65. *Studies on Tuberculin Active Peptide. I* *Purification, Crystallization, and Properties of* *Tuberculin Active Peptide*

By Yuichi YAMAMURA, Seisi MORIZAWA, Atsushi TANAKA,
and Kenji SHOJIMA

Department of Biochemistry, School of Medicine,
Kyushu University, Fukuoka, Japan

(Comm. by Y. KOTAKE, M.J.A., June 12, 1959)

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. Concerning the active principle from the bodies of *Mycobacterium tuberculosis*, a number of studies have been reported by Heidelberger and Menzel,¹⁾ Heckley and Watson,²⁾ Iland et al.,³⁾ and Seibert et al.⁴⁾

Throughout these reports, the following special characteristics of the tuberculin active principle were noted.

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 considerably stable against the heating.

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

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 minutes. Fifty grams of the washed and acetone-dried bacterial cells was extracted with 10 volumes (500 ml) of 0.1 N HCl at 37°C for 72 hours under frequent stirring.

The extract was filtered through the 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. On standing at room temperature for 2 hours or more, the flocculent white precipitate was removed by filtration.

The clear filtrate was added with an equal volume of a saturated solution of picric acid. Yellow precipitates appeared after standing at

room temperature overnight. The precipitate was collected by centrifugation and extracted overnight in 200 ml of 3 per cent HCl solution of 95 per cent ethanol under stirring at room temperature. The insoluble material was removed by filtration and the filtrate was concentrated to one-tenth of its original volume in vacuo. The concentrate was added with 10 volumes of acetone. The resulting flocculent white precipitate was collected by centrifugation after standing for two hours 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 mg to 75 mg.

The dried white powder was resuspended in 50 ml of one per cent HCl solution of 95 per cent methanol and extracted under vigorous stirring overnight at room temperature. After removing the insoluble material by filtration, the filtrate was added with 10 volumes of acetone, the precipitate was 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.

A five per cent solution of purified tuberculin peptide in 3 per cent HCl in methylalcohol was concentrated below 30°C in vacuo, and then was added with dried tetrahydrofuran. Then glistening, pale yellow, laminated crystals appeared. Crystalline peptide was also obtained by the addition of saturated methanol solution of purified peptide to a concentrated peptide solution of methanol.

Further, flavianate or picrolonate of the peptide was easily crystallized by the addition of flavianic acid or picrolonic acid to the peptide solution.

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, xantoprotein, biuret, Millon's, and Sakaguchi's reaction of the peptide was all positive, while Molisch, diphenylamine, and orcinol test was negative.

Spectrophotographic absorption. A 0.4% solution of the peptide was studied in the Beckmann DU spectrophotometer in the ultraviolet range. The maximum absorption was at wave length of 2,800 Å.

Electrophoresis. Zone electrophoresis of Kunkel's vertical type apparatus was used. In order to avoid the irreversible adsorption on cellulose, acid-ethyl alcohol treated cellulose powder according to the method of Flodin and Kupke⁵⁾ was used.

The amount of the peptide for electrophoretic analysis was usually 10 mg. Electrophoresis was carried out with 7 milliamperes current for 12 hours in acetate buffer, pH 4.7 and 0.05 ionic strength. Elution was carried out with the same buffer and each eluate was analysed with ninhydrin and Sakaguchi's reaction. Recovery was 90–95%.

It was shown that the purified peptide was found to be consisted of a single component only, even after 12 hours of electrophoresis. The peak remained almost the same shape concerning the ninhydrin and Sakaguchi's reaction.

Ultracentrifugal analysis. Ultracentrifugal analysis was undertaken in several concentrations of peptide (0.5–2.0%) in acetate buffer (pH 4.7, μ 0.05) using a Spinco Model E ultracentrifuge. The result indicated that the peptide sediments too slowly to calculate the sedimentation constant of the substance by the movement of the maximum gradient of refractive index and it was suggested that the molecular weight of peptide is considerably small.

Amino acid analysis. Quantitative amino acid analysis was carried out by the Dowex 50 column chromatographic method of Moore and Stein.⁶⁾ The following amino acid composition of the peptide was shown.

Arg₁₀ His₁ Lys₂ Phe₁ Tyr₁ Leu₃ Ileu₂ Val₅

Ala₉ Gly₆ Glu₈ Pro₅ Ser₂ Thr₃ Asp₆ Try₁

The minimal molecular weight calculated from this result is 7180.

Other chemical properties. The purified or crystalline peptide is easily soluble in water, considerably soluble in methanol and ethanol but insoluble in acetone, ether, and chloroform. It is dialysable through cellophane membrane.

Biological properties. Skin reaction on humans. Purified peptide preparation was tested intradermally on patients and students in the primary and middle schools. Intradermal injections of 0.1 ml amounts of proper dilutions of the peptide were made in the volar surface of the forearms approximately 2 inches below the elbow. Peptide was compared with the standard 2000 fold diluted Old Tuberculin, given in the opposite arm. After twenty-four and forty-eight hours the amount of induration of the reaction was measured as two diameters at right angles to each other and the mean value was described.

In groups of patients of pulmonary tuberculosis, the reaction to 0.1 μ g of the peptide was more remarkable than that to standard 2000 fold diluted Old Tuberculin. However, in groups of students in primary and middle schools, the reaction to the peptide approximately equaled that with tuberculin. These results indicate that the purified peptide has a strong tuberculin activity comparable to the Old Tuberculin.

Discussion. The results in the present study show that there is a peptide in the tubercle bacilli, which has a potent tuberculin activity. Several studies concerning the tuberculin active peptide were reported.^{7,8)} 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 given in this study that

the peptide fraction isolated from the bacillary bodies has a high potency in tuberculin reaction. Moreover, the method here used for isolation this peptide is simple, reproducible and readily yields considerable quantities of the peptide in pure and potent form.

Summary. A highly potent basic peptide was isolated in pure form from the bodies of tubercle bacillus. Crystallization was done from methanol solution of the purified peptide. Crystals of flavianate and picrolonate of the peptide were also obtained.

Chemical and biological properties of the purified peptide are described. The tuberculin skin potency tests on humans indicated that the purified peptide was potent comparable to purified protein isolated from bacillary bodies or culture filtrate of bacilli.

Acknowledgments. The writers express their thanks to Prof. K. Kotake for his encouragement and criticism throughout all of this work.

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