CRYSTALLIZATION AND PROPERTIES OF β-TYROSINASE FROM *ESCHERICHIA INTERMEDIA*

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Kakihara and Ichihara (1953) identified tyrosine as the source of phenol produced by bacterial cultures. The tyrosine-inducible enzyme which is responsible for this conversion, was subsequently named β-tyrosinase by Uchida et al. (1953). Yoshimatsu (1957) reported that β-tyrosinase catalyzed the stoichiometric conversion of tyrosine to phenol, pyruvate and ammonia (Reaction I) and required pyridoxal phosphate as a cofacter.

$$\text{L-Tyrosine} + H_2O \longrightarrow \text{pyruvate} + NH_3 + \text{phenol} \quad (I)$$

The present communication describes a method for obtaining a crystalline preparation of β-tyrosinase from cells of Escherichia intermedia, and some properties of the crystalline enzyme.

Purification and Crystallization.

Escherichia intermedia (AKU 0010) was grown in 30 liters of a medium containing 0.5% peptone, 0.5% meat extract, 0.2% NaCl, 0.05% yeast extract and 0.2% tyrosine, with aeration, for 14 hours at 30°. The cells were harvested, washed and suspended in a dilution buffer which contained 0.01 M potassium phosphate, pH 6.0, and 0.005 M mercaptoethanol. All subsequent procedures were performed at 0-5°. Step I. The cell suspension was subjected to ultrasonic oscillation (20 kc, for 30 minutes) and centrifuged.

Step II. The supernatant solution was fractionated with ammonium sulfate (30-70% saturation), followed by dialysis against the dilution buffer. Step III. One tenth volume of a 6% protamine sulfate solution at pH 7.0 was added to the dialyzate and the precipitate formed was centrifuged off. Step V. The supernatant solution was applied to a DEAE sephadex column (6 x 53 cm) equilibrated with the dilution buffer. After the column was washed with 2 liters of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol, the enzyme was eluted with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol and 0.1 M KCl. The active fractions were combined and concentrated by the addition of ammonium sulfate (70% saturation). The precipitate was collected and dialyzed against the dilution buffer. Step IV. The dialyzate was applied to a hydroxylapatite column (5 x 10 cm) equilibrated with the dilution buffer. After the column was washed with 500 ml of 0.03 M potassium phosphate buffer, pH 6.0, containing 0.005 M mercaptoethanol, the enzyme was eluted with 0.1 M potassium phosphate buffer, pH 6.0, containing 0.005 M mercaptoethanol. The active fractions were combined and concentrated with ammonium sulfate (70% saturation). The precipitate was collected and dissolved in a minimum amount of the dilution buffer. Step V. The enzyme solution was passed through a sephadex G-150 column (2 x 100 cm) equilibrated with the dilution buffer. The active fractions containing enzyme of specific activity greater than 1.0 were combined and concentrated with ammonium sulfate (70% saturation). The precipitate was dissolved in a minimal amount of the dilution buffer. Step VI. Finely powdered ammonium sulfate was cautiously added to the enzyme solution until it became slightly turbid, and the mixture was placed in an ice bath. Crystallization began after about 6 hours and was virtually complete within a week. A photomicrograph of the crystalline enzyme is shown in Fig. 1. Recrystallization was carried out by repeating the last step. A summary of typical purification procedures is presented in Table I.

Table I. Purification of β-Tyrosinase from Escherichia intermedia

β-Tyrosinase was assayed in a reaction mixture containing enzyme, 5 μmoles of tyrosine, 0.4 μmoles of pyridoxal phosphate, 200 μmoles of potassium phosphate buffer, pH 7.8, in a total volume of 4 ml. The reaction was carried out at 30° for 30 minutes, with gentle shaking, and stopped by the addition of 1 ml of 30% trichloroacetic acid. The amount of pyruvate formed was determined with the deproteinized filtrate by a modification of the method of Friedemann and Haugen (1943). A unit was defined as the amount of enzyme which catalyzed the formation of 1 μmole of pyruvate per minute under the assay conditions. The protein concentration was determined spectrophotometrically by measuring the absorbancy at 280 mμ. An $\underline{E}$ value of 8.37 for 1 mg per ml and for 1-cm light path which was obtained by absorbancy and dry weight determinations, was used throughout.

Step	Fraction	Total protein (mg)	Total units	Specific activity
I	Crude extract	155,000	2,750	0.0177
II	Ammonium sulfate	68,600	2,200	0.0321
III	Protamine sulfate	57,000	2,190	0.0384
IV	DEAE-sephadex	3,840	660	0.172
V	Hydroxylapatite	464	363	0.782
VI	Sephadex G-150	179	242	1.35
VII	Crystals (I)	84	157	1.86
	Crystals (II)	62	120	1.94

Properties.

The crystalline β-tyrosinase preparation gave a single band on disc gel electrophoresis carried out at pH 8.3 (Davis, 1964). The enzyme preparation sedimented as a single symmetric peak under ultracentrifugation performed at pH 6.0 in the dilution buffer. Extrapolation of the data obtained from four ultracentrifuge runs to zero protein concentration gave an $S^0_{20,w}$ of 7.77×10^{-13} (cm/sec). A diffusion constant, $D_{20,w}$, of 4.42×10^{-7} (cm^2/sec) was determined for a 7.80 mg/ml solution of protein. A value of 170,000 was calculated for the molecular weight of the enzyme assuming a partial specific volume of 0.75.

