Polymerization of phenolic intermediates of pesticides by a fungal enzyme

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Summary. The fungus *Rhizoctonia praticola* produces an extracellular phenol oxidase (laccase) which polymerizes phenolic intermediates of various pesticides. The enzyme catalyzes the formation of oligomeric products from halogenated phenolic intermediates of phenoxyalkanoate herbicides and from naphtholic products derived from carbamate insecticides. These findings permit further investigations into the mechanism and role of oxidative coupling leading to the incorporation of xenobiotic compounds into soil organic matter.

The soil habitat is the primary receptacle for pesticides and their decomposition products, however, the fate of these chemicals in such a complex environment is often difficult to determine. Therefore, laboratory experiments with single microbial isolates or with purified enzymes are often used to establish pathways of pesticide metabolism. Results from this model system approach have established hydroxylation, epoxidation, reduction, hydrolysis, dealkylation and dehalogenation among others, as important reactions in the transformation of pesticides[1].

As the result of our investigations, a previously unconsidered reaction may be added to this list, as we have observed that phenolic intermediates of pesticides can be polymerized by an enzyme produced by a soil fungus. In our studies the enzyme was isolated from the growth medium of the fungus *Rhizoctonia praticola* (Vaartaja No. 1347) by DEAE-cellulose column chromatography and Sephadex G-200 gel filtration[2,3].

Material and methods. An assay was established with 2,6-dimethoxyphenol as a substrate[3]; the enzyme solution (0.1 ml) was added to 3.24 μmoles 2,6-dimethoxyphenol in 3,4 ml 0.1 M phosphate buffer, pH 6.9, and incubated at 23 °C. One unit of enzyme was defined as that amount which caused a change in OD of 1.0/min at 468 nm, the λ_{max} of the product, 3,5,3′,5′-tetramethoxydiphenoquinone. The compounds tested were added to a solution of 0.5 units/ml of enzyme in 0.1 M phosphate buffer, pH 6.9 at a concentration of 50 μg/ml. The incubation mixtures were extracted with diethyl-ether, and the product extract was analyzed by mass spectrometry at an ionization potential of 70 eV and at a temperature of 350 °C using the direct insertion probe.

Results and discussion. The tested intermediates as well as the pesticides from which they are derived are listed in the table, and oligomeric products, as detected by mass spectrometry, are also presented. 2-Chlorophenol and 4-chlorophenol were readily polymerized by the enzyme and the major mass spectral peaks of the product extract correspond to dimeric, trimeric, and tetrameric oligomers. Isotopic pattern analysis revealed the possible involvement of a dechlorination reaction in the formation of a tetrameric product from 4-chlorophenol.

The mass spectrum of the 2-chlorophenol product mixture is reproduced in the figure and possible oligomers from

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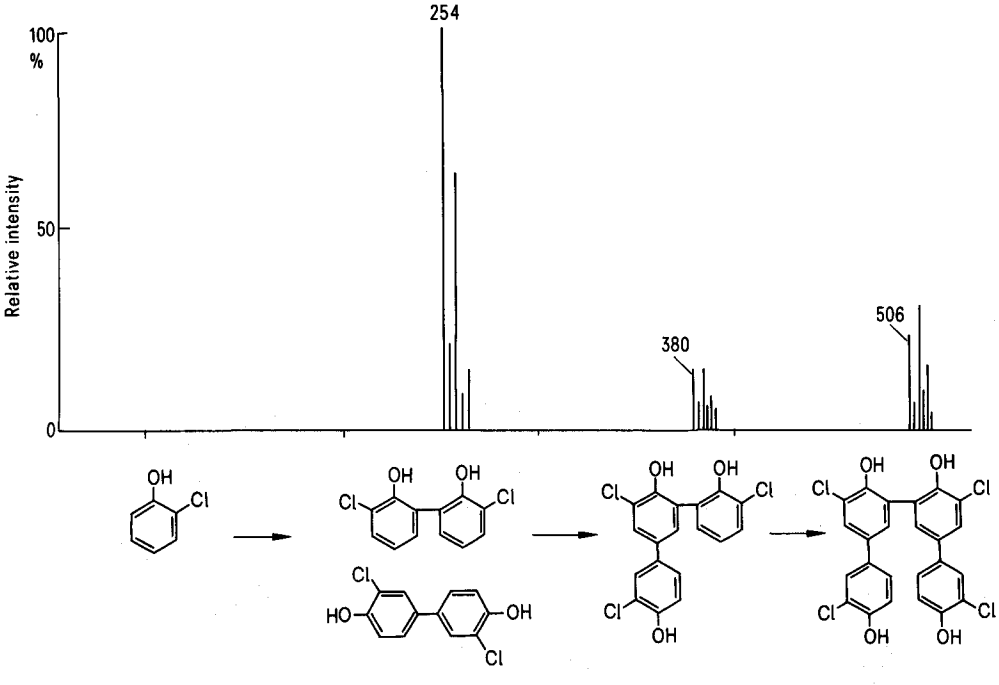

