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Interaction of reactive and inert chemicals in the presence of oxidoreductases: Reaction of the herbicide bentazon and its metabolites with humic monomers

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Abstract

The herbicide bentazon (3-isopropyl-1*H*-2,1,3-benzothiadiazine-4(3*H*)-one-2,2-dioxide), a relatively inert chemical, and some of its metabolites were incubated with a laccase or a peroxidase in the presence or absence of humic monomers to evaluate the incorporation of the herbicide and its metabolites into humic material by oxidative enzymes. Guaiacol and ferulic acid were used as representative electron donor co-substrates in most of the oxidative coupling reactions. Bentazon and its metabolites, with the exception of hydroxy metabolites, underwent little or no transformation by the two enzymes in the absence of guaiacol and ferulic acid, but in the presence of these co-substrates transformation occurred. The reaction of bentazon with guaiacol in the presence of the laccase or a peroxidase was almost complete in 30 min. 6-Hydroxy- and 8-hydroxy-bentazon were completely transformed by each enzyme both with and without co-substrates. At pH 3.0 and in the presence of laccase and guaiacol, the concentrations of bentazon and its metabolites 2-amino-N-isopropyl-benzamide (AIBA), des-isopropyl-bentazon and 8-chloro-bentazon decreased by 27, 57, 20 and 4%, respectively. The corresponding levels of transformation with peroxidase at pH 3.0 were 9, 70, 30 and 5%, respectively. The extent of transformation decreased with increasing pH. At low pH, the hydroxy-bentazons were completely transformed, followed by (in order of percentage transformation) AIBA, des-isopropyl-bentazon, bentazon and 8-chloro-bentazon. Transformation of bentazon by the laccase increased with increasing guaiacol concentration. In the presence of the peroxidase, the most effective co-substrates for transformation of bentazon were (in decreasing order) catechol, vanillic acid, protocatechuic acid, syringaldehyde and caffeic acid, while in the presence of the laccase, catechol was most effective, followed by caffeic acid, protocatechuic acid and syringaldehyde.

Introduction

An important problem for environmental research continues to be the removal or degradation of pesticides applied to the field or accidentally spilled in the environment. One way by which detoxification can be achieved is through the binding of pollutants to soil constituents, in particular humic substances. Because of the structural resemblance between humic acid precursors and certain pesticides or their degradation products, binding of pesticides to soil humic substances can occur during the humification process and be mediated by oxidative coupling reactions (Bollag et al. 1992). These reactions are catalyzed biologically by polyphenol oxidases and peroxidases of plant or microbial origin, and chemically by certain metal oxides and clay minerals (Bollag et al. 1992; Shindo & Huang 1982). The coupling reactions produce free radicals of the substrate or co-substrate and result in the formation of C-C, C-O, C-N and N-N linkages between humic substances and the xenobiotics or their degradation products (Bollag et al. 1992).

Research efforts to characterize the binding of soil constituents and pesticides are complicated by the heterogeneous nature of soil (Ruggiero et al. 1996; Sparks 1995). Thus model systems have proved useful for determining the types of chemical bonds and reaction mechanism involved. Typically a free enzyme is incubated with a single xenobiotic substrate in the presence of a humus constituent as co-substrate (Bollag & Myers 1992). This design was derived from the finding that the reactivity of many pollutants is enhanced by the addition of compounds that are easily oxidized by oxidative enzymes.

Bentazon, which contains benzothiadiazine, is a contact-selective herbicide for post-emergence use mainly against dicotyledonous weeds. It is known to be transformed in soil by hydroxylation or SO_2 elimination; the resulting degradation products can then be incorporated into soil humic substances (Huber & Otto 1994). The half-life (average DT_{50} value) of bentazon in soil under laboratory conditions is about 46 days. The bentazon residues left in edible plant parts at the time of harvest are bentazon, 6-hydroxy-bentazon and 8-hydroxy-bentazon. Traces of 2-amino-N-isopropyl-benzamide (AIBA) can be found in soil in addition to the hydroxy-bentazons (Otto et al. 1978). However, because these three metabolites of bentazon are degraded microbially more rapidly than they are formed, they are not easily detectable.

Although bentazon incorporated in humus is not bioavailable to plants and earthworms (Lee et al. 1988; Ebert 1992), it is not yet clear which interactions take place between the herbicide or its metabolites and humic substances. Therefore the objective of this study was to determine whether bentazon, a relatively inert chemical and its metabolites can be transformed in the presence of humic monomers and bound to humic monomers in the presence of oxidative enzymes. In the model system employed, guaiacol and ferulic acid, phenolic acids derived from lignin degradation, were used as representative electron donor co-substrates in the oxidative coupling reactions.

