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Formation and Properties of Organo-Phosphatase Complexes by Abiotic and Biotic Polymerization of Pyrogallol−Phosphatase Mixtures

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In this paper, the catalytic efficacy of peroxidase and manganese oxide, both commonly present in soil, to catalyze the formation of pyrogallol−phosphatase complexes was compared. The influence of several factors (e.g., the concentration of pyrogallol, the amount of catalysts, the nature of manganese oxide, birnessite, or pyrolusite, the incubation time, and the pH) on the transformation of pyrogallol and the characteristics and properties of the pyrogallol−phosphatase interaction products were investigated. The pyrogallol transformation mediated by both catalysts was very fast and increased by increasing the catalyst concentration. The nature of the catalyst also influenced the size and the molecular mass of the formed complexes. When polymerization of pyrogallol occurred with high intensity, a loss of phosphatase activity occurred, and it strongly depended on the pH at which the process was carried out and the catalyst. In particular, with peroxidase, the phosphatase activity was much lower in either suspensions or supernatants and not measurable in the insoluble complexes as compared to that measured in the presence of manganese oxides.

KEYWORDS: Pyrogallol; birnessite and pyrolusite; peroxidase; acid phosphatase; oxidative polimerization; soil enzymes

INTRODUCTION

Exogenous substances of different chemical and structural complexities as well as endogenous inorganic and organic soil components (e.g., clays, oxides, humus constituents or their precursors, or even enzymes secreted by microorganisms and/or plant roots) may be simultaneously present in soil environments. In this complex scenario, various interactions among these components may occur. They may be governed by different mechanisms and lead to different reaction products.

Among exogenous compounds arriving to the soil, phenols (mono- and polysubstituted phenols) cover an important role because of their negative, toxic effects (e.g., carcinogenic and mutagenic activities) on soil-living organisms and their main involvement in the formation of humus material (*1* and references therein).

In the environment, phenolic monomers may be transformed into polymeric products or simpler compounds in the presence of either Fe_2O_3, MnO_2, soil, and clays or phenoloxidases and peroxidases (PODs), that is, oxidoreductase enzymes produced by plants, bacteria, and fungi, behaving as abiotic or biotic catalysts, respectively (*2*). Both of the processes may be affected by the pH, temperature, catalyst amount, contact time, and simultaneous presence of several catalysts. The net resulting effect of the process may be an efficient removal of the (toxic) phenols (e.g., a natural decontamination of the environment) and a concomitant formation of more complex products such as polymers or polymeric aggregates (e.g., the formation of humic substances) (*3*). Besides polyphenols, other molecules present in the soil, but extraneous to the oxidative process, may be involved in it and/or affect to a variable extent the whole process. In particular, if active proteins are involved, protein−organic or protein−organo mineral complexes with different structural and functional properties may form.

In this paper, we compared the catalytic efficiency of a biotic catalyst, POD, and an abiotic catalyst, manganese oxide, to form acid phosphatase (AP)−pyrogallol (P) interaction products under different environmental conditions. The influence of several factors (e.g., the concentration of P, the amount of catalysts, the nature of the manganese oxide, birnessite (Bir) or pyrolusite (Pyr), the incubation time, and the pH) on the oxidative transformation of P and the characteristics of the P and P−phosphatase reaction products were investigated.

The interest for this type of interactions relies on several considerations. To our knowledge, no comparative investigations on the catalytic efficacy of POD and manganese oxides on the formation and the catalytic properties of P−phosphatase complexes have been performed. P is a very common humus starting material and is frequently present in soil because it is a derivative of lignin transformation (*4*). PODs are important oxidative enzymes, catalyzing the oxidation of numerous compounds, and are very effective agents in the oxidation and transformation of phenolic compounds (*1*). They are widespread in nature because they are produced by plants and microorganisms. In soils, these enzymes may play an important role in the rhizosphere (*5*), and they usually increase in the presence of phenolic

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compounds (*6*). Manganese oxides are very reactive components, widely distributed in soils of different origins. They are known to be very effective oxidants with respect to the abiotic formation of humic substances from natural phenolic compounds in the environment over the pH range common in soil (*7*). In soil, phosphatases, extracellularly secreted by plants and microorganisms, play a key role in the phosphorus cycle, allowing the formation of inorganic phosphorus, the only phosphate form taken up by plants and microorganisms. The synthesis of extracellular phosphatases is usually the response of plants to phosphorus deficiency. These enzymes are usually not free in solution but are associated with soil constituents.

MATERIALS AND METHODS

Chemicals. AP (E.C. 3.1.3.2) from potato tubers was a commercial preparation [1 mg mL^{-1} water solution, 50 enzymatic units (EU), where 1 EU hydrolyzes 1 μmol of *p*-nitrophenylphosphate (*p*NPP) per min at pH 4.8 at 37 °C] produced by Sigma Aldrich (Italy).

P was in the form of amorphous powder by Sigma Aldrich (Italy). All other chemicals, reagent grade, were supplied by Analar, BDH Ltd. (Germany), unless otherwise stated. All solutions were prepared with high-performance liquid chromatography (HPLC) grade water.

Catalysts. POD (EC 1.11.1.7, MM of 44 kDa) was a commercial preparation from horseradish purchased from Sigma Aldrich (Italy), containing 1.100 EU for mg of solid [1 EU oxidizes 1 μmol of 2,2′-azino-bis-(ethylbenzothiazoline-6-sulfonic acid) (ABTS) per min at 25 °C and pH 5.0]. Two manganese oxides were used. A commercial MnO_2 preparation from Aldrich (Italy) showing X-ray diffraction peaks characteristic of Pyr (γMnO_2) and a manganese oxide were synthesized as described by McKenzie (*8*). The material was characterized by X-ray diffraction analysis and showed a poorly crystalline nature and peaks characteristic of Bir (δMnO_2).

P−Phosphatase Complexes by POD and Manganese Oxide Catalysis. P, phosphatase, and POD (P-AP-POD samples) were incubated in different trials according to the procedure by Sarkar and Burns (*9*). Four milliliters of 1% hydrogen peroxide and 5.0 mL of P solution (1.5 mg mL^{-1} in 0.1 M Na-acetate buffer at pH 5.0) were added to 0.05 mL of POD solution at different initial concentrations (0.05, 0.20, 0.45, 0.90, and 2.0 mg mL^{-1}) in the same buffer. Thereafter, 0.05 mL of 1 mg mL^{-1} AP and 0.9 mL of buffer solution were added to have a final volume of 10 mL.

