APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1977, p. 906-910

Vol. 33, No. 4
Printed in U.S.A.

Oxidative Coupling of Aromatic Pesticide Intermediates by a Fungal Phenol Oxidase[1]

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Received for publication 8 October 1976

The soil fungus *Rhizoctonia praticola* produced an enzyme that accumulated in the growth medium and caused the polymerization of phenolic and naphtholic intermediates of various pesticides. The dialyzed crude enzyme was purified by ion-exchange column chromatography with diethylaminoethyl-cellulose, followed by gel filtration with Sephadex G-200. The enzyme, a phenol oxidase, was capable of polymerizing 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, and 4-bromo-2-chlorophenol. 1-Naphthol, 2-naphthol, and some of their derivatives formed oligomers or polymers when incubated with the enzyme, but 4-nitrophenol and 2,4-dinitrophenol were not oxidized. Chlorinated and brominated anilines, which are derivatives of herbicides, were not altered by the phenol oxidase from *R. praticola*, but 4-methoxyaniline was transformed by the enzyme to 2-amino-5-*p*-anisidinobenzoquinone-di-*p*-methoxyphenylimine. The formation of polymeric products was determined by mass spectrometric analysis.

Many pesticides are aromatic compounds that are degraded chemically or biologically to phenolic intermediates or to aromatic amines. It is well established that naturally occurring aromatic compounds are polymerized in the formation of humus and other biological products by the activity of phenol oxidases (4), but little is known about the activity of these enzymes on aromatic intermediates of xenobiotic compounds.

Oxidative coupling reactions with aromatic compounds result in the formation of polymers. This process is catalyzed not only by iron- or copper-containing phenol oxidases, but also by a wide range of oxidizing chemicals such as ferric chloride and cupric hydroxide (15). In the oxidative coupling of phenol, for example, aryloxy or phenolate radicals are formed by removal of one electron and a proton from the hydroxyl group. The resulting phenolate radicals then couple to yield stable dimerized and polymerized products.

It has recently been shown that 1-naphthol, the major chemical and biological decomposition product of the insecticide carbaryl (1-naphthyl-*N*-methylcarbamate, Sevin), can be polymerized at least to a pentameric compound by an extracellular phenol oxidase that was isolated from the culture filtrate of the fungus *Rhizoctonia praticola* (14). The enzyme has an activity similar to previously isolated laccases (1, 9), which cause the polymerization of phenolic compounds like *o*-methoxyphenol, *p*-methylphenol, and 2,6-dimethoxyphenol.

This report further describes the activity of the fungal enzyme when it is incubated with some substituted phenolic compounds and aromatic amines that are established intermediates of pesticide degradation, or which may reach the environment through industrial waste materials.

MATERIALS AND METHODS

Fungal growth and enzyme preparation. *R. praticola* (Vaartaja no. 1347) was grown in a modified Czapek Dox medium as previously described (3). After the cultures were grown at 25°C on a rotary shaker (150 oscillations/min) for 7 to 8 days, 1-naphthylamine (Fisher Scientific Co., Fair Lawn, N.J.) was added at a final concentration of 5×10^{-4} M to stimulate enzyme production by the fungus. Prior to its addition, the inducer was dissolved in ethanol and sterilized by membrane filtration (0.22-μm pore size).

After 2 to 3 more days of incubation, the mycelium was separated from the growth medium by filtration, and the culture filtrate was dialyzed in the cold against distilled water for 24 h. The dialyzed filtrate was then passed through a column (2 by 15 cm) of diethylaminoethyl (DEAE)-cellulose (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with distilled water. The column, containing the adsorbed enzyme, was washed with distilled water until no further pigmented material was

[1] Paper no. 5173 of the Journal Series of the Agricultural Experiment Station, University Park, Pa.

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eluted. The enzyme was then removed from the ion-exchange resin by eluting the column with 0.05 M tris(hydroxymethyl)aminomethane(Tris) buffer (Sigma Chemical Co., St. Louis, Mo.), pH 7.1. The active eluate was subsequently lyophilized, and the freeze-dried fraction was then dissolved in 0.1 M phosphate buffer, pH 6.9, before gel filtration.