Materials and methods

Chemicals

Bentazon and its metabolites, 6-hydroxy-bentazon, 8-hydroxy-bentazon, 2-amino-N-isopropyl-benzamide (AIBA), 8-chloro-bentazon and des-isopropyl-bentazon, were obtained from BASF AG (Germany). The chemical structures are shown in Figure 1. Guaiacol, caffeic acid, phloroglucinol and vanillic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI), and ferulic acid and protocatechuic acid were from Sigma Chemical Co. (St. Louis, MO). *p*-Hydroxybenzoic acid, resorcinol, syringaldehyde, syringic acid and vanillin were from Fluka AG (Buchs, Switzerland). Catechol and pyrogallol were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

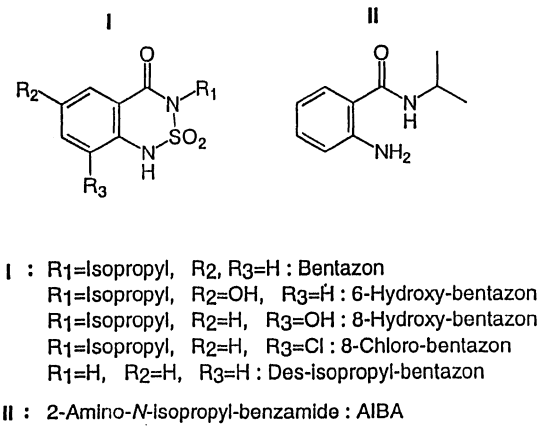

Figure 1. Chemical structures of bentazon and its metabolites.

Enzymes

Laccase of the fungus *Polyporus pinsitus* was obtained from Novo Nordisk Bioindustrials, Inc. (Danbury, CT). Laccase activity was determined spectrophotometrically by using 2,6-dimethoxyphenol as substrate. One unit was defined as the amount causing a change in optical density of 1.0/min at 468 nm and 20 °C. Horseradish peroxidase (HRP) (EC 1.11.1.7) (Rz 1.1) was purchased from Sigma (St. Louis, MO). Reactions catalyzed by HRP were performed with an enzyme concentration of 0.087 purpurogallin units/ml. A purpurogallin unit is defined as the amount of enzyme that forms 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 and 20 °C as determined from its absorption at 420 nm. One percent hydrogen peroxide was added as a co-enzyme to the HRP reactions.

Enzymatic assays

Incubations were carried out at 25 °C with 1 mM bentazon or it metabolites in 5 ml 0.1 M citrate-phosphate buffer at various described levels of pH. The reaction mixes contained 4 units/ml *P. pinsitus* laccase or 6

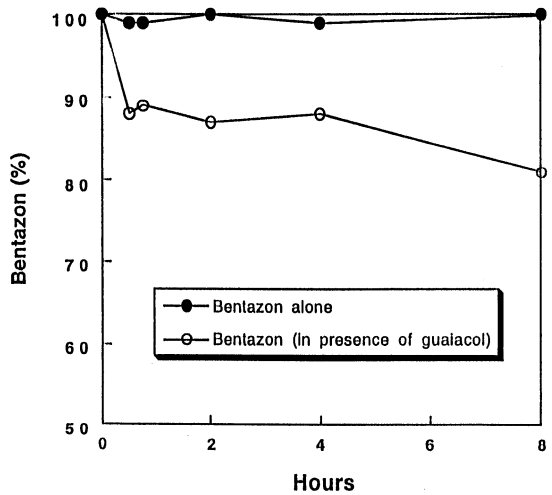

Figure 2. Time course for the reaction of bentazon with guaiacol in the presence of the *P. pinsitus* laccase at pH 4.0.

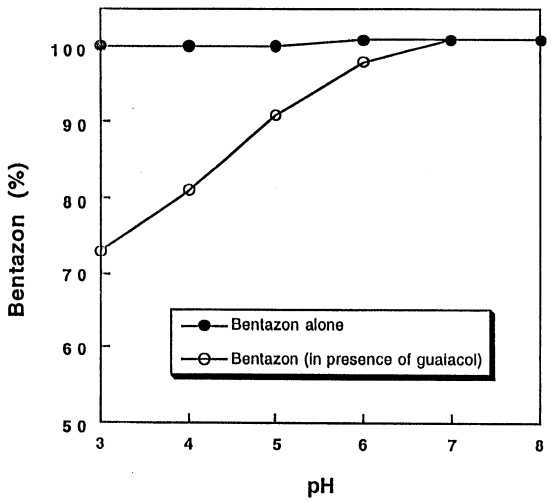

Figure 3. Reaction of bentazon with guaiacol in the presence of the *P. pinsitus* laccase as a function of pH.

