Influence of Side-Chain Substituents on the Position of Cleavage of the Benzene Ring by *Pseudomonas fluorescens*[1]

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Pseudomonas fluorescens was grown on mineral salts media with phenol, *p*-hydroxy-benzoic acid, *p*-hydroxy-phenylacetic acid, or *p*-hydroxy-*trans*-cinnamic acid as sole carbon and energy source. Each compound was first hydroxylated, *ortho* to the hydroxyl group on the benzene ring, to give catechol, protocatechuic acid (3,4-dihydroxy-benzoic acid), homoprotocatechuic acid (3,4-dihydroxy-phenylacetic acid), and caffeic acid (3,4-dihydroxy-*trans*-cinnamic acid), respectively, as the ultimate aromatic products before cleavage of the benzene nucleus. Protocatechuic acid and caffeic acid were shown to be cleaved by *ortho* fission, via a 3,4-oxygenase mechanism, to give β-substituted *cis*, *cis*-muconic acids as the initial aliphatic products. However, catechol and homoprotocatechuic acid were cleaved by *meta* fission, by 2,3- and 4,5-oxygenases, respectively, to give α-hydroxy-muconic semialdehyde and α-hydroxy-γ-carboxymethyl muconic semialdehyde as initial aliphatic intermediates. Caffeic acid: 3,4-oxygenase, a new oxygenase, consumes 1 mole of O_2 per mole of substrate and has an optimal pH of 7.0. The mechanism of cleavage of enzymes derepressed for substituted catechols by *P. fluorescens* apparently changes from *ortho* to *meta* with the increasing nephelauxetic (electron donor) effect of the side-chain substituent.

Aromatic compounds are oxidatively dissimilated by two distinct mechanisms: (i) cleavage of a catechol between adjacent carbon atoms bearing hydroxyl groups (11, 13) and (ii) cleavage of the ring between a carbon atom bearing a hydroxyl group and the adjacent nonhydroxylated carbon atom (3, 4, 8, 13). It is generally accepted that a particular species of *Pseudomonas* employs either *ortho* or *meta* ring fission for a given aromatic substrate and that both types of oxygenase are not derepressed simultaneously by one inducer (15). *Ortho* or *meta* cleavage of protocatechuic acid has been used as one of the parameters to classify pseudomonads (15).

In this communication, we report that, in the case of *P. fluorescens* [this organism was classified as *P. fluorescens* according to Stanier, Palleroni, and Doudoroff (15)], the electron-donating or electron-withdrawing capacity of the side-chain substituent appears to determine whether *ortho*- or *meta*-cleaving enzymes are derepressed; the whole molecule seems to be unimportant. In this

organism, both types of ring fission mechanism are not derepressed simultaneously as is found with some microorganisms (6, 9). Details of the properties of the ring fission products from catechol, protocatechuic acid, and homoprotocatechuic acid have been published elsewhere (3, 5, 12). However, caffeic acid: 3,4-oxygenase has not been reported, and details of the properties of this enzyme and those of the ring fission product are presented.

MATERIALS AND METHODS

Culture methods. *P. fluorescens* was grown on mineral salts media containing, per liter: KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4$, 10 mg; and $FeSO_4$, 5 mg. The desired carbon source was added in the following amounts, per liter: phenol, 0.3 g; *p*-hydroxybenzoic acid, 1.0 g; *p*-hydroxy-phenylacetic acid, 0.7 g; and *p*-hydroxy-*trans*-cinnamic acid, 0.7 g. Cultures were grown with forced aeration at 30 C in 16-liter carboys, by stepwise increase in the volume of the culture from 50 ml to 1 to 16 liters.

Preparation of cell-free extracts. Cells were harvested in the late exponential phase in a Sharples centrifuge and were washed in 0.05 M tris(hydroxy-

methyl)aminomethane (Tris) buffer (*p*H 7.0); crude extracts were prepared by exposing 1 g (wet weight) of bacteria per 2.0 ml of 0.05 M Tris buffer (*p*H 7.0) to the maximal output of a Branson sonic probe for 2 min at 0 C. Cell debris was removed by centrifugation at 23,000 × *g* for 20 min at 0 C. Protein was determined by the method of Lowry et al. (10).

