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AN AROMATIC 3,4-OXYGENASE FROM *TILLETIOPSIS WASHINGTONENSIS*-OXIDATION OF 3,4-DIHYDROXYPHENYLACETIC ACID TO β-CARBOXYMETHYLMUCONOLACTONE

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Abstract—An aromatic 3,4-oxygenase with a broad spectrum of substrate specificity has been partially purified from acetone-dried powders of the fungus, *Tilletiopsis washingtonensis*. The enzyme catalyzed the oxidation of 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxycinnamic acid, 3,4-dihydroxyphenylpropionic acid and 3,4-dihydroxyphenylalanine. The lactone of the primary fission product of 3,4-dihydroxyphenylacetic acid (*β-carboxymethyl*-cis-cis *muconic acid*) was isolated as a dimethyl ester and identified as *β-carboxymethylmuconolactone*.

INTRODUCTION

3,4-DIHYDROXYPHENYLACETIC acid (homoprotocatechuic acid) is known to be a metabolite of tyrosine[1] and phenylacetic acid[2–4] in microorganisms, and is formed by the hydroxylation of *p*-hydroxyphenylacetic acid.[4,5] A 2,3-oxygenase from *Pseudomonas ovalis*, which catalyzes the oxidative fission of the aromatic nucleus of homoprotocatechuic acid, leading to the formation of α-hydroxy-δ-carboxymethylmuconic semialdehyde has been crystallized.[6,7] Dagley and Wood[8] reported that cell extracts of a *Pseudomonas* grown on phenylacetic acid, oxidized homoprotocatechuic acid to pyruvate, formate and acetoacetate. The bond cleavage in this case occurred between carbon atoms 4 and 5 of homoprotocatechuic acid. The initial product was expected to be α-hydroxy-γ-carboxymethylmuconic semialdehyde. In the present communication we report the oxidation of homoprotocatechuic acid to *β-carboxymethylmuconic acid* by an aromatic 3,4-oxygenase from the basidiomycete, *Tilletiopsis washingtonensis* (UBC 907). The enzyme can also effect the fission of the aromatic ring of several substituted catechols.

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RESULTS AND DISCUSSION

Purification of the Aromatic 3,4-*Oxygenase*

A summary of the purification procedure is given in Table 1. The final enzyme preparation showed an 18-fold increase in specific activity with a 30% recovery.

TABLE 1. PURIFICATION OF THE AROMATIC 3,4-OXYGENASE FROM *T. washingtonensis*

	Step	Total volume (ml)	Total protein (mg)	Total activity	Specific activity*	Recovery (%)
I.	Crude enzyme	380	635	51	80	100
II.	Protamine sulphate	420	450	38	84	74
III.	35–55% Ammonium sulphate	60	61	26	426	51
IV.	Alumina C γ gel eluate	60	45	21	466	41
V.	DEAE-Cellulose eluate	40	10	15	1490	30

* mμmoles Homoprotocatechuic acid oxidized/mg protein/hr.
Standard assay conditions were used.

Properties of the 3,4-*Oxygenase*

The general properties of the aromatic 3,4-oxygenase from *Tilletiopsis* were the same as those reported from other fungi[9,10] and bacteria[11,12] for protocatechuic acid 3,4-oxygenase. The oxygenase from *Tilletiopsis* was studied in cultures grown in the absence of an aromatic inducer. The addition of 0·05% tyrosine to the growth medium produced no significant effect on the amount or substrate specificity of the oxygenase. The enzyme has a broad pH curve with an optimum at pH 8. This is similar to the pH activity curve reported for the crystalline protocatechuic acid-3,4-oxygenase from *Pseudonomas aeruginosa.*[12] The 3,4-oxygenase from *Tilletiopsis* showed no requirement for any cofactor or metal ions. Extensive dialysis against water or EDTA did not affect the enzyme activity. Metal chelating agents such as EDTA, 8-hydroxyquinoline, α,α'-dipyridyl and *o*-phenanthroline did not inhibit the oxygenation reaction.

The most significant feature of the 3,4-oxygenase obtained from *T. washingtonensis* is its ability to attack a wide range of substituted catechols. The results in Table 2 suggest that the *Tilletiopsis* oxygenase attacks only *para*-substituted catechols with a free carboxyl group. The enzyme activity decreases as the chain length of the substrate increases.

