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Microbial Synthesis of L-Dopa (L-3, 4-dihydroxy L-phenylalanine) by a *Pseudomonad* Mutant D101†

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Abstract

The *in vitro* synthesis of L-dopa from L-tyrosine by a mutant D **101** (by 3-hydroxylase enzyme) of a soil isolate, *Pseudomonas* (PL-strain) was carried out. The synthesized L-dopa was identified and estimated by chromatographic and colorimetric methods. The synthesis had a pH optimum at 6.0. The *N*-formyl derivative of L-tyrosine was a better substrate for L-dopa formation compared to L-tyrosine. Presence of Cu^{2+}, cysteine and ascorbic acid increased the total synthesis of L-dopa when L-tyrosine or *N*-formyl L-tyrosine were used as substrates. *N*-Formyl-L-tyrosine more prominently increased the synthesis of L-dopa compared to L-tyrosine.

Introduction

L-Dopa can be synthesized either starting from a variety of aldehydes and a two carbon molecule or starting with L-tyrosine and hydroxylating it chemically or biosynthetically with a microorganism or enzyme.[1)]

For a long time, because of high deaminase activity in microorganisms, L-dopa was not detectable as a metabolite of tyrosine metabolism.[2)] Recently, several L-dopa producing microorganisms have been screened.[2,3)] Also, enzymatic preparations of L-dopa of microbial origins have been worked out.[4~6)] The present investigation deals with the preparation of L-dopa from a *Pseudomonad* mutant D101.

Materials and Methods

Isolation of mutant D101 A soil *Pseudomonas* (PL-strain 433) obtained from National Chemical Laboratory, Poona, was grown on nutrient agar slant, consisting of glucose (0.5%), peptone (0.5%), beef extract (0.3%) and agar (1.5%), for 24 hours at 27°C. The growth on a slant was taken out by washing with water and diluted to a concentration of 10^5 cells per ml. The suspension was shaken for 1 hour at 27°C, to cause consumption of all the internal metabolites. The suspension was then irradiated under ultraviolet lamp and aliquots were taken out at different time intervals (10, 30, the 60, 120 and 300 sec) and plated out. In this particular case, the 120-sec irradiated sample gave 30~40 colonies per plate. Colonies were picked up at random and allowed to grow on nutrient agar slants. A

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mutant D101 was thus obtained which synthesized considerable amounts of L-dopa.

Preparation of cell suspension Mutant D101 was grown at 27°C for 24 hours, in shaking flasks containing the nutrient broth mentioned earlier. After growth of the mutant, the cells were collected by centrifugation in the cold at 16,000×*g* for 30 min. The cells were washed with normal saline and suspended in chilled 0.05 M phosphate buffer (pH 7.0) to make a 2% (w/v) suspension.

L-Dopa synthesis by the mutant The medium used for L-dopa synthesis was prepared according to the method of Soubert,[7] with L-tyrosine (1.0%) and 1.0 ml of 2% (w/v) cell suspension.

Identification of L-dopa L-Dopa was detected by chromatographic and colorimetric methods. Thin layer chromatography was carried out on cellulose plate employing *n*-butanol: acetic acid: water (4:1:10) as a solvent system. The plate was developed for 1~2 hours in the solvent system and spots were detected after spraying with ninhydrin. Paper chromatography (descending) was done on Whatman No. 1 paper using *n*-butanol: acetic acid: water (60:15:25) as a solvent system. The chromatogram was developed for 20~24 hours. Identification of spots was done by ninhydrin spray. The spots were cut out and eluted from paper by 75% ethanol containing 5 mg of copper sulphate per 100 ml. Color intensity was compared at 540 mμ. In a colorimetric determination,[8] an intense orange color was obtained with nitrite-molybdate reagent in alkaline condition and was read at 540 mμ.

Preparation of *N*-formyl-L-tyrosine The formyl derivative of L-tyrosine was prepared by treating it with 98% formic acid and acetic anhydride in the cold. It was distilled under vacuum and the derivative was recrystallized with aqueous ethanol. Formyl group was removed by treatment with 5 N HCl for 8 hours at room temperature.[2]

Results

The synthesis of L-dopa by mutant D101 was studied using L-tyrosine or *N*-formyl-L-tyrosine as substrates and incubations were carried out under sterile conditions. After, incubation, the reaction mixture was centrifuged at 10,000×*g* for 30 min. The supernatant was analyzed for L-dopa.

Effect of time on L-dopa synthesis The cells of mutant D101 were added to a medium containing L-tyrosine in flasks. The flasks were kept on a rotary shaker at 27°C for 2 to 48 hours. At different time intervals, the flasks were removed and analyzed for L-dopa. The results are presented in Fig. 1. The relationship between L-dopa synthesis and incubation period was linear up to 6 hours, after which the synthesis leveled off. Maximum synthesis took place in 6 hours and the yield was 20%. L-Dopa is decarboxylated by the relatively non-specific L-amino acid decarboxylase,[9~11] or by specific L-dopa decarboxylase.[12] L-Amino acid decarboxylase activity was present in the mutant. Among the three substrates L-tyrosine, L-lysine and L-dopa, tested for decarboxylase activity, melanin formation took place rapidly with L-dopa (data not reported). This observation clearly indicates that dopa decarboxylase was very active in the mutant, thus degrading synthesized L-dopa to further metabolites. This is probably the reason for the significant decrease in L-dopa, which almost disappeared after 24 hours.