For the preparation of P−phosphatase−manganese oxide mixtures (P-AP-Bir/P-AP-Pyr samples), increasing quantities (5, 10, and 20 mg) of either Pyr or Bir were added to 5.0 mL of P (1.5 mg mL^{-1} in 0.1 M acetate buffer) supplemented with 0.05 mL of 1 mg mL^{-1} AP and with 4.95 mL of buffer for a final volume of 10 mL. The sequence of component addition should have allowed the enzymatic molecules to reach the system when the P polymerization by POD or MnO_2 was already started (*9*). Five binary mixtures, P-AP, AP-POD, AP-MnO_2, P-POD, and P-MnO_2, and two controls containing only P or AP were incubated under the same conditions to evaluate the fate of each component during the incubation period.

After a preincubation at 10 °C, the samples were manually shaken and incubated in a thermostatic bath at 10 °C and in the dark, to avoid any interference by light on the hydrogen peroxide decay and the P polymerization. After 4 h of incubation, the mixtures were centrifuged at 10000 rev min^{-1} for 30 min using a Sorvall SA600 Rotor in a Sorvall RC-5B Refrigerated Super Speed Centrifuge. Solid (precipitates) and liquid (supernatants, Sn) phases were separated and further analyzed. The solid phases, when present, were suspended in 4 mL of acetate buffer (1:2.5 diluted with bidistilled water to ensure the ratio water:buffer of the initial solutions) for further measurements.

The Sn of the P-AP-POD and P-AP-Bir/P-AP-Pyr mixtures were analyzed for UV−visible spectra, soluble manganese and residual P by UV−vis spectrometry, atomic absorption spectroscopy (AAS), and HPLC, respectively, as described below. Before HPLC analysis, the Sn were filtered through Acrodisc LC13 PVDF 0.45 μm (Millipore) filters, which specifically do not adsorb phenols and phenol derivatives. The residual activity of POD in the Sn was also measured by incubating suitable amounts of samples with ABTS as the substrate. The standard conditions adopted were as follows: 10 mM ABTS and 0.01% (w/w) H_2O_2 in 0.1 M Na-acetate buffer at pH 5.0 and 25 °C. A sample with only ABTS and buffer served as the control. P-enzyme complexes by POD or Pyr catalysis were also prepared at pH 4.0 and processed as described above.

Fractionation of Soluble P−Phosphatase Polymerization Products. The Sn of P-AP-POD and P-AP-Bir/P-AP-Pyr samples were ultrafiltered by using Amicon Ultra-4 Centrifugal Filter Units (Millipore) with cellulose regenerated membranes with molecular mass cutoffs of 50000 and 100000 Da in a Hermle Z364 centrifuge. Phosphatase activity assays were run on each permeate and retentate obtained with the two membranes. A similar procedure was applied to AP-POD and AP-Bir/P-Pyr samples.

Phosphatase Activity Assays. The activity of free and immobilized phosphatase was measured by incubation at 10 °C in a suitable volume of free enzyme solution or immobilized enzyme suspension (generally 0.030 or 0.050 mL) with 1 mL of 6 mM PNP in 0.1 M Na-acetate buffer at pH 5.0, optimal pH (*10*). Both the substrate and the enzymatic solutions were previously thermostatted at 10 °C. After 20 min of incubation, 1 M NaOH was added to stop the hydrolysis reaction, and the concentration of *p*-nitrophenol (*p*NP) (molar extinction coefficient, 18.5 mM^{-1} cm^{-1}) was determined by direct reading of the absorbance at 405 nm, the maximum absorbance wavelength of *p*NP. One unit of enzyme activity was the amount of enzyme that hydrolyzes 1.0 μmol of PNP per min per mL at pH 5.0 and 10 °C.

Interference Tests. P and its polymeric products, obtained after catalytic oxidative transformation, were tested for possible interference (i.e., increase or decrease of optical density as function of their presence) with the analytical method adopted for evaluating *p*NP concentration. Increasing concentrations of P, ranging from 0 to 0.7 mM (the highest concentration achieved in the *p*NP assay, see below) were added to *p*NP solutions at concentrations ranging from 0.015 to 0.052 mM. The concentrations of *p*NP were those possibly formed from *p*NPP under the experimental conditions adopted in phosphatase activity assays. Both of the solutions were previously thermostatted at 10 °C. Afterward, 1 mL of 1 M NaOH was added, and the absorbance of the *p*NP-P-NaOH solutions was measured at 405 mM; UV−vis spectra of the various solutions were also run to evaluate possible bathocromic and hypsochromic effects. The absorbance of *p*NP-P-NaOH solutions was also measured at 405 nm at different incubation times (0−24 h) from the addition of NaOH.

Inhibition Tests. The influence of P on the phosphatase activity was determined by carrying out at 10 °C and pH 5.0 activity assays with *p*NPP concentrations ranging from 0.2 to 6 mM in the presence of increasing P (0−0.7 mM) concentrations. The experimental data were elaborated according to the Michalis−Menten equation, and the kinetic parameters were calculated by a computed nonlinear regression analysis.

HPLC and UV Analyses. P was determined by HPLC analysis. The HPLC analysis was performed with a Shimadzu instrument equipped with a pump and a variable wavelength absorbance detector set at 280 nm for P. A Spheri-5-RP18 220 mm × 4.6 mm C18−80 BrownLee (Chebios. Italy) column with a 5 μm particle size, and a BrownLee Spheri-5-RP300 7 μm particle size (30 mm × 4.6 mm) guard column were used. Isocratic elution was performed at a flow rate of 1 mL min^{-1} with a mobile phase composed of acidified water (3 mL of H_3PO_4 in 1 L of water, adjusted at pH 2.95 with 0.5 M NaOH) and acetonitrile at a ratio of 70:30 (v:v). The retention time for P was 3.5 min.