A column (4 by 45 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., New Market, N.J.), equilibrated with the phosphate buffer, was used in the next purification step. The void volume was 180 ml, and the flow rate was 1 ml/min, with fractions of 8 ml being collected.

Enzyme assay. The enzymatic activity of each fraction after Sephadex chromatography was measured on a spectrophotometer (model PMQ II, Carl Zeiss, Inc., New York, N.Y.) with 2,6-dimethoxyphenol as the substrate. When this chemical was used as a substrate, 3,5,3',5'-tetramethoxydiphenoquinone was the sole enzymatic product (14).

The incubation mixture consisted of 3.24 μmol of 2,6-dimethoxyphenol in 3.4 ml of 0.1 M phosphate buffer, pH 6.9. The enzyme solution (0.1 ml) was added to the incubation mixture, and the solution was thoroughly mixed. One unit of enzyme was defined as that amount which caused a change in optical density of 1.0 per min at 468 nm, the λ_{max} of 3,5,3',5'-tetramethoxydiphenoquinone. The initial velocity of the reaction at 23°C was measured from the linear portion of the curve.

Incubation of substrates. The compounds to be tested were dissolved in ethanol and added to a 0.1 M phosphate buffer solution (pH 6.9) containing 0.5 units of the isolated enzyme per ml. The final substrate concentrations ranged from 20 to 100 μg/ml, depending on the particular compound being tested (Table 1), and the final ethanol concentration in any assay never exceeded 4%. All incubations were performed at 23°C, and incubation times varied with the particular substrate (Table 1). Only a small volume of enzyme solution was needed to determine whether a substrate was transformed by the enzyme; however, enzyme solution volumes of 100 to 200 ml were used if products were to be isolated and identified. Enzyme controls boiled for 5 min were employed for each substrate and were found to be inactive in all cases.

Isolation of products. Equal volumes of diethyl ether were used to extract products from the enzyme solution after incubation; however, in assays with 4-nitrophenol and 2,4-dinitrophenol as substrates, the incubation mixture was acidified to pH 2.5 by addition of HCl before extraction with ether.

Thin-layer chromatography (TLC) of the ether extracts was used to assess the enzymatic activity of the individual compounds, as well as for the isolation of products. Precoated TLC plates (Brinkmann Instruments, Inc., Westbury, N.Y.) with a thickness of 0.50 and 0.25 mm of Silica Gel F-254 were used for the preparative thin-layer analyses and for routine separations, respectively. Compounds separated by TLC were either colored materials or could be made visible by exposure to ultraviolet light at 254 nm. All products were removed from the TLC plate by scraping, followed by extraction from the silica gel with acetone. An ether-hexane (4:1, vol/vol) solvent system was effective in separating the mixture of products that resulted when the enzyme was found to be active on a particular substrate. A methylene chloride-methanol-ammonium hydroxide (80:20:3, vol/vol) solvent system was used when 4-nitrophenol served as the substrate, and a chloroform-methanol-acetic acid (95:6:1, vol/vol) system was employed with 2,4-dinitrophenol. In the assay with 4-nitrophenol, 0.006 μCi of 4-nitrophenol-^{14}C (specific activity, 120 μCi/mg) was added per ml of incubation mixture.

TABLE 1. *Concentration, incubation times, and detection of oxidative coupling of various compounds incubated with the isolated phenol oxidase*

Substrate	Concn (μg/ml)	Incubation time (h)
2-Chlorophenol	50	6
4-Chlorophenol	50	6
2,4-Dichlorophenol	50	12
4-Bromo-2-chlorophenol	50	12
1-Naphthol	50	3
4,4'-Bi-1-naphthol	50	6
4-Chloro-1-naphthol	100	5
2-Naphthol	50	3.5
4-Nitrophenol	20	16
2,4-Dinitrophenol	20	16
Aniline	50	4
2-Chloroaniline	50	12
3-Chloroaniline	50	12
4-Chloroaniline	50	4
2,4-Dichloroaniline	50	4
3,4-Dichloroaniline	50	12
2-Bromoaniline	50	12
3-Bromoaniline	50	12
4-Bromoaniline	50	4
4-Methoxyaniline	100	3

Radioactivity was determined with a liquid scintillation counter (Isocap 300, Searle Analytic Inc., Des Plaines, Ill.). Samples were measured in a modified Bray solution composed of 60 g of naphthalene, 100 ml of methanol, and 8 g of Omnifluor (New England Nuclear Corp., Boston, Mass.) in 1 liter of dioxane.