Protocatechuic acid 3,4-oxygenase isolated from fungi[10] and bacteria[11] was reported to be specific for protocatechuic acid. In all these instances, the substrate specificity was evaluated with compounds such as catechol, 2,3-, 2,4- and 2,5-dihydroxybenzoic acids. Fujisawa and Hayaishi[12] reported that the crystalline protocatechuate-3,4-oxygenase obtained from *Pseudomonas aeruginosa*, oxygenated pyrogallol, catechol, 3-methylcatechol, 4-methylcatechol, homoprotocatechuic acid and 3,4-dihydroxymandelic acid at low rates. The activities for homoprotocatechuic acid and 3,4-dihydroxymandelic acid relative to protocatechuic acid were 0·2 and 0·1 respectively, as compared to values of 22 and 9·4

[9] R. B. CAIN, R. F. BILTON and J. A. DARRAH, *Biochem. J.* **108,** 797 (1968).
[10] S. R. GROSS, R. D. GAFFORD and E. L. TATUM, *J. Biol. Chem.* **219,** 781 (1956).
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TABLE 2. PROTOCATECHUIC ACID 3,4-OXYGENASE FROM *Tilletiopsis washingtonensis*—SUBSTRATE SPECIFICITY

Substrate	Relative activity
Protocatechuic acid	100·0
Homoprotocatechuic acid	22·0
3,4-Dihydroxymandelic acid	9·4
Caffeic acid	8·1
Dihydrocaffeic acid	4·3
Dopa	1·3
Dopamine	0
o-Pyrocatechuic acid	0
Gentisic acid	0
Homogentistic acid	0
Catechol	0
Protocatechualdehyde	0
Gallic acid	0

observed with our enzyme preparation from *Tilletiopsis*. Adachi *et al.*[5] reported that homoprotocatechuic acid-2,3-oxygenase isolated from *Pseudomonas ovalis* did not oxidize protocatechuic acid, homogentisic acid and catechol. However more recent studies with crystalline enzyme showed that protocatechuic, dihydrocaffeic acid, dopa and other related compounds were oxidized at extremely low rates.[13]

The stoichiometry of the reaction catalyzed by the partially purified protocatechuic acid-3,4-oxygenase from *Tilletiopsis* is shown in Table 3. One mole of molecular oxygen is consumed per mole of substrate.

TABLE 3. PROTOCATECHUIC ACID 3,4-OXYGENASE FROM *Tilletiopsis washingtonensis*—STOICHIOMETRY

Substrate	Substrate added (μmoles)	Substrate disappeared (μmoles)	Oxygen uptake (μmoles)
Protocatechuic acid	3·0	3·0	3·3
Homoprotocatechuic acid	3·0	2·9	3·2
Caffeic acid	3·0	2·5	2·4

Oxygen uptake studies were carried out in Warburg flasks at 30°. The reaction mixture consisted of 1 ml of 0·05 M Tris-HCl buffer, pH 7·5, partially purified protocatechuic acid, 3,4-oxygenase (1·5 ml) and substrate (0·3 ml) in a final volume of 3 ml. The reaction was started by the addition of substrate from the side-arm. When the oxygen consumption reached completion (after 2 hr), aliquots were taken for colorimetric estimation of substrate.

Identification of the Oxidation Products of the 3,4-Oxygenase

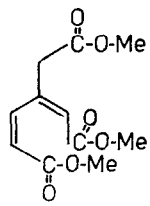
(1) *Protocatechuic acid as substrate.* Incubation of protocatechuic acid or homoprotocatechuic acid with partially purified preparations from *Tilletiopsis* resulted in the formation of oxidation products which showed characteristic peaks at 253 nm and 266 nm respectively

[13] S. SENOH, H. KITA and M. KAMIMOTO, *Biological and Chemical Aspects of Oxygenases*, p. 378, Maruzen, Tokyo (1966).

at pH 7·5. The existence of keto-enol tautomerism was ruled out since there was no significant change in the spectra either at pH 2 or at pH 11. The primary product of protocatechuic acid oxidation was identified as β-carboxy-*cis,cis*-muconic acid by comparing its properties with those of an authentic sample obtained as a generous gift from Dr. R. Y. Stanier.[14]

Enzyme preparations from Step IV rapidly oxidized protocatechuic acid and β-carboxy-*cis,cis*-muconic acid to a compound whose spectral characteristics were similar to those of β-carboxymuconolactone (absorption maximum at 217 nm in 0·1 M phosphate buffer, pH 7·5).