units/ml HRP, and in many cases included guaiacol, ferulic acid or other humic monomers as indicated. Unless otherwise specified, the concentration of these co-substrates was 1 mM, and the incubation times for laccase and HRP were 24 and 2 hr, respectively. Boiled enzymes were used as controls. Reactions were stopped with acetic acid, and reaction mixtures were centrifuged at about 12,000 g for 10 min to remove precipitates. The supernatants were filtered twice with 2 ml methanol through a 0.45 μm nylon membrane and adjusted to a final volume of 10 ml with Milli Q water. The filtrate was analyzed by high-performance liquid chromatography (HPLC). Reaction conditions were varied to determine the effects of pH, incubation time, and type and concentration of co-substrates.

HPLC analysis

Analysis was performed on a Waters Associates HPLC system (Milford, MA) equipped with a Model 6000A and a Model 501 solvent delivery system, a U6K septumless injector and a Lambda-Max Model 440 Absorbance Detector operating a 254 nm. A 4.6 mm $\times$ 25 cm reverse-phase column (Nucleosil 120 5C18, Bischoff) was used. An isocratic system was employed, with a mobile phase of 50/50 (v/v) methanol/1% acetic acid and a flow rate of 0.8 ml/min.

Results

When bentazon alone was incubated with the laccase at pH 4.0 for 24 hrs, no transformation occurred, but in the presence of guaiacol the concentration of bentazon was reduced by 12% in 30 min and 19% in 8 hrs (Figure 2). The time course for the reaction of bentazon by laccase in the presence of guaiacol demonstrated that after a rapid initial decrease in bentazon concentration, a much slower secondary phase of continued removal occurred over 8 hrs.

Over the pH range 3.0 to 8.0, bentazon was not transformed by the *P. pinsitus* laccase in the absence of guaiacol, but addition of guaiacol led to pH-dependent transformation (Figure 3). Bentazon concentration decreased by 27% at pH 3.0, 19% at pH 4.0, and 9% at pH 5.0, while negligible transformation occurred at pH 6.0, 7.0 and 8.0.

When the effect of guaiacol concentration (0 to 50 mM) on bentazon transformation was examined at pH 4.0 in the presence of the laccase, it was found that bentazon concentration decreased by 19, 25, 33, 46 and 57% at guaiacol concentrations of 1, 5, 10, 20 and 50 mM, respectively (Figure 4). Thus transformation was lowest when bentazon and guaiacol were equimolar at 1 mM.

These experiments were extended to include the study of bentazon metabolites, and the results of incubations both with and without co-substrates are presented in Table 1. Like bentazon, des-isopropyl-bentazon, 8-chloro-bentazon and AIBA were not trans-

Table 1. Reaction of bentazon and its metabolites with guaiacol or ferulic acid in the presence of the *P. pinsitus* laccase (incubation time: 24 hours) and horseradish peroxidase (incubation time: 2 hours)

Compound	% Transformed by laccase						% Transformed by peroxidase					
	Control[a]		Guaiacol		Ferulic acid		Control[a]		Guaiacol		Ferulic acid	
	pH 3.0	pH 5.6	pH 3.0	pH 5.6	pH 3.0	pH 5.6	pH 3.0	pH 6.0	pH 3.0	pH 6.0	pH 3.0	pH 6.0
Bentazon	0	0	27	0	9	0	6	0	9	0	19	0
6-Hydroxy-bentazon	100	100	100	100	100	100	100	100	100	100	100	100
8-Hydroxy-bentazon	100	100	100	100	100	100	100	100	100	100	100	100
AIBA	6	0	57	34	16	40	10	11	70	10	30	40
Des-isopropyl-bentazon	0	0	20	3	12	0	10	12	30	5	15	13
8-Chloro-bentazon	0	0	4	3	0	0	5	0	5	0	10	12

[a] Without co-substrate.

