculin antigens: the need for purification. Am. Rev. Respir. Dis. **113**:717–719.
7. **Daniel, T. M., and P. A. Anderson.** 1978. The isolation by immunoabsorbent affinity chromatography and physicochemical characterization of *Mycobacterium tuberculosis* antigen 5. Am. Rev. Respir. Dis. **117**:533–539.
8. **Daniel, T. M., J. J. Ellner, L. S. Todd, D. W. McCoy, V. D. N. Payne, P. A. Anderson, and F. T. Bhe.** 1979. Immunobiology and species distribution of *Mycobacterium tuberculosis* antigen 5. Infect. Immun. **24**: 77–82.
9. **Daniel, T. M., and L. E. Ferguson.** 1970. Purification and characterization of two proteins from culture filtrates of *Mycobacterium tuberculosis* H37Ra strain. Infect. Immun. **1**:164–168.
10. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. **42**:84–113.
11. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121**:404–427.
12. **Diena, B. B., H. Yugi, R. Wallace, J. Carriere, and L. Greenberg.** 1968. The bentonite flocculation test in the serology of tuberculins. I. Purification of BCG antigens. Can. J. Microbiol. **14**:881–885.
13. **Dubios, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
14. **Elson, L. A., and W. T. J. Morgan.** 1933. Colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. **27**:1824–1828.
15. **Ferguson, H. R., J. K. McClatchy, T. R. Sharpton, and P. Minden.** 1978. Immunological method to differentiate between antigens of tubercle bacilli, other mycobacterial species, and non-acid-fast bacteria. Infect. Immun. **22**:101–106.
16. **Goodwin, T. W., and R. A. Morton.** 1946. The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. **40**:628–632.
17. **Gray, W. R.** 1972. End-group analysis using dansyl chloride. Methods Enzymol. **25**:121–138.
18. **Gupta, K. C., and S. Landi.** 1978. Isolation of tuberculin peptides from tuberculin purified protein derivative (PPD). Can. J. Microbiol. **24**:1242–1249.
19. **Gupta, K. C., and S. Landi.** 1980. Isolation, characterization, and biological properties of a tuberculin-active peptidoglycan isolated from the culture filtrate of *Mycobacterium tuberculosis*. Infect. Immun. **27**:344–350.
20. **Harboe, M., O. Closs, and J. Deverill.** 1976. Production of monospecific antisera against antigenic components of *Mycobacterium bovis* (BCG). Scand. J. Immunol. **5**: 861–866.
21. **Harboe, M., O. Closs, K. Svindahl, and J. Deverill.** 1977. Production and assay of antibodies against one antigenic component of *Mycobacterium bovis* BCG. Infect. Immun. **16**:662–672.
22. **Laguerre, M., and R. Turcotte.** 1975. Purification and characterization of tuberculin-active components from BCG. Can. J. Microbiol. **21**:2019–2027.
23. **Laguerre, M., and R. Turcotte.** 1977. Use of sepharose-conjugated BCG antibodies for the purification of tuberculin-active components. Can. J. Microbiol. **23**:695–700.
24. **Larson, C. L., R. E. Baker, and M. B. Baker.** 1970. Comparison of antigenic fractions obtained by Seibert and Affronti method from protoplasm and culture filtrates of *Mycobacterium bovis* (BCG). Am. Rev. Respir. Dis. **101**:979–983.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Matsumoto, J., K. Kohda, S. Nagai, and T. Nagasuga.** 1979. Various antigenic reactivities in delayed hypersensitivity among crystalline proteins from *Mycobacterium phlei*. Am. Rev. Respir. Dis. **120**:619–624.
27. **Nagai, S., T. Nagasuga, and J. Matsumoto.** 1980. Tuberculin peptide from culture filtrate of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **121**:551–557.
28. **Nagai, S., T. Nagasuga, J. Matsumoto, and K. Kohda.** 1974. Isolation of tuberculin skin-test reactive proteins from heated culture filtrate of *Mycobacterium tuberculosis* H37Rv. Am. Rev. Respir. Dis. **109**:17–28.
29. **Orr, M. D., R. L. Blaklay, and D. Panagon.** 1972. Discontinuous buffer systems for analytical and preparative electrophoresis of enzymes on polyacrylamide gels. Anal. Biochem. **45**:68–85.
30. **Ouchterlony, O.** 1958. Diffusion in gel-methods for immunological analysis. Prog. Allergy **5**:1–9.
31. **Runyon, E. H.** 1976. Tuberculins and other mycobacterins. Am. Rev. Respir. Dis. **113**:715.
32. **Seifter, S., S. Dayton, B. Novic, and E. Muntwyler.** 1950. The estimation of glycogen with the anthrone reagent. Arch. Biochem. **25**:191–200.
33. **Turcotte, R., and Y. Des Ormeaux.** 1972. Influence of the age of mycobacterial cultures on the protein and carbohydrate composition of tuberculins. Can. J. Microbiol. **18**:637–645.
34. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**: 4406–4412.
35. **Yoneda, M., Y. Fukui, and T. Yamanouchi.** 1965. Extracellular proteins of tubercule bacilli. V. Distribution of α and β antigens in various mycobacteria. Biken J. **8**:201–223.