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PURIFICATION, PROPERTIES AND INDUCTION OF A SPECIFIC BENZOATE-4-HYDROXYLASE FROM *ASPERGILLUS NIGER* (UBC 814)

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Summary

An inducible benzoate-4-hydroxylase has been partially purified from crude extracts of the mycelial felts of *Aspergillus niger*. This enzyme catalyzes the transformation of benzoate to *p*-hydroxybenzoate with equimolar consumption of NADPH and O_2. It requires tetrahydropteridine as a prosthetic group. The optimum activity was found at pH 6.2 with a K_m value at 30 °C of $1.6 \cdot 10^{-4}$ M for NADPH and $1.3 \cdot 10^{-4}$ M for benzoate. Fe^{2+} (iron) is required for the enzyme activity.

The enzyme is stabilized by the inclusion of benzoate, EDTA and glutathione in the extracting buffer. The enzyme is specific for benzoate as substrate. Sulfhydryl group(s) are essential for enzyme activity as indicated by *p*-chloromercuri-benzoate and *N*-ethylmaleimide inactivation. Benzoate-4-hydroxylase activity is decreased in the mycelial felts of *Aspergillus niger* grown in the presence of higher concentrations of benzoate. Maximum activity of the enzyme was observed at 36 h after inoculation.

Introduction

The degradation of benzoic acid follows different routes in various microorganisms. Certain bacteria have been shown to oxidatively decarboxylate benzoate to catechol which may undergo either ortho or meta cleavage [1,2]. It is suggested that 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid may be an intermediate in the oxidation of benzoate to catechol [3]. In *Pseudomonas convexa* var. *hippuricum* and *Azotobacter vinelandii* there is some evidence for the formation of salicylic acid as an intermediate in the oxidation of benzoate to catechol [4,5]. But so far an enzyme catalyzing the 2-hydroxylation of benzoate to give salicylic acid has not been isolated. *Trichoderma lignorum*, *Pseudomonas testosteronii* and *Pseudomonas acidivorans* seem to hydroxylate ben-

zoate in the 3-position, but no definite evidence for the existence of a benzoate-3-hydroxylase has been obtained [6,7].

Fungi, in general are known to degrade benzoate to protocatechuate via 4-hydroxybenzoate [8—11]. The enzyme responsible for the conversion of benzoate to *p*-hydroxybenzoate was demonstrated in cell-free extracts of *Aspergillus niger* (UBC 814) [12] and *Polyporus hispidus* [13]. Benzoate-4-hydroxylase was shown to require NADPH as an electron donor. However, the detailed study of the enzyme has not been done in any system. The present paper is concerned with the purification, properties and induction of benzoate-4-hydroxylase in *A. niger* (UBC 814).

Materials and Methods

Chemicals

Benzoic acid was purchased from BDH Company, Bombay, India. *p*-Hydroxybenzoate was obtained from K & K Laboratories Inc., California, U.S.A. These chemicals were recrystallized from hot water. Protamine sulfate, DEAE-cellulose, $NADP^+$, glucose 6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (Type IV), 2-mercaptoethanol, glutathione and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 6,7-Dimethyl 5,6,7,8-tetrahydropteridine, tetrahydrofolic acid, biopterin and alumina Cγ gel were from Calbiochem, Los Angeles, California, U.S.A. Tricalcium phosphate gel was prepared by the method of Keilin and Hartree [14]. Other chemicals used were of analytical grade available commercially.

Organism and conditions of cultivation

A. niger (UBC 814) was obtained from R.J. Bandoni, Department of Botany, University of British Columbia, Canada. The organism was grown on a synthetic medium [15] supplemented with 0.1% (w/v) benzoate. The final pH of the medium was adjusted to 5.5 with dilute NaOH. *A. niger* was grown in one litre flasks for 36 h at 30°. Stock cultures were maintained on slants of the same medium solidified with 1.5% agar. The mycelium was washed thoroughly with glass distilled water before enzyme extraction.

