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PURIFICATION AND PARTIAL CHARACTERIZATION OF VANILLATE HYDROXYLASE (DECARBOXYLATING) FROM *SPOROTRICHUM PULVERULENTUM*

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SUMMARY

An intracellular enzyme, vanillate hydroxylase, which oxidatively decarboxylates vanillic acid to methoxyhydroquinone has been isolated from the lignin-degrading white-rot fungus *Sporotrichum pulverulentum*. Hydroxylation is dependent on reduced pyridine nucleotide and the highest activity was observed when assay mixtures were supplemented with NADPH and FAD. The enzyme has a molecular weight of approximately 65,000 and is inhibited by Tiron (3,5-pyrocatecholdisulphonic acid), heavy metals, chloride ions and *p*-chloromercuribenzoate (PCMB). Inhibition by PCMB is partially reversed by addition of reduced glutathione or dithiothreitol. Substrate specificity of vanillate hydroxylase was studied using crude and partially purified mycelial extracts.

INTRODUCTION

Vanillic acid is widely reported to be present in extracts of wood that has undergone varying degrees of microbial degradation and is generally accepted as being a breakdown product of the lignin component[1]. Further catabolism of vanillate may proceed via several routes: through a demethylation step to protocatechuate followed by cleavage of the aromatic ring[2–4], by a non-oxidative decarboxylation to guaicol[5] or by an oxidative decarboxylation reaction to methoxyhydroquinone[6–8]. Reduction to vanillin and vanillyl alcohol has also been observed[8].

Methoxyhydroquinone was identified in culture filtrates of the lignin-degrading white-rot fungus *Sporotrichum pulverulentum* grown in the presence of vanillic acid[8]. The enzyme catalysing the oxidative decarboxylation reaction has been partially purified from mycelial extracts[6]. This paper describes the purification of the enzyme, vanillate hydroxylase, and some of its properties.

EXPERIMENTAL

Organism and culture conditions for enzyme production

A description of the organism and the cultivation conditions have been given previously[6]. In this paper, crude extracts refer to the supernatant fraction after the

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initial centrifugation at 30,000 *g* for 30 min. Partially purified extracts describe the material obtained after the phenyl-Sepharose step.

Vanillate hydroxylase assay

Crude and partially purified enzyme was routinely assayed at 30°C by measuring either oxygen consumption with an oxygen electrode (Rank, Cambridge, Great Britain), or the decrease in absorbance at 340 nm due to NADH or NADPH oxidation. The pH optima for hydroxylase activity in crude extracts were determined by measuring methoxyhydroquinone production by gas–liquid chromatography of its silylated derivative using a glass capillary column containing SE-30 (25 m × 0.36 mm I.D.). Syringyl was used as the internal standard.

Purified enzyme was assayed by measuring $^{14}CO_2$ evolution from [carboxy-^{14}C]vanillate. Evolved $^{14}CO_2$ was trapped in sodium hydroxide and measured in Picofluor 30 containing 1 % of Carbosorb as described earlier[6,8].

One unit of vanillate hydroxylase is the amount of enzyme that converts 1 μmol of vanillic acid into methoxyhydroquinone and carbon dioxide per minute per millilitre at 30°C.

Isoelectric focusing on flat-bed polyacrylamide gel

The chemicals, apparatus, preparation of gels and technique for isoelectric focusing were as described by Vesterberg[9] and Ayers *et al.*[10]. An 80-μl volume of the enzyme sample, containing about 0.5 mg/ml of protein, was applied to the gel surface by using glass-fibre strips (4 × 40 mm with a thickness of 1 mm).

Purification of vanillate hydroxylase

The purification procedure was carried out in three steps starting from a crude intracellular material: (1) precipitation with 1 *M* potassium phosphate buffer; (2) fractionation on a phenyl-Sepharose bed; (3) chromatofocusing; and (4) affinity chromatography on phenyl-Sepharose.

Step 1: crude extract

The preparation of crude enzyme solution was carried out as described earlier[6], each batch starting from approximately 120 g net weight of frozen mycelium. After dialysis against 0.001 *M* potassium phosphate buffer (pH 7.4), the enzyme solution was concentrated by freeze-drying and stored at −20°C. The freeze-dried material was dissolved in 250 ml of 0.1 *M* potassium phosphate buffer (pH 7.0) so that a total absorbance at 280 nm of 9495.5 was obtained.

