Biocatalysis and Biotransformation, 2004 VOL. 22 (1). pp. 35–44

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Magnetite-supported hematin as a biomimetic of Horseradish peroxidase in phenol removal by polymerization

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(Received for publication 21 May 2003; Accepted for publication 18 December 2003)

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Phenol removal using HRP and hematin as a biomimetic of HRP has been studied under various conditions at room temperature. The best results were obtained with treatment in two steps, with double addition of HRP or hematin and a final treatment with activated carbon. This two-step treatment achieved a minimum of 90% conversion of the initial phenol, under conditions commonly found in wastewaters (from 400 up to 1500 ppm phenol). Other additives such as chitosan, cellulose or polyethylene glycol (PEG) gave no satisfactory results. Hematin and magnetite-supported hematin showed comparable activities in phenol removal from aqueous solution. The supported hematin is an interesting alternative to HRP for practical application of a biomimetic catalyst for phenol removal.

Keywords: Phenol removal; Biomimetic Catalysts; Supported Hematin; Hematin; Magnetite

INTRODUCTION

Peroxidase (EC 1.11.1.7) catalyzes the oxidation of aqueous aromatic compounds by hydrogen peroxide, generating phenoxide radicals at high reaction rates. The free radicals polymerize and the products can be removed by sedimentation and filtration. This approach is being studied as an alternative for the treatment of wastewaters containing aromatic contaminants (Yu *et al*., 1994). Peroxidase from *Horseradish* or *Armoracia rusticana* has been demonstrated to be especially effective for phenol removal (Buchanan and Nicell, 1997). However, the high cost of this enzyme has encouraged the search of alternative, biomimetic catalysts. Moreover, an accumulation of toxic, soluble products occurred during the enzymatic reaction for treatments conducted with peroxidases from soybeans (SBP) and horseradish (HRP), at intermediate and high levels of phenol initial. The use of polyethylene glycol (PEG) as an additive to protect the enzyme resulted in an increase in the quantity of soluble products but it did not have a significant effect on the residual toxicity of treated solutions (Ghiourelitis and Nicell, 1999). The use of *Arthromyces ramosus* peroxidase (ARP) has been reported by Villalobos *et al*. (2002). Buchanan and Han (2000) reported that the inactivation of ARP differs from that of HRP. ARP seemed to be more sensitive to the presence of excess hydrogen peroxide.

Another problem is the need to immobilize the enzyme to reduce the costs for industrial application. The supported enzyme can be re-used after adequate washing. A suitable biomimetic catalyst must provide the same facility to be economically feasible.

Activated carbon has been used for phenol removal by adsorption (Samaras *et al*., 1995). Although this adsorbent is very effective the cost of activated carbon is very high and the problem of the disposal/treatment of the residual phenol-containing carbon is important. Moreover, the procedure for activation and the source of the carbon are very critical because of the heterogeneity of the carbon surface for adsorption (Hsieh and Teng, 2000).

Hematin or ferriprotoporphyrin hydroxide has been studied recently as a suitable catalyst for phenol polymerization (Akkara *et al*., 2000). It offers several advantages over HRP, especially in terms of cost. Hematin is almost 100 times cheaper on a weight basis.

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ISSN 1024-2422 print/ISSN 1029-2446 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10242420410001661231

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This work presents results of phenol elimination from aqueous solutions in order to compare the effectiveness of alternative catalysts to HRP: hematin and magnetite-supported hematin as biomimetic catalysts for phenol elimination by polymerization under selected conditions. This work does not try to optimize the reaction condition variables, but presents important results using an HRP-biomimetic catalyst such as Hematin. Glutaraldehyde was considered as a spacer between the hematin and the magnetite surface. Direct adsorption of hematin on magnetite seemed unsuitable because the pH conditions for adsorption would be different from the reaction conditions. As a double-functionalized spacer, glutaraldehyde is highly attractive because it can react with lateral groups of protoporphyrin structure and Bronsted acidic sites at the magnetite surface (Fe-OH) or even strong Lewis acidic sites (coordinative unsaturated Fe^{+3}). The heme lateral groups which react with glutaraldehyde may be the olefinic double bond in carbon 4 or the terminal carboxyl groups in carbons 6 and 7. They could react by a modified Prins reaction or by addition, forming a partially reduced anhydride bond. The Prins reaction is the acid-catalyzed addition of olefins to formaldehyde to give 1,3-diols, allylic alcohols or meta-dioxanes (Snider, 1991). The reaction of lateral groups in heme has been known for a long time (Smith, 1975, 2000).

Magnetite was selected because of its magnetic properties. This property is very useful for separating the catalyst from the effluent of wastewater treatment, enabling recovery of the catalyst, washing and reuse. The effect of different additives under selected conditions has been evaluated (cellulose, chitosan and activated carbon). The adsorption of residues from polymerization using activated carbon was tested under several conditions, as an additional step in the phenol removal procedure.