Assay of benzoate-4-hydroxylase

Reaction mixtures (1 ml) contained the following components in μmol: Tris/maleate buffer, pH 6.2 (28), sodium benzoate (0.2), ferrous sulfate (0.01), dimethyl tetrahydropteridine (0.02), NADPH generating system (NADP (0.2), glucose 6-phosphate (0.4) and glucose 6-phosphate dehydrogenase 15 munits (The components of the NADPH generating system were preincubated for 10 min prior to the addition to the reaction mixture) and enzyme protein was incubated at 30°C for 30 min. The enzyme was preincubated with benzoate and buffer for 5 min before adding other components of the reaction mixture. The reaction was terminated by the addition of 0.2 ml of 0.5 M HCl and the precipitated protein was sedimented by centrifugation and discarded. The supernatant was extracted with 3 ml of peroxide-free diethyl ether, samples of ether layer (2 ml) were taken to dryness and the *p*-hydroxybenzoate was determined by the colorimetric method of Bray et al. [16].

Isolation of the reaction products

For identification of the reaction products, 100 standard reaction mixtures were pooled, acidified to pH 2 with 0.5 M HCl and extracted with twice the volume of peroxide-free ether. After removing the solvent under vaccum, the residue was dissolved in 0.5 ml ethyl acetate and chromatographed on silica gel G plates (0.5 mm thickness) using benzene/acetic acid/water (10 : 7 : 3 v/v/v) as the solvent system. The R_F value of *p*-hydroxybenzoate was determined after spraying the plate with diazotized *p*-nitroaniline reagent [17]. The band corresponding to *p*-hydroxybenzoate (R_F = 0.4) was scraped from the plate and the product was eluted with peroxide-free ether. The eluate was evaporated to dryness and rechromatographed in the same solvent system as described above. The pure enzymatic product thus obtained was characterized by determining the ultraviolet and infrared spectra, and also by comparing its R_F values on paper chromatography in different solvent systems with those of an authentic sample.

Assay of p-hydroxybenzoate-3-hydroxylase

Reaction mixtures (1 ml) contained the following components in μmol: Tris/maleate buffer, pH 6.2, (28) *p*-hydroxybenzoate (.02), FAD (0.02), NADPH generating system (as described) and enzyme protein was incubated at 30 °C for 30 min. The reaction was terminated and the mixture extracted as previously described, and the protocatechuate formed was determined colorimetrically [18].

NADPH oxidase assay

Oxidation of NADPH was determined spectrophotometrically by following the decrease in absorbance at 340 nm. The total reaction mixture (1 ml) consisted of the same components mentioned under benzoate-4-hydroxylase assay except that benzoate was deleted from the mixture and NADPH was used in place of NADPH generating system.

Definition of enzyme activities

The unit of enzyme activity is defined as that amount of the enzyme which catalyzed the formation of 1 μmol of product per min under standard conditions of the assay.

Specific activity is expressed as munits of enzyme per mg of protein.

The protein concentrations were determined by the method of Lowry et al. [19], with bovine serum albumin as standard.

Oxygen uptake

Oxygen consumption was determined by the conventional Warburg technique.

Results

Purification of benzoate-4-hydroxylase

All operations were carried out at 0—4 °C.

(1) Preparation of crude extract. The mycelium (30 g) was macerated in a

chilled mortar with an equal weight of alumina for 15 min and extracted with 60 ml of 0.025 M Tris/maleate buffer, pH 6.2 containing 0.2 mM EDTA, 0.1 mM benzoate and 0.1 mM glutathione, hereafter referred to as Standard buffer. The extract was passed through a cheese cloth and centrifuged at 15 000 × *g* for 30 min. The supernatant solution containing 10—12 mg protein per ml was used for further purification.

(2) Treatment with protamine sulfate. A 2% solution of protamine sulfate in 0.025 M standard buffer (6 ml) was added to the crude extract (54 ml) with gentle mechanical stirring. After 10 min, the mixture was centrifuged at 12 000 × *g* for 5 min to remove the precipitated nucleoproteins.