The UV−vis spectra of products remaining in the Sn after reaction of the P and P-phosphatase mixture with either POD or MnO_2 were obtained with scanning from 900 to 200 nm using a Perkin-Elmer spectrophotometer Lambda 25. The ratios between absorbance measured at 465 and 664 nm (E_4/E_6) (*11*) were also determined.

AAS Analyses. AAS measurements of soluble Mn were performed at 279.5 nm with a Perkin-Elmer Analyst 700 by using Flam Adsorption Spectroscopy. Before each investigation, standard solutions (0.5, 1.0, and 2.0 mg L^{-1}) were freshly prepared by diluting the $MnCl_2 \cdot 4H_2O$ standard solution in HCl. The detection limit for Mn^{2+} determination was 0.0082 mg L^{-1}. All of the experiments were carried out at least in triplicate, and the relative standard deviation was determined.

RESULTS AND DISCUSSION

Interference and Inhibition Tests. *Interference between P or P Products and pNP.* Preliminary experiments were devoted to verify the possible interference, if any, of the P and P products

Table 1. Residual P (% ±SD) with Time by Different Amounts of POD in the Absence and Presence of AP

	P (%)									
	POD (mg mL^{-1})									
	0.05		0.20		0.45		0.90		2.0	
time (min)		+ AP		+ AP		+ AP		+ AP		+ AP
0	100 ± 4	100 ± 5	100 ± 3	100 ± 2	100 ± 2	100 ± 3	100 ± 2	100 ± 3	100 ± 2	100 ± 2
5	37 ± 2	52 ± 2	19 ± 0	23 ± 1	3 ± 0	30 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
20	42 ± 2	49 ± 1	13 ± 0	20 ± 0	4 ± 0	12 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
40	43 ± 1	43 ± 2	11 ± 1	21 ± 1	5 ± 0	17 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
60	39 ± 1	43 ± 3	10 ± 1	21 ± 1	8 ± 0	20 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
120	53 ± 2	41 ± 2	9 ± 0	15 ± 0	3 ± 0	20 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
180	34 ± 2	31 ± 1	10 ± 1	15 ± 0	0 ± 0	14 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
240	47 ± 1	32 ± 1	8 ± 1	15 ± 1	0 ± 0	12 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

obtained by biotic and abiotic oxidative polymerization with the analytical measurement of *p*NP, the end product of the AP action.

As described in the Materials and Methods, *p*NP, the product of *p*NPP hydrolysis, is quantitatively measurable by the absorbance at 405 nm of its anionic form, *p*-nitrophenate, colored in yellow and formed by the addition of NaOH. However, at alkaline pH, P and its polymeric derivatives may give rise to colored products, possibly also absorbing at 405 nm. An overestimation of *p*NP concentration and in turn of AP activity may result.

Absorbance values at 405 nm of alkaline *p*NP-P mixtures and *p*NP-P Sn obtained after POD or MnO_2 action, see below, did not differ from those of *p*NP solutions. Moreover, UV−vis spectra of the mixtures indicated that no detectable bathochromic or hypochromic effects occurred by P on the *p*NP maximum absorbance (data not shown). Results clearly indicate that no significant interference between *p*NP and P or P polymeric derivatives occurs, at least within the range of the concentrations that we investigated.

These results can be considered reliable only when the absorbance measurements were carried out within 1 h of the NaOH addition. Indeed, the absorbance of alkaline *p*NP-P mixtures, measured at different relative concentrations of the two phenols and at different times since NaOH addition, demonstrated that absorbance at 405 nm was stable within 1 h. Thereafter, it started to decline with time. After 24 h of incubation, a decrease ranging from 18 to 25% of the initial absorbance was monitored. The effect was similar whether the test tube was hermetically sealed by parafilm or not. Therefore, all experimental runs reported below absorbance at 405 nm were measured within 1 h from the alkalinization procedure.

Inhibition of P on AP Activity. When the activity of AP was assayed at both increasing substrate (0.0−0.6 mM) and P (0.15, 0.3, 0.5, and 0.7 mM) concentrations, all data conformed to Michaelis−Menten kinetics, thus allowing the kinetic parameters V_{max} and K_m to be calculated (data not shown). The activity and kinetics of free phosphatase were not significantly influenced by P in the range 0.15−0.3 mM. At these concentrations, no changes of V_{max} and K_m (18.6 μmol mg^{-1} min^{-1} and 0.23 mM, $r^2 = 0.999$, respectivley) values were detected. By contrast, at P concentrations higher than 0.5 mM, an inhibition effect was measured. At 0.7 mM P, V_{max} decreased from 18.6 to 16.8 μmol mg^{-1} min^{-1} and K_m increased from 0.23 to 0.37 mM. According with the classical kinetic inhibition models, P behaved as a noncompetitive inhibitor of the AP. Similar inhibitory effects on AP activity were observed with other phenolic molecules such as tannic acid. Typical enzymatic inhibition studies showed that 2.0×10^{-2} mM tannic acid gave 50% inhibition of AP (*12*). A similar inhibition was measured at tannic acid concentrations of 5.0×10^{-2} and 4.8×10^{-1} mM with urease and invertase enzymes, respectively (*12*, *13*). A great affinity between tannic acid and AP was also demonstrated by Hsu et al. (*14*), who used tannic acid in the purification of AP from potato.

A complexation phenomenon of AP by phenolic compounds (*15*) is suggested to explain their inhibition effects, usually prevented to a different extent by the presence of carbohydrate residues in the protein molecule. Evidently, AP, a glycoprotein with only 17% of carbohydrate content, presented weak resistance to the complexation phenomenon by P and tannic acid (*15*). To avoid any inhibition effect of P on the AP activity, all experiments reported below were run at concentrations of P lower than 0.3 mM in the activity assay specimens.

P Polymerization by POD and Manganese Oxides with or without AP. *Tests with POD*. The residual P concentrations detected in the samples incubated at pH 5.0 and 10 °C with different concentrations of POD (0.05, 0.2, 0.45, 0.9, and 2.0 mg mL^{-1}) with and without phosphatase and at different incubation times are reported in **Table 1**. P transformation was very fast independently of the catalyst concentration. Indeed, 37% of P remained in solution in less than 5 min and with the smallest quantity of POD (0.05 mg mL^{-1}).