The dissolved material was precipitated by addition of an equal volume of 2.0 *M* potassium phosphate buffer (pH 7.0). The precipitate obtained was stirred at room temperature for 30 min and centrifuged at 10,000 rpm in a Sorvall RC-5B centrifuge at 4°C and the supernatant was retained.

Step 2: phenyl-Sepharose chromatography

The supernatant from step 1 was passed through a phenyl-Sepharose bed (40 × 50 mm; Pharmacia, Uppsala, Sweden) equilibrated with 1.0 *M* potassium phosphate buffer (pH 7.0) at a rate of 8 ml/min. Under these conditions, vanillate hydroxylase was quantitatively retained on the phenyl-Sepharose. The bed was washed with

1.0 *M* potassium phosphate buffer (pH 7.0) and then successively with 500 ml of 0.5 *M* potassium phosphate and 500 ml of 0.25 *M* potassium phosphate until the absorbance at 280 nm of the washing solution had decreased to zero in both instances. The enzyme was finally eluted from the phenyl-Sepharose bed with a mixture of 0.2 *M* potassium phosphate buffer (pH 7.0) and an equal amount of ethylene glycol and the eluate was dialysed against distilled water for 2 h to remove the ethylene glycol. The dialysed solution was concentrated with Immersible Molecular Separators (Millipore, Bedford, MA, U.S.A.) to a final volume of 15 ml.

Step 3: chromatofocusing

Chromatofocusing is a new column chromatographic method for separation of proteins according to differences in their isoelectric points. For this purpose a range of new products, Polybuffers and Polybuffer exchangers, have been developed (Pharmacia). The principles of the technique are as follows.

A standard chromatographic column is packed with a specially designed gel, PBE (Pharmacia) and equilibrated to the desired pH value by using a Tris buffer. The protein sample to be separated is added to the column, which is then eluted with Polybuffer. The method takes advantage of the positively charged groups on the gel and the amphoteric character of the eluent, the Polybuffer and the proteins to be separated. When these species migrate down the column, the most acidic components bind to the basic anion exchanger. As the elution progresses, the pH at each point in the column is gradually lowered as more Polybuffer is added to the column. The technique offers a high resolution of proteins with small differences in isoelectric points combined with a high capacity to separate a high load of proteins (several hundred milligrams).

The chromatofocusing was used here as step 3 in the purification of vanillate hydroxylate and was carried out as follows.

A standard chromatographic column (200 × 9 mm I.D.) was packed with the Polybuffer exchanger PBE 94 (Pharmacia) and equilibrated with 200 ml of 20 m*M* Tris buffer (pH 8.0). The concentrated enzyme solution, obtained by pooling the active fractions from the phenyl-Sepharose fractionation (purification step 2), concentrated by ultrafiltration using Immersible Molecular Separators and run on a PD-10 column (Pharmacia) to eliminate the salts, was applied to the PBE 94 column. The column was then eluted with Polybuffer 96 until adjusted to pH 6.0 with glacial acetic acid.

Step 4: affinity chromatography on phenyl-Sepharose

Adsorption of vanillic acid on phenyl-Sepharose gel. Vanillic acid (200 mg) dissolved in 20 ml of acetone was added to 13 g of phenyl-Sepharose gel suspended in 50 ml of acetone. The gel was kept in suspension by end-over-end rotation and diluted stepwise with water. The equilibrium time after every step was 1 h and the acetone–water concentrations were 100, 66, 33, 17 and 8%, respectively. After the last step the acetone was removed by washing with water and finally equilibrated with 1 *M* potassium phosphate buffer (pH 7.0).

Fractionation on the Phenyl-Sepharose–vanillic acid gel. The active fractions from step 3 were pooled and concentrated to 1.5 ml by ultrafiltration using Immersible Molecular Separators. The concentrated enzyme was applied to the equili-

brated phenyl-Sepharose–vanillic acid column (120 × 6 mm I.D.) and eluted with a linear gradient (total volume 200 ml), simultaneously decreasing from 1.0 to 0.05 *M* potassium phosphate (pH 7.0) and increasing from 0 to 50% ethylene glycol.