(3) Negative adsorption onto tricalcium phosphate gel. Tricalcium phosphate gel (500 mg) was equilibrated with 0.025 M Tris/maleate buffer, pH 6.2 and centrifuged. To the gel thus obtained was added the supernatant from protamine sulfate treatment (50 ml) and the mixture was stirred for 20 min. The suspension was centrifuged at 12 000 × *g* for 5 min and the supernatant was collected.

(4) DEAE-cellulose treatment. DEAE-cellulose was washed according to the procedure described by Peterson and Sober [20] and equilibrated in 0.025 M Tris/maleate buffer, pH 6.2, prior to filtering through a Buchner funnel. To the supernatant from the previous step (40 ml) were added 5 g of the washed DEAE-cellulose cake and the mixture was stirred mechanically for 20 min. The suspension was filtered through a chilled Buchner funnel and the filtrate was collected.

(5) Adsorption onto and elution from tricalcium phosphate gel. The enzyme preparation from the previous step (30 ml) was treated with 1.08 g of tricalcium phosphate gel. After stirring for 15 min, the suspension was centrifuged and the precipitate was washed with 30 ml of 0.025 M standard buffer. The enzyme was finally eluted from the gel by stirring it for 20 min twice with 30 ml (15 ml each time) of 0.5 M Tris/maleate buffer, pH 6.2.

(6) Alumina Cγ gel treatment. The enzyme preparation obtained from the previous step (20 ml) was treated with alumina Cγ gel (300 mg) and stirred for 15 min. The suspension was centrifuged at 15 000 × *g* for 10 min and the supernatant was collected. The clear supernatant was used as benzoate-4-hydroxylase for all subsequent studies unless otherwise stated.

Stabilization of the enzyme

The mycelium can be stored at −20°C for 10 days without appreciable loss in activity. The enzyme activity is rapidly lost during purification. Aromatic substrate analogs, EDTA and thiol compounds have profound action on stabilization of the enzyme, EDTA, benzoate and glutathione being most effective.

The balance sheet for the purification of benzoate-4-hydroxylase is shown in Table I. Crude extracts of mycelium showed very low benzoate-4-hydroxylase activity. These preparations were found to contain a potent NADPH oxidase and *p*-hydroxybenzoate-3-hydroxylase. The preparation, obtained after tricalcium phosphate gel treatment (Step 3) was free from *p*-hydroxybenzoate-3-hydroxylase activity but NADPH oxidase activity was observed. Indeed *p*-hydroxybenzoate-3-hydroxylase activity was observed in the eluate of trical-

TABLE I

PURIFICATION PROCEDURE OF BENZOATE-4-HYDROXYLASE FROM *A. NIGER*

Procedure	Volume (ml)	Activity (munits)	Protein (mg/ml)	Specific activity (munits/ mg protein)	Total enzyme activity recovered (munits)	Purification (fold)
1. Crude extract	60*	3.7	10.8	0.34	222	1
2. Protamine sulfate	54	16.0	7.0	2.3	864	7
3. Tricalcium phosphate gel	50	25.6	4.4	5.8	1280	17
4. DEAE-cellulose	40	42.0	2.25	18.7	1680	55
5. Tricalcium phosphate gel	30	17.3	0.5	34.0	519	102
6. Alumina Cγ gel	20**	16.0	0.18	88.8	320	261

* Experimental details are given in the text.

** The total activity of the one step does not represent the total activity of the previous step.

cium phosphate gel treatment (Step 3). Even in the presence of a sufficient excess of NADPH-generating system (NADP 1 μmol, glucose 6-phosphate 1.25 μmol and glucose-6-phosphate dehydrogenase 50 munits) an increase in enzyme activity was not observed in the preparations obtained after Step 3. However, the total munits of benzoate-4-hydroxylase activity still further increased during the purification step 4 from 1280 to 1680 and this increase seems not to be attributable to the interfering action of the NADPH-oxidase activity still present in step 3 extracts (see above). We therefore tentatively suggest that a third endogenous inhibitor is present apart from the two interfering enzymes mentioned earlier. Attempts to purify the enzyme to homogeneity have been limited by its extreme instability. The enzyme was inactivated when treated with alcohol or acetone. Ammonium sulfate precipitation could not be used as one of the steps in the purification, because dialysis or passage through Sephadex G-25 column to remove ammonium sulfate inactivated the enzyme. The enzyme was inactivated by about 40% at 4 °C after 24 h.

