OXIDATIVE DECARBOXYLATION OF VANILLIC ACID BY *SPOROTRICHUM PULVERULENTUM*

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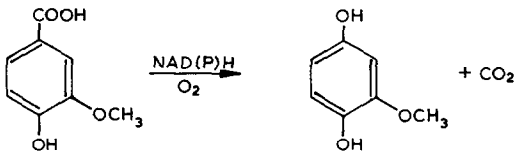
1. Introduction

Catabolism of vanillate in bacteria and certain fungi normally proceeds via a demethylation step to protocatechuate followed by cleavage of the aromatic ring [1–3]. More recently, an alternative transformation involving non-oxidative decarboxylation of vanillate to guaiacol by strains of *Bacillus megaterium* and a *Streptomyces* sp. was reported [4]. A third degradative route through methoxyhydroquinone was described in *Lenzites trabea* and *Polyporus dichrous* [5]. This compound has also been identified in culture filtrates of the lignin-degrading white-rot fungus, *Sporotrichum pulverulentum,* grown in the presence of vanillate [6]. We now report some characteristics of a partially purified enzyme from *S. pulverulentum* which catalyses the oxidative decarboxylation of vanillate to methoxyhydroquinone according to the scheme:



2. Materials and methods

Sporotrichum pulverulentum, Novobranova (ATCC 32629) synonym, *Phanerochaete chrysosporium* (Burdsall and Eslyn) was obtained from Dr T. Nilsson [7].

The fungus was grown in shake culture at 28°C in a medium containing per litre: 2.0 g $(NH_4)H_2PO_4$, 0.6 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.4 g K_2HPO_4, 74 mg $CaCl_2 \cdot 2\ H_2O$, 12 mg ferricitrate, 6.6 mg $ZnSO_4 \cdot 7\ H_2O$, 5 mg $MnSO_4 \cdot 4\ H_2O$, 1 mg $CoCl_2 \cdot 6\ H_2O$, 0.1 mg thiamine, 2.5 g glucose, and 0.1 g yeast extract (Difco). The pH was adjusted to 5.5 with orthophosphoric acid and vanillate (3 mM) added as a filter-sterilized solution. A spore suspension (~2.5×10^6 spores) served as inoculum. Mycelial pellets were harvested after 50 h and either used immediately or stored frozen until required.

For the preparation of extracts, mycelia were suspended in 4 vol. 0.1 M KH_2PO_4–NaOH buffer (pH 7.4) and broken in a homogenisator at 0–5°C. Homogenates were clarified by centrifugation at 30 000 × *g* for 30 min and the supernatant fraction (crude extract) concentrated by $(NH_4)_2SO_4$ precipitation. The redissolved protein (0–60% saturation) was dialysed and partially purified by preparative flat-bed electrofocusing (LKB) in Utrodex granulated gel.

Enzymic activity was assayed at 30°C by measuring oxygen consumption or the release of $^{14}CO_2$ from carboxyl-labelled vanillic acid (6.2×10^6 dpm/mg). Evolved $^{14}CO_2$ was absorbed in 1 ml 1 N NaOH, and the radioactivity measured in a Packard, model 3255 Tri Carb Liquid Scintillation Spectrometer using a liquid scintillation solution containing Pico-fluor 30 and 1% Carbosorb (both from Packard).

Oxygen consumption was measured with a Clark oxygen electrode, and NADPH utilization from the rate of decrease in A_{340}. Vanillate was estimated from the A_{254}. Methoxyhydroquinone, as the trimethylsilyl derivative, was identified by gas chromatography (GLC) and by gas chromatography – mass spectrom-

etry. Protein was determined spectrophotometrically and by the method in [8].

3. Results

Extracts of *S. pulverulentum* grown in the presence of vanillate catalysed the oxidative conversion of vanillate to methoxyhydroquinone and CO_2. Evolution of $^{14}CO_2$ from [*carboxyl*-^{14}C]vanillate, using partially purified extracts, is shown in fig.1. Enzymic activity was dependent on NADPH as coenzyme; NADH was ~85% as effective under normal assay conditions. In addition, a small (up to 15% in different experiments) but reproducible stimulation of $^{14}CO_2$ evolution occurred when FAD was included in reaction mixtures containing NADPH. Most of the enzymic activity resided in the soluble fraction although twice-washed particulate material from which unbroken mycelia and coarse debris had been removed also converted vanillate to methoxyhydroquinone. No extracellular activity was detected. Oxidative decarboxylation was strongly inhibited by 1 mM Tiron, a specific chelator for Fe^{3+}, but *a,a'*-dipyridyl, EDTA and diethyldithiocarbamate had no effect.

Decarboxylase activity was detectable in extracts of mycelia grown on glucose as the sole carbon source, but levels were increased up to 20-fold by the presence of vanillate. Under laboratory growth conditions the specific activity of the decarboxylase enzyme in crude extracts began to increase after 24 h and reached maximum levels after 40–50 h (fig.2).

High rates of oxygen consumption were observed when several analogues were substituted for vanillate in reaction mixtures containing partially purified fungal extract (table 1). Results indicate the enzyme to be specific for compounds with a hydroxyl group situated *para* to a carboxyl substituent attached directly to the aromatic ring.