Dodecylsulphate–polyacrylamide gels

Electrophoresis in slab gels of polyacrylamide containing dodecylsulphate was performed with an apparatus similar to that described by Reid and Bieleski[11] using the procedure of Laemmli[12] with 10% gels. Samples contained 10–30 μg of protein. The gels were stained with Coomassie brilliant blue according to the procedure described by Holbrook and Leaver[13]. Molecular weight determinations were based on a comparison of the migration rate of the purified enzyme with that of the following protein standards: phosphorylase B (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), soybean trypsin inhibitor (MW 20,100) and α-lactalbumin (MW 14,400). The protein standards were obtained from an Electrophoresis Calibration Kit (Pharmacia).

RESULTS

Enzyme purification

The results of a typical purification procedure for vanillate hydroxylase are presented in Table I. The starting amount of frozen mycelium was 120 g. After extraction and precipitation, as described under Experimental, the supernatant was adsorbed on a phenyl-Sepharose bed, washed and eluted again. After dialysis and concentration, the enzyme solution was applied to a chromatofocusing PBE 94 column and eluted with Polybuffer 96. The fractionation obtained with this new technique is illustrated in Fig. 1.

Tubes 22–27 were pooled, concentrated and further purified by affinity chromatography on a phenyl-Sepharose column to which vanillic acid had previously been adsorbed. The enzyme was desorbed from this column by applying a linear phosphate–ethylene glycol gradient. The elution pattern (Fig. 2) shows an enzymic activity curve coinciding with the A_{280} curve. These steps gave rise to an approximately 240-fold purification with an overall yield of 13.3%.

TABLE I

RESULTS OF A TYPICAL PURIFICATION PROCEDURE FOR VANILLATE HYDROXYLASE

Step	*Volume (ml)*	*Total absorbance, A_{280} (nm)*	*Total amount of enzyme (mU)*	*Specific activity (mU/A_{280})*	*Purification factor*	*Yield (%)*
Crude mycelial extract	250	9495.5	63.1	$6.64 \cdot 10^{-3}$	1.0	100
After precipitation with phosphate buffer	500	6086.0	45.2	$7.43 \cdot 10^{-3}$	1.0	71.6
Phenyl-Sepharose chromatography	52	145.6	32.2	$2.21 \cdot 15^{-1}$	33.5	51.1
Chromatofocusing	15	8.0	11.6	1.45	218.4	18.3
Affinity chromatography	10	5.2	8.4	1.61	243.1	13.3

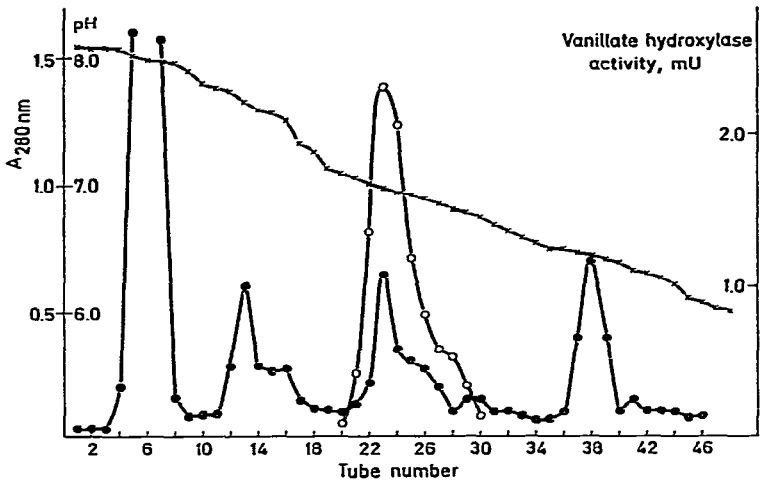
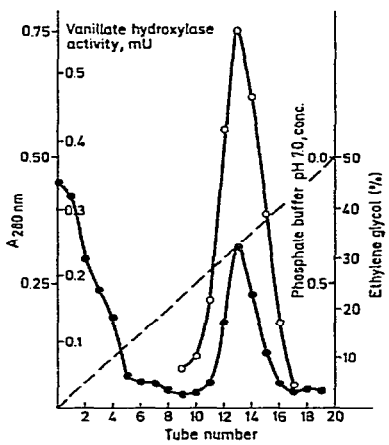


Fig. 1. Purification of vanillate hydroxylate by chromatofocusing. ●, Absorbance at 280 nm; ○, vanillate hydroxylase activity; ×, pH development during the elution process.