The amount of P transformed increased by increasing the catalyst concentration. At the highest POD concentrations (0.9 and 2 mg mL^{-1}), the phenol transformation was complete, regardless of the presence of AP or not. The process was practically constant at more elevated incubation times. The corresponding chromatograms of P-POD and P-AP-POD samples, as referred to the controls P and AP, showed the complete absence of the peak at P retention time (i.e., the complete removal of P), and several intense peaks in the range between 0 and 5 min retention time appeared (data not shown), indicative of products at higher polarity (*16*).

At low POD concentrations and short incubation times, a greater concentration of P usually remained in the solution in the presence of phosphatase, thereby indicating that the interaction between P and POD was possibly hindered by phosphatase molecules (**Table 1**). As reported above, phenolic molecules may give rise to stable interactions with proteins. Preferential interactions of P with phosphatase rather than with POD might have occurred. As a result, a minor number of P molecules were available for reacting with POD and remained in solution. The effect was overwhelming at higher POD concentrations.

When the POD activity was measured in the Sn of P-POD and P-AP-POD samples, the residual POD activity was completely absent at any used POD concentration, indicating a complete loss of the POD activity after the P polymerization process (*17*). Similar results were observed with laccase, another oxidative enzyme, and phenolic molecules. A reduction of laccase activity

was usually detected in treatments showing a measurable removal of phenolic substrates (*18*−*21*). The greater the phenol transformation, the higher the laccase activity reduction was (*19*−*21*). In this case, the disappearance of active protein molecules complementary to the polyphenol oxidation may be hypothesized. Active enzymatic molecules may be removed from the solution and incorporated (entrapped, adsorbed, and/or bound) into the newly formed polymeric products. A partial and/or complete loss of enzymatic activity may result from the immobilizing process. A direct denaturation by the phenolic compounds toward the enzyme cannot be, however, ruled out.

Figure 1 shows the UV−vis spectra of the Sn of P-AP and P-AP-POD samples obtained at different POD concentrations. With respect to the control P-AP, P-AP-POD samples showed a bathocromic effect with absorbance peaks at 239 nm varying in their intensity at the different catalyst concentrations. A broad, very intense absorbance area at 289−490 nm was also visible. This effect can be attributed to the increase of quinonic intermediates and their byproducts that have maximum absorbance at 240−255 and 285−295 nm (*22*). Furthermore, the occurrence of the broad, very intense absorbance area at 289−490 nm could still be indicative of the presence of products with highly condensed aromatic rings and their derivatives (*23*).

Correspondingly, elevated E_4/E_6 values were measured in the various supernatant solutions. They ranged from 24 and 28 for P-POD and P-AP-POD at 0.9 mg mL^{-1} POD up to 30 at 2 mg mL^{-1} POD. As early indicated by Chen et al. (*11*), the E_4/E_6 ratios give information on the size and molecular mass of humic substances. The greater the E_4/E_6 ratio, the lower the degree of condensation of aromatic constituents was.

Tests with Bir and Pyr. Also, with the abiotic catalysts, the transformation of P was rather fast at the beginning of reaction,

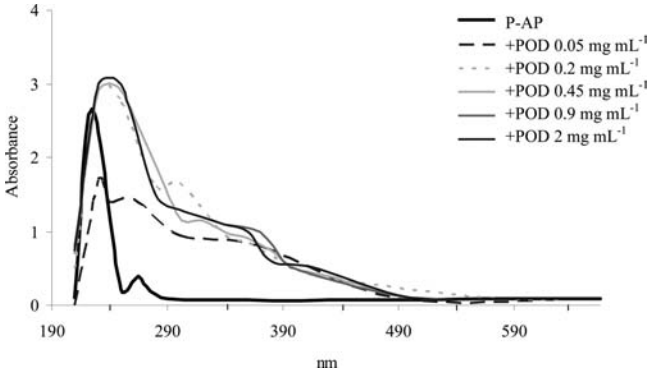

Figure 1. UV−vis spectra of the Sn (1:10 diluted) of the P-AP and P-AP-POD samples obtained at different POD concentrations and at pH 5.0.

and it increased proportionally to the increase of the catalyst concentration (**Table 2**). After 5 min of incubation, P disappeared by 39 and 13% with 0.5 mg mL^{-1} of Bir and Pyr, respectively; the phenol disappearance increased up to 51 and 64% in the presence of 1 and 2 mg mL^{-1} of Bir and Pyr, respectively. The polymerization process continued at much reduced speed as confirmed by experimental tests performed at incubation times even longer than 4 h (from 24 h to a month) (data not shown).

A similar behavior was observed by Pal et al. (*24*). The rate of transformation of P, catechol, and syringic acid by 0.25−1 mg mL^{-1} Bir was proportional to the amount of Bir during the first hour of exposure, but subsequently, little or no transformation occurred.

The presence of phosphatase did not influence significantly the transformation of P (**Table 2**). After the same 5 min reaction time, 36−37, 48−49, and 61−62% transformation of P was measured in the presence of 0.5, 1, and 2 mg mL^{-1} of Pyr and Bir, respectively. Also, in this case, the removal of the phenolic molecules did not increase when the incubation time increased.

Results of **Table 2** clearly indicate that both manganese oxides at the used concentrations promoted the polymerization process of P. Lower percentages of P were, however, transformed by Pyr, indicating its lower catalytic power than Bir (*25*).

Accordingly, in the chromatograms of the samples P-Bir, P-Pyr, and P-AP-Bir and P-AP-Pyr mixtures (data not shown), the intensity of the P peak remarkably diminished revealing the P disappearance from the reaction mixtures. No changes were observed with AP in the mixtures. Simultaneously, peaks in the 0−5 min interval also appeared, and their intensity proportionally increased by increasing the catalyst concentration. When the tests were performed with larger quantities of catalyst (1 and 2 mg mL^{-1} of Bir or Pyr) and at longer incubation times (1 month), the total disappearance of the P peak occurred, corresponding to the complete phenol transformation (data not shown).

