SYNTHESIS OF 3,4-DIHYDROXYPHENYL-L-ALANINE FROM L-TYROSINE AND PYROCATECHOL BY CRYSTALLINE β-TYROSINASE

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Received December 9, 1968

β-Tyrosinase is an enzyme which catalyzed the α,β-elimination of L-tyrosine into phenol, pyruvate and ammonia, in the presence of pyridoxal phosphate (Equation I) (Uchida *et al.*, 1953; Yoshimatsu, 1957). The enzyme has been crystallized in our laboratory (Yamada *et al.*, 1968), from cells of *Escherichia intermedia* (AKU 0010) and its properties established in some detail. In the course of further investigation on catalytic properties of the enzyme, we have found that 3,4-dihydroxyphenyl-L-alanine (L-dopa) is synthesized from L-tyrosine and pyrocatechol *via* β-replacement catalyzed by the enzyme (Equation II).

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

$$\text{L-Tyrosine} + \text{pyrocatechol} \longrightarrow \text{L-dopa} + \text{phenol} \qquad \text{(II)}$$

Crystalline β-tyrosinase was prepared from cells of *E. intermedia* grown in a bouillon-peptone medium supplemented with L-tyrosine, according to Yamada *et al.* (1968).

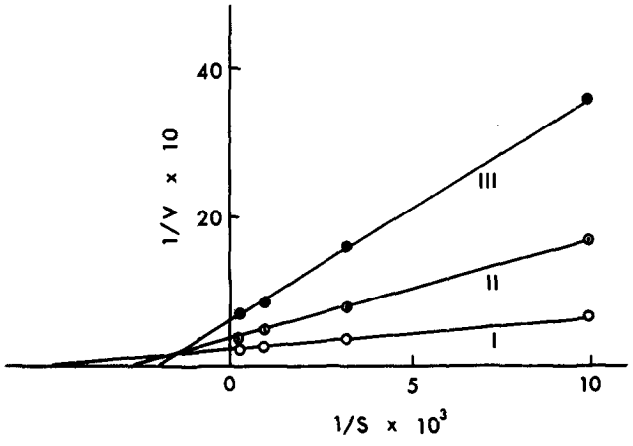

Fig. 1. Inhibition of pyruvate formation by pyrocatechol and phenol. Incubations were carried out at 30° for 10 minutes in reaction mixtures containing L-tyrosine as indicated, 0.4 μmoles of pyridoxal phosphate, 0.2 mmoles of potassium phosphate buffer, pH 7.8 and 17 μg of the recrystallized enzyme, in total volume of 4.0 ml. The mixtures (II) and (III) also contained 5 μmoles of pyrocatechol and 1 μmole of phenol, respectively. The amount of pyruvate formed was determined using a modification of the method of Friedemann and Haugen (1943). Velocity was expressed as μmoles of pyruvate formed per minute of incubation.

The formation of pyruvate by the recrystallized β-tyrosinase (Equation I) was inhibited by pyrocatechol and phenol. Fig. 1 shows Lineweaver-Burk plots (Lineweaver and Burk, 1934) of pyruvate formation in the presence or absence of pyrocatechol or phenol. The inhibition was found to be of a mixed type and the K_i values were 4.6×10^{-4} and 3.56×10^{-5} M for pyrocatechol and phenol, respectively.

Chromatographic examination of a similar incubation mixture on paper and ion-exchange resin (Fig. 2) showed the presence of a new ninhydrine-positive compound which cochromatographed exactly with authentic L-dopa.

The enzymatically synthesized L-dopa was isolated from the large scale incubation mixture, as its tri-benzoyl ethyl ester. Incubation was carried out at 30° for 2 hours in a reaction mixture containing L-tyrosine (3.7 mmoles), pyrocatechol (18.5 mmoles), pyridoxal phosphate (0.1 mmole), potassium phosphate buffer (50 mmoles, pH 7.8) and 12 mg of the recrystallized enzyme, in a total volume of one liter. Under these conditions,