EXPERIMENTAL

Materials

HRP was kindly donated by Amano Inc. (USA), with an activity of 240 U per mg and a molecular mass of near 38.5 kDa. Activity was measured using the Amano method. This method is based on the appeareance of quinoneimine-dye formed by coupling of 4-aminoantipyrine and phenol, which is measured at 500 nm by spectrophotometry. One unit is defined as the enzyme quantity which oxidizes one μmole of hydrogen peroxide per minute under the assay conditions. Hematin and 25% aqueous glutaraldehyde were obtained from Sigma Chemical Co. Buffer pH 7/pH 11 and analytical grade chemicals for the preparation of magnetite ($FeNO_3$) were obtained from Meck. H_2O_2 (30 vol% or 9.1% w/v) Proforma was used. Polyethylene glycol 35.0 kDa was provided by Fluka, commercial chitosan by Primex and cellulose by Theorore Munchen, Germany. Activated carbon- from coal- (density 1.8–2.1 g/cm^3) was provided by Alfa-Aesar and used without a previous heat pretreatment.

Methods

The 4-amino Antipyrine (4AAP-AmNH$_2$) Method for Activity Assay

Enzyme or Hematin activity was determined by a phenol-4AAP colorimetric assay containing a solution of HRP or Hematin, phenol, 4AAP and added hydrogen peroxide as color generating substrates (Nicell *et al*., 1995). With excess amounts of phenol, H_2O_2 and 4 AAP the initial rate of the reaction is directly proportional to the amount of active enzyme/hematin present in solution. The rate of color development (red) at 510 nm of non-precipitating product was measured. The assay mixture contained the following: 0.2 mL of phenol solution (650 mg phenol in 50 mL buffer pH 7), 0.2 mL 4 AAP (100–105 mg 4 AAP in 10 mL buffer pH 7), 0.001 mL of HRP (7–10 mg in 10 mL buffer pH 7) or 0.05 mL hematin solution (9.6 mg hematin in 10 mL dimethylformamide-DMF) for a final volume at the cuvette of 3.55 mL. Variable amounts of H_2O_2 of different concentrations (from 0.091 to 9.1% w/v) were added to avoid undesirable volume changes at the cuvette (3.5–3.8 mL was the final volume). The final peroxide concentration at the cuvette was from 1.507×10^{-4} M to 3.34×10^{-2} M. For HRP the test was done at pH 7. When hematin was analyzed, pH 7 and pH 11 were used. The ε used to obtain the initial rate was 7210.00 M^{-1} cm^{-1}. The rates for hematin and HRP at the same conditions of low hydrogen peroxide concentration ($3-6 \times 10^{-4}$ M) differ by a factor of almost 100. The rate of phenoxide generation at the highest hydrogen peroxide concentration was almost the same for HRP and hematin. The reported data were obtained in duplicate or occasionally triplicate and were all quite reproducible ($\pm 1\%$ in U/mg).

The rate of color generation at 510 nm was calculated using linear regression of the linear portion of the absorbance versus reaction time data. The rate of H_2O_2 consumption in the activity assay (Ms^{-1}) was calculated according to the following equation

$$d[H_2O_2]/dt = -dA_{510}/dt\varepsilon^{-1}L^{-1}$$

where the rate of color generation is expressed in absorbance units per second, ε is the molar absorptivity and L is the pathlength of the cuvette (1 cm). Initial Reaction rate is expressed as μmoles per minute per mg (hematin or peroxidase) or U/mg.

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Test for Residual Phenol
Residual phenol after the reaction was tested using the absorbance at 270 nm. A calibration curve was constructed to test it. The ε obtained was 1490.00 M^{-1} cm^{-1} at neutral pH (7) or 1767.00 using pH 2. An aliquot of the remaining solution after precipitation by flocculation was diluted to 5 mL with distilled water and tested for residual phenol. This method has been reported as very useful to test the suitability of different reaction conditions on phenol removal and to analyze minimal conversion. Several soluble oligomers of phenol can absorb at this wavelength. Our aim was to test the suitability of the treatment in two steps with two different additions of HRP and hematin (soluble and supported). The UV method provides a conservative estimate of phenol removal. Spectra shown in Figs. 5 and 6 were obtained using 1 mL of the remaining solution after flocculation with HCl and dilution to 6 mL, before and after the step of treatment with activated carbon. Percentage of conversion of phenol is defined as initial concentration minus residual concentration related to the initial concentration X100.

Role of Additives

After the addition of HCl and precipitation of insoluble products, different additives were added: PEG, cellulose, chitosan and activated carbon. Because PEG has been reported as unsuitable as an additive for HRP because of toxicity, this additive was used with hematin. In the case of chitosan, the basic structure is the same as cellulose, but with one OH group at C_2 is replaced by NH_2. Cellulose and activated carbon were tested with HRP whereas chitosan, PEG and activated carbon were used with hematin. The masses added varied between 20 up to 150 mg.