As compared with P-AP samples, the UV−vis spectra of the Sn of the samples P-AP-Bir showed a hyperchromic effect at 266 nm at the higher Bir concentration and a hypsochromic shift with a decrease in absorption intensity at 218 nm, attributable to P and to an aromatic ring containing a phenyl group, respectively (**Figure 2a**). A third band at 292 nm, which indicates the presence of phenolic chromophores produced by Bir, was also observed (*26*). In the spectra of samples obtained with higher Bir concentrations (3 and 5 mg mL^{-1}), the band at 218 nm disappeared, while several bands in the region between 240 and 340 nm were observed (Del Gaudio, unpublished results). According to what was reported by Naidja et al. (*26*), this suggests the formation of quinons and their derivatives, showing hydroxyquinone absorbance bands at 292 nm and antraquinons and their derivatives with a maximum of absorbance around 240 nm.

Table 2. Residual P (% ±SD) with Time Affected by Different Amounts of Bir and Pyr in the Absence and Presence of AP

	P (%)											
	0.5 mg mL^{-1}				1 mg mL^{-1}				2 mg mL^{-1}			
	Bir		Pyr		Bir		Pyr		Bir		Pyr	
time (min)		+ AP		+ AP		+ AP		+ AP		+ AP		+ AP
0	100 ± 2	100 ± 3	100 ± 3	100 ± 2	100 ± 3	100 ± 1	100 ± 3	100 ± 2	100 ± 1	100 ± 1	100 ± 3	100 ± 2
5	61 ± 2	63 ± 3	87 ± 2	64 ± 2	49 ± 2	51 ± 1	49 ± 2	62 ± 1	36 ± 1	38 ± 2	36 ± 1	39 ± 1
20	49 ± 2	51 ± 2	53 ± 2	56 ± 1	46 ± 2	46 ± 2	44 ± 1	49 ± 1	35 ± 2	32 ± 1	34 ± 0	30 ± 0
40	51 ± 1	50 ± 1	47 ± 1	56 ± 1	47 ± 1	34 ± 1	44 ± 1	49 ± 2	29 ± 0	29 ± 0	30 ± 1	31 ± 1
60	54 ± 1	52 ± 1	60 ± 2	59 ± 1	48 ± 2	35 ± 2	56 ± 2	49 ± 1	29 ± 1	27 ± 1	31 ± 1	32 ± 2
120	48 ± 2	48 ± 2	59 ± 2	53 ± 2	49 ± 1	37 ± 1	51 ± 2	50 ± 2	28 ± 1	26 ± 1	30 ± 2	28 ± 1
180	50 ± 2	49 ± 1	51 ± 1	52 ± 1	37 ± 0	39 ± 1	43 ± 0	50 ± 1	20 ± 0	27 ± 0	25 ± 1	26 ± 0
240	44 ± 1	45 ± 1	45 ± 1	46 ± 0	38 ± 1	39 ± 0	49 ± 1	45 ± 1	25 ± 0	27 ± 0	26 ± 1	27 ± 0

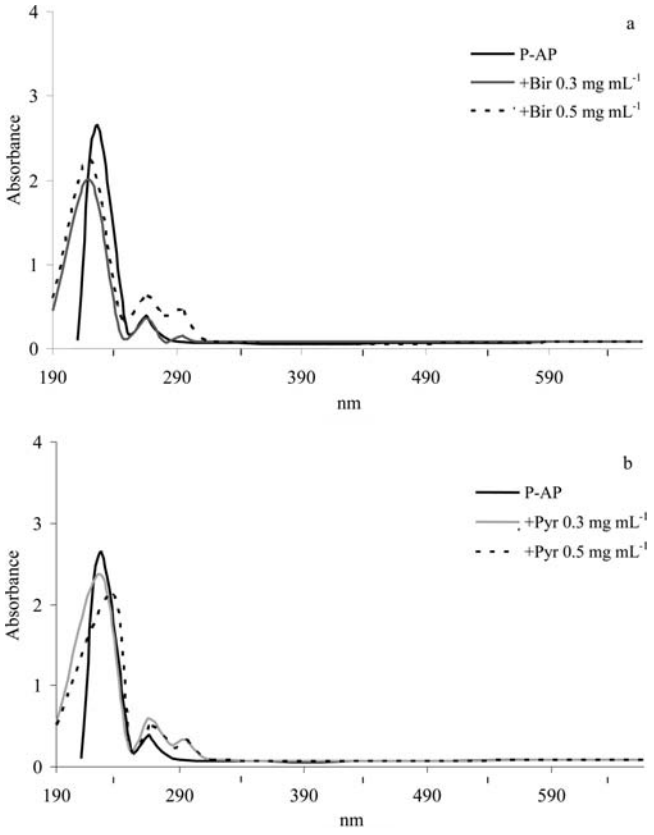

Figure 2. UV−vis spectra of the Sn (1:10 diluted) of P-AP and (**a**) P-AP-Bir and (**b**) P-AP-Pyr samples obtained at different manganese oxide concentrations and at pH 5.0.

As for the Bir, the presence of Pyr produced a change in the spectra of the mixtures P-AP-Pyr when compared to the reference sample (**Figure 2b**). A diminution of the intensity of the band at 218 nm and the appearance of a third band at 292 were also observed. With higher concentrations of Pyr, the absorbance spectra changed similarly to what were observed for P-AP-Bir samples (Del Gaudio, unpublished results).

The E_4/E_6 values of Sn of samples prepared with Bir and Pyr were also quite high, even if lower than those calculated with POD. For instance, at the maximum amount of the two catalysts (2 mg mL^{-1}), the E_4/E_6 values were 22 for Bir and 12 for Pyr.

The oxidative process catalyzed by manganese oxides is characterized by the release of soluble manganese in the reaction mixture. Detectable, even high, quantities of soluble Mn^{2+} were measured in the P-Bir/Pyr and P-AP-Bir/Pyr samples at each oxide concentration in comparison with relative controls. Results obtained with 0.5 mg mL^{-1} Bir and Pyr are shown in **Figure 3**. With Bir, a sudden release of Mn^{2+} (by more than 90% of the initial amount) was measured after 5 min of reaction, and it slightly, not significantly, decreased with time. A measurable, although very low, quantity (0.1−0.14 mg L^{-1}) of soluble manganese was released also in the Bir controls, indicating the spontaneous solubilization of the oxide (**Figure 3**). Similar phenomena occurred with Pyr, although spontaneous Pyr solubilization was significantly higher than Bir and greatly increased by increasing the incubation time (**Figure 3**). Although phosphatase did not remarkably affect the solubilization of both of the manganese oxides, its presence produced contrasting effects on the two catalysts. Indeed, the solubilization of Bir was not significantly affected, while that of Pyr remarkably decreased and much more after 4 h of incubation. Moreover, in samples with AP, a precipitate visible to the naked eye was obtained to