Characterization of the enzymatic product

The enzymatic product was identified as *p*-hydroxybenzoate by comparing its chromatographic mobilities and spectral properties with those of an authentic sample. The R_F values on paper chromatography in benzene/acetic acid/water (10 : 7 : 3, v/v/v upper phase) and 2% formic acid were 0.40 and 0.52 respectively. The enzymatic product, like authentic *p*-hydroxybenzoate melted at 211 °C (uncorrected) with decomposition. There was no change in melting point on admixture. The ultraviolet spectrum of the isolated enzymatic product showed an absorption maximum around 254 nm in 95% ethyl alcohol. A bathochromic shift from 254 to 275 nm was observed in alkaline solution. These values were exactly identical to that of authentic *p*-hydroxybenzoate. The enzymatic product showed characteristic peaks at 3450 cm^{-1} (OH free), 3000 cm^{-1} (aromatic), 1700 cm^{-1} (carboxyl), 1605 cm^{-1} (aromatic), 1360 cm^{-1} (phenolic OH), 840 cm^{-1} (aromatic 1,4-disubstituted) in the infrared spectrum. The infrared spectra of enzymatic product and authentic *p*-hydroxybenzoate were superimposable.

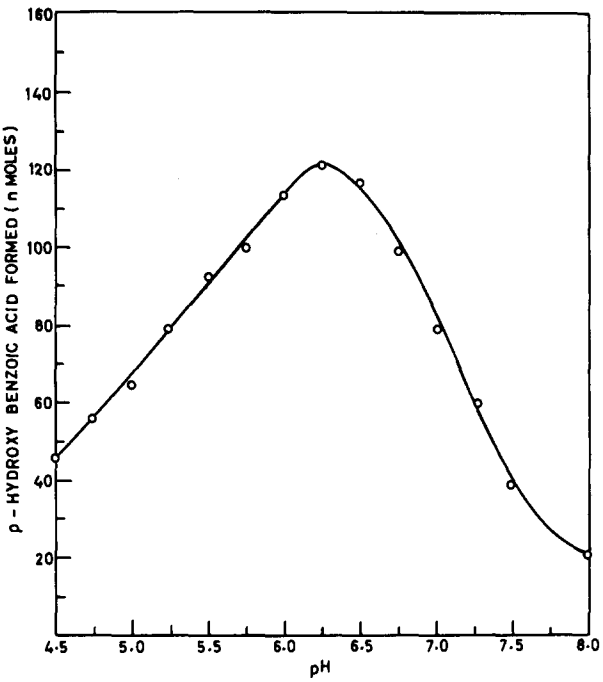


Fig. 1. Effect of pH on benzoate-4-hydroxylase activity. Standard assay conditions as described in Materials and Methods were used escept that the reaction was carried out at different hydrogen concentration for 15 min at 30°C. The buffers used for the different pH ranges are: (1) Citrate/phosphate buffer (pH 4.0 to 5.5); (2) Tris/maleate buffer (pH 5.5 to 7.5); (3) Tris · HCl buffer (pH 7.5 to 9).

Properties of benzoate-4-hydroxylase

Effect of pH on enzyme activity. The optimum pH of the reaction was determined by using 0.1 M citrate/0.2 M sodium phosphate, 0.1 M Tris/maleate and 0.1 M Tris · HCl buffers. As shown in Fig. 1, the enzyme exhibits a maximum of activity around pH 6.2. The enzyme was stable between pH values 5.5 and 7.5 on storage.

Effect of temperature. Enzyme activity was maximum around 30 °C with a rapid decrease in the activity above 38 °C and below 25 °C.

Effect of enzyme concentration. The reaction rate was found to be linear upto a protein concentration of 150 μg.