Phenol Removal Conditions

The reaction was performed at room temperature in 10 mL vials, using three different phenol concentrations, taking into account the common range of contamination found in wastewaters (up to 1500 ppm). From 0.7 to 0.175 mL phenol (650–690 mg phenol in 50 mL) were used for a final volume of 10 mL, using buffer pH 7/pH 11. From 0.33–1 mg hematin/HRP was added. To compare the costs of both catalysts the same amounts were used, leading obviously to different molar concentrations because of the different molecular weights of hematin and HRP. Porcine hematin has a commercial price 100 times lower than HRP on a weight basis. Hydrogen peroxide at a molar ratio H_2O_2/Phenol = 1.5 was selected using data reported by Nicell *et al*. (1992, 1995) for HRP, whereas a molar ratio of near 11 was selected when hematin was used. This molar ratio has been reported as adequate in the case of hematin as a catalyst in phenol polymerization (Akkara *et al*., 2000). The stoichiometry consumption of hydrogen peroxide to phenol was reported to be near 1:1 (Nicell *et al*., 1992). Although this molar ratio appears to be inconsistent with that predicted by the mechanism of HRP (2 moles of phenol oxidized per mole of H_2O_2 consumed) other authors proposed explanations based on free radical interactions during polymerization (Santimone, 1975). The peroxide was added in the first 30 minutes of the reaction in three steps. At selected times of reaction, and one or two steps of pretreatment with catalyst, 1 mL of 37% HCl was added to precipitate the polyphenol. The time of reaction varied with the catalyst used. With HRP the reaction time for the first step was 2 hours, whereas the reaction time with hematin was 18 h. When a second step was analyzed, it was tested for 2 hours, whatever the case. After this, the flocculant was added and polyphenol precipitated. Finally, the additive was included at different concentrations and left for 1 hour at room temperature. It should be noted that additive was added at very acidic pH (near 2). The solids obtained by sedimentation/filtration/evaporation gave colors from clear brown to deep black. The solutions before the additive treatments were a yellowish-brown color. After the treatment with activated carbon, the solutions remained clear and transparent.

When higher temperatures were selected for phenol removal (50°C), after the addition of peroxide, the increase of temperature under the conditions used was almost 15°C. After 15 minutes reaction, the temperature decreased to 50°C. Bubble production (O_2) is evident at this reaction temperature in the stirred media, using hematin and magnetite-supported hematin. This time, the conversion was high after 3 h reaction and we included a second step, hematin/H_2O_2 (see Tables II and VII).

Synthesis of Magnetite-supported Hematin

Magnetite synthesis
Magnetite was prepared through a controlled oxidation in alkaline media, using 36.80 g $FeSO_4 \cdot 7H_2O$, 14.00 g NaOH and 4.53 g $NaNO_3$ (Sidhu *et al*., 1978). To 300 mL of NaOH 0.35 M and $NaNO_3$ 0.05 M, 250 mL of $FeSO_4 \cdot 7H_2O$ 0.53 M were added, with a constant rate of near 2.8 mL/min and magnetic stirring. A green-brownish gel was obtained, probably of structure $Fe(OH)_2 \cdot H_2O$ (Regazzoni, 1984). This gel was left for 12 days at room temperature. The final color was dark black. The sample was carefully washed 10 times with double-distilled water and centrifuged twice.

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Supported Biomimetic catalyst Preparation
Magnetite was contacted with 1 mL 25% glutaraldehyde in 20 mL buffer pH 7. After 2 h in reflux at 80°C the resulting gray-black solid was filtered. 160 mg of magnetite were contacted with 20-mg hematin in buffer, pH 4. The pH of the reaction media was selected to provide the conditions to attack the O of C=O from glutaraldehyde, where COO^- groups from hematin would be the nucleophiles to react with the C of carbonyl group. After 2 hr at 40°C the slurry was filtered using acetate filters due to the small particle size of this mixed iron oxide. The efficiency of this step was near 86%. Therefore, an amount near 17.2 mg hematin was supported on the recovered solid. The amount of hematin was 9.5–10% of the total amount of catalyst recovered. This was determined using the 1–10 phenanthroline method to test iron in solution, using a calibration curve ($\varepsilon = 9159.00\ M^{-1}cm^{-1}$). FTIR studies (not shown) demonstrated the presence of glutaraldehyde bands of C=O near 1720 cm^{-1} and from CH_2 near 1470 and 2800–3000 cm^{-1}, that decreased upon contact with hematin.

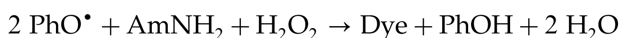
FTIR Characterization of Products

After flocculation, a solid can be recovered by filtration. This solid was analyzed using a Nicolet 550 FTIR Spectrophotometer using Transmission Infrared Spectroscopy.

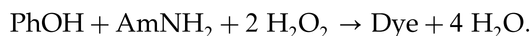
RESULTS

Kinetic Study of Phenoxide Formation

The global reaction for the formation of the dye is

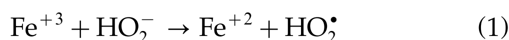


$$2\ PhO^{\bullet} + AmNH_2 + H_2O_2 \rightarrow Dye + PhOH + 2\ H_2O$$



$$PhOH + AmNH_2 + 2\ H_2O_2 \rightarrow Dye + 4\ H_2O.$$

Hematin
Figure 1 shows the initial rate of hydrogen peroxide decomposition vs hydrogen peroxide concentration at two different pHs: 7 and 11. It is clear that the reaction was faster initially at pH 11. The rates at low $[H_2O_2]$ look very similar at both pHs. When pH 7 buffer was used, there was a complex dependence on the hydrogen peroxide concentration. It seems that along the flat portion, equilibrium is involved in the reaction, which is displaced to the left at pH 11. Considering two equations from the mechanism proposed for hydrogen peroxide decomposition by ferric ions (Baxendale, 1952)