Heat treatment. *Meta*-cleaving enzymes from *P. fluorescens* were found to be heat stable, but enzymes for the further degradation of the semialdehyde product were totally inactivated by treatment at 70 C for 5 min. The protein precipitated by heat treatment was removed by centrifugation, and the supernatant fluids were shown to contain active catechol 2,3-oxygenase (EC 1.13.1.2; phenol-grown cells) or homoprotocatechuic acid: 4,5-oxygenase (*p*-hydroxyphenylacetic acid-grown cells).

Separation of ortho-cleaving enzymes. Protocatechuic acid: 3,4-oxygenase (from *p*-hydroxy-benzoate-grown cells) was shown to accumulate β-carboxy *cis*,*cis*-muconic acid when crude extracts were diluted to a level at which the lactonizing enzyme, which converts β-carboxy *cis*,*cis*-muconic acid to γ-carboxy-muconolactone, would not function but at which the protocatechuate 3,4-oxygenase was still quite active.

Crude extracts from cells grown with *p*-hydroxy-*trans*-cinnamic acid were fractionated with a saturated solution of (NH₄)₂SO₄ (*p*H 7.0). The fraction which precipitated at between 50 and 70% saturation was shown to contain caffeic acid: 3,4-oxygenase. Dialysis of this fraction for 18 hr against distilled water resulted in a preparation containing caffeic acid: 3,4-oxygenase but lacking the enzymes necessary for the subsequent degradation of the ring fission product.

Enzymatic assays. Oxygen uptake was determined with a Gilson differential respirometer. Warburg flasks contained (in a total volume of 3.0 ml) 1.8 ml of 0.05 M Tris buffer (*p*H 7.0), 0.5 ml of enzyme (15 to 20 mg of protein), and 0.5 ml of 0.01 M substrate. Carbon dioxide was removed with 0.2 ml of 20% KOH on Whatman 342 filter paper in the center well. Specificity of enzymes was checked on a Gilson Oxygraph (model K) oxygen electrode. Caffeic acid: 3,4-oxygenase could be conveniently assayed by following the change in absorbancy at 320 nm with a Zeiss PMQ II spectrophotometer (Fig. 1). Typical assay conditions consisted of 0.8 ml of 0.05 M Tris buffer (*p*H 7.0), 0.1 ml of fractionated enzyme preparation containing 1 to 2 mg of protein, and 0.1 ml of 0.001 M caffeic acid in a cuvette having a 1-cm light path.

Isolation of catechols from culture filtrates. An 18-liter amount of culture filtrate of *P. fluorescens* grown with *p*-hydroxy-*trans*-cinnamic acid, in the absence of Fe⁺⁺, was acidified with 20 ml of concentrated H_2SO_4 and extracted with 12 liters of ethyl acetate. The ethyl acetate was dried by filtration through anhydrous Na_2SO_4 before evaporation to dryness. The residue was taken up in a small volume of chloroform and filtered. The material that was not chloroform-soluble was dissolved in diethyl ether and subjected to silica gel column chromatography. A silica gel (Merck; 0.05 to 0.2 mm) column (3 by 60 cm) was prepared with diethyl ether as the solvent.
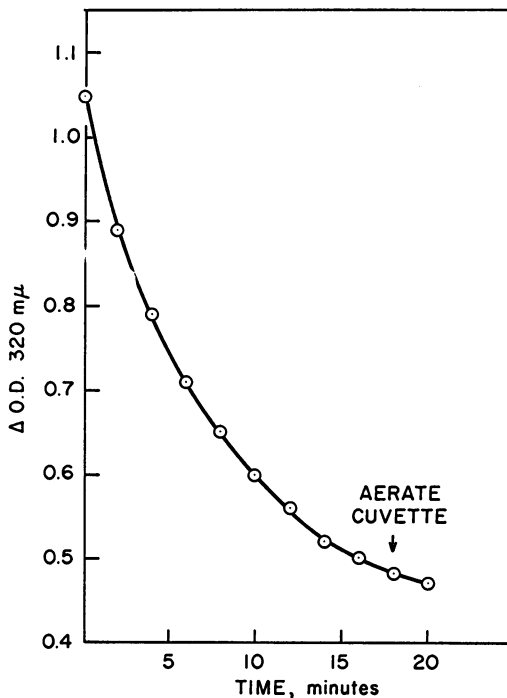