Substrate specificity. Benzoate-4-hydroxylase showed an absolute requirement for NADPH as an electron donor. Among the other electron donors tested, NADH, glutathione, 2-mercaptoethanol, ascorbate, cysteine and dithiothreitol, only ascorbate showed about 30% of the activity.

Reduced pteridine cofactor was also essential for the enzyme reaction, no non-pterine compound could substitute for it. Of the three pterine cofactors tested, biopterine, tetrahydrofolic acid and 6,7-dimethyl 5,6,7,8-tetrahydropterine, the last compound showed maximum activity, tetrahydrofolic acid giving about 60% of the activity.

Benzoate was readily hydroxylated to give *p*-hydroxybenzoic acid. Substrate analogs like phenyl acetate, trans-cinnamate, phenyl lactate, phenyl alanine, benzaldehyde and methyl benzoate ester were tested and found inactive.

Monohydroxybenzoates such as salicylate and *m*-hydroxybenzoate could not be hydroxylated by this enzyme.

Substrate affinities. A study of the effect of different concentrations of NADPH and of benzoate on the rate of formation of *p*-hydroxybenzoate gave hyperbolic curves. The K_m values for NADPH and benzoate were determined by Lineweaver-Burk plots to be $1.6 \cdot 10^{-4}$ M and $1.3 \cdot 10^{-4}$ M, respectively. The benzoate concentrations present in the enzyme preparation were taken into account while calculating the affinity constants.

Requirement of molecular oxygen. The oxygen requirement of the hydroxylase was suggested by relating the amount of *p*-hydroxy-benzoate formed in the presence and absence of air (Table II). In the presence of atmospheric oxygen, the formation of *p*-hydroxy-benzoate was increased about 7-fold over that formed in the anaerobic system. Oxygen could not be replaced by other electron acceptors such as phenazine methosulfate, methylene blue, and 2,6-dichlorophenolindophenol.

Stoichiometry. The stoichiometry of benzoate-4-hydroxylase reaction is summarized in Table III. For the formation of 1 mol of *p*-hydroxybenzoate from benzoate, there was a consumption of 1 mol of oxygen and oxidation of 1 mol of NADPH. From the foregoing data it is evident that the overall reaction proceeds according to the following equation:

$$\text{Benzoate} + \text{NADPH} + \text{H}^+ + \text{O}_2 \xrightarrow[\text{cofactor}]{\text{pteridine}} p\text{-hydroxybenzoate} + \text{NADP}^+ + \text{H}_2\text{O}.$$

Metal ion requirements. Benzoate-4-hydroxylase reaction was activated by Fe^{2+}. The activity was inhibited by iron-chelators such as 8-hydroxyquinoline, α,α'-dipyridyl and *o*-phenanthroline (Table IV). The enzyme activity was considerably reduced when the organism was grown on an iron dificient medium. Other divalent metal ions such as Zn^{2+}, Cu^{2+}, Hg^{2+}, Mo^{2+}, Mg^{2+} and Mn^{2+} caused complete inhibition even at low concentrations ranging from 10^{-6} to 10^{-5} M.

Effect of sulfhydryl reagents. As might be expected from the protective effect by thiol compounds, the hydroxylation was inhibited by sulfhydryl

TABLE II

FORMATION OF *p*-HYDROXYBENZOATE UNDER AEROBIC AND ANAEROBIC CONDITIONS

Each reaction mixture contained in the main compartment of Thunberg tube, benzoate (0.4 μmol), Tris/maleate buffer, pH 6.2 (56 μmol), $FeSO_4$ (0.02 μmol) and 16 munits of enzyme. In each side bulb 0.5 μmol of NADPH and 0.04 μmol of dimethyltetrahydropteridine. The Thunberg tubes were then alternatively flushed with nitrogen gas and evacuated for 10 min. The solution from side tube was then tipped into the main compartment bringing the final values of the reaction mixture to 2 ml and the reaction was run at 30°C for 20 min and was arrested by adding 0.4 ml of 0.5 M HCl. *p*-Hydroxybenzoate formed was determined as described in Methods and Materials. In aerobic system immediately after tipping the solution from side bulb, air was admitted into the tube.