Mass spectrum obtained from the diethyl-ether extract of an assay with an extracellular enzyme of *R. praticola* and 2-chlorophenol as substrate. Below the mass spectrum is one possible scheme for the formation of oligomeric products.

this substrate are represented below their respective ion groupings. The oligomers result from oxidative coupling, and enzymes that catalyze this reaction are known as phenol oxidases. In the catalytic event, aryloxy radicals are produced from phenolic compounds, and polymerization occurs as these radicals couple through the sites ortho or para to the hydroxyl group with the meta-positions being nonreactive[4].

Dimerized products were formed when 2,4-dichlorophenol and 4-bromo-2-chlorophenol were incubated with the phenol oxidase from *R. praticola*, and 4-chloro-2-methylphenol was polymerized to dimeric, tetrameric, pentameric, and hexameric compounds with the higher polymers exhibiting varying degrees of dehalogenation. A trimer was not observed in the mass spectrum.

Oligomeric products were not formed from nitrophenols (4-nitrophenol, 2,4-dinitrophenol). This is probably due to the strong electron-attracting characteristic of the nitro-substituents, which may effectively prevent the removal of an electron from the phenolic hydroxyl group.

Several factors must be kept in mind when the technique of mass spectrometry is used to analyze mixtures of compounds. First, higher mol. wt polymers may well be present but too involatile to be detected. Also, one is not always sure whether ion peaks represent molecular ions or fragments of higher mol. wt compounds.

For example, the mass spectrum of the material extracted from the enzyme-naphthol reaction showed ions at m/e value corresponding to coupling of 2–5 monomeric units, but these peaks might have represented fragment ions from the pentamer. However, by TLC of this extract on silica gel plates using triethylamine or ether-hexane (4:1, v/v) as solvent systems, 2 dimers (m/e 286), a trimer (m/e 428), and a tetramer (m/e 570), were isolatable[3]. A dimer was also separated from the product mixture produced from 4-chlorophenol.

Hydroxylated carbaryl metabolites[5] were also oxidatively coupled by the fungal enzyme. It appears that these compounds were hydrolyzed to the corresponding free naphthols prior to the coupling reaction. The mass spectra of these products indicated that the observed dimers or trimers had undergone further oxidation or dehydration corresponding to loss of H_2 or water. The isomeric 2-naphthol behaved similarly to 1-naphthol.

Although phenol oxidases are instrumental in the biosynthesis of a wide range of naturally occurring compounds such as lignins, antibiotics, alkaloids, melanins, and pigments, 'knowledge concerning their properties is scarcer than for any other group of enzymes of such biological importance[6]. Further, there are only a few studies that attempt to establish the importance of phenol oxidases in soil[7]. Laccases, peroxidases, and tyrosinases comprise the group of metal-containing enzymes that catalyze phenol coupling, and the distinction between these classes is not always clear. Yet, the enzyme isolated from *R. praticola* can be classified as a laccase since tyrosine is not oxidized nor is a hydroxylation reaction observed with any substrate. Further, hydrogen peroxide is not required for activity, and is even found to be inhibitory (unpublished observations).