FIG. 1. *Spectrophotometric assay for caffeic acid: 3,4-oxygenase with fractionated extracts of P. fluorescens grown with p-hydroxy-trans cinnamic acid.*

The ether-soluble material was applied to the column followed by elution with a methanol-ether mixture of increasing polarity. Fractions (50 ml) were collected, and caffeic acid was eluted in fractions 27 to 30 (10% methanol, 90% ether). After evaporation of the solvent, this catechol was crystallized from an ether-petroleum mixture to give a total yield of 25.1 mg.

Thin-layer chromatography. Eastman Kodak chromogram cellulose plates with fluorescent indicator were used for thin-layer chromatography of reaction products. Benzene-dioxane-acetic acid (45:12:2.5) was found to be the most satisfactory solvent system. Spots were located under ultraviolet light. Preparative thin-layer chromatography was performed with silica gel plates (20 by 20 cm) containing a surface of 20 g of Silica Gel G (Merck). Benzene-ethyl acetate (90:10) was found to be the most satisfactory solvent system for this procedure.

RESULTS

Characterization of catechols. Catechol, protocatechuic acid, and homoprotocatechuic acid have been established as intermediates in the oxidative degradation of phenol, *p*-hydroxy-benzoic acid, and *p*-hydroxy-phenylacetic acid (or phenylacetic acid) in *P. fluorescens* (3, 5). Extracts of *P. fluorescens* grown with *p*-hydroxy-*trans*-cinnamic acid oxidized both caffeic acid and proto-

catechuic acid with the consumption of 1 mole of O_2 per mole of substrate (Fig. 2). Since side-chain attack on p-hydroxy-*trans*-cinnamic acid may occur to give protocatechuic acid as the ultimate aromatic product before ring cleavage, it was necessary to determine which of these two substituted catechols was the natural inter-
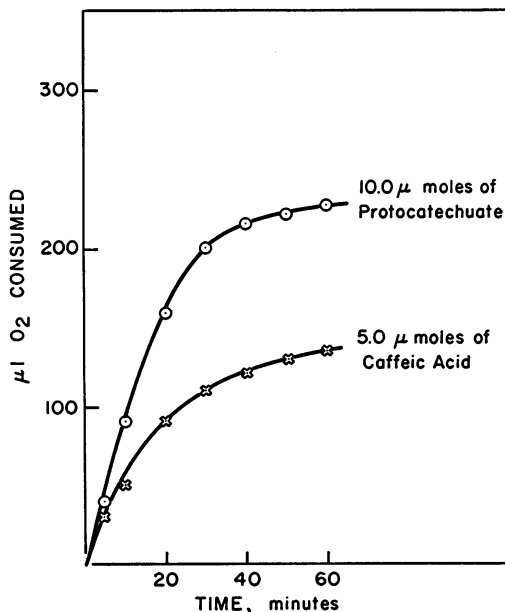

FIG. 2. *Oxidation of protocatechuic acid and caffeic acid by cell-free extracts of P. fluorescens grown with p-hydroxy-trans-cinnamic acid.*

mediate in this metabolic pathway. When cultures were grown with p-hydroxy-*trans*-cinnamic acid medium, in which Fe^{++} was omitted, caffeic acid was isolated in significant yield, but protocatechuic acid could not be detected even on thin-layer chromatographic analysis. Caffeic acid could be detected in lower yields by thin-layer chromatography from culture filtrates in which iron was included. This unsaturated acid is readily distinguishable from protocatechuic acid, since the former compound fluoresces under ultraviolet light. Caffeic acid was characterized by a melting point of 195 C (no depression on admixture with authentic caffeic acid) and by infrared spectral analysis in a Nujol mull (Fig. 3). These data indicate that caffeic acid is most likely the natural intermediate in the degradation of p-hydroxy-*trans*-cinnamic acid.