Condition	*p*-Hydroxybenzoate formed (nmol/2 ml)
Aerobic	262
Anaerobic	38
Air admitted after 5 min of anaerobic condition	248

TABLE III

TIME COURSE AND STOICHIOMETRY OF BENZOATE-4-HYDROXYLASE ACTIVITY

The experiments were carried out under standard assay conditions as described in Methods and Materials, except that NADPH was used instead of NADPH generating system. In experiment No. II, the reaction mixture (2 ml) consisted of Tris/maleate buffer, pH 6.2 (58 μmol), benzoate-4-hydroxylase (16 munits), benzoate (0.4 μmol), $FeSO_4$ (0.02 μmol), pteridine cofactor (0.04 μmol), and NADPH. After oxygen consumption was measured, 1 ml of the reaction mixture was acidified with 0.2 ml of 0.5 M HCl and extracted with 2.5 ml of peroxide-free ether. The residue obtained by taking to dryness 2 ml aliquot of the ether layer was taken up in 1 ml of 0.1 M Tris/maleate buffer, pH 6.2 and the absorbance at 229 nm was determined for estimation of benzoate.

Time	Benzoate utilized (nmol)	*p*-Hydroxy-benzoate formed (nmol)	NADPH oxidised (nmol)	Oxygen consumption (nmol)
Experiment I				
5	— *	56	58	—
10	—	90	95	—
15	—	120	126	—
20	—	145	160	—
30	—	186	192	—
Experiment II				
30	360	340	—	354

* Not determined.

reagents such as *p*-chloromercuribenzoate and *N*-ethyl maleimide. The inhibition could be reversed by adding excess amounts of sulfhydryl compounds.

Studies on induction of benzoate-4-hydroxylase in A. niger

Benzoate-4-hydroxylase was induced by DL-mandelate, benzoylformate, benzaldehyde and benzoate in *A. niger*. Benzoate was found to be the most potent inducer. No other substrate analogs like phenylacetate, phenyllactate, cinnamate could serve as inducer for the synthesis of benzoate-4-hydroxylase. Neither *p*-hydroxy-benzoate nor protocatechuate could induce this enzyme.

TABLE IV

EFFECT OF METAL-CHELATING AGENTS ON BENZOATE-4-HYDROXYLASE

The NADPH-free standard reaction system was first incubated for 10 min with the test compound and the reaction was started by the addition of NADPH. Assay of the enzyme reaction was done as described in Materials and Methods.

Chelator	Concentration (M)	Inhibition (%)
8-Hydroxy quinoline	$2.0 \cdot 10^{-3}$	80
8-Hydroxy quinoline	$5.0 \cdot 10^{-3}$	100
Diethyl dithiocarbamate	$2.0 \cdot 10^{-3}$	70
Diethyl dithiocarbamate	$5.0 \cdot 10^{-3}$	95
o-Phenanthroline	$2.0 \cdot 10^{-5}$	40
o-Phenanthroline	$5.0 \cdot 10^{-5}$	70
α,α′-Dipyridyl	$2.0 \cdot 10^{-5}$	30
α,α′-Dipyridyl	$5.0 \cdot 10^{-5}$	50
Oxalate	$2.0 \cdot 10^{-3}$	20
EDTA	$2.0 \cdot 10^{-3}$	0

