STUDIES ON TUBERCULIN ACTIVE PEPTIDE

I. THE ISOLATION, CRYSTALLIZATION AND PROPERTIES OF TUBERCULIN ACTIVE PEPTIDE FROM TUBERCLE BACILLUS

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SUMMARY

A highly potent basic peptide was isolated in pure form from the bodies of tubercle bacilli. Crystallization was carried out using a methanol solution of the purified peptide. Crystals of flavianate and picrolonate of the peptide were also obtained.

The chemical and biological properties of the purified peptide are described. The tuberculin skin potency tests on human subjects indicated that the purified peptide was of a potentcy comparable to that of purified protein isolated from bacillary bodies or culture filtrate of bacilli.

INTRODUCTION

Numerous attempts have been made to isolate a biologically active protein fraction either from the culture filtrate of tubercle bacilli or from the bacillary bodies[1-6].

From these reports, the following points emerged about the tuberculin active principle.

(1) The evidence for the protein nature of the active principle has been conclusive and potent tuberculin protein molecules of many different sizes, shapes and complexities have been identified.

(2) The active protein is rather stable towards heating.

(3) Pepsin digestion of a tuberculin preparation is not able to destroy its potency completely.

These results seemed to indicate that some tuberculin activity resides even in very small protein or peptide molecules. The present study deals with the extraction, purification and crystallization of a tuberculin active peptide and some of its chemical and biological properties.

EXPERIMENTAL

Purification and crystallization of tuberculin active peptide from tubercle bacilli

Tubercle bacilli of human strain, H37Rv, were grown on Sauton's synthetic medium for seven to ten weeks and sterilized by heating at 100° for 30 min. The bacterial cells were filtered off and washed repeatedly with distilled water. After being

washed several times with cold acetone, the bacilli were dried *in vacuo* and stored in a desiccator as an acetone-dried powder. About 50 g of the dried bacilli were extracted with 10 volumes (500 ml) of 0.1 *N* HCl at 37° for 72 h with frequent stirring. The mixture was filtered through filter paper and the residue was washed 2–3 times with 100 ml of 0.1 *N* HCl. The filtrate and washings were combined and filtered through a Seitz filter to remove the bacilli. The slightly opalescent filtrate was brought to pH 7.0 by the cautious addition of 5.0 *N* NaOH. It was left standing at room temperature for 2 h or more, and the flocculent white precipitate was removed by filtration.

The clear filtrate was added to an equal volume of a saturated solution of picric acid. A yellow precipitate was obtained after the mixture was left at room temperature overnight. As much of the yellow supernatant as possible was then removed by suction and the precipitate was collected by centrifugation. The precipitate was extracted overnight in 200 ml of 3 % HCl solution of 95 % ethanol, with stirring, at room temperature. The insoluble material was removed by filtration and the filtrate was concentrated *in vacuo* to one-tenth of its original volume. The concentrate was added to 10 vol. of acetone. The resulting flocculent white precipitate was collected by centrifugation after standing for 2 h at room temperature. The precipitate was washed repeatedly with acetone and dried *in vacuo*. The yield at this stage from 50 g of acetone-dried bacilli was between 50 and 75 mg.

The dried white powder was resuspended in 50 ml of a 1 % HCl solution of 95 % methanol and extracted overnight, with vigorous stirring, at room temperature. After the insoluble material was removed by filtration, the filtrate was added to 10 vol. of acetone. The precipitate was collected by centrifugation, dried *in vacuo*, and stored as "purified tuberculin peptide". The yield at this stage was about 20 mg from 50 g of acetone-dried bacilli. The preparation at this stage of purification was used for the study of biological activity.

