MANDELIC ACID-4-HYDROXYLASE, A NEW INDUCIBLE ENZYME FROM PSEUDOMONAS CONVEXA

by

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Summary: A soluble fraction of Pseudomonas convexa catalyzed the hydroxylation of mandelic acid to p-hydroxymandelic acid. The enzyme had a pH optimum of 5.4 and showed an absolute requirement for Fe^{2+}, tetrahydropteridine, NADPH. p-Hydroxymandelate, the product of the enzyme reaction was identified by paper chromatography, thin layer chromatography, UV and IR-spectra.

1. Introduction

The metabolism of mandelic acid to intermediates of tricarboxylic acid cycle has been extensively worked out in the bacterial system (1,2,3,4). Jamaluddin et al (5) have studied the degradation of the same compound in the fungal system (5). The pathway for the degradation of p-hydroxymandelic acid has also been established by Gunter (6). However, the involvement of p-hydroxymandelic acid in the catabolism of mandelic acid has not been reported so far. The present paper deals with a new inducible enzyme, mandelic acid-4-hydroxylase, which catalyzes the conversion of mandelic acid to p-hydroxymandelic acid in the presence of pteridine cofactor, NADPH and Fe^{2+}.

2. Methods

2.1 Preparation of cell-free extract.

A strain of Pseudomonas convexa obtained by enrichment with DL-mandelic acid was used in the present study. It was grown at 30 ± 1° in a chemically defined medium as described by Seubert (7) supplemented with 0.5% glucose and 0.1% DL-mandelic acid (final pH of the medium was adjusted to 7.4 by the addition of 1 N NaOH). The cells were harvested in the midexponential growth phase and washed twice with 0.1 M sodium phosphate buffer pH 7.0. The harvested cells were resuspended in 4 volumes of 0.025 M sodium phosphate buffer pH 7.0 and then subjected to sonication for 3 min. at 0-5° in a Branson sonifier by keeping the current strength at 5 amps. The sonicate was centrifuged at 10,000 g for 10 min. The supernatant was designated as the crude extract which usually contained 10-12 mg of protein per ml.

The crude extract was then centrifuged at 100,000 g for one hr in a Beckman model L-3-50 preparative ultracentrifuge. The cream coloured pellet thus obtained was resuspended in 0.025 M sodium phosphate buffer pH 7.0 and again centrifuged at 100,000 g for one hr. The pellet obtained was uniformly dispersed in 0.025 M sodium phosphate buffer pH 7.0 with the help of a fine hypodermic syringe. This preparation is referred to as the particulate fraction and the earlier 100,000 g supernatant fluid as the supernatant fraction.

2.2 Assay of mandelate-4-hydroxylase activity.

Mandelate-4-hydroxylase activity was assayed by measuring the amount of p-hydroxymandelate formed using a modification of the colorimetric method of Bray et al (8). Assays were conducted as mentioned in Table I and the reaction was terminated by the addition of trichloroacetic acid to a final concentration

of 6% and centrifuged to remove denatured protein. An aliquot (0.5 ml) was pipetted out and diluted to 1 ml with water. To this, 1 ml of 95% ethanol, 0.2 ml of diazotized p-nitroaniline (freshly prepared by mixing 25 ml of 0.3% p-nitroaniline solution in 0.8 N HCl and 1.5 ml of 5% $NaNO_2$) and 2 ml of water were added. After 2 min, the mixture was made alkaline by adding 1 ml of 5% Na_2CO_3 solution. The red colour developed was read immediately or within 5 min. against a reagent blank in a Klett-Summerson photoelectric colorimeter using No.54 filter (500-570 nm). The response to increasing concentration of p-hydroxymandelic acid in the test solution was linear upto 160 n moles.

.3 Isolation and Identification of the product.