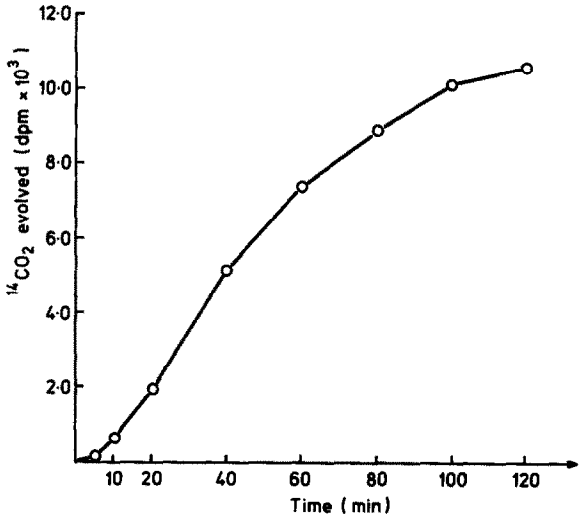


Fig.1. Evolution of $^{14}CO_2$ from [*carboxyl*-^{14}C]vanillate by partially purified extracts of *S. pulverulentum*. Reaction mixtures contained in 3 ml, 200 μmol phosphate buffer (pH 7.4), 46 μg cell extract protein, and 3 μmol NADH. 29 nmol [*carboxy*-^{14}C]Vanillate was added to initiate the reaction. Temp. 30°C. Values are corrected for background counts.

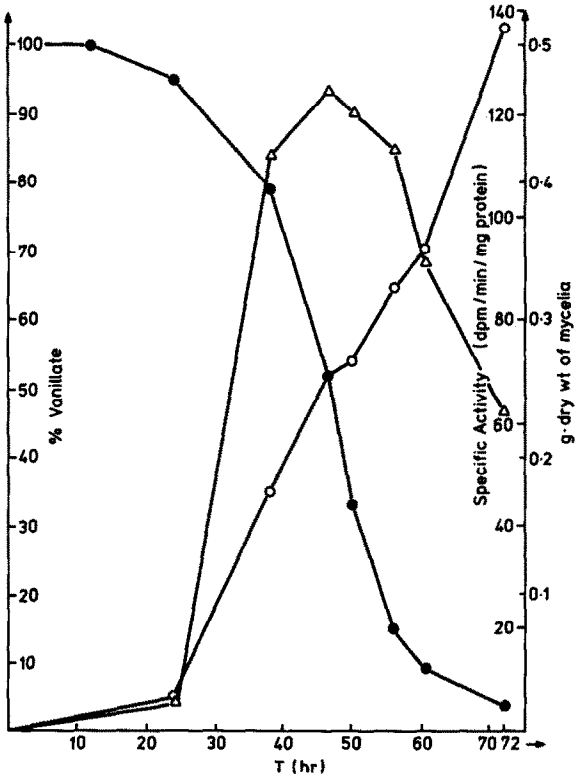


Fig.2. Induction of decarboxylase activity during growth of *S. pulverulentum* in the presence of vanillate. Mycelial dry weight (○), decarboxylase activity in crude extracts (△), vanillate concentration (●).

Table 1
Specificity of decarboxylase activity in partially purified extracts of *S. pulverulentum* grown in the presence of vanillate

Substrate	Specific activity (nmol O_2.min^{-1}.mg prot.$^{-1}$)	% Activity (vanillate = 100%)
Vanillate	68.1	100
Protocatechuate	63.8	94
Gallate	39.2	58
2,4-Dihydroxybenzoate	50.7	74
4-Hydroxybenzoate	46.3	68
Gentisate	1.7	2
Syringate	4.5	7
Ferulate	6.1	9
Veratrate	6.0	9
Homovanillate	9.6	14
2,3,4-Trihydroxybenzoate	18.1	27
4-Methoxybenzoate	3.3	5
3-Methoxybenzoate	n.d.	0
Benzoate	n.d.	0

The reaction mixture contained in 3 ml: 250 μmol phosphate buffer (pH 7.4); 0.32 mg cell extract protein; 1.0 μmol NADH; 1.0 μmol substrate. Temp. 30°C. Values are corrected for O_2 consumption in the absence of substrate. n.d., not detected

4. Discussion

Oxidative decarboxylation of vanillate to methoxyhydroquinone by mycelial pellets of *P. dichrous* has been reported [5]. In higher plants, the methoxylated phenols, methoxyhydroquinone and 2,6-dimethoxyhydroquinone, are formed through oxidative decarboxylation of the corresponding substituted benzoic acids, vanillate and syringate [9].

The oxidative decarboxylating enzyme described here requires oxygen and NAD(P)H for activity and is probably a mono-oxygenase, although this has not yet been firmly established. Mono-oxygenases which catalyse the oxidative decarboxylation of salicylate have been isolated and purified from bacteria [10–12]. Purification of the fungal enzyme is currently underway to enable a more complete characterization and further comparisons to be made. Vanillate decarboxylation catalysed by laccase-type enzymes from *Polyporus versicolor* and *Polystictus sanguineus* has been reported [13–15]. Although phenoloxidases are produced by *S. pulverulentum* [16,17] the intracellular location of the decarboxylase activity, and the requirement for NAD(P)H as cosubstrate, indicates an alternative mechanism to a phenoloxidase-mediated decarboxylation.

The role of methoxyhydroquinone as an intermediate in vanillate degradation remains unclear. We have not yet been able to demonstrate further catabolism of this compound using mycelial extracts although it disappears from fungal growth cultures.

Acknowledgements

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