$$Fe^{+3} + HO_2^- \rightarrow Fe^{+2} + HO_2^{\bullet} \qquad (1)$$

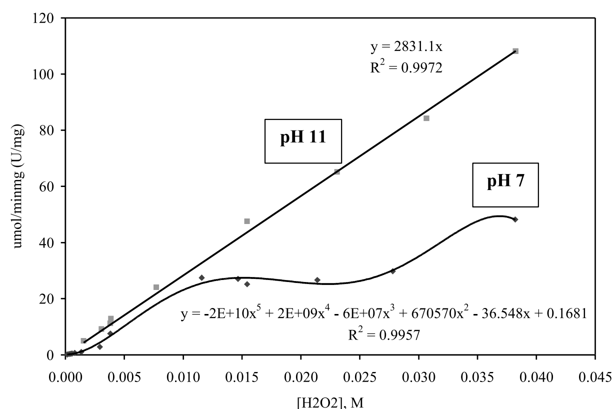
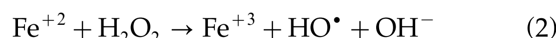


FIGURE 1 Initial rate of H_2O_2/PhOH decomposition using the method of 4AAP– Hematin pH 7 and 11. Conditions presented in experimental.



$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + HO^{\bullet} + OH^- \qquad (2)$$

The second reaction could be at equilibrium at pH 7, but displaced to the left at pH 11. Because of enhanced hydrogen peroxide dissociation at pH 11, the HO_2^- concentration is increased. Reaction of Fe^{+2} with further $HO_2^{\bullet}$ produces more HO_2^- and Fe^{+3} again.

In strongly alkaline solution, there is a good evidence that heme forms a compound with two hydroxyl groups. Hematin, but not heme gives a complex with hydrogen peroxide which has been represented as $H_2O_2 \cdot Fe \cdot OH$ (George, 1952). Different reaction schemes for ferric reaction with hydrogen peroxide lead to the equation $-d[H_2O_2]/dt = K\,[Fe^{+3}]^{1/2}[H_2O_2]^{3/2}/([H+] + K_h)^{1/2}$. It is clear that the rate increases at lower [H+] concentration or alkaline conditions and higher $[H_2O_2]$. The kinetic profile at pH 7 is similar to that found for the system chromate/hydrogen peroxide under acidic conditions ($[HNO_3] = 5.03 \times 10^{-3}$ M). In this case the catalytic decomposition is very complex. Participation of Cr^{+3} and Cr^{+6} is certain, but other intermediates have been proposed, even with +4 and +5 states of oxidation (Baxendale, 1952). The flat portion of the velocity curve was assigned to an equilibrium involving two different intermediates. We think that a similar situation takes place in our case at pH 7.

HRP
Figure 2 shows the initial rate vs hydrogen peroxide concentration at pH 7. The optimum concentration of hydrogen peroxide is between 4 and 6×10^{-4} M. The initial rate decreases at $[H_2O_2]$ higher than 3–6×10^{-4} M due to 10^{-4} suicide inactivation of peroxidase (Valderrama *et al.*, 2002). The horizontal line represents the maximum initial rate with hematin, measured over a range of pH values. The

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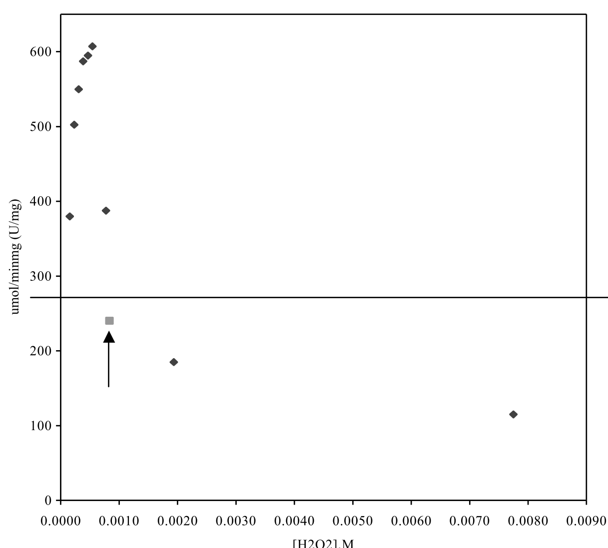

FIGURE 2 Initial rate of H_2O_2/PhOH decomposition using the method of 4AAP– HRP pH 7. 80 Units L-1 Conditions presented in experimental. The arrow shows the activity reported by Amano Inc., at low concentration of H_2O_2.

maximum activity of HRP is near by 5 times higher than that with hematin, but this is attained at very low hydrogen peroxide concentration. The HRP activity determined by the suppliers Amano Inc, is also shown. When activities are compared, the maximum activity found for HRP is nearly 601 U/mg, whereas for hematin at pH 7.0 is 48.2 U/mg. HRP is 12.5 times more active than hematin at the best selected condition in peroxide concentration, but it has several drawbacks when high peroxide concentration must be removed. The cost of purified HRP in a per gram basis is near 3900 US$, whereas the cost for hematin is 38 US$.