The complex product mixture from 4-chloro-1-naphthol was separated by the successive use of the following thin-layer solvent systems: benzene-chloroform (1:1), ether-hexane (4:1), benzene, and ether-hexane (1:4).

Mass spectrometry was used as the major tool for the assessment of polymerization, and analysis was often performed on the product mixture extracts as well as on isolated compounds. Mass spectra were determined using an AEI MS-902 mass spectrometer at an ionization potential of 70 eV and at a temperature of 350°C with use of the direct insertion probe.

Chemicals. 2-Chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 4-bromo-2-chlorophenol, 2,4-dinitrophenol, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, 3,4-dichloroaniline, 2,4-dichloroaniline, 4-chloro-1-naphthol, and 2,6-dimethoxyphenol were purchased from Aldrich Chemical Co. (Milwaukee,

Wis.). Aniline, 2-bromoaniline, 3-bromoaniline, 4-bromoaniline, and 4-methoxyaniline were obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.), and 2-naphthol was from Eastman Organic Chemicals (Rochester, N.Y.) 4-nitrophenol-^{14}C was supplied by CIBA-GEIGY (Basel, Switzerland), and the Chemistry Department at The Pennsylvania State University was the source of 4-nitrophenol. All chemicals that were not of sufficient purity, as determined by TLC, when obtained were purified by charcoal addition, subsequent filtration, and recrystallization. 4,4'-Bi-1-naphthol was synthesized from 1-naphthol according to the technique reported by Dianin (8).

RESULTS

In our previous studies, it was demonstrated that *R. praticola* produces an extracellular enzyme that was found to be active in the transformation of phenolic compounds (3, 14). A three- to fourfold increase in enzyme activity (3 U/ml) was observed in the present study when 1-naphthylamine was added, at a final concentration of 5×10^{-4} M, to the growth medium of a well-established culture of the fungus. The enzyme was concentrated from the culture filtrate by adsorption to DEAE-cellulose, and elution from the column with 0.05 M Tris buffer (pH 7.1) could be visually followed, since the active enzyme formed a blue band. Contaminating yellow pigments were also present in the active eluate after ion-exchange chromatography; however, they were separated from the blue-colored enzyme by subsequent fractionation on Sephadex G-200. The active fractions were determined by the previously described assay with 2,6-dimethoxyphenol as the substrate, and all further experiments with the various substrates were performed using an enzyme concentration of 0.5 U/ml.

Products from phenolic intermediates of phenoxyalkanoic herbicides. Since a large number of microorganisms are able to convert phenoxyalkanoic herbicides to the corresponding phenols, the isolated enzyme was incubated with some of these intermediates. The formation of oligomers from several phenoxyalkanoic herbicide intermediates is presented in Table 2.

When 2- and 4-chlorophenol were incubated with the isolated phenol oxidase from *R. praticola*, subsequent mass spectral analysis of the product extracts revealed major peaks at *m/e* 254, 380, and 506, corresponding to molecular ions of the dimeric, trimeric, and tetrameric compounds. All *m/e* values reported for molecular ions are the ^{35}Cl and ^{79}Br isotope. Peak ratios were in agreement with the normal isotopic patterns expected. A white solid with an R_f value of 0.42 was isolated from the product mixture of 4-chlorophenol by TLC in an ether-hexane (4:1, vol/vol) solvent system, and the mass spectrum (molecular ion, $M^+ = 254$) of this compound confirmed that it was a dimerized product.

2,4-Dichlorophenol and 4-bromo-2-chlorophenol were also oxidized by the phenol oxidase from *R. praticola*, and TLC analysis (with ether-hexane) of the product extract showed that a compound with an R_f value of 0.20 was formed in good yield from each of the substrates. Both chemicals were white solids, and subsequent mass spectral analysis showed that the chemicals were dimeric derivatives of their respective substrates (product of 2,4-dichlorophenol: $M^+ = 322$; product of 4-bromo-2-chlorophenol: $M^+ = 410$).