A 5 % solution of purified tuberculin peptide in 1 % HCl in methyl alcohol was

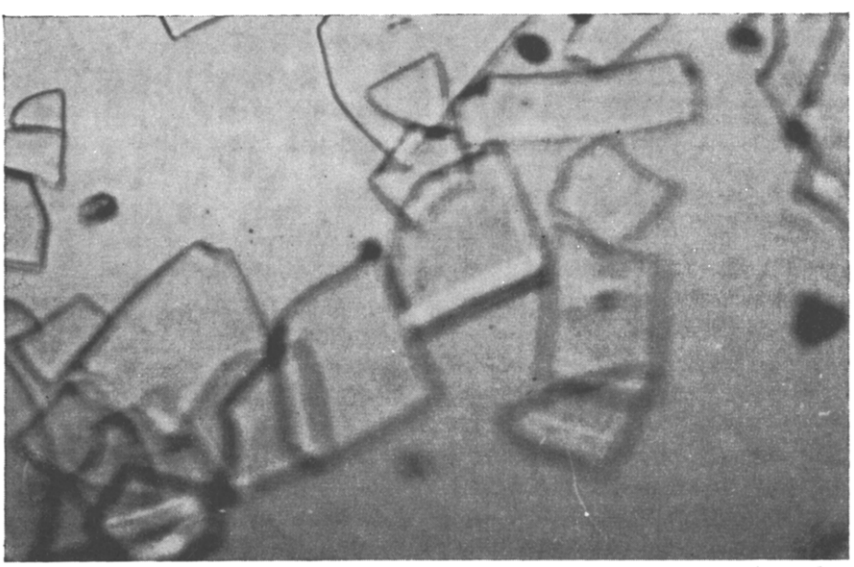
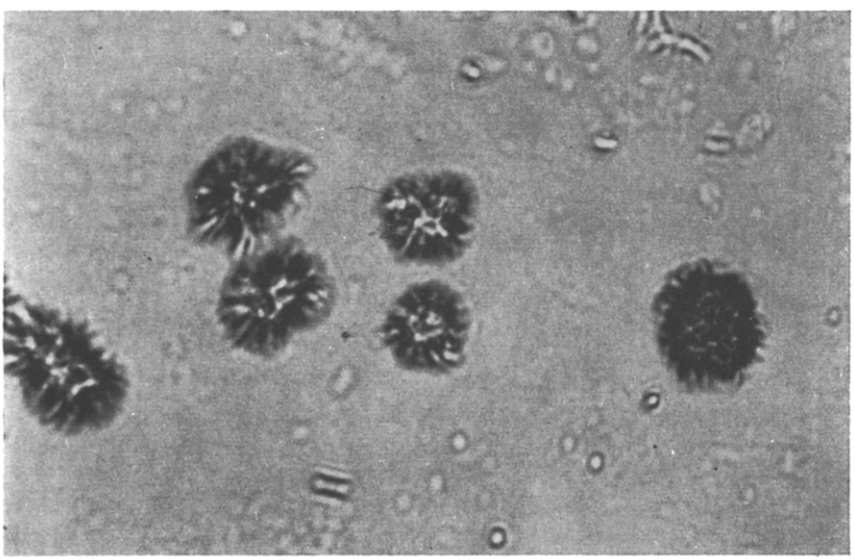


Fig. 1. Photomicrograph of crystals of tuberculin active peptide from *Mycobacterium tuberculosis* (60 ×).

concentrated below 30° under reduced pressure and then was added to 100 ml of dried tetrahydrofuran. Glistening, pale yellow, laminated crystals appeared. Fig. 1 shows the crystalline peptide thus prepared.

Several other successful attempts have been made to crystallize purified tuberculin peptide as follows. Twenty mg of purified peptide was dissolved in 10 ml methanol, concentrated *in vacuo*, and added to methanol solution saturated with purified peptide. Glistening, laminated crystals as described above then appeared.

Thirty mg of peptide were dissolved in 3 ml of distilled water and added to 0.5 ml of 5 % flavianic acid solution. The mixture was allowed to evaporate slowly in a desiccator. Crystals of flavianate appeared after about one week. The solution was filtered and the crystals were washed once with 0.25 % cold flavianic acid solution and three times with cold water, and lyophilized. Ten mg of crystals were obtained. Fig. 2 shows crystalline flavianate of a purified tuberculin peptide.



Twenty mg purified peptide was dissolved in 2.5 ml distilled water and added to 0.5 ml of 1 % solution of picrolonic acid. The resulting yellow precipitate (picrolonate) was collected by filtration and washed several times with cold distilled water. The precipitated picrolonate was redissolved in 2 ml distilled water, filtered and stored in a desiccator. After 3 days yellow crystals of picrolonate of tuberculin active peptide were obtained.

CHEMICAL PROPERTIES

Chemical analysis

The nitrogen content of the purified tuberculin peptide was determined by the micro-Kjeldahl method and found to be 15.9 %. Ninhydrin, xanthoprotein, biuret, Millon's and Sakaguchi's reactions were all positive when applied to the peptide, while

the Molisch, diphenylamine and orcinol tests were negative. From these results it will be concluded that there was neither sugar nor nucleic acid in the peptide.

Electrophoresis

The zone electrophoretic technique was used. In order to avoid the irreversible adsorption on cellulose, an attempt was made to use acid–ethyl alcohol-treated cellulose powder according to the method of FLODIN AND KUPKE[7]. Good quality cellulose powder (200–300 mesh) was treated by refluxing for 24 h in 10 vol. of 0.1 *N* HCl–absolute ethanol, washed several times with ethanol and distilled water, and allowed to settle until the supernatant was quite clear. The precipitated cellulose powder was washed again with ethanol and dried. For the zone electrophoresis, Kunkel's vertical type[8] apparatus was used, having a column of 2 × 40 cm.