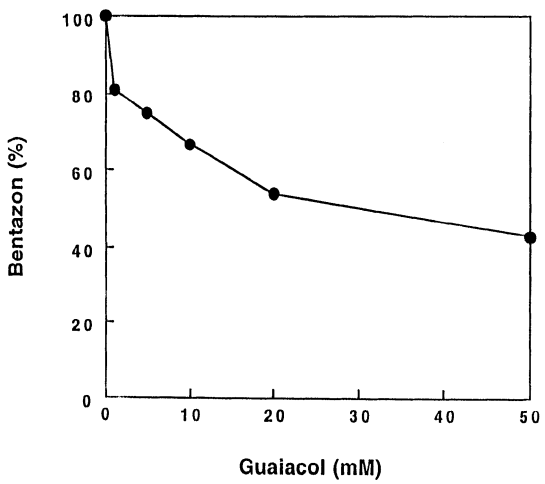

Figure 4. Effect of guaiacol concentration on the transformation of bentazon in the presence of the *P. pinsitus* laccase at pH 4.0.

formed by the laccase in the absence of guaiacol or ferulic acid, at either pH 3.0 or pH 5.6. However, in the presence of guaiacol and at pH 3.0, transformation levels for bentazon, AIBA, des-isopropyl-bentazon and 8-chloro-bentazon by the laccase were 27, 57, 20 and 4%, respectively. At pH 5.6, transformation of AIBA was 34%, and that of bentazon, des-isopropyl-bentazon and 8-chloro-bentazon was negligible. At pH 3.0 and in the presence of ferulic acid, the transformation by *P. pisitus* laccase was 9% for bentazon, 16% for AIBA and 12% for des-isopropyl-bentazon. 8-Chloro-bentazon was not transformed under these conditions. At pH 5.6 and in the presence of ferulic acid, AIBA was 40% transformed by the laccase, while no transformation of bentazon, des-isopropyl-bentazon and 8-chloro-bentazon occurred. Transformation of 6- and 8-hydroxy-bentazon by the laccase was complete under all experimental conditions indicated.

A similar pattern was observed for reactions with horseradish peroxidase (Table 1). When bentazon, AIBA, des-isopropyl-bentazon and 8-chloro-bentazon were incubated with HRP in the absence of humic monomers, only slight or no transformation occurred, whereas 6- and 8-hydroxy-bentazon were completely transformed regardless of pH and the presence or absence of humic monomers. In the presence of guaiacol, transformation of bentazon was very low at pH 3.0 and non-existent at pH 6.0. Transformation levels for AIBA, des-isopropyl- and 8-chloro-bentazon at pH 3.0 were 70, 30, and 5%, respectively, but at pH 6.0, transformation was negligible. As in the case of laccase, removal of AIBA by peroxidase was higher than that of bentazon. In the presence of ferulic acid, 19% of bentazon was transformed at pH 3.0, and no transformation occurred at pH 6.0. Thirty and 40% transformations of AIBA were observed at pH 3.0 and 6.0, respectively. Slight transformation of des-isopropyl- and 8-chloro-bentazon occurred in the presence of ferulic acid at both pH values.

The effect of a variety of additional co-substrates, differing as to chemical structure, was also evaluated (Table 2). Several of these proved to be more effective than guaiacol and ferulic acid. Bentazon transformation in the presence of laccase was greatest for catechol (100%), followed by 59% for caffeic acid, 40% for protocatechuic acid and 39% for syringaldehyde. In the presence of HRP, bentazon was transformed 90% by catechol, 94% by vanillic acid, 65% by protocatechuic acid, 49% by syringaldehyde and 27% by caffeic acid.

Table 2. Reaction of bentazon with humic monomers in the presence of a laccase (incubation time: 24 hours) or a peroxidase (incubation time: 2 hours)

Humic monomers	Bentazon transformed (%)	
	Laccase (pH 4.0)	Peroxidase (pH 3.0)
Caffeic acid	59	27
Catechol	100	95
Ferulic acid	9	19
Guaiacol	18	9
p-Hydroxybenzoic acid	0	8
Phloroglucinol	7	0
Protocatechuic acid	40	65
Pyrogallol	0	0
Resorcinol	2	0
Syringaldehyde	39	49
Syringic acid	0	3
Vanillic acid	6	94
Vanillin	6	13

Discussion

In previous work Kim et al. (1996) observed that, after a 56-day incubation of ^{14}C-bentazon with soil, approximately 70% of the initially applied compound was bound to the soil matrix. Forty-two percent of bound residues were detected in fulvic acid, 30% in humic acid, and 28% in humin. It has also been shown that chlorophenols, chloroanilines and phenolic acids can be incorporated into humic substances in the presence of oxidative coupling enzymes during the humification process (Bollag et al. 1992). In studies such as these, an understanding of reaction mechanisms can be facilitated through the use of model systems in which a pesticide substrate is incubated with an oxidative enzyme and single humus constituent (Bollag & Myers 1992). The present research applied this approach to examine the transformation of bentazon and its metabolites.

