A TRYPTOPHAN HYDROXYLASE INHIBITOR PRODUCED BY A STREPTOMYCETE: 2,5-DIHYDRO-L-PHENYLALANINE

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Inhibitors of enzymes which are involved in animal functions also can be found in microbial culture filtrates. As already reported, inhibitors of tyrosine hydroxylase[1], dopamine β-hydroxylase[1], histidine decarboxylase[2] and DOPA decarboxylase[3] have been found in culture filtrates of microorganisms. Tryptophan hydroxylase is the first enzyme in the biosynthetic route to serotonin from tryptophan. A specific inhibitor of this enzyme may be a useful tool for the study of the biological role of serotonin. After testing 4,000 freshly isolated strains of actinomycetes, we found three strains which produced inhibitors of this enzyme. One of these compounds was isolated and identified as 2,5-dihydro-L-phenylalanine which had been chemically synthesized[4] and has been reported to be produced by streptomycetes[5~8]. The method of screening for tryptophan hydroxylase inhibitors and the isolation of 2,5-dihydro-L-phenylalanine and its activity are reported in this paper.

Experimental

Materials

Tryptophan hydroxylase was partially purified from homogenates of bovine pineal gland by the method of ICHIYAMA *et al.*[9] up to the step of ammonium sulfate fractionation. Aromatic amino acid decarboxylase was partially purified from homogenates of hog kidney cortex. ^{14}CO-OH-L-tryptophan was purchased from New England Nuclear Corp., Boston, and purified by the method of ICHIYAMA *et al.*[10] Radioactive 5-hydroxy-L-tryptophan, labeled at the C-1, C-2 and C-3 of the side chain, was prepared from uniformly labeled L-serine (purchased from Radiochemical Center, Amersham, England) by the method of ICHIYAMA *et al.*[10] 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) was purchased from Calbiochem, California.

Assay for inhibition of tryptophan hydroxylase

Tryptophan hydroxylase was activated[9] by incubation in 100 μl of Tris-acetate buffer (10 mM, pH 8.1) containing 12.5 μg of catalase, 2.5 μmoles of dithiothreitol and 3.3 nmoles of ferrous ammonium sulfate at 0°C for 120 minutes in a nitrogen atmosphere. The reaction mixture for the assay of tryptophan hydroxylase activity consisted of 20 μmoles of potassium phosphate buffer (pH 7.0), 3.75 nmoles of ^{14}COOH-L-tryptophan, 9.2 nmoles of ferrous ammonium sulfate, 5 μl of distilled water or a test sample solution, 250 nmoles of $DMPH_4$ (25 μl) and 100 μl of the activated enzyme solution in a total volume of 250 μl. The reaction mixture except for $DMPH_4$ and the enzyme solution was incubated at 37°C for 3 minutes. The activated enzyme solution and $DMPH_4$ were added and incubation was continued for an additional 15 minutes. The reaction mixture was heated in a boiling water bath for 1 minute, and adjusted to pH 8.8 by the addition of 100 μl of Tris-acetate buffer (1 M, pH 8.8). This solution was mixed with 10 μl of cold 5 mM 5-hydroxy-DL-tryptophan aqueous solution, 20 μl of 5 mM pyridoxal phosphate aqueous solution (adjusted to pH 7.0 with NaOH), 20 μl of 5 mM disodium ethylenediaminetetraacetate aqueous solution, 15 m units hog kidney decarboxylase (100 μl) and incubated for 20 minutes at 37°C. Addition of 200 μl of 18% perchloric acid to the reaction mixture liberated $^{14}CO_2$ which was determined by the method described by ICHIYAMA *et al.*[10] The percent inhibition was calculated from measurements of $^{14}CO_2$ produced with and without addition of a test sample. The activity of aromatic amino acid decarboxylase using radioactive 5-hydroxy-L-tryptophan (labeled at C-1, C-2 and C-3 of the side chain) as the substrate was determined by the method of ICHIYAMA *et al.*[10]

Production and isolation of 2,5-dihydro-L-phenylalanine

Strain ME238-AG4, found to produce an inhibitor of tryptophan hydroxylase in its culture filtrate, was most closely related to *Streptomyces*

