Purification and Properties of Tuberculin-active Protein from Mycobacterium tuberculosis

(Received for publication, September 12, 1973)

SEISHI KUWABARA

From the Institute of Medical Science, University of Tokyo, Tokyo 108, Japan

SUMMARY

When Mycobacterium tuberculosis was grown on Sauton medium, intracellular tuberculin-active protein was produced. This product was purified by chromatography on DEAE-cellulose and Sephadex G-200 and was obtained in crystalline form. The crystals were plates, somewhat irregular in shape, about 50 µm in length and 25 µm in width. The yield corresponded to a 1.5% over-all recovery of total protein. Ultracentrifugal analysis showed only one major component with a calculated molecular weight of 9700. Sedimentation velocity analysis gave a sedimentation coefficient at 20°C ($s_{20,w}$) of 1.73 S. The estimated specific activities of tuberculin-active protein were 6.33 × 10⁹ tuberculin units per mg of protein-nitrogen for sensitized guinea pigs and 6.33 × 10¹⁰ tuberculin units per mg of protein-nitrogen for humans. This is the most potent tuberculin-active protein that has yet been obtained.

Old tuberculin was first observed by Koch (1, 2) in the culture medium of human-type tubercle bacilli, Mycobacterium tuberculosis. In humans or laboratory animals immunized with tubercle bacilli, 0.1 ml of a solution of old tuberculin given intracutaneously can produce an allergic reaction within 24 to 48 hours. Haas (1958) showed that old tuberculin is a protein and can be separated into at least three distinct protein bands by zone electrophoresis. The material was purified by Seibert (4-7) and called purified protein derivative. PPD⁴ is widely used and is presently the most popular tuberculin-active protein preparation.

I have been able to obtain intracellular tuberculin-active protein from the cells of tubercle bacilli instead of from culture fluid. It exhibits a higher tuberculin activity than PPD (8), with 10 µg equivalent to 1 TU, as compared to 20 ng of PPD required for a positive Mantoux skin test of a sensitized person.

**Buffer Used in Purification and Assay**—Tris buffer was used in the purification of tuberculin-active protein. Stock Tris buffer consisted of 0.1 m Tris-HCl, pH 7.0, containing 1 mM EDTA. Diluted buffers (10 mM and 1 mM in Tris) were also used. In the diluted buffers, the final concentration of EDTA was always 1 mM. Phosphate buffered saline (PBS)-stock buffer (0.1 m Na₂HPO₄, 0.06 m K₂HPO₄, pH 7.3) was diluted 10 times with 0.85% NaCl solution before use.

**Determination of Protein**—Protein was determined by the method of Lowry et al. (9) with bovine serum albumin (Koch/Light Laboratory Ltd., Colnbrook, Buckinghamshire, United Kingdom) as a standard. Protein-N was calculated on the assumption that the protein measured contained 15.8% nitrogen. In some cases determination of protein and nucleic acid was made by the spectrophotometric method of Warburg and Christian (10).

**Amino Acid Analysis**—Amino acids in acid hydrolysates of tuberculin-active protein (200 to 700 µg) were determined as described by Moore et al. (11) and Spackman et al. (12) with an amino acid analyzer. Hydrolysis was performed in 6 M of HCl at 110°C for 24, 48, and 72 hours. Cystine and methionine were determined as cysteic acid and methionine sulfone as described by Hiro (18). Identification of the NH₂-terminal residues of tuberculin-active protein was determined by the method of Archibald (21).

**Determination of Molecular Weight**—The molecular weight of tuberculin-active protein was determined by the method of Archibald (21) in a synthetic boundary cell in order to create a boundary away from the meniscus. A solution of tuberculin-active protein (0.3 mg per ml) dialyzed against 0.1 m Tris was put in a synthetic boundary cell, and the solvent was layered on top in the centrifuge. The boundary was left to diffuse until it formed a bell-shaped curve in the schlieren optics, and a photograph was taken. This was done at full speed, and at a $B$ form was obtained from a series of pictures of log $r$ versus $t$ was plotted—slope is $ω_0$. The area under the curve was measured. The experiments were repeated with 0.1 m Tris in the synthetic boundary cell, again using a double sector cell with the same solvent in the second half. Pictures were taken at the same bar angle (60°) as used in the synthetic boundary run. The area under the curve between the meniscus and place where the curve reaches the base-line was measured. This was subtracted from the area under the curve in the synthetic boundary run, and designated area $A$ in square centimeters. The height ($H$) in centimeters of the line from the base-line at the meniscus was measured. The molecular weight, $M$, was cal-