In order to obtain a sufficient quantity of the enzymatic product, a large scale incubation (50 ml) was carried out under the conditions of the assay procedure with 20 mg of DL-mandelic acid, 25 ml of enzyme extract (260 mg protein) and proportionate amount of cofactors. After incubation for 90 min. at 30° the pH of the reaction mixture was adjusted to 2 with 2 N HCl and it was extracted twice with distilled peroxide-free ether. The ether layers were pooled, dried over anhydrous sodium sulphate and evaporated to dryness under suction. The residue was dissolved in a small volume of ethyl acetate and then chromatographed on silica gel G plates (0.5 mm thickness) using benzene-methyl isobutyl ketone-formic acid (60:37:3 v/v) as the solvent system. The enzyme product was detected on the chromatogram by exposing to iodine vapours, the area corresponding to p-hydroxymandelate was scraped out from the plate and the product was eluted with peroxide-free ether. The eluate was evaporated to dryness and rechromatographed twice in the

TABLE - I

Requirement for Mandelic Acid Hydroxylation by Cell-free Extracts of Pseudomonas convexa.

The reaction mixture contains 70 μmoles of citrate-phosphate buffer, pH 5.4; 0.1 μmole of ferrous sulphate; 0.25 μmole of DL-mandelic acid; 0.02 μmole of 2-amino-4-hydroxy-6,7-dimethyl tetrahydropteridine (in 0.1 ml of 10^{-3}M β-mercaptoethanol); 0.1 ml of TPNH-regenerating system (TPNH-regenerating system contains 50 μmole of sodium phosphate buffer, pH 7.0; 2.0 μmole of NADP; 2.5 μmole of glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase in a final volume of 1 ml and incubated at 30° for 15 min.) and 100,000 g supernatant fraction (4 mg protein) in a final volume of 1.5 ml. Incubation was carried out in a metabolic shaker with air as the gas phase for 30 min. at 30°.

Component	p-Hydroxymandelic acid formed (nmoles)
Complete system	89.0
Without enzyme	7.0
Without Fe^{2+}	0.0
Without mandelic acid	0.0
Without NADPH	5.0
Without tetrahydropteridine	8.0
Complete but boiled enzyme	5.0

same solvent system as described above. The pure enzymatic product thus obtained was identified as follows:

1) It was subjected to ascending paper chromatography

on Whatman No.3 filter paper and the chromatograms were developed using the following solvent systems:-

A. formic acid - water (2:98 v/v).

B. benzene - methanol - water (45:8:4 v/v).

C. isopropanol - ammonia - water (20:1:2 v/v).

D. benzene - acetic acid - water (4:4:1 v/v, organic phase).

The product was located by spraying the dried chromatograms with diazotized p-nitroaniline, followed by 10% aqueous NaOH.

2) Thin layer chromatography on silica gel G plates (0.25 mm thickness) using the following solvent systems:-

E. benzene-methylisobutylketone-formic acid (60:37:3 v/v).

F. benzene-ethylmethyl ketone-formic acid (80:18:2 v/v).

3) By taking ultraviolet spectrum in ethyl acetate using Unicam SP-700 A recording spectrophotometer.

4) By recording the Infrared spectrum in nujol mull with Perkin-Elmer 700 spectrophotometer.

Results and Discussion.

As shown in Table-I the requirements for mandelic acid hydroxylation by the cell-free extract of the Pseudomonas convexa were substrate, ferrous ion, tetrahydropteridine, NADPH, oxygen and enzyme. When any one of these compounds was omitted, either the amount of the reaction product formed decreased or there was no enzymatic reaction. All preparations of the enzyme showed an absolute requirement for ferrous ion. The optimum pH for hydroxylation was found to be 5.4. The activity was proportional to the amount of the enzyme added upto 5.2 mg

TABLE - II

Intracellular Distribution of Mandelic Acid-4-Hydroxylase

Fraction	p-Hydroxymandelic acid formed (nmoles)
Crude	62
Particulate	0
Supernatant	89

protein and the reaction proceeded linearly for 45 min. Extracts from cells grown on glucose were devoid of hydroxylase activity.

The crude enzyme was subjected to differential centrifugation as described under methods and each fraction was assayed for hydroxylating activity (Table II). The particulate fraction was found to be completely devoid of enzymatic activity.