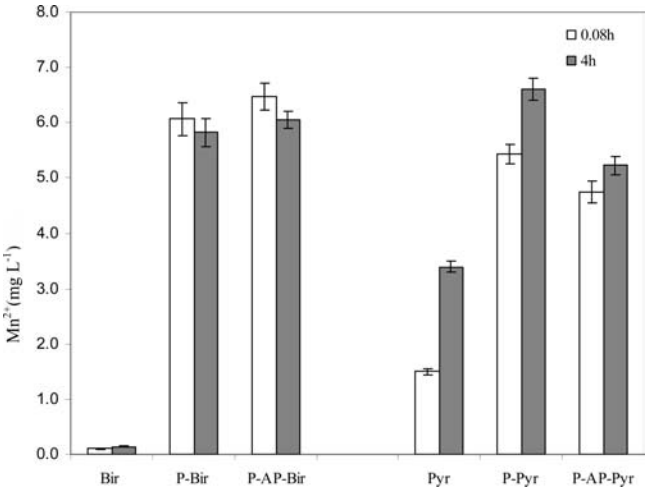

Figure 3. Release of soluble Mn^{2+} from Bir, P-Bir, P-AP-Bir, Pyr, P-Pyr, and P-AP-Pyr samples obtained with 0.5 mg mL^{-1} Bir or Pyr.

indicate the formation of P-phosphatase products (copolymers?) so big and heavy to precipitate only because of gravity. On the contrary, the polymerization products formed in the absence of AP were so much smaller and lighter to precipitate only after centrifugation of the samples.

When the phosphatase was also present in the reaction mixture along with P and Bir or Pyr, a rather complicated interplay of physically mediated processes and chemical reactions probably occurred, thereby leading to a very complex sequence of pathways and products being formed. Indeed, the enzyme may have interacted with (i) oxide alone, (ii) P polymers alone, (iii) oxide partly covered by P polymers, and (iv) oxide and P. Along with the formation of soluble P polymers, the involvement of $-NH_2$ and −OH groups of the enzymatic protein in cross-coupling reactions with phenoxy radicals produced upon manganese oxide catalysis is likely to occur. Several findings have demonstrated that amino acids, humic substances, and phenols may produce cross-coupling products during the oxidative polymerization of phenols promoted by Bir (*2*, *27*), thus favoring the entrapment of the enzyme in P-manganese oxide insoluble fractions.

Influence of pH on P Polymerization by POD and Pyr with or without AP. Differences in P polymerization process as well as in the properties of P-phosphatase complexes (see below) were observed when the polymerization of P by either POD or manganese oxide with and without AP was carried out at pH 4.0. Comparison studies were performed between complexes formed after incubation for 4 h at pH 5.0 or pH 4.0 prepared with 0.9 mg mL^{-1} POD and 0.5 mg mL^{-1} Pyr.

The amount of P polymerized by the catalysts was not significantly influenced by the pH except for the sample P-AP-Pyr, where only 21% ± 1 P was transformed at pH 4.0 against 28% ± 2 measured at pH 5.0. Conversely, different UV−vis spectra were observed for the Sn of the samples prepared at the two pH values. For instance, peaks at 298 and 358 nm appeared with POD at pH 4.0 (data not shown), whereas a uniform decrease of absorbance has been observed at pH 5.0 in the same wavelength range (**Figure 1**). With Pyr at pH 4.0, no peak at 358 nm was observed, whereas a less intense peak was still present at 298 nm. The corresponding E_4/E_6 ratios of the Sn obtained with both the catalysts were always greater than those of samples prepared at pH 5.0 (**Table 3**), thus suggesting that at pH 5.0 soluble polymers and copolymers characterized by greater size and mass and lower variability of chromophore groups remained in solution. Correspondingly, the solutions achieved different colors classifiable by

Table 3. E_4/E_6 Values, Released Mn^{2+}, Colors, and Corresponding Munsell Codes of Samples Prepared with 0.9 mg mL^{-1} POD or 0.5 mg mL^{-1} Pyr at pH 4.0 and 5.0 after 4 h of Incubation Time

samples	pH	E_{465}	E_{665}	E_4/E_6	soluble Mn^{2+} (mg L^{-1})	color	Munsell code
				biotic			
P-POD	4.0	2.250	0.080	28		yellow-reddish	7.5YR/7/8
P-AP-POD		1.790	0.050	36		yellow-reddish	5YR/6/8
				abiotic			
P-Pyr		0.590	0.030	20	6.32 ± 0.28 (5.91 ± 0.15)[a]	intense yellow	5Y/8/8
P-AP-Pyr		0.380	0.020	19	8.01 ± 0.35 (4.53 ± 0.41)	intense yellow	5Y/8/8
				biotic			
P-POD	5.0	2.625	0.110	24		red-yellowish	5YR/5/8
P-AP-POD		2.480	0.090	28		red-yellowish	5YR/5/8
				abiotic			
P-Pyr		0.490	0.050	10	6.60 ± 0.31 (5.43 ± 0.21)	intense yellow	5Y/7/8
P-AP-Pyr		0.590	0.070	8	5.22 ± 0.20 (4.74 ± 0.32)	intense yellow	5Y/7/8

[a] Data in parentheses are the amounts of Mn^{2+} measured at zero time.

Munsell codes and ranging from yellow-reddish for samples with POD to intense yellow for those with Pyr (**Table 3**).

The charge as well as intra- and intermolecular electrostatic repulsion increase as acidic groups are ionized with increasing pH, restricting aggregation phenomena (*28*). At pH 4.0 and with the biotic catalyst, it is likely assumed that during the incubation process bigger and heavier polymers and copolymers formed, which easily precipitated, leaving in the solution smaller and lighter polymers and copolymers. To support this assumption, the samples were centrifuged, and the masses of precipitates (when present) were weighted. A precipitate (0.7 mg) was detected either with or without the phosphatase at pH 4.0, whereas no solid phase was separated at pH 5.0.