Phenol removal

Hematin

Tables I and II present the conversion data for different conditions using a one/two step treatment with hematin and PEG, chitosan or activated carbon. The 24 hour duration for the first step was selected on the basis of the work of Akkara *et al*. (2000). The second step had a higher efficiency because the relative concentration of phenol was lower. Evidently, there are some inactivation reactions in the case of hematin, that inhibit further reaction. However, hematin is highly resistant to hydrogen peroxide concentration. This can be an additional advantage because high concentrations of H_2O_2 are desirable to decompose compounds other than phenol, present in wastewater.

TABLE II Polymerization of phenol at pH 11 using hematin in 2 steps and activated carbon at room temperature. 20 mg activated carbon. First step: 24 hours, Second step: 24 hours, Step with carbon added: 2 hours

[PhOH], M	[Hematin], M	$[H_2O_2]$, M	% conversion
0.0168	9×10^{-5}	0.188	85
0.00838	4.5×10^{-5}	0.0937	85
0.00419	2.25×10^{-5}	0.0469	71

HRP

Tables III, IV and V present the data obtained for HRP in one step/two steps and using cellulose or activated carbon as additives. Increasing the amount of activated carbon from 20 to 150 mg did not significantly change the remaining amount of phenol (from 89 to 91%), but the solution changed color from yellowish to uncolored.

Magnetite-supported hematin

Table VI resumes the results for magnetite-supported hematin. The amount of hematin used was the same as in the soluble and the supported system. Qualitative tests of supported hematin using the 4AAP method gave positive results: phenoxide radicals were formed, even in a media with a dispersed solid. Table VII shows the results for the reaction at 50°C. Phenol removal was almost 100%, even when carbon was not added when supported hematin was used. However, when non-supported hematin was used, the conversion seemed to be lower that at lower temperatures (compare Tables II and VII).

FTIR Characterization of Polyphenol

Voltammetry and FTIR techniques have been employed to investigate polyphenol films formed on Pt

TABLE I Polymerization of phenol using hematin at pH 7 and pH 11 – Percentage conversion (%)

[PhOH], M	[Hematin], M	$[H_2O_2]$, M	At pH 7	At pH 11	% [a] conversion	% [b] conversion
0.0168	9×10^{-5}	0.187	39	41.4	47	30 (11.5 mg)
0.00838	4.5×10^{-5}	0.0937	27	26.3	41	25 (17.2 mg)
0.00419	2.25×10^{-5}	0.0469	25	30	32	–

[a] At pH 11 – Conversion obtained after two steps of treatment with chitosan-for [PhOH] = 0.0168 M we used 33.6 (first step)/41 mg (second step) for [PhOH] = 0.00838 we used 14.1 (first step)/23 mg (second step) and for [PhOH] = 0.00419 we used 6 (first step)/12 mg (second step). First step: 24 h at room temperature-Second step = 2 h at room temperature.
[b] At pH 11 after a step of treatment with PEG of 24 hours-in brackets, amount of PEG added.

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TABLE III Polymerization of phenol using HRP Amano – 250/125/62.5 U – pH 7

[PhOH], M	Amount HRP, mg	[H_2O_2], M	Buffer pH 7, mL	% conversion
0.0168	1.04	0.025	7.2	57
0.00838	0.52	0.0125	8.6	53
0.00419	0.26	0.0063	9.3	65

TABLE IV Second step phenol polymerization 1 hour – 131 U HRP added after 2 hours reaction – Mass of additive: 60–65 mg

[PhOH], M	Amount HRP, mg	[H_2O_2], M	Additive	% conversion
0.0168*	0.576	5.35E-03	Activated C	87
0.0168*	0.576	5.35E-03	Cellulose	76
0.0168*	0.576	5.35E-03	Blank	84

*Initial phenol concentration at the first step.

TABLE V Effect of amount of activated C added after the second step – HRP130 U

[PhOH], M	Amount HRP mg	[H_2O_2], M	Amount Additive, mg	% conversion
0.0168*	0.576	5.35E-03	20	89.5
0.0168*	0.576	5.35E-03	100	91.1
0.0168*	0.576	5.35E-03	150	90.2

*Initial phenol concentration at the first step.

surfaces by electrochemical oxidation of phenol (Lapuente *et al*., 1998). The polyphenol presents several groups of bands:

- two medium intensity bands associated with the in-plane and out of plane aromatic ring deformation vibrations are observed in the 475–550 cm^{-1} range. Other bands at 700–850 cm^{-1} are associated with aromatic out-of plane stretching vibrations of C–H
- Several bands appear related to the interaction of OH deformation and C–O stretching vibrations in the 1260–1350 cm^{-1} region.
- Several bands appear associated with the aromatic C–C stretching vibration in the 1400–1600 cm^{-1} region (the best resolved at 1500 cm^{-1}).
- A broad band at 330 cm^{-1} is attributed to the O–H stretching vibration
- A strong, broad band at 900–1150 cm^{-1} can be associated with ether C–O symmetric and asymmetric stretching vibration (=C–O–C= ring).
- The strong bands in the 2800–3000 cm^{-1} region can be associated with the C–H stretching vibration, expected for multisubstituted benzenes.