Relationship between L-tyrosine concentration and L-dopa synthesis L-Tyrosine was added at different concentrations ranging from 0.2 to 2.0%. The results are presented in Fig. 2.

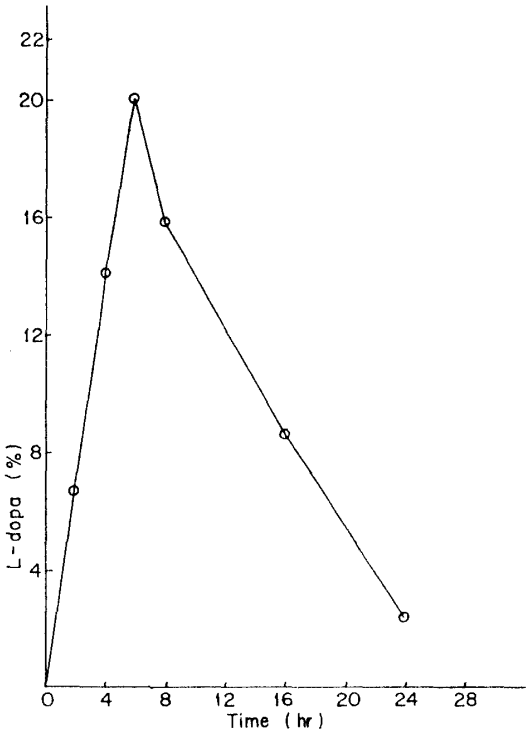

Fig. 1. Relationship between incubation time and L-dopa synthesis.

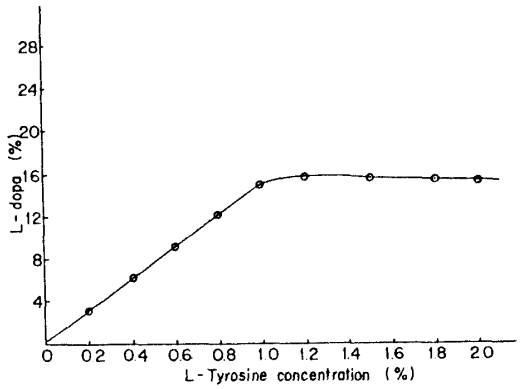

Fig. 2. Relationship between L-tyrosine concentration and L-dopa synthesis.

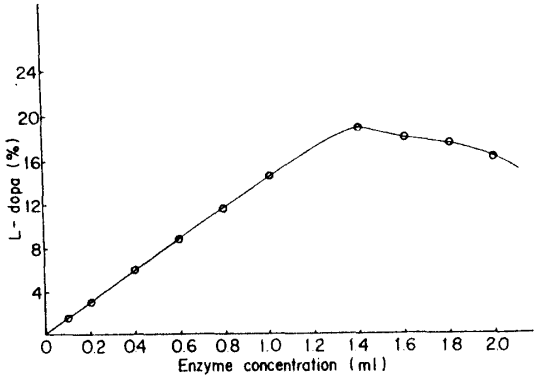

Fig. 3. Relationship between cell concentration and L-dopa synthesis.

The data clearly show a linear relationship between L-dopa synthesis and L-tyrosine concentration. This relationship held up to 1.0% concentration. At higher concentrations, no significant increase in synthesis was observed.

Relationship between enzyme concentration (cell suspension) and L-dopa synthesis Different aliquots of 2% enzyme (w/v) ranging from 0.1 to 2.0 ml in terms of cells suspensions were plotted against L-dopa synthesis (Fig. 3). A straight line relationship was found between the parameters up to 1.0 ml of cell suspension. At higher concentratoins, L-dopa synthesis decreased.

Relationship between pH and L-dopa synthesis The synthesis of L-dopa was tested at different pH levels ranging from 4.0 to 5.5 with acetate buffer and 6.0 to 8.0 with phosphate buffer (0.1M). The optimum pH for L-dopa synthesis was 6.0, on either side of which, total synthesis was significantly lower (Fig. 4).

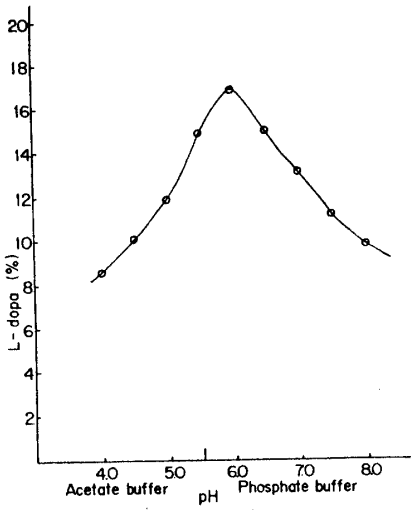

Fig. 4. Relationship between pH and L-dopa synthesis.