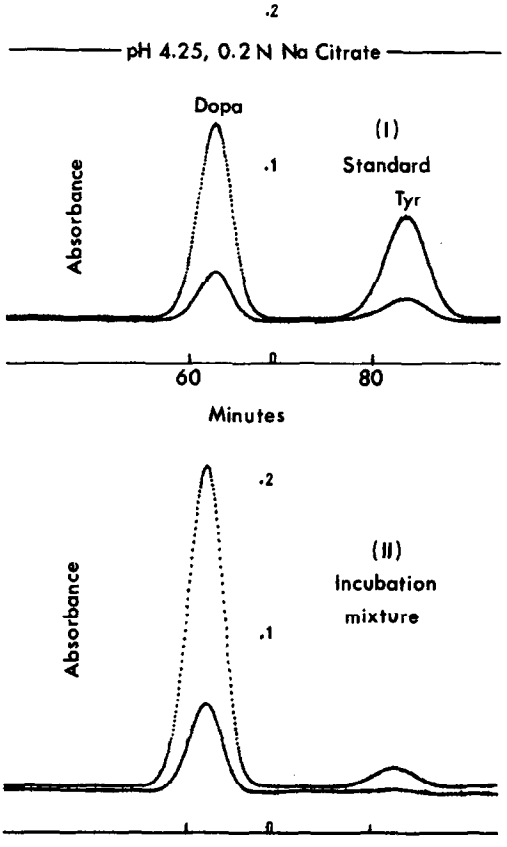

Fig. 2. Ion-exchange chromatography of an incubation mixture of L-tyrosine and pyrocatechol with β-tyrosinase. The chromatography was done with a Yanagimoto model LC-5S automatic amino acid analyzer. Samples were placed on a 70 x 0.9-cm column of Aminex A-4 resin and eluted with 0.2 N sodium citrate buffer, pH 4.25. Samples were (I), standard (46 mμmoles of L-tyrosine and 72 mμmoles of L-dopa); (II), incubation mixture (0.16-ml aliquot of a 4 ml mixture). Incubation was carried out at 30° for 30 minutes in a reaction mixture similar to that described in Fig. 1, except that it contained 5 μmoles of L-tyrosine, 75 μmoles of pyrocatechol and 164 μg of the enzyme.

0.81 mmoles of L-dopa were found to be synthesized in the mixture by ion-exchange chromatographic analysis. The incubation mixture was applied to an aluminum oxide column (Merck, neutral, 4 x 20 cm). This column was eluted first with distilled water (one liter), then with 0.3 N acetic acid. The acetic acid eluate was evaporated to dryness at 50° under reduced pressure. The pale brown solid obtained was refluxed for 2 hours with

thionyl chloride (2 ml) in ethanol (10 ml). The mixture was evaporated to a syrup, which was treated with benzoyl chloride (4.5 g) in pyridine (20 ml) for 30 minutes at room temperature. The syrup obtained was applied to a silicic acid column (Mallinckrodt, 3 x 20 cm). The column was first eluted with benzene (500 ml), then with chloroform. The chloroform eluate was evaporated to dryness. The syrup obtained was subjected to a preparative thin layer chromatography (Merck PF 254) which was developed with benzene-ethyl acetate (4:1). The tri-benzoyl ethyl ester fraction (R_f 0.60-0.70) was collected and recrystallized from ethanol, yielding colorless crystals (m.p. 168-169°). The crystals were identical in all respects (melting point, IR- and NMR-spectra) with the authentic ethyl ester of 3,4-di-benzoyl-N-benzoyl-dopa derived from L-dopa according to the same procedure. The tri-benzoyl ethyl ester derived from DL-dopa melted at 153-154°.

The synthesis of L-dopa by β-tyrosinase was found to be pyridoxal phosphate dependent and to proceed linearly with incubation time, enzyme and pyrocatechol concentrations (Table I). The pH activity curve for this synthesis has the same shape as for pyruvate formation with an optimum around 8.0.

The L-dopa synthesis can be explained from the reaction scheme previously proposed (Morino and Snell, 1967). In catalysis of the pyruvate formation, L-tyrosine interacts with β-tyrosinase to form, reversibly, the enzyme-bound α-aminoacrylate, by elimination of H^+ and $phenol^-$ The enzyme-bound α-aminoacrylate hydrolyzes irreversibly to yield pyruvate and ammonia and regenerate β-tyrosinase (Equation I, α,β-elimination). In the presence of pyrocatechol, the enzyme-bound α-aminoacrylate, rather than hydrolysis, can occur yielding L-dopa by reversal of the reactions (Equation II, β-replacement). More generally, the addition of other groups than pyrocatechol may also be catalyzed by β-tyrosinase to yield

Table I. Effect of Pyrocatechol Concentration on Synthesis of L-Dopa

Incubations were carried out at 30° for 30 minutes in reaction mixtures similar to that described in Fig. 1, except that they contained 5 μmoles of L-tyrosine, pyrocatechol as indicated and 24.2 μg of the enzyme. L-Dopa synthesized was determined by the ion-exchange chromatographic method described in Fig. 2.

Pyrocatechol added (μmoles)	L-Dopa synthesized (μmoles)
0	0
5	0.22
10	0.38
25	0.59
50	0.65

the corresponding amino acid. However, the rate of such replacement reactions would depend on the affinity of β-tyrosinase for these groups. Detailed investigation on the replacement reactions catalyzed by β-tyrosinase is currently in progress in our laboratory.

Acknowledgments

We wish to thank Prof. Y. Morita, Kyoto University, for his interest and advice during the course of this work. We also thank Mr. T. Higasa for his assistance in the amino acid analysis.

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