A pH effect exists in the electropolymerization mechanism of phenol on platinum electrodes; C–C bonds are mainly formed in acid medium and C–O bonds in alkaline medium. The enzymatically-synthesized polyphenol often has a structure consisting of a mixture of phenylene and oxyphenylene units. Generally, in polyphenol bands appear at 3430 cm^{-1} (OH of phenolic group), 1219 and 1170 cm^{-1} (asymmetric vibrations of the C–O–C linkage and to the C–OH) and a peak at 1101 cm^{-1} due to the ether bond. Appearance of these peaks suggests the presence of phenylene and oxyphenylene units (Akkara *et al*., 1991; Uyama *et al*., 2002). The spectra of polypara-phenylphenol obtained with HRP shows peaks at 697, 781 834, 878, 1030, 1120, 1218, 1403, 1482, 1599, 1684, 3028 cm^{-1} and a broad band centered in 3200 cm^{-1} plus another peak at 3600 cm^{-1}. All the bands cited above are present in this polymer.

Hematin

Figure 3 shows the FTIR trace of the polyphenol obtained at pH 7 and 0.008375 M of initial phenol concentration after two steps of treatment.

The spectrum shows bands at 3200 cm^{-1}, 1720 and 1680 cm^{-1}, 1480, 1450 and 1400 cm^{-1}, 1200 and 1150 cm^{-1}, 900 and 720 cm^{-1}.

HRP

Figure 4 shows the FTIR trace of the polyphenol obtained using two steps of treatment with HRP. The bands are not so well resolved as in the case of the polymer obtained with hematin. All the bands mentioned above appear but with much lower importance. The clear band at 1100 cm^{-1} is one of

TABLE VI Polymerization of phenol using magnetite supported hematin in two steps and activated carbon – Conversion of initial phenol (%)

[PhOH], M	Catalyst, mg [Hematin], M	[H_2O_2], M	% conversion Step one	Mg Catalyst added Second step	% conversion After step two
0.0168	15.8 [$9*10^{-5}$]	0.188	26	12.4	78
0.00838	7.5 [$4.5*10^{-5}$]	0.0937	27	6.5	78
0.00419	3.8 [$2.25*10^{-5}$]	0.0469	< 1	3.5	80

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TABLE VII Activity of hematin soluble and magnetite supported hematin at 50 C. Step 1 – 2 h 50 C, Step 2 – 2 h 50 C, Step 3 – Carbon step-18 h 25–27 mg activated carbon

[PhOH], M	Catalyst, mg [Hematin], M	$[H_2O_2]$, M	Mg Catalyst added Second step	$[H_2O_2]$, M	% Conversion Before	After
0.0168	17.7 mg [9*10^{-5}]	0.188	17.2	0.177	95	> 95
0.0168	1.2 mg [10^{-4}]	0.188	1.2 mg	0.177	73	75

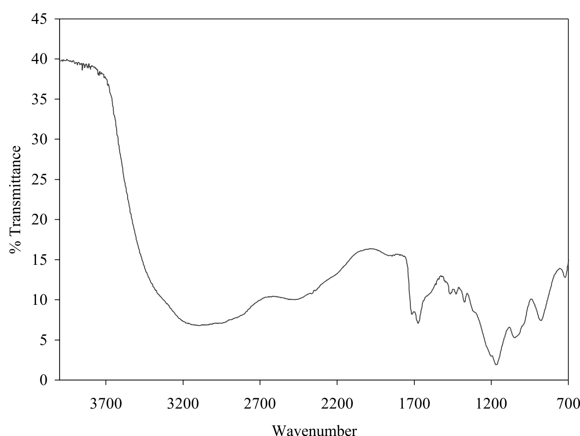


FIGURE 3 FTIR of polyphenol obtained with hematin pH 7.

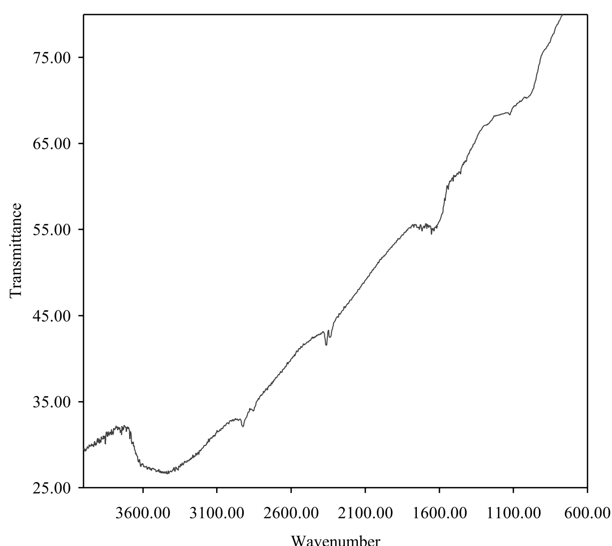


FIGURE 4 FTIR of polyphenol obtained with HRP at pH 7.