---

¹ The abbreviations used are: PPD, purified protein derivative; TU, international tuberculin unit which is specified as equal to 20 ng of PPD; BCG, Bacillus bili de Calmette et Guérin, strain Tokyo 172.
calculated from the equation,

\[
\frac{H - m}{r - A} = \frac{M(1 - \theta)\omega^{2}}{RT}
\]

in which \(m\) is the magnification of the optical system in a horizontal direction, \(r\) is the distance of the meniscus from the center of rotation in cm, \(\theta\) is the partial specific volume of the protein, \(\rho\) is the density of the solution, \(\omega\) is the angular speed of the rotor (radians per s), \(R\) is the gas constant, and \(T\) the absolute temperature.

Preparation of Cells of Mycobacterium Tuberculosis Aoyama/B and BCG—The human-type tubercle bacillus, strain Aoyama/B, obtained from the Department of Tuberculosis, National Institute of Health in Tokyo, was inoculated in veal infusion broth and, when sufficiently grown, transferred to Sauton's synthetic medium (22) in 1-liter bottles containing about 200 ml each. After incubation at 37.5°C for 6 to 8 weeks, the cultures were shaken and heated in a sterilizer at 120°C for 30 min. The bacilli were filtered from the culture fluid through a Buchner funnel, and stored at -15°C. The avirulent bovine type strain BCG, a stock culture of the National Institute of Health in Tokyo, was cultured in the supernatant was collected, dissolved in a small amount of 1 mM Tris, and dialyzed against the same buffer overnight at 4°C. Assay of the resulting solution showed that the specific activity of tuberculin-active protein had increased 4-fold (Table I, Stage 4).

Stage 5: First DEAE-cellulose Column—The resulting solution (283 mg, 10 ml) was added to a DEAE-cellulose column (100 x 1 cm) and eluted at room temperature with 1 mM Tris buffer containing 1 mM NaCl. Fractions (2 ml) were collected every minute, and the active fractions were combined (tubes 0 to 15). The protein recovered was 5.67% of the total (Fig. 1, left). The material at this stage contained 2.9% nucleic acid, showing an \(E_{280/260}\) value of 1.11. The specific activity of tuberculin-active protein was increased about 14-fold at this stage.

Stage 6: Second DEAE-cellulose Column—The solution from Stage 5 (74.6 mg) was brought to 55% saturation with solid \((\text{NH}_{4})_{2}\text{SO}_{4}\) at pH 7.0. The mixture was stirred for 30 min at 4°C and centrifuged for 15 min at 14,000 x g. The concentration of \((\text{NH}_{4})_{2}\text{SO}_{4}\) in the supernatant was raised to 96.6% saturation, and the mixture was stirred and centrifuged as before. The precipitate was dissolved in a small volume (5 ml) of 10 mM Tris buffer. The resulting solution was chromatographed on a

TABLE I

| Stage | Material from | Total protein (mg) | Protein recovered (l) | \(E_{280/260}\) | 10\(^{9}\) Sp. activity units/mg of protein-N | Purification |
|-------|---------------|--------------------|-----------------------|-----------------|-------------------------------------------|-------------|
| 1     | Heated cells, Aoyama/B | (519000) * | 100 | 0.90 | 2.06 | 100 | 0.10 | 1 |
| 2     | Acetone-dried powder from heated cells | (500000) * | 1319 | 100 | 0.90 | 2.06 | 100 | 0.10 | 1 |
| 3     | Cell-free extracts, treatment with streptomycin | 283 | 215 | 0.99 | 1.48 | 72 | 0.39 | 4 |
| 4     | Eluate from first DEAE-cellulose column | 74.6 | 5.7 | 1.11 | 1.58 | 77 | 1.34 | 14 |
| 5     | Eluate from second DEAE-cellulose column | 37.0 | 2.8 | 1.55 | 1.61 | 78 | 2.75 | 28 |
| 6     | Eluate from Sephadex G-200 | 25.9 | 1.8 | 1.80 | 1.69 | 82 | 4.45 | 45 |
| 7     | First crystallization | 20.0 | 1.5 | 2.00 | 2.00 | 97 | 6.33 | 64 |