Characterization of ring fission products. Heat-treated extracts of cells of *P. fluorescens* grown either with phenol or p-hydroxy-phenylacetic acid, were reacted with catechol and homoprotocatechuic acid, respectively. After the consumption of 1 mole of O_2 per mole of substrate, reactions were stopped; then the protein was precipitated with 0.5 ml of 5 N HCl and removed by centrifugation. Samples (0.1 ml) of supernatant liquid were taken and subjected to spectral analysis in acid (2 N HCl) and alkali (5 N NaOH) in a Cary model 14 recording spectrophotometer. The spectral properties of these ring fission products were found to be identical to those reported for α-hydroxy-muconic semialdehyde and α-hydroxy-γ-carboxymethyl muconic semialdehyde (3, 5; Table 1).
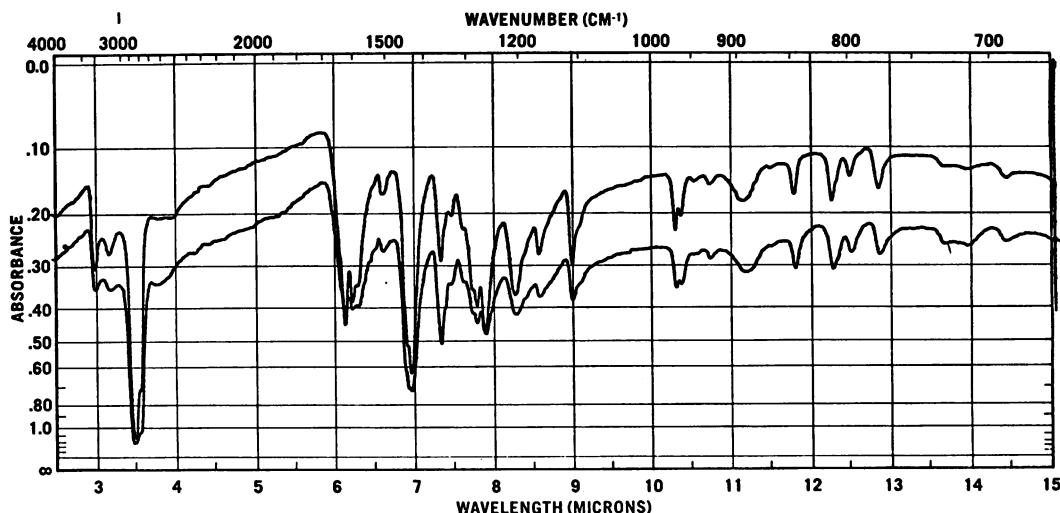

FIG. 3. *Infrared spectra of authentic caffeic acid (upper spectrum) and the catechol isolated from culture filtrates of cells grown with p-hydroxy-trans-cinnamic acid.*

TABLE 1. *Ring fission mechanism of oxygenases of P. fluorescens grown with different aromatic substrates*

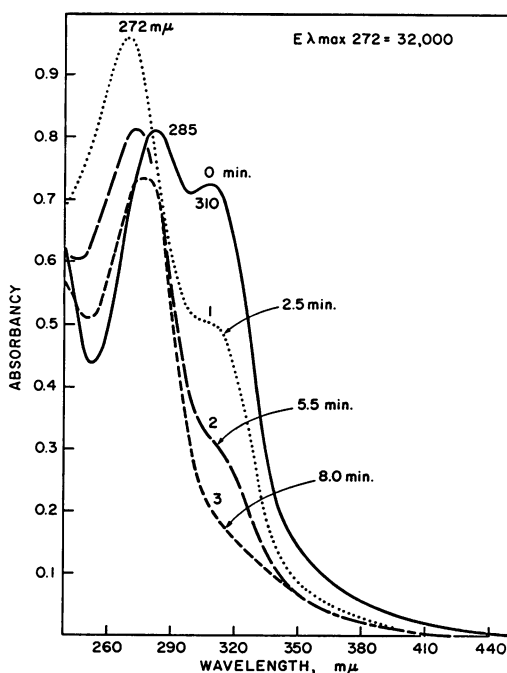| Growth substrate	Catechol utilized	Absorption maxima of ring fission products		Ring fission mechanism
		Acid	Alkali	
		nm	*nm*	
Phenol	Catechol	317	375	*Meta*
p-Hydroxy-benzoic acid	Protocatechuate	230	257	*Ortho*
p-Hydroxy-phenylacetic acid	Homoprotocatechuate	320	380	*Meta*
p-Hydroxy-*trans*-cinnamic acid	Caffeic acid	242, 272	235, 345	*Ortho*