Homogeneity and molecular weight

To obtain an estimate of the effectiveness of each purification step, samples representing the various stages were examined by analytical isoelectric focusing and electrophoresis on slab gels in the presence of dodecylsulphate. In both methods, different concentrations of the purified enzyme protein gave a single band.

Molecular weight determinations on vanillic hydroxylase were carried out using dodecylsulphate–polyacrylamide slab gels. The estimated molecular weight for vanillate hydroxylase is 65,000, based on protein molecular weight standards (Fig. 3).



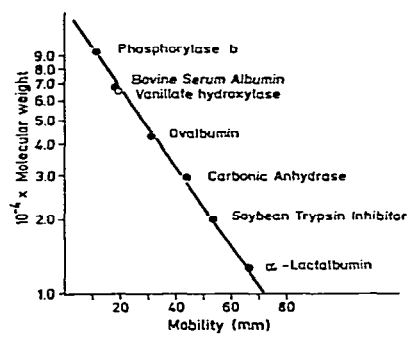


Fig. 2. Affinity chromatography on phenyl-Sepharose–vanillic acid column. ●, Absorbance at 280 nm; ○, vanillate hydroxylase activity; broken line, linear gradient for phosphate buffer (pH 7) and ethylene glycol concentrations.

Fig. 3. Determination of molecular weight of vanillate hydroxylase by dodecylsulphate–polyacrylamide gel electrophoresis.

pH optimum of enzyme

In crude extracts the enzyme was active over a wide pH range (5.8–8.0) when measured in potassium phosphate buffer, and activity peaks were observed at pH 6.6 and 7.8. There was considerable inhibition of the enzyme in Tris–hydrochloride buffer (40% inhibition compared with the activity in potassium phosphate at pH 7.2), as has been reported for other aromatic hydroxylases[14,15]. Inhibition was almost certainly due to chloride ions. Addition of 0.1 *M* potassium chloride or sodium chloride to assay mixtures using potassium phosphate buffer resulted in an approximately 70% reduction of enzyme activity.

Effect of cofactor supplementation

Both NADH and NADPH would serve as electron donors for vanillate hydroxylase but under the conditions for the $^{14}CO_2$ evolution assay the rate with NADH was only 85% of that with NADPH (Table II). As reported previously[6], the activity was increased still further when FAD was incorporated in reaction mixtures together with NADPH. No stimulation was observed when NADH replaced NADPH in the FAD-containing assay system (in fact, a small decrease in $^{14}CO_2$ evolution occurred), or when FMN replaced FAD.

TABLE II

EFFECT OF COFACTOR SUPPLEMENTATION ON PURIFIED VANILLATE HYDROXYLASE

The reaction mixture contained the following in 3 ml: 250 μmol of phosphate buffer (pH 6.8); 1.0 μmol of NADH or NADPH; 25 nmol of FAD or FMN where indicated and purified enzyme. The reaction was initiated by addition of 29 nmol of [carboxy-^{14}C]vanillate ($6.2 \cdot 10^6$ dpm/mg). $^{14}CO_2$ evolution was measured after incubation for 1 h at 28°C. Values represent the means of three separate incubations.

Cofactor supplement	*$^{14}CO_2$ evolved (dpm)*
None	35
NADH	2050
NADPH	2411
FAD	85
FMN	36
NADH, FAD	1823
NADH, FMN	1802
NADPH, FAD	3403
NADPH, FMN	2332

Stoichiometry of the reaction with vanillate

Vanillate hydroxylase was presumed to catalyse a typical mono-oxygenase reaction:

$$\text{Vanillate} + O_2 + NADPH_2 \rightarrow \text{methoxyhydroquinone} + NADP + H_2O$$