Products of various naphthols. As was previously mentioned, 1-naphthol has been shown to be polymerized by the enzyme from *R. praticola* (14). Therefore, it became of interest to determine whether other naphtholic compounds would be oxidized and coupled in a similar manner. Table 3 summarizes the results obtained when naphtholic compounds were incubated with the enzyme isolated from the culture filtrate of *R. praticola*.

When 2-naphthol served as substrate, it could be clearly established, by analyzing the product mixture with mass spectrometry, that the chemical was polymerized by the fungal enzyme after a 3.5-h incubation period. Major peaks were seen at *m/e* 286, 428, and 570, indicating that 2-naphthol was polymerized at least to the tetramer. TLC analysis of the product extract in triethylamine as solvent system dem-

TABLE 2. *Oxidative coupling of phenolic intermediates of phenoxyalkanoic herbicides by R. praticola phenol oxidase*

Herbicide	Phenolic intermediate	Enzymatic products (detected by mass spectrometry)	M^+ of major oligomeric peaks
2-Chlorophenoxyacetic acid	2-Chlorophenol	Dimer, trimer, tetramer	254, 380, 506
4-Chlorophenoxyacetic acid	4-Chlorophenol	Dimer, trimer, tetramer	254, 380, 506
2,4-Dichlorophenoxyacetic acid	2,4-Dichlorophenol	Dimer	322
4-Bromo-2-chlorophenoxyacetic acid	4-Bromo-2-chlorophenol	Dimer	410

TABLE 3. *Polymerization of naphtholic compounds by R. praticola phenol oxidase*

Naphtholic compound	Mol wt	M^+ of major oligomeric peaks
1-Naphthol	144	286, 428, 570, 712
2-Naphthol	144	286, 428, 570
4-Chloro-1-naphthol	178.6	332, 336, 478
4,4'-Bi-1-naphthol	286	570

onstrated clearly that several compounds were present, but the isolation of individual oligomers was not attempted.

When 4-chloro-1-naphthol was incubated with the fungal enzyme, three compounds with molecular ions of *m/e* 332, 336, and 478, respectively, were isolated. However, it was evident that many more products, which could be separated by TLC, were formed. Mass spectral analysis of the product mixture did not reveal the presence of simply coupled oligomers, and the masses of some isolated compounds indicated that a more complex pattern of coupling is occurring, with the possible involvement of a dehalogenation reaction. From these indications, it can be concluded that 4-chloro-1-naphthol does not follow the same pattern of polymerization observed with the other naphthols.

4-Nitrophenol and 2,4-dinitrophenol as enzyme substrates. In an enzyme assay with the phenol oxidase of *R. praticola*, it was determined that neither 4-nitrophenol nor 2,4-dinitrophenol was oxidized. TLC of the acid-ether extract of the incubation mixtures of the nitrophenols was performed at 0, 2, 4, and 16 h of incubation and, in both cases, only in the R_f area of the substrate could a spot be detected. In the case of 4-nitrophenol, where 4-nitrophenol-^{14}C was added, all the radioactivity after TLC analysis was localized in the R_f area of the substrate.

Products from various anilines. Numerous agriculturally important herbicides are substituted aniline derivatives, and aromatic amines are often employed industrially in the manufacture of dyes. When aniline and several halogenated anilines were incubated with the isolated phenol oxidase, it was not possible to detect a change in the substrates as indicated by TLC of the ether extract of the incubation solutions.

The aromatic amines not oxidized were aniline, 2-, 3-, and 4-chloroaniline, 2-, 3-, and 4-bromoaniline, 2,4- and 3,4-dichloroaniline.

When 4-methoxyaniline was incubated with the enzyme, a major product with an R_f value of 0.58 in ether-hexane (4:1, vol/vol) was isolated. The compound crystalized as red leaflets from ethanol and was identified by mass spectral analysis and melting point (164°C) to be 2-amino-5-*p*-anisidinobenzoquinone-di-*p*-methoxyphenylimine (Fig. 1). This product is also formed in 80% yield when 4-methoxyaniline is incubated with peroxidase (6).