* Determined as wet weight. ** International tuberculin unit (TU) which is specified as equal to 20 ng of PPD. The values given for total units are those obtained with guinea pigs.
DEAE-cellulose column (100 x 1 cm). Elution of protein from the column was carried out with a sodium chloride gradient obtained by using 500 ml of 1 mM Tris buffer in a mixing chamber with a magnetic stirrer and an equal volume of the same buffer containing 0.1 M sodium chloride in a reservoir. Fractions (2 ml each) were collected every minute (Fig. 1, right). Virtually all of the activity was found in Fractions 4 to 9, with peak activity in Fraction 5. The active protein was precipitated by the addition of (NH₄)₂SO₄ to saturation. The precipitate was separated by centrifugation and dialyzed against 10 mM Tris buffer for 12 hours. The protein recovery was 2.8%. The material at this stage contained 0.5% nucleic acid and showed an $E_{280}$ value of 1.55. The specific activity of tuberculin-active protein in the resulting solution was about 2.0 times that at Stage 5.

Stage 7: Sephadex G-200 Column—The solution of protein prepared as described above was added to a Sephadex G-200 column (100 x 1 cm). Elution was carried out with 10 mM Tris buffer and fractions (0.1 ml) were collected every minute at room temperature (Fig. 2). Fractions 28 to 50 were combined. The over-all recovery of protein in the combined fractions was 1.5%, and its $E_{280}$ value (1.8) indicated that it was free of nucleic acid. The resulting solution contained $4.44 	imes 10^9$ TU per mg of protein-N and the over-all number of units recovered was 82%. At this stage the active protein was pure enough for crystallization.

Stage 8: First Crystallization—The resulting solution from Stage 7 was dialyzed against 10 mM Tris buffer at 4°C overnight. Solid (NH₄)₂SO₄ was added to the dialyzed protein solution (5 ml), to 85% saturation, and kept in a cold room (4°C) for 2 weeks in tightly sealed 10-ml Pyrex bottles. The white precipitate was separated from the solution, cold 50% acetone was gently added without mixing, and the upper acetone-rich layer was discarded. The resulting white precipitate was again treated with cold acetone, dissolved in 50% cold acetone, and left at room temperature. Immediately after the above treatment, a silvery sheen began to appear on the surface of the mixture, and the first crystals of the tuberculin-active protein began to form. The crystals were slightly soluble in water and soluble in a salt solution such as 1 mM or higher concentration of Tris buffer, or a solution of 50% saturated (NH₄)₂SO₄. The crystals were somewhat irregularly shaped plates, about 50 μm in length and 25 μm in width (Fig. 3). They contained $6.33 	imes 10^8$ TU per mg of protein-N as assayed in guinea pigs and $6.33 	imes 10^9$ TU per mg of protein-N as assayed in guinea pigs.
in humans. The yield corresponded to 1.5% over-all recovery of total protein and 97% over-all recovery of tuberculin activity. Recrystallization was carried out in essentially the same manner as in Stage 8. The specific activity, shape, and size of the crystals did not change significantly on recrystallization. The crystals were in 50% saturated (NH₄)₂SO₄, pH 7.0, in 10 mM Tris buffer. When kept under the conditions at 2-4°C and at a concentration of 10 mg per ml, tuberculin-active protein was stable for months. Purification of tuberculin-active protein from Mycobacterium tuberculosis Aoyama/B is summarized in Table 1.

Purification of Tuberculin-active Protein From Avirulent Bovine-type BCG Strain

This procedure was the same as in the purification of tuberculin-active protein from human-type Aoyama/B strain except that intact cells from the avirulent bovine-type living BCG strain were used as starting material in Stage 1. The purification of tuberculin-active protein from this source is summarized in Table II.