The stoichiometry of the reaction is difficult to establish unequivocally, as the product undergoes non-enzymic oxidation to its quinone, which in turn is reduced by any excess of NADPH present in the reaction mixture. The rate of quinone formation is relatively slow at slightly acidic pH values and the presence of crude or partially

purified fungal extract retarded the rate of non-enzymic oxidation even further, an effect observed previously with bacterial extracts and the autoxidation of hydroxy quinol[16]. Even so, an uptake of slightly more than 1 nmol of oxygen per nanomole of vanillate was observed in reaction mixtures at pH 6.8, containing crude extract, NADPH and 100–200 nmol of vanillate. However, vanillate hydroxylase oxidatively decarboxylates protocatechuate and 2,3-dihydroxybenzoate to hydroxyquinol (see below) and crude mycelial extracts contain a dioxygenase which catalyses the intradiol ring cleavage of hydroxyquinol to maleyl acetate[17]. Here, in reaction mixtures containing crude extract which catalysed both hydroxylation and ring fission, and using 100–200 nmol of protocatechuate or 2,4-dihydroxybenzoate, approximately 2 nmol of oxygen was consumed for every nanomole of protocatechuate or 2,4-dihydroxybenzoate metabolized. By analogy, oxidative decarboxylation of vanillate would consume 1 nmol of oxygen per nanomole of vanillate converted to methoxyhydroquinone. The stoichiometry of NADPH consumption was not determined because of the asymptotic nature of the decrease in absorbance at 340 nm.

Substrate specificity

Oxidation of several substrate analogues by partially purified vanillate hydroxylase indicated the enzyme to be specific for compounds with a hydroxyl group located *para* to a carboxyl substituent attached directly to the aromatic ring[6]. The ability of various aromatic acids with this configuration to serve as substrates for the hydroxylase in crude and partially purified extracts is shown in Table III.

TABLE III

SUBSTRATE SPECIFICITY OF CRUDE AND PARTIALLY PURIFIED VANILLATE HYDROXYLASE

The reaction mixture contained the following in 3 ml: 250 μmol of phosphate buffer (pH 6.8); cell extract protein; 1.0 μmol of NADH and 0.5 μmol substrate added to initiate the reaction. Values are corrected for NADH oxidation in the absence of substrate. Temperature: 30°C.

Substrate	*Relative enzymic activity**	
	Crude	*Partially purified*
Vanillate	100	100
Protocatechuate	100	113
p-Hydroxybenzoate	98	115
2,4-Dihydroxybenzoate	96	105
Syringate	8	6
Gallate	48	73
3-O-Methylgallate	54	75
2,3,4-Trihydroxybenzoate	23	33
2,4,6-Trihydroxybenzoate	74	65

* Crude enzyme was the 30,000 *g* supernatant from disrupted mycelia passed through a PD-10 G-25 column and partially purified enzyme was the dialysed fraction eluted from phenyl-Sepharose.

Apart from syringic acid, all compounds tested were capable of promoting high rates of NADH oxidation. NADH was used instead of NADPH as oxygen consumption in excess of that expected for a mono-oxygenase was observed with

several analogues in the presence of crude extract. This suggested that the products of hydroxylation may serve as a substrate for the aromatic ring-cleavage enzyme in this fungus, which in turn may have given rise to substrates for a NADPH-dependent maleyl acetate reductase[17]. Thus, misleading levels of cosubstrate oxidation would have been observed.

Although the reason is not clear, when hydroxylase activity was assayed spectrophotometrically higher values were routinely observed towards most substrate analogues tested than when rates were determined from oxygen uptake measurements (compare Table III with ref. 6).

Effect of inhibitors on purified vanillate hydroxylase (Table IV)

Tiron and heavy metals (copper, silver, mercury) at 1 n*M* concentrations and 0.1 m*M* *p*-chloromercuribenzoate completely inhibited vanillate hydroxylase. Inactivation by *p*-chloromercuribenzoate was partially reversed by addition of stoichiometric amounts of reduced glutathione or dithiothreitol. Cyanide (1 n*M*) and α,α′-dipyridyl (1 n*M*) also depressed the enzyme activity by about 15% and 30%, respectively, but arsenite, azide, EDTA and diethyldithiocarbamate at 1 n*M* concentrations had no significant effect.