FIG. 4. *Kinetic study of the disappearance of caffeic acid with the concomitant accumulation of ring fission product with an absorption maximum at 272 nm.*

FIG. 5. *Spectral properties of the ring fission product from caffeic acid.*

The formation of β-carboxy *cis,cis*-muconic acid from protocatechuic acid was demonstrated with cells grown with both p-hydroxy-benzoate and p-hydroxy-*trans*-cinnamic acid by following the procedure of Ornston and Stanier (12). A 1.0-μmole amount of protocatechuic acid was allowed to react with 0.5 to 1.0 mg of crude extract in a total volume of 1.0 ml of 0.1 M phosphate buffer (pH 7.0). The disappearance of protocatechuic acid which absorbs at 290 nm and the appearance of the ring fission product which absorbs at 257 nm were observed by repeatedly scanning the reaction mixture in a Cary model 14 spectrophotometer. Spectral characteristics of the product at pH 7.0 and in 2 N acid are presented in Table 1. The above procedure was used
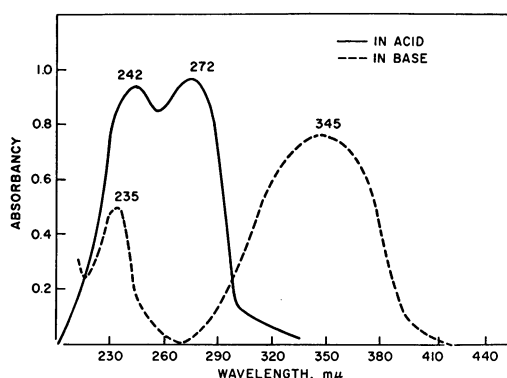
to follow the cleavage of caffeic acid, except that fractionated extracts were required in this experiment (Fig. 4). This ring fission product showed no keto-enol tautomerism, having no functional keto, aldehyde, or hydroxyl groups (7, 14). Thin-layer chromatography of the ring fission product in the benzene-dioxane-acetic acid solvent system gave one spot ($R_F = 0.23$), which was more polar and readily distinguishable from caffeic acid ($R_F = 0.43$). Elution of the compound with an R_F value of 0.23 followed by spectral analysis gave absorption maxima at 272 and 242 nm in acid and at 345 and 235 nm in alkali (Fig. 5). When the ring fission product was allowed to stand in 5 N HCl overnight, a product having the infrared spectrum of a γ,β-unsaturated γ-lactone (1,750 cm^{-1}) was isolated. Also, further infrared analysis of this compound showed a carbonyl absorption band at 1,720 cm^{-1} as well as —C—O—C stretch at 1,090 cm^{-1}. These data indicate that caffeic acid is degraded via an *ortho* cleavage mechanism to form a tricarboxylic acid which may lactonize to form a substituted muconolactone (Fig. 6).

The specific activity of caffeic acid:3,4-oxygenase was determined as the change in absorbancy at 320 nm in 5 min per milligram of protein.
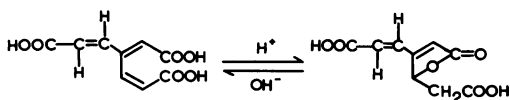
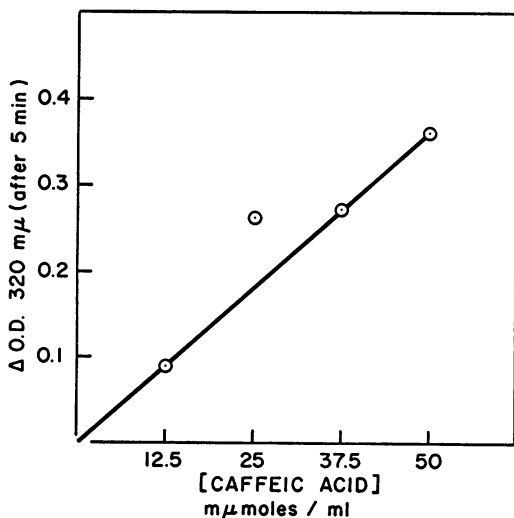FIG. 6. *Degradation of caffeic acid via an ortho cleavage mechanism to form a tricarboxylic acid.*