The amount of the peptide used for electrophoretic analysis was usually 10 mg. Electrophoresis was carried out with 7 mA current at 400 V for 12 h in acetate buffer, pH 4.7 and 0.05 ionic strength. Most runs were made at room temperature; a temperature increase of 1–2° was usually observed at the end of the experiment. Elution was carried out with the same buffer; 1 ml samples were collected. Two methods, based on the ninhydrin reaction and Sakaguchi's reaction respectively, were used for the peptide determination. Independent of the reaction used purified peptide was found to consist of a single component, even after 12 h of electrophoresis. The total recovery of the peptide was 90–95 %.

Ultracentrifugal analysis

Ultracentrifugal analyses were undertaken in several concentrations of peptide (0.5–2.0 %) in acetate buffer (pH 4.7, $\mu = 0.05$) using a Spinco Model E ultracentrifuge. Schlieren photography showing the sedimentation of purified tuberculin peptide at various concentrations indicated that the boundary remains sufficiently close to the meniscus, as shown in Fig. 3. This result indicated that the peptide sediments too slowly to allow the sedimentation constant of the substance to be calculated from the movement of the maximum gradient of refractive index, and it was suggested that the molecular weight of peptide is rather small.

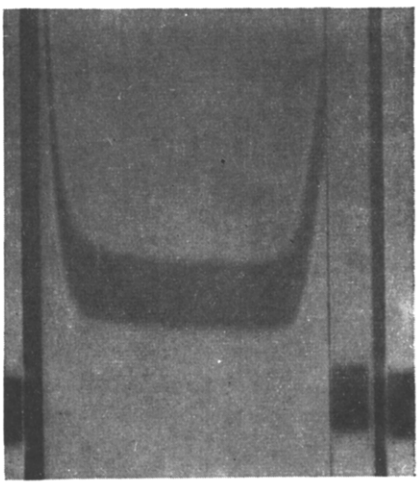


Fig. 3. Sedimentation diagram of tuberculin active peptide. Peptide concentration was 1.0 % in acetate buffer pH 4.7, ionic strength 0.05. Speed 56,100 rev./min. Exposure was taken 40 min. after reaching full speed.

Other chemical properties

The purified or crystalline peptide is easily soluble in water and rather soluble in methanol and ethanol. When diluted alkaline solution was added to peptide solution, the solution became cloudy near pH 8.5 and precipitates appeared in the vicinity of pH 9.0. Neutral solution of the peptide was heat-stable (70°, 15 min) with respect to the tuberculin activity and dialysable through cellophane membrane, indicating that the peptide is not as large a molecule as the protein component separated by SEIBERT *et al.*[6].

BIOLOGICAL PROPERTIES

Skin reaction on human subjects

Purified peptide preparation was tested intradermally on patients and students in primary and junior school. Intradermal injections of 0.1 ml amounts of proper dilutions of the peptide were made in the volar surface of the forearm approximately 2 in. below the elbow. Peptide was compared with the standard Old Tuberculin, given in the opposite arm. After 24 and 48 h the amount of induration of the reaction was measured as two diameters at right angles to each other and the mean value was described.

The comparative data of the activity of 2000-fold diluted Old Tuberculin and 0.1 μg purified peptide is shown in Figs. 4, 5 and 6. In groups of patients with

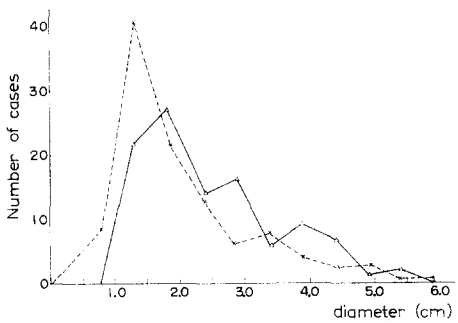


Fig. 4.

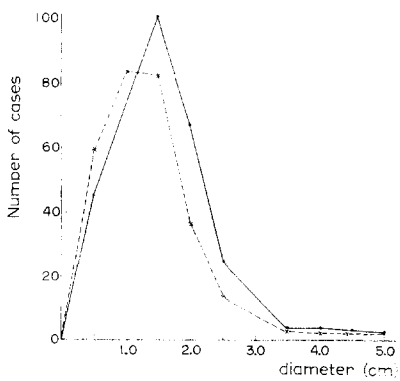


Fig. 5.