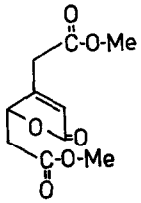
(2) *Homoprotocatechuic acid as substrate.* An enzyme preparation from Step V was incubated with homoprotocatechuic acid (50 mg) for 8 hr. The reaction products were extracted and methylated with ethereal diazomethane. After purification, an oil (22 mg) was obtained and was termed compound A. This compound had R_f 0·62 in ethyl acetate–petrol (40–60°) (3:2), and R_f 0·49 in ether–petrol (40–60°) (1:1) on silica gel G plates. It had u.v. max. at 266 nm and ϵ 33,720 (acetonitrile). Examination of the i.r. spectrum of compound A showed peaks at 2964 cm^{-1} (—C—H stretch), 1730 cm^{-1} (ester), 1610 cm^{-1} (C=C), 1440 cm^{-1} ($CH_2C(=O)$), 1166 cm^{-1} (C=CH—) 1000 cm^{-1} (C—O). The mass spectrum of compound A showed a parent ion, M^+ at *m/e* 242, and fragment ions at *m/e* 227 (M-15), 211 (M-31), 183 (strong, M-59), 179 (M-63), 151 (M-91), 147 (M-95), 124 (M-118), 95 (M-147), 65 (M-177), 59 (M-183). No metastable ions were observed. The mass spectral data and the data from the u.v. and i.r. spectra are consistent with the structure I, namely the trimethyl ester of β-carboxymethylmuconic acid.



The proposed structure for compound A was further confirmed by the preparation of β-carboxymethylmuconolactone, using an enzyme preparation from Step IV, with homoprotocatechuic acid and compound A as substrates. After methylation of the reaction products a clear oil (compound B) was obtained. This compound had u.v. max. at 207 nm and ϵ 10,773 in acetonitrile. The i.r. spectrum showed peaks at 2995 cm^{-1} (—C—H), 1765 cm^{-1} (γ lactone), 1650 cm^{-1} (—CH=CH_2), 1445 cm^{-1} ($CH_2C(=O)$), 1180 cm^{-1} (—C—O stretch), 1030 cm^{-1} (C—C stretch). The mass spectrum of B showed a parent ion at *m/e* 228, and fragment ions at *m/e* 197 (M-31), 169 (M-59), 137 (M-91) and 110 (M-118). The NMR spectrum shows two OCH_3 (τ = 6·30–6·33), one proton on a double bond (τ = 3·94) one other proton (τ = 4·56), two CH_2 (τ = 6·5/6·54; 7·22/7·24). The integration shows the presence of 12 protons. The u.v. and i.r. spectra indicate that the C=C is

[14] D. L. MACDONALD, R. Y. STANIER and J. L. INGRAHAM, *J. Biol. Chem.* **210**, 809 (1954).

conjugated with the lactone group. From this evidence the structure II is proposed for compound B.



The previously unknown compounds A and B could only have been obtained in this reaction by a 3,4-oxidation of the homoprotocatechuic acid.

EXPERIMENTAL

Chemicals

Chemicals were obtained commercially and, where necessary, were purified by crystallization.

Media and Conditions of Cultivation of the Organism

Tilletiopsis washingtonensis (UBC 907) was grown on a medium containing dextrose, 5 g; asparagine, 2 g; $CaCl_2.2H_2O$, 1 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0·5 g; $FeCl_3.6H_2O$, 20 mg; $ZnSO_4.7H_2O$, 10 mg; $MnSO_4.4H_2O$, 3mg; $Na_2MoO_4.2H_2O$, 1·5 mg; $CuSO_4.5H_2O$, 1 mg and vitamin solution, 1 ml (containing biotin, 50 μg; thiamine, 100 μg; pyridoxine, 100 μg and inositol, 50 mg) per litre. Stock cultures were maintained on slants containing malt extract (1·5%), yeast extract (0·5%) and bacto-soytone (0·25%) solidified with 1·5% agar. Inoculum was prepared by growing the organism on malt extract–yeast extract–soytone medium for 72 hr with shaking at room temp. 20 ml inoculum was added per litre of the synthetic medium. After growing for 72 hr on a shaker at room temp, the cells were harvested by filtration through a Buchner funnel and washed thrice with distilled water and stored as wet packed cells at −20°.

Purification of the Oxygenase from Tilletiopsis

All operations were performed at 0–4° unless otherwise stated.

Frozen cells were brought to a temperature of 0–4° and a thick suspension was made by the addition of water. Three volumes of cold acetone (−20°) were added to the cell suspension and rapidly filtered through a Buchner funnel. The residue was washed several times with cold acetone, dried under suction and stored at −20°.