FIG. 7. *Linear dependence of the rate of dissappearance of caffeic acid on substrate concentration.*

With this assay, this oxygenase was shown to have a linear dependence on substrate concentration (Fig. 7) and a pH optimum of 7.0 (Fig. 8). Further studies are in progress on the complete degradative sequence for the metabolism of p-hydroxy-*trans*-cinnamic acid.

DISCUSSION

The strain of *P. fluorescens* used in this study is unique in its ability to derepress the synthesis of enzymes which cleave substituted catechols either by *ortho* or by *meta* mechanisms. The versatility of this organism is demonstrated in Fig. 9. Both *ortho-* and *meta-*cleaving enzymes are not derepressed by growth on any one substrate. Thus far, this study has been limited to four alternative pathways; however, on the basis of these four substrates, a pattern of cleavage influenced by the nephelauxetic effect of side-chain substituents is apparent. A broader survey of substrates is required before this hypothesis can be adopted as a general rule. However, it is of interest to note that studies with different organisms grown with cresols and with other substrates containing good electron-donating, side-chain substituents invariably show a derepression of *meta*-cleaving enzymes (1, 2, 13).

Caffeic acid:3,4-oxygenase, a new oxygenase, has been fractionated and a suitable assay to follow its activity has been developed. This enzyme may be useful for studying the mechanism of O_2 insertion by a dioxygenase; binding studies could be fruitful because caffeic acid is a fluorescent substrate but its ring fission product does
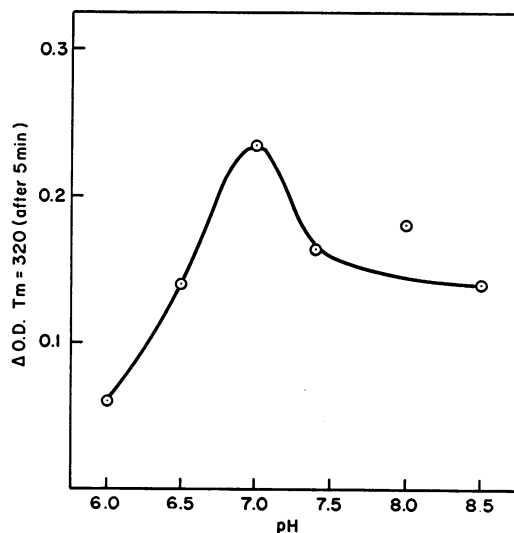

FIG. 8. *pH optimum of caffeic acid: 3,4-oxygenase. The assays at pH 6.0 and 6.5 were done in 0.05 m phosphate buffer, and assays at pH 7.0, 7.5, 8.0, and 8.5 were done in 0.05 m Tris buffer.*
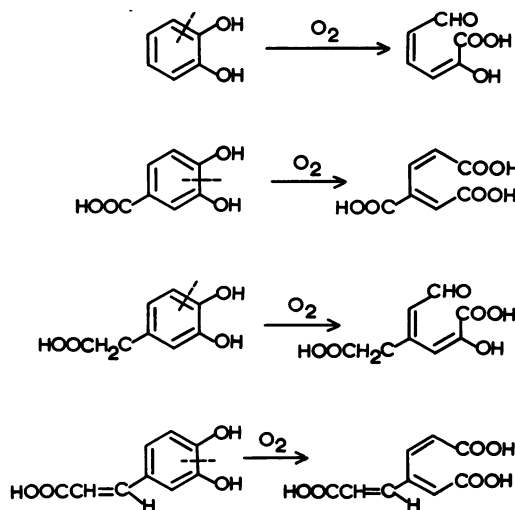

FIG. 9. *Versatility of the strain of P. fluorescens used in this study as shown by its ability to derepress the synthesis of enzymes which cleave substituted catechols either by ortho or by meta mechanisms.*

not fluoresce. Studies are in progress on the purification and mechanism of caffeic acid: 3,4-oxygenase.

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