Ultracentrifugal Analysis of Tuberculin-active Protein

The results of ultracentrifugal analysis of crystalline tuberculin-active protein from Aoyama/B and BCG indicate only one major component. Sedimentation velocity showed that the coefficient at 20°C (S₂₀,w) in 0.1 M Tris buffer was 1.73 S for tuberculin-active protein from Aoyama/B strain (Fig. 5). As shown in Fig. 4, such a low S value was very difficult to measure for the estimation of the molecular weight and required the use of synthetic boundary cells in order to create a boundary away from the meniscus. An analysis of the material at the meniscus gave 9700 for the molecular weight using a partial specific volume of 0.734 (23). Neither value was appreciably dependent on concentration.

Electrophoretic Analysis

Electrophoretic analysis was carried out on a Perkin-Elmer electrophoresis apparatus using a 2-ml cell and a 8.9 mM Na-
HPO₄-1 mM EDTA mixture in 0.17 M NaCl. Fig. 6 shows schlieren patterns of a 1% solution of tuberculin-active protein from Aoyama/B in the descending limb of the Tiselius cell. The schlieren pattern remains a sharp, single peak after 120 min.

**Amino Acid Composition of Tuberculin-active Protein**

The amino acid compositions of the tuberculin-active protein from two strains of *Mycobacterium tuberculosis* are given in Table III. No amino sugars nor carbohydrate was detected in the protein of either strain. The amino acid composition of the Aoyama/B strain protein is based on a molecular weight of 9700 (1). The molecular weight of BCG strain is not known, so the amino acid composition of this protein is based on the assumption of 1 mol of histidine per mol of protein. The amino acid compositions of the tuberculin-active protein from the two strains are very similar except that the protein of the BCG strain has less tyrosine, methionine, and half-cystine, and more arginine than the Aoyama/B protein. No free sulphydryl groups were detected by the Ellman procedure (24). Oxidation of cystine and methionine at -10°C for 2½ hours with performic acid yielded 92% each of cysteic acid and methionine sulfone. The NH₂-terminal analysis by the Edman procedure (14, 15) revealed a single NH₂-terminal residue (arginine), confirms that tuberculin-active protein has been isolated in a homogenous form.

**Tuberculin Activity of Tuberculin-active Protein**

Guinea Pigs—Various doses of tuberculin-active protein from Aoyama/B strain or BCG dissolved in 0.1 ml PBS buffer, were injected into guinea pigs intracutaneously. As little as 1.0 ng was sufficient to produce a tuberculin-positive skin test in guinea pigs immunized by heat-killed Aoyama/B strain (Fig. 7). This amount of tuberculin-active protein is equivalent to 1 TU, which is equal to 20 ng of PPD (Fig. 7). No skin reaction was observed in guinea pigs without sensitization by Aoyama/B strain or BCG. There is no difference in tuberculin activity between tuberculin-active protein from BCG and that from Aoyama/B.

**Table III**

Amino acid composition of tuberculin-active protein from *Mycobacterium tuberculosis* Aoyama/B

