NOTES

A POTENT PROLYL HYDROXYLASE INHIBITOR, P-1894B, PRODUCED BY A STRAIN OF *STREPTOMYCES*

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Prolyl hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate: oxygen oxidoreductase, EC 1. 14. 11. 2) is a mixed function oxygenase that catalyzes the hydroxylation to 4-hydroxyproline of specific prolyl residues in the peptide precursor of collagen. Prolyl hydroxylase is known as one of the key enzymes in collagen biosynthesis and its activity is enhanced in tissues of various pathological fibrosis[1~6]. A specific inhibitor of this enzyme, therefore, could have clinical application in the treatment of fibrotic diseases.

Several types of inhibitors of this enzyme are known, *i.e.*, metal chelating agents, certain divalent cations, SH-enzyme inhibitors, synthetic peptide analogs of the substrate as well as others. Recently, compound M-96[7] was isolated from the culture broth of *Chaetomium aureum* as an inhibitor of this enzyme.

In the course of our screening studies for a prolyl hydroxylase inhibitor from microbial sources, *Streptomyces* strain No. 1894, isolated from a soil sample, was found to produce a potent inhibitor. The present paper describes production, isolation, identification and biological properties of the inhibitor, P-1894B.

Prolyl hydroxylase was purified from chick embryo extracts as described by TUDERMAN *et al*[8]. The enzyme was entirely pure when examined by polyacrylamide gel electrophoresis. The enzyme activity was measured by the method of RHOADS *et al*[9]. The standard reaction mixture (total volume, 1.5 ml) contained 0.1 μmole [1-^{14}C]sodium α-ketoglutarate (0.016 μCi), 1.5 μmole ascorbic acid, 0.1 μmole ferrous ammonium sulfate, 4 mg heat-denatured bovine serum albumin (Sigma), 0.1 mg bovine liver catalase (Boehringer Mannheim), 0.45 mg (Pro-Pro-Gly)$_5 \cdot 4H_2O$, 0.1 μg enzyme and 50 μmole tris-HCl buffer (pH 7.8). The quantitative analysis of P-1894B was conducted by a high performance liquid chromatography equipped with a steel column (4 mm inside diameter, 300 mm length) packed with Unisil Q C_{18} 5 μm(Gasukuro Kogyo) in a reverse phase of acetonitrile - water (7: 3). Chromatography was monitored by a UV detector (254 nm) and the flow rate was maintained at 1.0 ml/minute.

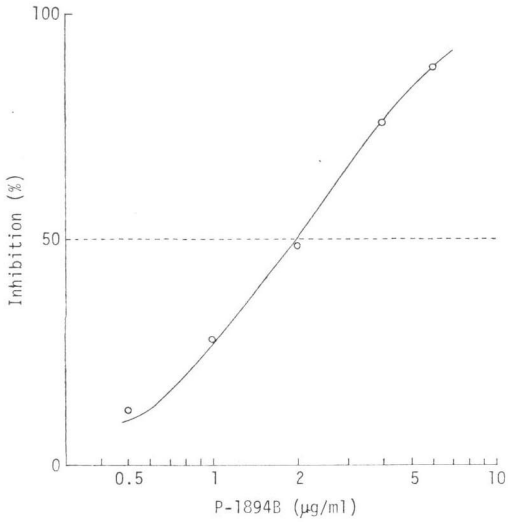
The strain No. 1894 was designated as *Streptomyces albogriseolus* subsp. No. 1894 from its taxonomic characteristics and has been deposited at the Institute for Fermentation, Osaka under the accession number of IFO 13881.

The strain No. 1894 was cultivated at 28°C for 7 days on a slant of yeast-KRAINSKY's agar. A loopful of spores was inoculated into 500 ml of a seed medium in a 2-liter Sakaguchi flask and incubated at 28°C for 2 days with reciprocal shaking. The seed medium contained (g/liter): glucose 20, glycerine 10, soy-bean flour 5, corn-steep liquor 5, Polypepton 3, NaCl 3, $CaCO_3$ 5, pH 7.0. The culture broth (1.5 liters) was transferred to 30 liters of the seed medium in a 50-liter fermentor and cultivated at 28°C for 2 days with an air-flow rate of 30 liter/minute and agitation at 280 rev/minute. The second seed culture (30 liters) was transferred to 300 liters of a production medium in a 500-liter fermentor and incubated at 28°C for 3 days with aeration (450 liter/minute) and agitation (240 rev/minute). The production medium contained (g/liter): dextrin 75, Polypepton 7, $FeSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 4 \sim 6H_2O$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 0.5, $CaCO_3$ 5, pH 7.0.

To 245 liters of the fermented broth adjusted to pH 5.0 with H_2SO_4, 245 liters of ethyl acetate was added. After stirring the mixture for 1 hour, mycelia were filtered off by a filter press. The solvent layer was separated and washed with 245 liters of water. The washed extracts were concentrated *in vacuo* to 800 ml and about 4 liters of *n*-hexane was added to the concentrated extracts. The resultant precipitate was collected, dissolved in 2 liters of the mixed solvent of chloroform - ethyl acetate (4: 1) and chromatographed on a column (5.5 × 52 cm) of silica gel using the same

Fig. 1. Inhibition of prolyl hydroxylase activity by P-1894B.

Assay was performed by the method of RHOADS *et al.* using $(Pro\text{-}Pro\text{-}Gly)_5$ as substrate. Each spot was mean value in triplicate assays.



solvent as the eluant. The active fractions were combined and concentrated *in vacuo* to give an orange powder. By crystallization from toluene-methanol, 100 g of crude crystals were recovered. On recrystallization with ethyl acetate, about 50 g of pure crystals of P-1894B were obtained(overall yield: about 58%).

Crystals of P-1894B are orange to orange-yellow needles with m.p. 164~166°C. This inhibitor, P-1894B, was found to be identical with OS-4742A_1[10] (vineomycin A_1) by direct comparing their physicochemical properties. Although the molecular formula of OS-4742A_1 was not known exactly, the most probable formula of P-1894B was estimated as $C_{49}H_{58}O_{18}$, M.W. 934.

The inhibition of prolyl hydroxylase by increasing concentration of P-1894B was found to give a sigmoid curve (Fig. 1) and the concentration required for 50% inhibition was about 2 μg/ml (2.2 μM). When excess amount of ferrous ions or ascorbic acid, each of which is a cofactor of prolyl hydroxylase, was added to the reaction mixture, the inhibition was slightly reversed. These findings indicate that P-1894B does not inhibit the enzyme activity by chelating ferrous ions or acting as antioxidant toward ascorbate.

The acute toxicity LD_{50} of P-1894B in a preliminary examination in rats was 100~200 mg/kg by intraperitoneal injection and more than 1,000 mg/kg by oral administration.

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