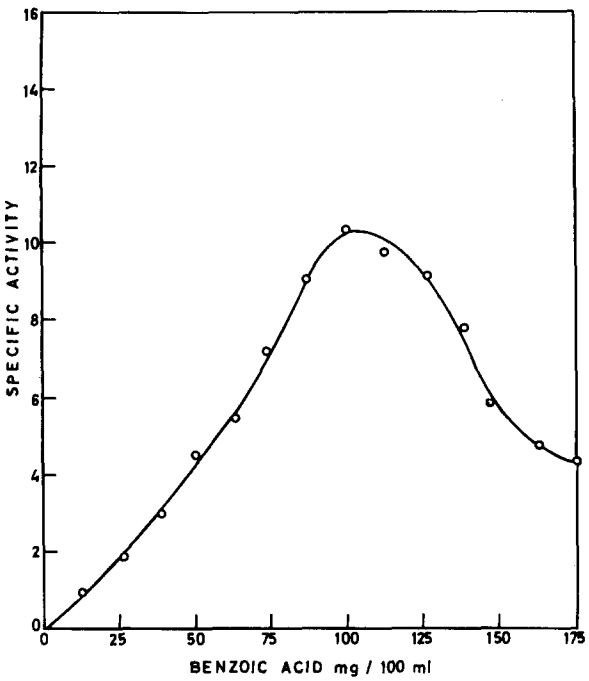


Fig. 2. Effect of benzoate concentration in the medium on the induction of benzoate-4-hydroxylase. *A. niger* was grown for 36 h on the standard medium supplemented with various concentrations of benzoate. Enzyme was prepared as described in the text upto Step 4 and assayed by standard procedures given in Materials and Methods.

Effect of benzoate concentration on induction of benzoate-4-hydroxylase.

The concentration of benzoate in the growth medium has a profound influence on the levels of benzoate-4-hydroxylase. As shown in Fig. 2 the benzoate-4-hydroxylase activity increased with an increase in the concentration of the benzoate and maximal activities of the enzyme were observed when the concentration of the benzoate was about 1 mg/ml of the medium. Further increase in the concentration of benzoate resulted in a considerable decrease in the hydroxylase activity as well as the growth of organism itself. There was considerable decrease in the growth of the organism with benzoate concentrations above 0.2% in the media.

Time-course of induction of benzoate-4-hydroxylase

The time course of induction of benzoate-4-hydroxylase in growing cultures of *A. niger* is shown in Fig. 3. Until 24 h after inoculation, there was no sizeable growth so as to permit the assay of the enzyme. The activity of the enzyme steadily increased and optimal activity could be observed at about 36 h. Thereafter the activity decreased, probably due to the inactivation of the enzyme. It was observed that the total activity decreased rapidly while the protein concentration remained same. The inducible nature of the enzyme was further confirmed by replacement culture [12] and inhibition studies using cycloheximide. Glucose grown mycelia were transferred to the standard medium supplemented with 0.1% benzoate. After a lag period of 1 h the activity

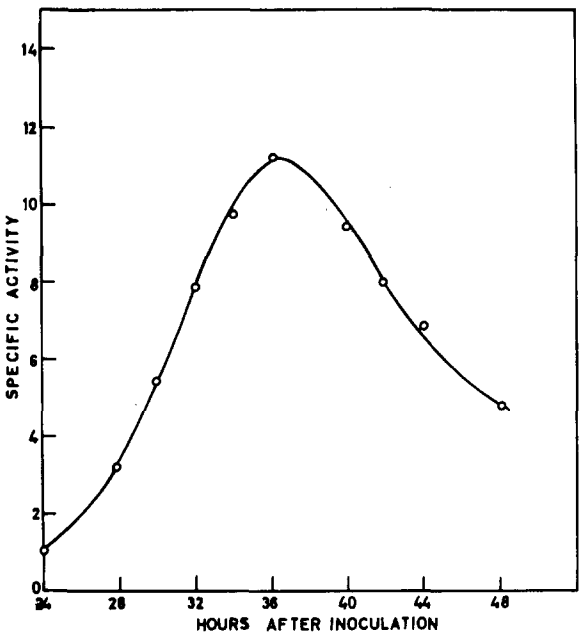
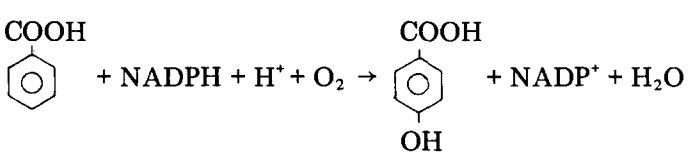


Fig. 3. Time course of induction of benzoate-4-hydroxylase during growth. *A. niger* was grown for different time intervals on the standard medium supplemented with 0.1% benzoate. Enzyme was prepared from mycelia harvested at different time intervals as described in the text upto Step 4. Assays were carried out by standard procedures given in Materials and Methods.

of benzoate-4-hydroxylase increased until about 6 h. When the replacement medium contained cycloheximide at a concentration of 10 mg/ml, the synthesis of the enzyme was inhibited to the extent of 80—90%.