| Amino acid     | 24 hours (residues/9700 g) | 48 hours (residues/9700 g) | 72 hours (residues/9700 g) | Average of extrapolated value | Best whole no. |
|----------------|-----------------------------|-----------------------------|----------------------------|-------------------------------|----------------|
| Lysine         | 4.11                        | 4.02                        | 4.12                       | 4.08                          | 4(4)           |
| Histidine      | 1.11                        | 1.02                        | 1.21                       | 1.11                          | 1(1)           |
| Arginine       | 3.89                        | 4.00                        | 5.92                       | 3.93                          | 1.04           |
| Aspartic acid  | 11.03                       | 11.03                       | 11.11                      | 11.06                         | 11(10)         |
| Threonine      | 5.05                        | 4.93                        | 4.88                       | 4.94                          | 5(5)           |
| Serine         | 3.95                        | 3.88                        | 3.76                       | 3.86                          | 4(4)           |
| Glutamic acid  | 0.90                        | 11.03                       | 10.90                      | 10.94                         | 11(11)         |
| Proline        | 5.01                        | 5.01                        | 5.11                       | 5.05                          | 5(4)           |
| Glycine        | 7.01                        | 7.11                        | 6.98                       | 7.03                          | 7(8)           |
| Alanine        | 21.01                       | 11.00                       | 11.11                      | 11.04                         | 11(10)         |
| Half-cystine*  | 1.94                        | 1.94                        | 1.94                       | 1.94                          | 2(1)           |
| Valine         | 7.89                        | 8.03                        | 8.21                       | 8.04                          | 8(7)           |
| Methionine**   | 7.89                        | 8.03                        | 8.21                       | 8.04                          | 8(7)           |
| Isoleucine     | 3.99                        | 4.04                        | 4.05                       | 4.02                          | 4(5)           |
| Leucine        | 6.83                        | 7.11                        | 7.19                       | 7.04                          | 7(8)           |
| Tyrosine       | 2.10                        | 1.98                        | 1.89                       | 1.98                          | 2(1)           |
| Phenylalanine  | 1.98                        | 1.97                        | 1.88                       | 1.94                          | 2(1)           |
| Tryptophan     | -                           | -                           | -                          | -                             | -              |

* Determined as cysteic acid. ** Determined as methionine sulfone. The relative numbers of residues per molecule of tuberculin-active protein from BCG strain given in parentheses. For details see text.
FIG. 7. Tuberculin activities of tuberculin-active protein from strain Aoyama/B and PPD in guinea pigs. The PBS buffers (0.1 ml) containing various doses of tuberculin-active protein (—whooping) and PPD (—△—△) were administered intracutaneously in the ventral side of guinea pigs immunized with complete Freund's adjuvant containing heat-killed Aoyama/B strain 6 weeks before. After 24 (---) to 48 hours (-----) the diameters of the erythematous areas were determined.

Humans—An injection of 0.1 ml of PBS buffer containing 10 pg of tuberculin-active protein from Aoyama/B was sufficient to produce a positive skin test in a man immunized with tubercle bacilli Aoyama/B strain or BCG (Fig. 8). The above amount of tuberculin-active protein is equivalent to 1 TU, which indicates that its activity is about 200 times higher than that of PPD. The sensitivity of humans to tuberculin-active protein is about 100 times higher than that of guinea pigs.

DISCUSSION

Intracellular tuberculin-active protein might be closely associated with the cytoplasmic membrane. Pollock (25) discovered two immunologically distinct types of β-lactamase in cultures of Bacillus cereus. Over 90% of the enzyme is of one type (α-type) and is found almost exclusively in the culture supernatant fluid. The other immunological type of β-lactamase, termed γ-type, is invariably bound to the cell. There is evidence (26) that the γ-type enzyme, in contrast to the small fraction of α-type enzyme absorbed in the cells, is firmly attached to the cytoplasmic membrane. According to one report (27) part of the γ-type appears to be bound to ribosomes. The enzyme is largely obtained in solution when the cells are extensively disintegrated (24), and it can be released from isolated membrane preparation by sonic disintegration or by treatment with organic solvents (25). Tuberculin-active protein and γ-type enzyme resemble each other in their localization and method of preparation. Although the extent of purification of tuberculin-active protein from the avirulent bovine-type BCG strain is much higher than from human-type Aoyama/B strain, this might be due to the heat used to kill the cells of the latter at the first stage of purification. The two preparations showed equal antigenic activity against guinea pigs that had been immunized with killed Aoyama/B strain. As a source of tuberculin-active protein, human-type Aoyama/B strain and bovine-type BCG are essentially the same.

An assessment of the significance of the apparent similarities and differences between tuberculin-active protein obtained by this method and PPD by Seibert (28, 29) derives from the sources of the starting materials. Whether intracellular tuberculin-active protein is the precursor of extracellular PPD by passage through the cell walls or cytoplasmic membranes remains to be ascertained. As shown in Fig. 7, a parallel relationship is obtainable between activities in both tuberculin-active protein and PPD when they are administered subcutaneously in guinea pigs sensitized with killed Aoyama/B strain or living BCG.