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Polymer formation as detected by mass spectrometry from phenolic pesticide intermediates after incubation with a phenol oxidase from the fungus *Rhizoctonia praticola*

Pesticide	Phenolic intermediate	Incubation (h)	Phenolic oligomers (m/e value of molecular ion) Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer
2-Chlorophenoxyacetic acid	2-Chlorophenol	6	[128][a]	254 (Cl_2)[b]	380 (Cl_3)	506 (Cl_4)		
4-Chlorophenoxyacetic acid	4-Chlorophenol	6	128 (Cl)	254 (Cl_2)[c]	380 (Cl_3)	472 (Cl_3) 506 (Cl_4)		
2,4-Dichlorophenoxy-acetic acid (2,4-D)	2,4-Dichlorophenol	12	162	322 (Cl_4)[c]				
4-Bromo-2-chlorophe-noxy-acetic acid	4-Bromo-2-chloro-phenol	12	206	410 (Br_2Cl_2)[c]				
4-Chloro-2-methylphe-noxy-acetic acid (MCPA)	4-Chloro-2-methyl-phenol	12	142 (Cl)	282 (Cl_2)	[422]	494 (Cl_2) 528 (Cl_3)	600 (Cl_2) 634 (Cl_3)	740 (Cl_3) 774 (Cl_4)
1-Naphthyl-N-methyl-carbamate (Carbaryl, Sevin)	1-Naphthol	3	144	286[c] 284 ($-H_2$)[d]	428[c]	570[c]	712	
1-Naphthyl-N-methyl-carbamate (Carbaryl, Sevin)	4-Hydroxy-N-methyl-carbamate →(1,4-Naphthalene-diol)[e]	6	160	314 ($-H_2O$)	472 ($-2H_2$) 456 ($-H_2$, H_2O)			
1-Naphthyl-N-methyl-carbamate (Carbaryl, Sevin)	5-Hydroxy-N-methyl-carbamate →(1,5-Naphthalene-diol)[e]	6	[160]	300 ($-H_2O$)				
ω-(2-Naphthyloxy)-n-alkylcarboxylic acids	2-Naphthol	3.5	144	286	428	570		

[a] M^+ values in brackets were not seen in the mass spectrum of the final product; [b] The number of halogens in oligomer as derived from the isotopic pattern is shown in parenthesis; [c] These oligomers were isolated by TLC; [d] M^+ values for oligomer after elimination of the elements shown in parenthesis; [e] The mass spectral data indicated that the 4- and 5-hydroxy-N-methylcarbamate were apparently hydrolyzed to the free naphthol during oligomerization.

The significance of phenol coupling in soils has not been assessed; however, it has been shown that pesticides and their transformation products can be readily bound to soil components[8]. Enzymatic oxidative coupling may be an important reaction that results in covalent bonding of phenolic intermediates to soil organic polymers. Anilinic residues of pesticides may also be similarly bound, since peroxidases are active in the oxidation and coupling of aromatic amines. Whether the binding reactions with phenolic pesticide intermediates should be viewed as resulting in the formation of recalcitrant molecules of unknown toxic properties or as mechanisms of environmental detoxication is still open to debate.

Our results form the basis for future investigations with agrochemicals in soils in which the degree of polymerization of these chemicals as well as the extent of their incorporation into soil organic matter may be determined under various conditions. It is our suggestion that the role of phenol oxidases in determining the fate of xenobiotic chemicals in the soil environment has been widely overlooked and probably underestimated.

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Synthesis and some characteristics of [^{13}C]-specially enriched tetragastrin and the related compound[1]

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Summary. [^{13}C]-enriched tetragastrin and the related compound were synthesized in solution. Conversion of S-[^{13}C]-methylated tetragastrin to the enriched tetragastrin gave 10.5 ppm upfield chemical shift of C^{ε} resonance. The potency of the synthetic tetragastrin to stimulate gastric acid secretion was virtually identical with that of pentagastrin (ICI).