DISCUSSION

Our results show that phenolic and naphtholic intermediates of pesticides and industrial pollutants can be polymerized by a phenol oxidase isolated from the fungus *R. praticola*.

In studies on the metabolism of phenoxyalkanoic herbicides, it has been shown that microorganisms cause cleavage of the ether linkage, which results in the formation of phenols (2). Thus, 4-chlorophenol is produced from 4-chlorophenoxyacetic acid, 2,4-dichlorophenol from 2,4-dichlorophenoxyacetic acid, etc. Even though further degradative pathways of the formed phenols have been established, it has to be assumed that other transformation processes such as the described polymerization reactions can take place in a natural habitat like the soil.

The polymerization of 1-naphthol, a hydrolysis product of carbaryl, has previously been reported (14). A similar reaction was observed with 2-naphthol, which is an intermediary product of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids (5), an antioxidant in the rubber industry, and is used for the preparation of dyes and medicinal organics. Since other naphtholic compounds were also polymerized, there is a clear indication of broad substrate specificity of the enzyme.

4-Nitrophenol is a hydrolytic product of Parathion (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) and other pesticides, and 2,4-dinitrophenol is used in fungicidal preparations (2). The two nitrophenols were not oxidized by the

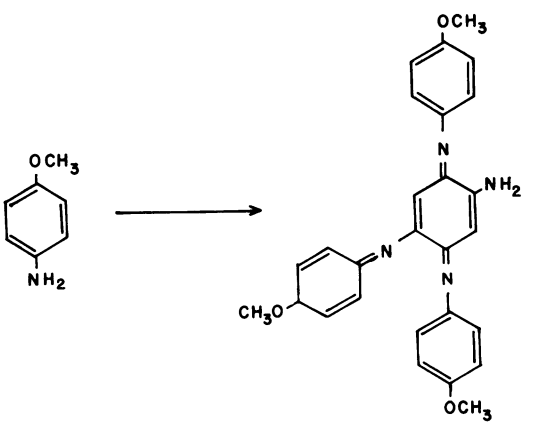

FIG. 1. *Oxidation of 4-methoxyaniline to 2-amino-5-p-anisidinobenzoquinone di-p-methoxyphenylimine.*

fungal enzyme. It could be theorized that the presence of the strong electron-attracting nitro-substituents can prevent the removal of an electron from the phenolic hydroxyl group, thereby rendering these compounds resistant to oxidative coupling.

The phenol oxidase from *R. praticola* was also not able to cause a polymerization reaction of the aniline-related substrates, with the exception of 4-methoxyaniline. It is well known that peroxidases are effective in producing condensed or coupled molecules from various anilines (7), and the inability of the enzyme isolated from *R. praticola* to transform many anilinic products clearly shows that it belongs to another group of phenol oxidases. Further, the blue color of the enzyme, as well as the observed absence of any hydroxylation reaction with the tested intermediates, would indicate that it is a laccase. It is well known that phenol oxidases—peroxidases, tyrosinases, and laccases—are common in soil. However, these enzymes have been relatively little studied in this environment (11), even though they appear to be essential for the humification process and formation of other important natural products.

Haider et al. (10) speculated that the participation of phenolase enzymes in the formation of polymers is of minor significance for several soil fungi of the Imperfecti group. They concluded that the appearance of polymers in the growth medium of the fungi is largely due to an autoxidative process. However, an enzyme from the mycelium of the fungus *Hendersonula toruloidea* transformed phenolic substances to polymers resembling humic acids (12).

The experiments described in this paper were performed with an isolated enzyme under typical laboratory conditions. It now appears of great interest to determine whether a similar reaction can also be established in the soil environment. It is conceivable that phenol oxidases play an important role in the transformation of a wide variety of aromatic chemicals; however, the polymerization reactions in soils have not yet been assessed. Our results should initiate further investigations in which the extent of polymerization, as well as the degree of incorporation of polymerized synthetic aromatic compounds into soil organic matter, can be evaluated.

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