At zero time incubation, an immediate solubilization of Pyr with the release of soluble Mn^{2+} also occurred at pH 4.0 after the contact between the P and the catalyst (P-Pyr samples) (values in parentheses in **Table 3**). No relevant differences were observed at the two pH values, although lower amounts of soluble Mn^{2+} were measured in the presence of phosphatase (P-AP-Pyr samples, **Table 3**). After 4 h of incubation and in the presence of phosphatase, the amount of Mn^{2+} raised up to 8.01 mg L^{-1}, while it slightly increased without the enzyme as it did at pH 5.0 with and without phosphatase (**Table 3**). This may suggest that different mechanisms were very likely involved in the redox process when phosphatase was present or when samples were prepared at the two pH values. At the lower pH (4.0), the oxidation process might have involved a high number of OH groups of the P molecule and/or the manganese would have participated as a less effective electron acceptor, by accepting, for instance, only one electron per Mn^{4+} ion rather than two as possibly occurred at pH 5.0. Indeed, at lower pH, nondissociated forms of phenols may prevail, and an increase of the reduction potential of MnO_2 is favored (*29*).

Properties of P−Phosphatase Complexes by POD and MnO_2 Catalysis. *Complexes with POD*. The suspensions (Sp) and the Sn of the P-AP-POD samples obtained at different POD concentrations and pH 5.0 showed phosphatase activity values (expressed as percentages of the initial EUs) very similar to each other, and they were very low in comparison to the activity of free phosphatase (AP sample in **Table 4**). Indeed, the activity was reduced by more than 90% in the sample prepared with 0.2 mg mL^{-1} of POD, and only with the lowest POD concentration (0.05 mg mL^{-1}), a detectable phosphatase activity (46−48%) was measured. No solid phase (Cp) was present at pH 5.0 at all (**Table 4**). No significant decrease of AP activity was detected in the AP-POD controls, thereby ruling out any possible detrimental effect by POD on the phosphatase activity.

Table 4. Residual Phosphatase Activity (%) of P−AP−POD (P-AP-POD) Complexes Obtained at Different POD Concentrations and pH 5.0

	POD (mg mL^{-1})	residual phosphatase activity (%)		
		Sp	Sn	Cp
AP		100 ± 3	100 ± 3	0 ± 0
P-AP		99 ± 4	99 ± 3	0 ± 0
AP-POD	0.05	91 ± 3	95 ± 2	0 ± 0
P-AP-POD		46 ± 2	48 ± 2	0 ± 0
AP-POD	0.2	98 ± 3	98 ± 3	0 ± 0
P-AP-POD		7 ± 1	2 ± 0	0 ± 0
AP-POD	0.45	109 ± 4	123 ± 4	0 ± 0
P-AP-POD		8 ± 0	4 ± 0	0 ± 0
AP-POD	0.9	89 ± 3 (91 ± 2)[a]	87 ± 3 (95 ± 3)	0 ± 0
P-AP-POD		11 ± 1 (9 ± 1)	12 ± 1 (1 ± 0)	0 ± 0 (11 ± 1)
AP-POD	2	84 ± 3	85 ± 2	0 ± 0
P-AP-POD		11 ± 1	11 ± 1	0 ± 0

[a] Values in parentheses refer to samples prepared at pH 4.0.

The marked reduction of phosphatase activity of the samples formed with POD can be explained assuming that during the polymerization process, the phosphatase molecules could have interacted with the formed P polymers, even being entrapped in them. A direct influence on the catalytic efficiency as the consequence of either a hindrance for the substrate to the active site or the modification of the structural conformation of the phosphatase protein may have resulted. Such behavior is confirmed by the numerous findings reported in the literature (*13* and *30* and references therein). Indeed, the partial or almost complete loss of enzyme activity in comparison to the same enzyme in the free form is one of the primary results of the immobilization process regardless of the matrix nature and the immobilization process.

Propriety of Complexes with MnO_2. The residual activities (expressed as percentage of the initial EUs) of the Sp, the Sn, and the insoluble complexes (Cp) of P-AP-Bir/Pyr samples prepared at 0.3, 0.5, and 2 mg mL^{-1} MnO_2 are reported in **Table 5**. The

Table 5. Residual Phosphatase Activity (%) of P−AP−Manganese Oxide (P-AP-Bir and P-AP-Pyr) Complexes Obtained at Different Bir or Pyr Concentrations and pH 5.0

		residual phosphatase activity (%)					
		Sp		Sn		Cp	
	MnO_2 (mg mL^{-1})	Bir	Pyr	Bir	Pyr	Bir	Pyr
AP		100 ± 3	100 ± 2	100 ± 3	100 ± 3		
P-AP		98 ± 2	99 ± 2 (91 ± 2)	98 ± 2	99 ± 3 (97 ± 2)		
AP-MnO_2	0.3	95 ± 1	82 ± 3	93 ± 2	95 ± 2	3 ± 0	4 ± 0
P- AP-MnO_2		20 ± 1	31 ± 1	28 ± 1	56 ± 1	2 ± 0	6 ± 1
AP-MnO_2	5	86 ± 3	88 ± 2 (93 ± 2)[a]	87 ± 2	87 ± 3 (94 ± 2)	6 ± 1	5 ± 1 (2 ± 0)
P- AP-MnO_2		33 ± 1	27 ± 1 (35 ± 2)	33 ± 1	42 ± 1 (32 ± 1)	3 ± 0	7 ± 1 (10 ± 2)
AP-MnO_2	2	37 ± 2	66 ± 2	39 ± 2	66 ± 2	17 ± 1	11 ± 1
P-AP-MnO_2		32 ± 1	10 ± 0	21 ± 1	9 ± 0	3 ± 0	7 ± 0

[a] Values in parentheses refer to samples prepared at pH 4.0.

activities of the controls (AP-Bir/Pyr) are also shown. The enzymatic activities of AP-Bir Sp decreased with the increase of Bir amount. The enzyme activity was differently distributed between the supernatant and the insoluble complex with a progressive increase of the last one in correspondence to the reduction of the supernatant activity. The reduction of activity was minimal at the lowest concentration of Bir, while it was rather remarkable when the Bir amount increased 7-fold, reaching about 60% of reduction in the presence of 2 mg mL^{-1} of Bir.