The enzymatic product was identified as p-hydroxymandelic acid by comparing its chromatographic mobilities and spectral characteristics with those of the authentic sample. As shown in Table III, the R_f values of p-hydroxymandelic acid and the enzymatic product were identical. Cochromatography of the authentic p-hydroxymandelic acid with the enzymatic product in any of the solvent systems gave a single spot. The ultraviolet

TABLE - III

R_f Values of Authentic p-Hydroxymandelic acid and Enzymatic Product in Different Chromatographic systems

Chromatogram	Solvent* system	R_f Values	
		Authentic p-hydroxy mandelic acid	Enzymatic product
Paper (ascending)	A	0.91	0.90
	B	0.21	0.21
	C	0.23	0.23
	D	0.07	0.07
Thin layer	E	0.26	0.26
	F	0.15	0.15

* Details in the text.

spectrum of the isolated compound showed an absorption maximum around 277 nm and a shoulder at 282 nm in ethyl acetate which was indistinguishable from that of authentic p-hydroxymandelic acid. The enzymatically produced compound gave an infrared spectrum identical with that given by the authentic p-hydroxymandelic acid. [ν_{max}^{nujol} 3450 cm^{-1}; 3225 cm^{-1} (OH free and hydrogen bonded) 3000 cm^{-1} (aromatic); 1700 cm^{-1} (carboxyl); 1605 cm^{-1} (aromatic); 1360 cm^{-1} (phenolic OH); 1260 cm^{-1} (secondary alcohol) and 840 cm^{-1} (aromatic 1,4-disubstituted)].

Mandelic acid-4-hydroxylase of this Pseudomonas species

bears a notable resemblance to the inducible phenylalanine hydroxylase of Pseudomonas species (ATCC 11299a) (9,10). Most prominent among these is the requirement of both the enzymes for tetrahydropteridine and Fe^{2+}. However, there were also striking points of difference in the properties of the two enzymes. For instance, mandelic acid-4-hydroxylase was not activated when preincubated with Fe^{2+}, Hg^{2+}, Cd^{2+}, Cu^{+} and Cu^{2+} ions. ~~(Ref)~~. NADPH serves as a better electron donor than NADH for mandelic acid-4-hydroxylase, whereas for the bacterial phenylalnine hydroxylase, NADH is the most suited one. In fact, NADPH is inhibitory to bacterial phenylalanine hydroxylase (11).

Such a type of ring hydroxylation prior to the degradation of side chain was reported in the metabolism of phenylacetate by Aspergillus niger (12). Investigations are under way to elucidate the oxidative degradation of mandelic acid involving p-hydroxymandelic acid, as the first intermediate in this strain of bacterium.

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REFERENCES

(1) I.C.Gunsalus, C.F.Gunsalus and R.Y.Stanier. J. Bacteriol., 66, 538 (1953).
(2) C.F.Gunsalus, R.Y.Stanier and I.C.Gunsalus. J. Bacteriol., 66, 548 (1953).

(3) R.Y.Stanier, I.C.Gunsalus and C.F.Gunsalus.
J. Bacteriol., 66, 543 (1953).
(4) G.D.Hegeman.
J. Bacteriol., 91, 1140 (1966).
(5) M.Jamaluddin, P.V.Subba Rao and C.S.Vaidyanathan.
J. Bacteriol., 101, 786 (1970).
(6) S.E.Gunter.
J. Bacteriol., 66, 341 (1953).
(7) W.Seubert.
J. Bacteriol., 79, 426 (1960).
(8) H.G.Bray, B.G.Humphris, W.V.Thorpe, K.White and
P.B.Wood. Biochem. J., 52, 416 (1952).
(9) G.Guroff and T.Ito.
J. Biol. Chem., 240, 1175 (1965).
(10) G.Guroff and C.A.Rhodes.
J. Biol. Chem., 242, 3641 (1967).
(11) G.Guroff and T.Ito.
Biochim. Biophys. Acta, 77, 159 (1963).
(12) M.Sugumaran, K.Ramanarayanan and C.S.Vaidyanathan.
FEBS Letters 29, 69 (1973).