Gastrin, a gastrointestinal hormone, is an acidic heptadecapeptide amide and exists in 2 forms, which are gastrin I with Tyr residue in position 12 and gastrin II with sulfated Tyr residue in the position[3]. In both forms, 4 amino acid residues in the C-terminal, i.e., H-Trp-Met-Asp-Phe-NH_2 (tetragastrin), are known to be responsible for the full range of physiological activity of the parent hormone[4]. A number of analogues of tetragastrin have been synthesized and subjected to the investigation of structure-function relationships in the active site of gastrin. Morely[5] assumed that the Trp, Met and Phe positions in the tetragastrin participate significantly in binding the molecule with the site of physiological action. The ^{13}C-NMR-spectroscopy has become a useful tool for the study of conformations of peptides and proteins in solution. Recently, Bleich et al.[6] reported ^{13}C- and ^{1}H-NMR-studies of the tetragastrin at natural abundance using perdeuterio dimethyl sulfoxide solution. In this paper an attempt was made to synthesize the tetragastrin in which the methyl group of the methionine residue was specially enriched with [^{13}C]: H-Trp-[S-methyl^{13}C]Met-Asp-Phe-NH_2 (**I**). For comparison, the [^{13}C]-enriched tripeptide amide H-[S-methyl^{13}C]Met-Asp-Phe-NH_2 (**II**) was also synthesized. Specific enrichment with ^{13}C of the C^{ε} of the methionine residue in the peptide was performed by a method that modified the one by Jones et al.[7], which consists of methylation at the methionine sulfur with [^{13}C]-enriched methyl iodide followed by random demethylation. For the synthesis of compound **I**, protected tetragastrin, t-Boc-Trp-Met-Asp-Phe-NH_2[8], was first constructed by stepwise procedure starting from H-Phe-NH_2. The synthetic route is shown in figure 1.

The protected tetragastrin was methylated in dimethylformamide with 10fold equivalent of 90% methyl-[^{13}C] iodide. The reaction mixture was kept at pH 7.0 and allowed to stand at room temperature in dark for 24 h. An-

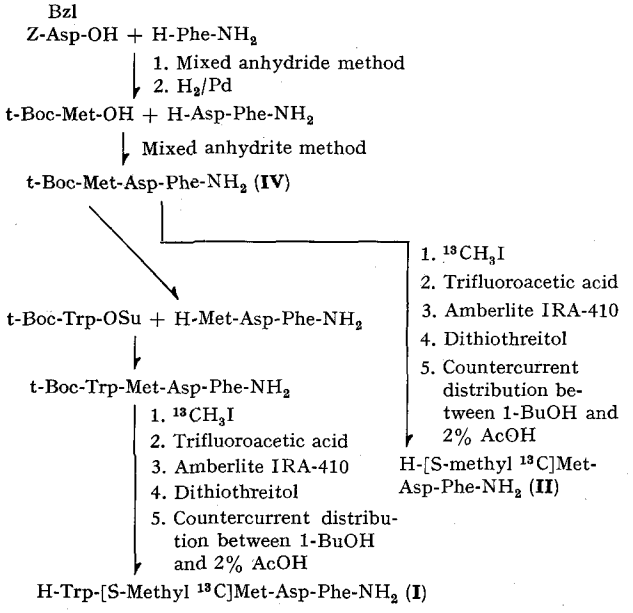

Fig. 1. Synthesis of [^{13}C]-enriched gastrin-related peptides.

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8 Abbreviations used are: t-Boc: tertiary butoxycarbanyl; BuOH: butanol; AcOH: acetic acid; DMSO-d_6: perdeuteriodimethyl sulfoxide; Bzl: benzyl ester; OSu: N-hydroxy succinimido ester; R_f^{I}- and R_f^{II}-values refer to the solvent systems: 1-BuOH-AcOH-H_2O(4:1:5) (upper layer) and 1-BuOH-pyridine-AcOH-H_2O(30:20:6:24), respectively.