Substrates tested for L-dopa synthesis Three substrates, L-tyrosine, L-phenylalanine and *N*-formyl-L-tyrosine, were tested for conversion to L-dopa. The results are recorded in Table 1.

Table 1. Substrate specificity for L-dopa synthesis by mutant D101

Substrate	L-Dopa synthesis (%)
L-Phenylalanine	0.0
L-Tyrosine	15.0
N-Formyl-L-tyrosine	23.0

Different substrates were tested at 1.0% final concentration.

With *N*-formyl-L-tyrosine as the substrate, *N*-formyl-L-dopa is produced.[2] The formyl group was removed by treatment with 5 N HCl for 8 hours at room temperature, thereby liberating L-dopa, which was assayed in the experiments.

With L-tyrosine and its formyl derivative 15 and 23% L-dopa synthesis occurred, respectively. L-Phenylalanine however could not act as a precursor because of the requirement of two hydroxylations. Increased synthesis of L-dopa with use of *N*-formyl-L-tyrosine may be due to protection of L-tyrosine from deamination.[2] The relationship between *N*-

formyl-L-tyrosine concentration and L-dopa synthesis was linear (Fig. 5). Higher concentrations of substrate did not cause much variation in amount of L-dopa synthesized.

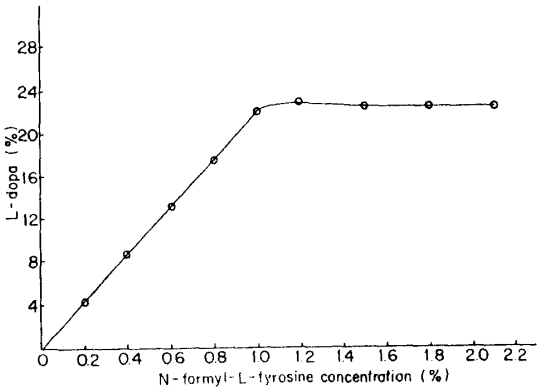

Fig. 5. Relationship between *n*-formyl-L-tyrosine concentration and L-dopa synthesis.

Effects of supplements Various supplements, e.g., copper sulfate, cysteine hydrochloride and ascorbic acid, were tested to determine their effect on L-dopa synthesis. These supplements were added at their optimal concentrations, predetermined for each case. As evident from Table 2, the synthesis of L-dopa was increased by 29, 51 and 87% using L-tyrosine and 35, 59 and 106% starting from *N*-formyl-L-tyrosine in the presence of copper sulfate, cysteine and ascorbic acid, respectively.

Table 2. Effect of supplements on L-dopa synthesis by mutant D101.

Supplement	Yield of L-dopa (%) using:		Increase in L-dopa synthesis (%) using:	
	L-tyrosine	*N*-formyl-L-tyrosine	L-tyrosine	*N*-formyl-L-tyrosine
Control	14.0	20.1	—	—
Copper sulfate	18.0	27.1	28.6	35.3
Cysteine hydrochloride	21.0	32.4	50.7	59.0
Ascorbic acid	26.2	41.5	87.1	106.4

The supplements were tested at their optimal concentrations, predetermined for each case. The optimal concentrations were 0.003, 0.4 and 0.5% for copper sulfate, cysteine and ascorbic acid, respectively.

Discussion

Madhyastha and Bhattacharyya reported that hydroxylation at the 3 position of benzene ring occurred when the organism (PL-strain 433) was grown on *p*-cymene as a sole carbon source by the enzyme 3-hydroxylase.[13] This property of the original strain was possibly retained by the mutant D101 which also hydroxylates L-tyrosine to L-dopa. Linear relationships exist between enzyme, and substrate concentrations and L-dopa synthesis. Linear relationships also exist between incubation time and synthesis up to 6 hours. Further, the synthesis was significantly retarded, by high decarboxylase activity. The optimum pH for L-dopa synthesis was found to be 6.0.

The fact that Cu^{2+} activates tyrosine hydroxylation reaction in general, has been well established.[14] The potentiation in L-dopa synthesis by Cu^{2+} also suggests that enzyme 3-hydroxylase needs Cu^{2+} ions for its activity. Besides this, a strong inhibition of L-dopa

decarboxylase enzyme by Cu^{2+} has been noticed.[15,16]

The synthesis of L-dopa can be affected by (a) deamination of the substrate to form products other than L-dopa[17] and (b) decarboxylation of L-dopa to dopamine which is further metabolized rapidly.[12] The presence of deaminase[17] and decarboxylase[9~11] are common among microorganisms. With *N*-formyl-L-tyrosine, the synthesis of L-dopa was increased by about 50% as compared to that with L-tyrosine. The formyl derivative of tyrosine protects L-tyrosine from deamination. Similar to L-tyrosine, L-dopa synthesis increases with increases in substrate concentration.

The synthesis either from L-tyrosine or *N*-formyl-L-tyrosine was increased significantly by cysteine and ascorbic acid. Dopa decarboxylase activity is known to be inhibited by sulfhydryl reagents[15] and ascorbic acid.[2] Besides decarboxylase, cysteine also inhibits L-dopa oxidation.[18,19]

Acknowledgements

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