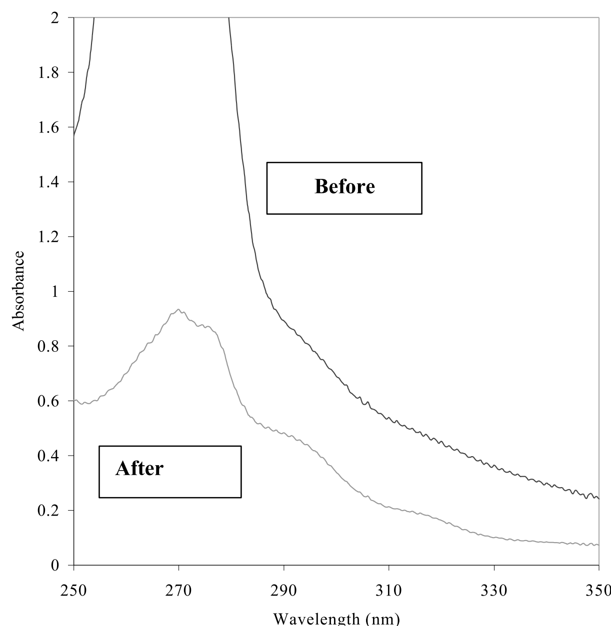


FIGURE 5 UV/Visible spectra of the solution from hematin – Before and after the treatment with carbon – Dilution 1/6 sample Table VII.

the main features of the spectrum, besides the absorption in the 1400–1800 cm^{-1}, 2800–3000 cm^{-1} and the strong absorption centered at 3400 cm^{-1}.

DISCUSSION

Activity Assay

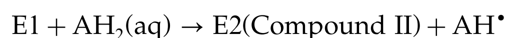
It is clear from the results that HRP gives a higher initial rate than hematin, whatever the pH. The problem is the inactivation of HRP with increasing peroxide concentration. The maximum rate was obtained from HRP at very low peroxide concentration. Moreover, hematin works better at pH 11 than at pH 7. The initial rate is linear with hydrogen peroxide concentration at pH 11, whereas a very complex dependence is evident at pH 7, at constant hematin concentration. A high hydrogen peroxide concentration, hematin at pH 11 is better than HRP. One point that must be taken into account is that peroxide dissociation is favored in alkaline medium.

As HO_2^- is especially important in the mechanism, it is clear that the rate is improved at high pH due to the concentration effect.

Mechanism for HRP

The mechanism of phenol polymerization using HRP involves several steps

$$HRP + H_2O_2 \rightarrow E1(\text{Compound I}) + H_2O$$



$$E1 + AH_2(aq) \rightarrow E2(\text{Compound II}) + AH^{\bullet}$$

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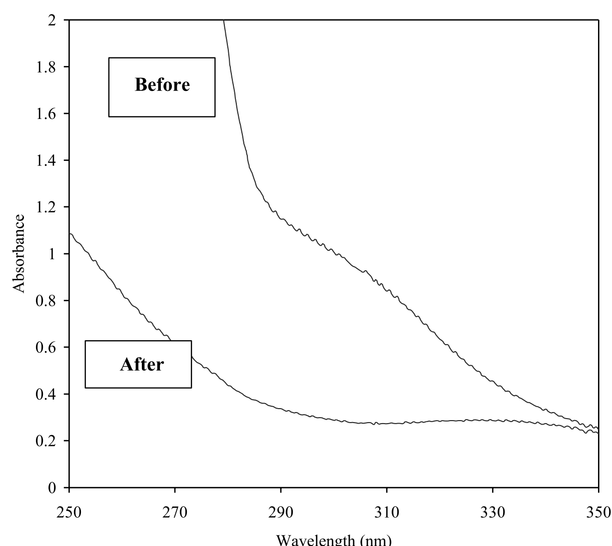
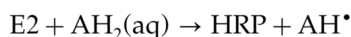


FIGURE 6 UV/Visible spectra of solution from hematin supported onto magnetite – Before and after the treatment with carbon – Dilution 1/6 sample Table VII.

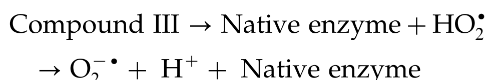


$$E2 + AH_2(aq) \rightarrow HRP + AH^{\bullet}$$

Compound E2 can be oxidized to compound III by hydrogen peroxide

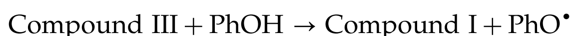
$$E2 + H_2O_2 \rightarrow E3(\text{Compound III}) + H_2O$$

Compound III is not a terminal inactivation of HRP. It decomposes to the native enzyme spontaneously according to



$$\text{Compound III} \rightarrow \text{Native enzyme} + HO_2^{\bullet} \rightarrow O_2^{-\bullet} + H^+ + \text{Native enzyme}$$