It is postulated that tuberculin-active protein has greater activity in a sensitized human subject than laboratory animals such as guinea pigs. When tuberculin-active protein is injected in humans, only 10 pg, the equivalent to 20 ng of PPD (1 TU), is required to get a positive Mantoux skin test. About 100 times more tuberculin-active protein, 1000 pg, is required to get a positive skin test with sensitized guinea pigs. PPD shows a wider range of tuberculin-activity, and 20 ng may be administered either to humans or guinea pigs with the same affinity. Another apparent difference between tuberculin-active protein and PPD is the relationship between the protein concentration...
and hypersensitivity in sensitized guinea pigs. Quite clear erythematous areas have been obtained even if a concentration of 1 ng tuberculin-active protein is administered to sensitized guinea pigs, but if less than 1 TU of PPD is administered no significant erythematous areas are obtainable.

The reasons why only tuberculin-active protein can respond at such a low concentration were not determined, but the difference in composition, the physicochemical properties, and the purity of these two proteins might give some indication.

Acknowledgments—The author is indebted to Dr. T. Tsumita for helpful discussion and advice. He is also indebted to Dr. T. Morohashi and his colleagues for assistance in assaying tuberculin activity.

REFERENCES

1. Koch, R. (1891) Dtsch. Med. Wochenschr. 17, 1198
2. Koch, R. (1912) Gesammelte Werke von R. Koch 1, 661
3. Haas, M. (1952) Zbl. Bakter. I. O. 158, 175-192
4. Seibert, F. B. (1950) J. Immunol. 55, 239-310
5. Seibert, F. B. (1949) Am. Rev. Tuberc. Pulm. Dis. 59, 86-101
6. Seibert, F. B. (1944) Chem. Rev. 34, 107-110
7. Long, E. R. & Seibert, F. B. (1926) Am. Rev. Tuberc. Pulm. Dis. 13, 448-453
8. Long, E. R. & Seibert, F. B. (1926) Am. Rev. Tuberc. Pulm. Dis. 13, 563-597
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
10. Warburg, O. & Christian, W. (1941) Biochem. Z. 210, 384-421
11. Moore, S., Spackman, D. H. & Stein, W. H. (1958) Anal. Chem. 30, 1190-1198
12. Spackman, D. H., Stein, W. H. & Moore, S. (1958) Anal. Chem. 30, 1190-1210
13. Hirs, C. H. W. (1956) J. Biol. Chem. 216, 611-621
14. Edman, P. (1950) Acta Chem. Scand. 4, 277-283
15. Edman, P. & Begg, G. (1963) Eur. J. Biochem. 1, 80-91
16. Fraenkel-Conrat, H. (1954) J. Am. Chem. Soc. 76, 3606-3607
17. Hedgely, E. T. & Overend, W. D. (1960) Chem. Ind. (London) 378
18. Sweeney, C. C. & Walker, B. (1963) Anal. Chem. 36, 1461-1466
19. Schachman, H. K. (1959) Ultracentrifugation in Biochemistry, Academic Press, New York
20. Svedberg, T. & Pederson, K. O. (1940) The Ultracentrifuge, Oxford University Press, New York
21. Archibald, W. J. (1947) J. Phys. Colloid Chem. 51, 1204-1241
22. Sauton, B. (1912) C. R. Acad. Sci. 154, 1869-1865
23. Hers, R. J. & Schachman, H. K. (1958) Virology, 6, 234-243
24. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-77
25. Pollock, M. R. (1956) J. Gen. Microbiol. 15, 154-169
26. Scheinin, R. (1950) J. Gen. Microbiol. 21, 124-134
27. Duerksen, J. D. & O'Connor, M. L. (1963) Biochem. Biophys. Res. Commun. 10, 34-39
28. Seibert, F. B. (1941) Am. Rev. Tuberc. Pulm. Dis. 44, 1 S
29. Seibert, F. B. & Glenn, J. T. (1941) Am. Rev. Tuberc. Pulm. Dis. 44, 9-25
Purification and properties of tuberculin-active protein from Mycobacterium tuberculosis.
S Kuwabara

J. Biol. Chem. 1975, 250:2556-2562.

Access the most updated version of this article at http://www.jbc.org/content/250/7/2556

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/7/2556.full.html#ref-list-1