Step I. Crude extract. The acetone powder (20 g) was extracted for 2 hr with 0·025 M sodium phosphate buffer, pH 7 (400 ml) by gentle stirring. The suspension was centrifuged at 20,000 *g* for 20 min and the clear supernatant was used as the crude enzyme.

Step II. Protamine sulphate. A 2% protamine sulphate solution in 0·025 M sodium phosphate buffer, pH 7 (40 ml) was added to the crude enzyme (360 ml) with gentle stirring. After 15 min the precipitate was removed by centrifugation.

Step III. Ammonium sulphate fractionation. The fraction from Step II (400 ml) was brought to 35% saturation by the addition of 83·36 g $(NH_4)_2SO_4$ stirred for 15 min and the precipitate was discarded by centrifugation. The supernatant was brought to 55 per cent saturation by the addition of 53·64 g $(NH_4)_2SO_4$. (A 40–50% $(NH_4)_2SO_4$ fraction was later found to give a purer enzyme preparation.) After stirring for 15 min, the precipitate was collected by centrifugation, suspended in 80 ml of water and dialysed overnight against 0·25 M phosphate buffer. Denatured protein was removed by centrifugation.

Step IV. Adsorption of Alumina C γ Gel. Alumina C γ gel (36 ml of a preparation containing 10 mg/ml) was added to the dialysed preparation from Step III (90 ml). The mixture was stirred for 15 min and the gel was separated by centrifugation and resuspended in 90 ml of 0·025 M sodium phosphate buffer, pH 7. After 20 min the gel was separated by centrifugation and the supernatant was used for further purification.

Step V. DEAE-Cellulose. The clear supernatant from Step IV (85 ml) was stirred for 30 min with 4·25 g (wet wt.) of DEAE-cellulose which had been washed according to the method of Peterson and Sober[15] and adjusted to pH 7 with 0·025 M sodium phosphate buffer. The suspension was filtered through a Buchner funnel. The DEAE-cellulose cake was resuspended in 0·05 M sodium phosphate buffer, pH 7 (85 ml) and stirred for 20 min. The suspension was again filtered through a Buchner funnel and the filtrate was used as partially purified oxygenase.

[5] E. A. Peterson and H. A. Sober, in *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan) Vol. 5, p. 3, Academic Press, New York (1962).

Assay of aromatic 3,4-Oxygenase

The oxygenase activity was measured by incubating a reaction mixture (1 ml) containing 0·3 ml of Tris-HCl buffer, pH 7·5 (30 μmoles), 0·1 ml of substrate (0·3 μmole) and 0·5 ml of partially purified oxygenase for 20 min at 30°. The reaction was stopped by the addition of 0·2 ml of 25% trichloroacetic acid and the amount of protocatechuic acid or any other catechol substrate was determined colorimetrically by the method described by Nair and Vaidyanathan.[16]

Determination of Protein

The protein concentration was estimated colorimetrically by the method of Lowry *et al.*[17] using bovine serum albumin as standard or spectrophotometrically by measuring the absorption at 280 and 260 nm.

Isolation of Products of Homoprotocatechuate Oxidation

The reaction mixture consisted of an enzyme preparation from Step V for the preparation of the tricarboxylic acid oxidation product or from Step IV for the preparation of the lactone. The reaction was started by the addition of 5 ml of a solution of homoprotocatechuic acid (25 mg) to 100 ml of an enzyme preparation (in 0·5 M phosphate buffer pH 7·2), and incubated at 30° with gentle agitation. The disappearance of homoprotocatechuic acid was followed colorimetrically by removing aliquots at intervals. After disappearance of the substrate, another 25 mg of homoprotocatechuic acid was added. The reaction was stopped by acidification to pH 2 with 6N HCl after 8 hr. The mixture was extracted with three 500-ml portions of ether, dried over anhydrous sodium sulphate and evaporated under reduced pressure. The products were methylated with ethereal diazomethane and purified by TLC on silica gel G in EtoAc–petrol (40–60°) (3:2). Both compounds A and B were further purified by sublimation at 80° under reduced pressure (0·1 mm). The i.r. spectra were recorded using NaCl discs and the mass spectra were determined on an A.E.I. MS9 instrument using an ion source temp. of 150°.

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[16] P. M. NAIR and C. S. VAIDYANATHAN, *Anal. Biochem.* **7**, 315 (1964).
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