Compound III can be reduced also by PhOH



$$\text{Compound III} + PhOH \rightarrow \text{Compound I} + PhO^{\bullet}$$

A new reaction of compound I with H_2O_2 regenerates the enzyme and provides O_2 and H_2O. Another possibility is the formation from compound I of an inactive enzyme in excess of hydrogen peroxide called verdohemoprotein (or P670 because of its characteristic peak at this wavelength in the UV-Visible spectrum) that may result from an uncharacterised reaction at the active site. The opening of the porphyrin-ring through reaction with peroxide can result in permanent damage for HRP. It has been published that HRP needs 265 molecules of hydrogen peroxide per active site for inactivation (Valderrama *et al*., 2002). This apparent resistance to hydrogen peroxide, when HRP is compared to ascorbate peroxidase (only 2 H_2O_2) is due to the low, although measurable, catalytic activity of HRP (Ruiz *et al*., 2001). The radicals from compound III decomposition can reorder and form hydroxyl radicals, which are more reactive than peroxyl ones. A chain reaction involving free radicals and protein damage can be propagated in the HRP structure. Additionally, inactivation of HRP occurs during phenol polymerization because the end-product polymer adheres to HRP molecules enclosing them and hindering the access of substrates to the active site.

Phenol polymerization in the H_2O_2–HRP system generates a precipitate when the peroxide is added, and the soluble HRP can become trapped in the precipitate because of the high initial rate of polymerization.

Mechanism for hematin

Ferreira (2003) proposed a mechanism for hematin-H_2O_2 interaction. When phenol is considered in this mechanism, the complete picture is similar to the mechanism of Valderrama *et al*. (2002) with the analogous formation of compounds I, II and III. Access of phenol to the active site is not hindered as in case of HRP because hematin is only solvated by water and the reaction takes place on the unoccupied side of the molecule. The main feature of this mechanism is that the hydroxide group of hematin is not lost. Moreover, the solution changes from yellow to brown but no precipitate is found until the addition of HCl. This reflects to the lower initial rate for hematin. The catalyst remains in solution during all the treatment steps and the same is for supported hematin: there is no trapping of the catalyst by polymerization products. Soluble hematin can not be separated before flocculation, but supported hematin can. This opens the possibility of reusing the supported biomimetic catalyst. Separation can be obtained by filtration or the magnetic properties of the support. A point to consider is the need for longer treatment times in the case of hematin, although higher temperatures (up to 60°C) might avoid this problem.

Phenol Removal

From Tables I and II it is evident that a treatment in two steps using soluble hematin and low amounts of activated carbon removes 71–85% of the total phenol. In the first step, pH 11 was better than pH 7 (Table I). Incorporation of PEG did not improve the conversion, but treatment with chitosan seems to improve the efficiency of this step. When lower amounts of phenol are considered, the efficiency decreases in the case of hematin. The FTIR data show that both catalysts produce polyphenol with phenoxide/oxyphenoxide structures. Lower rates of phenoxide generation with hematin give polyphenol structures with same regularity. Branched structures seem to be present in case of HRP.

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When HRP is considered, the efficiency of the first step is higher than with hematin at pH 11. Final conversion reaches the same level as hematin (85%) after the second step. Adding of activated carbon improves only slightly the conversion but it can be important to decrease the presence of soluble, toxic species. With the magnetite-supported hematin, the final conversion was 78–80% at r.t. but almost 100% at 50°C. Thus, glutaraldehyde treatment did not destroy the structure of the catalytically active, protoporphyrin, as in the case of soluble hematin. Moreover, the final conversion was very similar between soluble and supported hematin at r.t.. Although the activated carbon was very useful for phenol removal, in this case, it also removed soluble phenol oligomers. Preceding the step of flocculation, the magnetite-supported hematin could be separated by filtration/magnetic interaction and then, reused. The possibility of reusing the magnetite - supported hematin and the effect of temperature in efficiency of supported hematin will be the focus of a forthcoming paper.

When the effect of additives is analyzed, it is clear that PEG addition did not improve the conversion. This suggests that the lower conversion reported for HRP is not due to an effect on the enzyme. It seems that in the case of hematin the effect is similar, with the polyphenol being soluble in the reaction media. Perhaps a non-identified effect on the polymerization products (coupling reaction through phenoxide radicals with PEG?) increases the solubility of the phenol polymerization products.

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CONCLUSIONS

Although the initial rates and mechanism of phenoxide formation are different between HRP and hematin, the latter is a very interesting lower cost alternative with a wider application range at alkaline pHs than HRP. The use of magnetite as a hematin support provides an additional improvement in order to facilitate product separation and reduce treatment costs, allowing reuse of the catalyst. The application of activated carbon at acidic pH, after flocculation, seems to be useful to complete the treatment. Treatment in two steps with hematin also avoids the use of PEG.

ABBREVIATION TABLE

HRP	Horseradish Peroxidase
SP	Soybean Peroxidase
PEG	Polyethyleneglycol
ARP	Arthromyces ramosus
4AAP	4 aminoantipyrine
DMF	Dimethylformamide
UV/Visible	Ultraviolet/Visible
E1	Compound I
E2	Compound II
E3	Compound III
r.t.	room temperature

Acknowledgements

This work was done through a grant from *Fundación Antorchas* (Argentina) to M. L. Ferreira. E. Rueda and M. L. Ferreira acknowledge the support from UNS and CONICET.

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