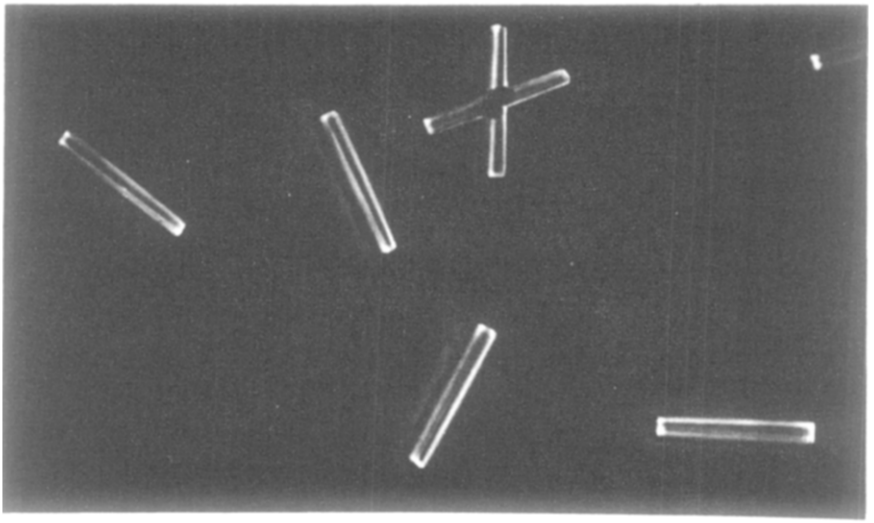

Fig. 1. Photomicrograph of crystalline β-tyrosinase (× 300).

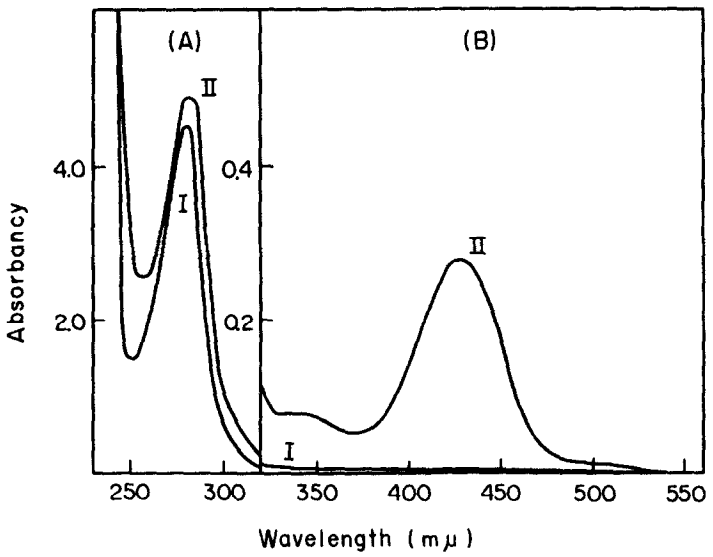

Fig. 2. Absorption spectra of crystalline β-tyrosinase in the presence and absence of pyridoxal phosphate. Recrystallized enzyme, 11.2 mg, was dissolved in 2 ml of 0.05 M potassium phosphate buffer, pH 7.0. Spectrum I was taken with 1 ml of the enzyme solution after dialysis against 0.05 M potassium phosphate buffer, pH 7.0. Spectrum II was taken with the second 1-ml solution after dialysis against the same buffer containing 3×10^{-5} M pyridoxal phosphate.

The crystalline enzyme prepared by the procedure in Table I showed negligible activity when assayed in the absence of added pyridoxal phosphate and, therefore, entirely represented the apoenzyme. The apoenzyme showed essentially no absorbance at 335 and 425 mμ. On association with pyridoxal phosphate, pronounced absorption maxima appeared at these two wave lengths (Fig. 2). The amount of pyridoxal phosphate bound by the enzyme was determined after dialysis of the enzyme against 0.05 M phosphate buffer, pH 8.0, containing 3×10^{-5} M pyridoxal phosphate, according to Newton et al. (1965). An excess concentration of pyridoxal phosphate was found inside the dialysis bag, corresponding to the binding of 2 moles of pyridoxal phosphate per mole of enzyme.

The concentration of pyridoxal phosphate required in the assay mixture to give the half-maximum rate of pyruvate formation from tyrosine was found to be 1.3×10^{-6} M.

The crystalline enzyme was found to catalyze the stoichiometric conversion of tyrosine, into phenol, pyruvate and ammonia (Reaction I). Serine and S-methylcysteine were also converted into pyruvate and ammonia. The concentration of these substrates to give the half-maximum rate of pyruvate formation was found to be 2.31×10^{-4} M, 3.45×10^{-2} M and 1.82×10^{-3} M, for tyrosine, serine and S-methylcysteine, respectively. The optimal pH range for these conversions was between 7.8 and 8.8 in both 0.05 M potassium phosphate and 0.1 M Tris-HCl buffers.

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