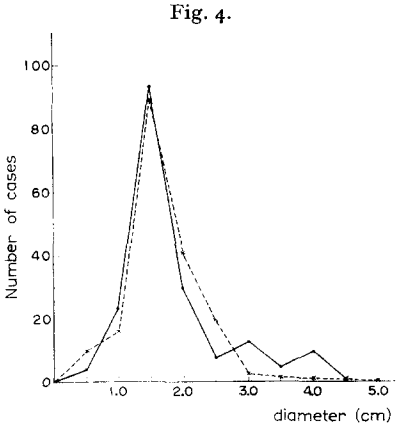


Fig. 6.

Fig. 4. Potency test of the tuberculin active peptide on tuberculous patients. Number of patients tested: 100. – – –, reaction with 0.1 ml of 2000-fold diluted old tuberculin; ——, reaction with 0.1 μg of tuberculin active peptide in 0.1 ml physiological saline solution.

Fig. 5. Potency test of tuberculin active peptide on primary school children. Number tested: 398. – – –, reaction with 0.1 ml of 2000-fold diluted old tuberculin; ——, reaction with 0.1 μg of tuberculin active peptide in 0.1 ml physiological saline solution.

Fig. 6. Potency test of the tuberculin active peptide on junior school pupils. Number tested: 191. – – –, reaction with 0.1 ml of 2000-fold diluted old tuberculin; ——, reaction with 0.1 μg of tuberculin active peptide in 0.1 ml physiological saline solution.

pulmonary tuberculosis, the reaction to 0.1 μg of the peptide was more remarkable than that to standard 2000-fold diluted Old Tuberculin. However, in groups of students in primary and junior school, the reaction with the peptide approximately equalled that with tuberculin. These results indicate that the purified peptide has a strong tuberculin activity comparable to the Old Tuberculin or the purified protein isolated from bacillary bodies and culture filtrate of bacilli.

DISCUSSION

The results in the present study show that there is a peptide in the tubercle bacilli that has a potent tuberculin activity. Several studies concerning the tuberculin active peptide were reported. TISELIUS AND GRÖNWALL[9] have found that PPD (Purified Protein Derivatives Tuberculin, Seibert) showed about one-tenth of the original tuberculin activity after digestion by pepsin and suggested that the tuberculin activity resides in small molecular peptide. TAKEYA[10] also obtained a tuberculin active principle from the digests of purified protein by trypsin, which was not precipitated by trichloracetic acid, but precipitated completely by phosphotungstic acid. KAIBARA *et al.*[11] extracted a tuberculin active substance from concentrated culture filtrate with methanol and termed it "μ". This substance was also precipitated by phosphotungstic acid and considered to be a peptide. Recently, KASUYA *et al.*[12] obtained several kinds of peptide by the hydrolysis of protein with performic acid and suggested that a peptide consisting of β-alanine, glutamic acid, glycine and cysteic acid had been proved to be the smallest unit having tuberculin activity. However, no critical study of the tuberculin potency in human and animals has so far been made with any of the preparations by these authors.

It is clear from the results of skin tests made in this study that the peptide fractions isolated from the bacillary bodies have a high potency in tuberculin reaction. Moreover, the method used here for the isolation of this peptide is simple and readily yields considerable quantities of the peptide in pure and potent form. Crystallization of a tuberculin active protein was reported by SEIBERT[13] in 1928 but did not seem to be reproducible. The method for crystallization of tuberculin active peptide described here is relatively easy and reproducible; it thus provides an excellent tool for the chemical study of the active principle of tuberculin. The amino acid composition and sequence of the purified peptide are under investigation and will be reported elsewhere.

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THE ACTIVE CENTER OF PLASMIN

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SUMMARY

Plots of the variation of pK_m and log V_{max} with pH for plasmin, determined in the absence and presence of either Ni^{++} or Ca^{++}, indicate that the active center of this enzyme is functionally identical to trypsin and that both of these enzymes differ significantly from thrombin. As with trypsin, some five groups are implicated in the active center or are affected by ES complex formation and subsequent decomposition; however, the probability exists that one or more of these groups in plasmin differ chemically from their functionally identical analogues in trypsin. Data are presented on the storage stability of plasmin and trypsin which also serve to emphasize their individuality.

INTRODUCTION

The studies reported here are a continuation of earlier research designed to identify the groups and group functions in the active centers of trypsin, thrombin and plasmin. The last report on trypsin and thrombin[1] presented data resulting from the application of the theoretical, kinetic treatment elaborated by DIXON[2] and by LAIDLER[3]. This report now extends that treatment to plasmin and makes comparison both with the previous results on thrombin and trypsin and with rechecked data on trypsin. Additional data on both the storage stability of plasmin and trypsin and as evidence of their non-identity are presented.

Abbreviation: TAME = N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester.

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