When bentazon, AIBA, des-isopropyl-bentazon and 8-chloro-bentazon were incubated with laccase in the absence of co-substrates, minimal or no transformation was observed. However, with addition of the humic monomers guaiacol or ferulic acid, transformation in most cases increased substantially. Thus, as found previously by Roper et al. (1995), introduction of co-substrates capable of reacting readily with the enzyme enhanced removal of the less active pollutants. This reaction with recalcitrant chemical was possible because of the involvement of free radicals which had been enzymatically generated from reactive compounds.

The extent of transformation depended upon the chemical structure of the substrate, the type and concentration of co-substrate, and pH of the reaction mixture. Bentazon, AIBA, des-isopropyl-bentazon and 8-chloro-bentazon were considerably less reactive than the hydroxy metabolites (Table 1), and results with a variety of different humic monomers were variable (Table 2). The difference in the reactivity of various substrates appears to be related to the coupling potential of the side groups; e.g., the hydroxyl group of hydroxy-bentazon is more reactive than a side chain such as a methyl group. Some co-substrates proved to be even more effective than guaiacol and ferulic acid; for example, bentazon transformation using catechol (with laccase and HRP) and vanillic acid (with HRP) equaled or exceeded 95%. For guaiacol, bentazon transformation was relatively low (19%) when both substrates were equimolar at 1 mM, but improved with increasing concentration of the humic acid monomer (Figure 4).

Maximum transformation of bentazon by the *P. pinsitus* laccase in the presence of guaiacol occurred at pH 3.0 (Figure 3). Optimal activity for this enzyme occurs over a rather narrow pH range and is substrate-dependent, previous work demonstrating that the most favorable pH levels for reactions with syringaldazine and 2,6-dimethoxyphenol are 5.6 and 4.0, respectively (Dec & Bollag 1990). The optimal pH for transformation by HRP was approximately 3.0 in the case of bentazon and 4.0 for AIBA (data not shown). Previous work demonstrated that optimal activity for HRP occurred at pH 5.0 (Dec & Bollag 1995); however, the optimal pH varied with different substrates (Dec & Bollag 1990). For example, the most effective pH values for 4-chlorophenol and 2,4-dichlorophenol transformation were 5.4 and 6.1, respectively. HRP is effective in removing a variety of chemical over a broad pH range (3.0–12.0) (Dec & Bollag 1990), a characteristic which allows HRP-mediated enzymatic coupling to occur in various soil environments.

The incubation time for maximal transformation of bentazon and its metabolites by laccase and HRP was found to be relatively short. For example, most of the bentazon transformation by the laccase in the presence of guaiacol was achieved within the first 30 min of incubation (Figure 2), a time frame similar to that reported by Dec & Bollag (1995) for removal of 2,4-dichlorophenol by purified HRP. In addition we found that partial transformation of AIBA and com-

plete transformation of 6- and 8-hydroxy-bentazon occurred within 5 min (data not shown).

It is probable that the transformation of bentazon, AIBA, des-isopropyl-bentazon and 8-chloro-bentazon in the presence of co-substrates involved a mechanism similar to that described by Bollag et al. (1995), who showed that in the first step of oxidative coupling reactions susceptible aromatic compounds are oxidized to form free radicals. These unstable intermediates then proceed to react with nearby molecules to form dimers and larger oligomers. Furthermore it has been suggested that free radicals generated from reactive substrates can couple not only to themselves but also to molecules of relatively resistant compounds (Klibanov et al. 1983; Bollag et al. 1992; Roper et al. 1995). Accordingly, we postulate that in the present study guaiacol and ferulic acid were rapidly oxidized to free radicals by the enzymes, and that these oxidation products reacted with bentazon and its metabolites to result in enhanced precipitation. The initial step in the oxidation of these phenolic co-substrates likely involved removal of an electron and hydrogen ion from the hydroxyl group, generating an alkoxy free radical capable of coupling with itself as other compounds. Such a reaction mechanism has been previously demonstrated with guaiacol by Simmons et al. (1988), and likely also underlies the rapid precipitation of hydroxy-bentazons in the present study, even in the absence of co-substrates. Confirmation of these hypotheses awaits further research.

In summary, we have shown that bentazon and several of its less reactive metabolites underwent negligible transformation when incubated with laccase and HRP unless reactive humic monomers such as guaiacol or ferulic acid were also present, in which case transformation was considerably enhanced. The combined use of oxidative enzymes and humic monomer co-substrates thus shows promise as a valuable tool for bentazon bioremediation.

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