As reported above, such data are reasonably explained if we assume that part of the enzymatic molecules could have been absorbed on the oxide surface in as much of a higher quantity as higher the oxide concentration is. The adsorption of proteins of either an enzymatic or a nonenzymatic nature on manganese oxides has been observed by several authors (*31*, *32*). Rao et al. (*32*) showed that 65 and 55% of a benign full-length recombinant purified ovine protein (PrP) and its C-terminal fragment were, respectively, removed from the solution and firmly adsorbed on Bir when the two proteins interacted with the oxide at pH 5.5. Tyrosinase, another oxidative enzyme occurring widely in nature, was tightly bound to the Bir surface (*31*). X-ray diffraction, Fourier transform infrared, and atomic force microscopy analysis showed that the enzyme was not intercalated in the mineral structure, while its molecular conformation was partly altered after binding to the mineral external colloid surfaces. Similar experimental evidence was found for bovine serum albumin when immobilized on Bir (*31*).

In the P-AP-Bir samples, in which the P oxidation occurred in association with the simultaneous formation of polymerization products, the activity of phosphatase greatly decreased in comparison with AP (i.e., the free enzyme) and AP-Byr controls, except at the higher Bir amount. In the three samples Sp, Sn, and Cp, the activity reduction was proportional to the Bir concentration, suggesting that the enzyme molecules were probably more and more involved in the polymerization process, which increased by increasing the quantity of the catalytic agent (**Table 5**).

Such loss of activity could be ascribed not only to the adsorption of the enzyme molecules on the Bir surface but also to their involvement in the polymerization process. Nevertheless, the phosphatase activity measured in the Sp at all Bir concentrations was quite similar to that measured in the Sn; therefore, it could be ascribable to soluble complexes present in the supernatant. Only with 2 mg mL^{-1} Bir, that is, with a relevant quantity of solid material, a detectable activity was also measured in the insoluble complexes.

Similar results were obtained with Pyr at low oxide concentrations (**Table 5**). On the contrary, the enzymatic activity of the AP-Pyr samples with 2 mg mL^{-1} oxide indicates that Pyr also adsorbed part of the enzyme but in a smaller amount since it is characterized by a lower specific area than that of Bir as well as by a higher PZC (7.3 against 1.6 of Bir) that determines at pH 5.0 a positively charged surface. Consequently, a smaller amount of enzymatic molecule may have interacted with the surface of this oxide, and higher activity values (66%) in Sn resulted in AP-Pyr samples (**Table 5**).

Comparison between the Complexes Obtained by Biotic and Abiotic Catalysis. The residual activity of P-AP-POD and P-AP-Pyr samples was at some degree affected by the pH (**Tables 4** and **5**). With POD, a higher recovery of enzymatic unities occurred in the solid complex at pH 4.0 than that obtained at pH 5.0. At pH 4, the POD-mediated polymerization process favored the formation of bigger insoluble copolymers, which precipitated and probably involved a larger number of enzymatic molecules. At pH 5.0, instead, the polymerization produced only soluble polymers and copolymers. No activity was measured in the insoluble complexes Cp, and higher phosphates activity levels were found in the supernatant Sn where more enzymatic molecules evidently remained. As suggested by the E_4/E_6 ratios, more condensed polymers possibly formed at pH 5.0. On the contrary, with the abiotic catalyst and at pH 4.0, a higher number of enzymatic molecules remained entrapped in the insoluble phases (Cp), and a slightly higher residual activity (10% ± 1 against 7% ± 1 at pH 5.0.) was measured.

After POD-mediated polymerization of P, the disappearance of P molecules from the solution either with or without phosphatase (**Tables 1** and **2**), the UV−visible spectra of Sn (**Figures 1** and **2**), the E_4/E_6 ratios (**Table 3**), and the absence of any activity of the insoluble complexes (**Table 4**) could be accounted for by the formation of small, soluble polymeric products. On the contrary, the results obtained with Bir or Pyr lead us to hypothesize that the two abiotic catalysts favored the formation of P-phosphatase products with a low polymerization index but with sizes and molecular masses greater than those of the polymeric products obtained by POD.

Further information on the size of the polymeric products was obtained by ultrafiltation experiments through membranes characterized by different cutoffs (50000 and 100000). As expected on the basis of the AP molecular mass and independently on the pH at which the experiment was conducted, the Sn of AP-POD and AP-Bir/Pyr samples freely flowed through the 100000 cutoff membrane (as assessed by the completely recovery of their phosphatase activity in the permeates), whereas they were both almost totally retained in the ultrafiltation cell with the 50000 cutoff membrane.

According to the results previously reported, at pH 5.0, a high residual phosphatase activity (80% of that initially added) was

present in the fraction of P-AP-POD samples > 100000 Da, and it was partly (about 10%) retained with the 50000 cutoff membrane. Conversely, the P-AP-Bir/Pyr samples slightly permeated the 100000 membrane and hundred percent of their activity was retained inside the ultrafiltration cell, thus confirming the production of P-AP-Bir/Pyr soluble polymerization products characterized by great size and high molecular mass.

In conclusion, the obtained results clearly indicate that the investigated catalysts showed a different behavior toward P polymerization and the formation of P-phosphatase polymerization products. The process differently proceeded depending on the nature of the catalyst (abiotic or biotic), its amount, the contact time, and the pH. The nature of the catalyst influenced the characteristics of the formed complexes, in particular their residual activity, their size, and molecular mass. The biotic catalyst favored the complete transformation of the P, producing the formation of soluble P-phosphatase complexes smaller in size, with lower molecular mass and characterized by low levels of enzymatic activity.

On the contrary, the abiotic catalysts transformed only part of the initially added P, and the formation of P polymers increased with an increase in the amount of the two catalysts and the incubation time. Soluble and insoluble P-phosphatase complexes bigger in size, with higher molecular masses but characterized by low polymerization indexes, were formed.

Overall, the results reported may contribute to improving our knowledge on the interactions possibly occurring in soil among organic and enzymatic components and involving biotic and abiotic catalysts. A more thorough knowledge of these processes may not only provide more insights in the still not completely clear formation of humus and enzyme complexes in soil but also could suggest and support the use of such biotic and abiotic catalysts as potential agents for the remediation of phenol-polluted soils.

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