TABLE IV

EFFECT OF INHIBITORS ON VANILLATE HYDROXYLASE

The reaction mixture contained the following in 3 ml: 250 μmol of phosphate buffer (pH 6.8); 100 μg of purified protein; 1.0 μmol of NADPH; 25 nmol of FAD; and inhibitor as shown. A 29-nmol amount of [carboxy-^{14}C]vanillate was added to initiate the reaction. Temperature: 30°C. Inhibitor was allowed to react with enzyme for 10 min prior to addition of substrate. The rate of activity was determined from the evolution of $^{14}CO_2$ after 30 min and values were corrected for background counts.

Inhibitor	*Final concentration (M)*	*Activity remaining (%)*
None	—	100
Arsenite	$1 \cdot 10^{-3}$	100
NaCN	$1 \cdot 10^{-3}$	84
Azide	$1 \cdot 10^{-3}$	99
$CuSO_4$	$1 \cdot 10^{-3}$	0
$AgNO_3$	$1 \cdot 10^{-3}$	0
$HgCl_2$	$1 \cdot 10^{-3}$	0
PCMB (*p*-chloromercuribenzoate)	$1 \cdot 10^{-3}$	0
PCMB	$1 \cdot 10^{-4}$	1
PCMB + $2 \cdot 10^{-3}$ *M* glutathione	$1 \cdot 10^{-3}$	46
PCMB + $2 \cdot 10^{-3}$ *M* dithiothreitol	$1 \cdot 10^{-3}$	44
α,α′-Dipyridyl	$1 \cdot 10^{-3}$	69
Tiron	$1 \cdot 10^{-3}$	0
Tiron	$5 \cdot 10^{-4}$	0
Diethyldithiocarbamate	$1 \cdot 10^{-3}$	100
EDTA	$1 \cdot 10^{-3}$	100
$Fe_2(SO_4)_3$	$1 \cdot 10^{-3}$	100

DISCUSSION

The enzyme vanillate hydroxylase is thought to catalyse a typical mono-oxygenase reaction, although the stoichiometry is difficult to establish directly because of

non-enzymic conversions undergone by the reaction product, methoxyhydroquinone. Both NADPH and NADH can serve as electron donors and maximal enzymic activity was observed with FAD included in assay mixtures. Many bacterial hydroxylases[14,18–21] and hydroxylase from the yeast *Trichosporon cutaneum*[15], which catalyse similar mono-oxygenase-type reactions, are known to be flavoproteins. However, purified vanillate hydroxylase does not exhibit a typical flavoprotein absorption spectrum and we have not been able to have somebody do the fluorescence study for us and we do not have access to adequate equipment ourselves. The role of this cofactor remains uncertain. Our enzyme appeared to be different from *Phanerochaete chrysosporium* vanillate hydroxylase[7], where only NADPH was reported to be active as an external electron donor*.

Vanillate hydroxylase has a wide specificity and several aromatic acids with a hydroxyl group located *para* to the carboxyl substituent attached directly to the benzene ring serve as substrates. However, only low rates of activity are observed with syringic acid, which may represent a steric hindrance effect due to the two methoxyl groups located either side of the hydroxyl substituent. Although the identities of reaction products from substrate analogues were not determined, some hydroxylation almost certainly takes place. Similar substrate specificities, based on oxygen uptake measurements, were exhibited by both crude and partially purified enzyme preparations. As the former were rich in catalase activity it is unlikely that analogues are simply acting as non-substrate effectors[14], although the possibility remains for combined substrate and non-substrate effector roles.

Purified vallinate hydroxylase was totally inhibited by 0.5 n*M* Tiron, a specific chelator for Fe^{3+}, and partially inhibited (*ca.* 30%) by α,α'-dipyridyl. Enzymic activity was also reduced by chloride ions, although the nature of this inhibition was not established.

Methoxyhydroquinone was first identified in an extract of a culture of *Lenzites trabea* to which vanillic acid had been added, and was found to be an intermediate in vanillate metabolism by *Polyporus dichrous*[22]. Recently, vanillate hydroxylase has been found in many brown-rot and white-rot fungi, but not in representative members of soft-rot fungi[23], and it would appear the route for vanillate catabolism via methoxyhydroquinone is widely distributed among the two former wood-decay groups.

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