Discussion

The benzoate-4-hydroxylase from *A. niger* catalyzes the stoichiometric conversion of benzoate to *p*-hydroxybenzoate. The overall reaction is as given below:



The typical monoxygenase (mixed function oxidase) nature of the enzyme is obvious from the above formulation. It is known that aromatic hydroxylases are generally unstable and in several cases attempts at the purification of the enzymes have been unsuccessful [7]. The benzoate-4-hydroxylase from *A. niger* is stabilised, as in the case of *p*-hydroxybenzoate hydroxylase from *Pseudomonas putida* [21] by the inclusion of an aromatic substrate (benzoate), EDTA and thiol compounds (glutathione) in extracting buffer. The presence of NADPH oxidase and endogenous inhibitors in the crude extracts were also observed in the case of *m*-hydroxybenzoate hydroxylase from *A. niger* [22].

From the balance sheet (Table I) it is observed that there was an increase of total enzyme units during purification thus giving an apparently high purification. Crude preparation showed very low enzyme activity. These preparations were found to contain at least three interfering factors of the assay system namely NADPH oxidase, *p*-hydroxybenzoate-3-hydroxylase and an endogenous inhibitor.

The pattern of inhibition of enzyme activity by metal chelation (Table IV) indicates that the enzyme is more sensitive to inhibition by Fe^{2+} (iron) chelators than it is by Fe^{3+} (iron) chelators. Thus *o*-phenanthroline which has high affinity for Fe^{2+} (iron) (Log K^a = 21.3) is a good inhibitor, whereas oxalate which has a high affinity for Fe^{3+} (iron) (log K^a = 18) is a very poor inhibitor [23]. This shows that Fe^{2+} is the activating metal ion involved in benzoate-4-hydroxylation. It is known that most of the external hydroxylases appear to share a common control phenomenon in which hydroxylatable substrates are good effectors [24]. Here the aromatic substrate acts as a regulator of electron flow from reduced nicotinamide nucleotides to oxygen. As it is observed that the enzyme is to be incubated with benzoate before starting the reaction in order to eliminate the lag period, benzoate may be acting as an effector.

Benzoate-4-hydroxylase from *A. niger* resembles the phenylalanine hydroxylase from *Pseudomonas* and mammalian liver [25,26]. The common features are the requirement for pteridine cofactor and Fe^{2+}. Benzoate-4-hydroxylase differs from the phenylalanine hydroxylase in its specificity towards pyridine nucleotide as an electron donor. There is an absolute requirement for NADPH in the case of benzoate-4-hydroxylase, whereas NADH also serves as electron donor for phenylalanine hydroxylase. The prerequisite structural requirements of a hydroxylatable substrate for benzoate-4-hydroxylase appears to be an unsubstituted benzene ring with an unmodified carboxyl group.

Cofactor requirement of benzoate-4-hydroxylase and some of the general properties shared with phenylalanine hydroxylase in mammalian liver [26] suggest that the mechanism of benzoate-4-hydroxylation may be similar to that of phenylalanine hydroxylase. Phenylalanine hydroxylation mechanism is of the rapid equilibrium random type and proceeds through a quarternary complex, involving the enzyme, oxygen, phenylalanine and tetrahydropteridine. Studies with various p-substituted derivatives of phenylalanine support the idea that a cationoid intermediate of phenylalanine is involved in the hydroxylation. Oxygen at the reduction level of peroxide may be an intermediate in the reaction. Superoxide anion involvement in benzoate-4-hydroxylation was demonstrated in the model system by Premkumar et al. [27]. However, it is too early to postulate any definite mechanism for the enzymatic benzoate-4-hydroxylation. Investigations are under way to elucidate the exact mechanism of p-hydroxylation of benzoate.

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