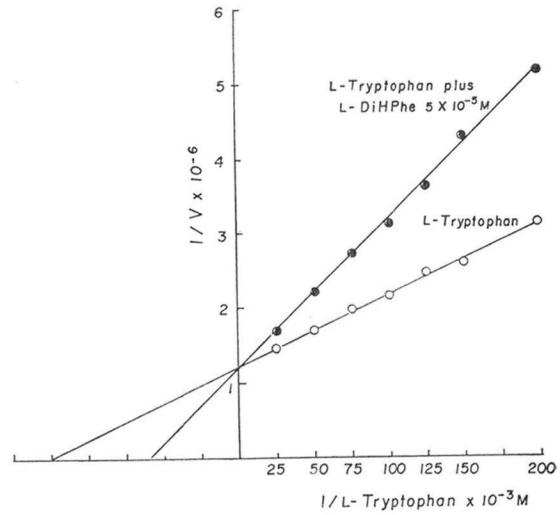
luteogriseus. Cultures of strain ME238-AG4 were grown in a medium consisting of glycerol 6.0%, peptone 1.0%, yeast extract 0.3%, $CaCO_3$ 0.2% (pH was adjusted to 7.4 before sterilization), at 27°C. Culture filtrate (5 μl) added to the reaction mixture caused 20~50% inhibition of tryptophan hydroxylase. The maximum production of the inhibitor was attained after 5 or 6 days of incubation. The active agent in the culture filtrate was isolated by the following procedure: clear filtrate (10 liters) was passed through a column (200 ml) of Dowex 50×8 (H^+ form). The column was washed with 1 liter of water, and eluted with 4 liters of 0.5 N NH_4OH. The active eluate was neutralized with 6 N HCl and passed through Amberlite XAD-4 column (700 ml). This column was also washed with 1.4 liters of water and eluted with 3.5 liters of 40% aqueous methanol. Active fractions were combined and concentrated under reduced pressure. This solution, adjusted to pH 2.5 with 1 N HCl, was passed through a Dowex 50×8 (200~400 mesh) column (3×60 cm) which was equilibrated with 0.1 M sodium-citrate buffer (pH 3.42). The active agent was eluted with 0.1 M sodium-citrate buffer (pH 4.25). In order to remove salts, the active fractions (about 1 liter) were passed through an Amberlite XAD-4 column (400 ml); the column was washed with 800 ml of water and the active agent was eluted with 2 liters of 40% aqueous methanol. The eluates were concentrated to about 50 ml under reduced pressure, the pH adjusted to 0.8 with 6 N HCl and the solution evaporated under reduced pressure to dryness. The hydrochloride salt of the active agent was crystallized from ethanol (about 20 ml). The overall yield was 2.12 g from 10 liters of culture filtrate; percent recovery, shown by the activity test, was 5.6. This active agent was identical with 2,5-dihydro-L-phenylalanine in all respects[4~8,11].

2,5-Dihydro-L-phenylalanine as an inhibitor of tryptophan hydroxylase

The degree of inhibition of tryptophan hydroxylase exhibited by various concentrations of 2,5-dihydro-L-phenylalanine was as follows: 23.9% at 10 μg/ml, 42.0% at 20 μg/ml, 50% at 28.8 μg/ml (calc.), 56.6% at 40 μg/ml, 73.6% at 80 μg/ml and 85% at 160 μg/ml. This compound did not inhibit aromatic amino acid decarboxylase at 160 μg/ml. The kinetics were studied in reaction mixtures containing tryptophan at varied concentrations. A LINEWEAVER-BURK plot of the data shown in Fig. 1 indicated that the inhibition was competitive with L-tryptophan. The *Ki* was 4.4×10^{-5}.

Fig. 1. LINEWEAVER-BURK plot of L-tryptophan-1-^{14}C concentration against rate of $^{14}CO_2$ formation with or without 2,5-dihydro-L-phenylalanine (L-DiHPhe) 5×10^{-5}.

The *Km* value obtained from the figure is 0.8×10^{-5} M and *Ki* value is 4.4×10^{-5} M.



Discussion

2,5-Dihydro-L-phenylalanine is very unstable and is easily converted into L-phenylalanine. We have confirmed that this compound remained unchanged after the enzymatic reactions. Phenylalanine was not detected by thin-layer chromatography [cellulose (Avicel) and 1-propanol - water (7: 3)] in the enzymatic reaction mixtures. The yield of crystalline 2,5-dihydro-L-phenylalanine was small (2 g from 10 liters of the culture filtrate), the culture broth at harvest was estimated to contain 30~40 g per 10 liters. Strain ME238-AG4 also produced L-phenylalanine. It is probable that phenylalanine resulted from dehydrogenation of 2,5-dihydro-L-phenylalanine.

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