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Biocatalytic oxidation of phenolic compounds by bovine methemoglobin in the presence of H_2O_2: Quantitative structure–activity relationships

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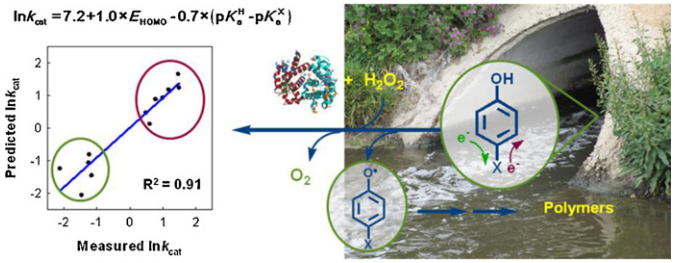
HIGHLIGHTS

- The kinetics of metHb-catalyzed oxidation of a group of phenols were analyzed.
- Unusual kinetic behaviour was observed for the phenols here tested.
- QSAR equations for a number of physicochemical parameters were established.
- A relationship between the peroxidase and catalase activities of metHb was found.
- Bovine metHb might represent a good economical alternative to other peroxidases.

GRAPHICAL ABSTRACT



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ABSTRACT

In the present work, 13 *p*-substituted phenols with different functional groups have been systematically evaluated as metHb substrates by means of HPLC analysis. Non-hyperbolic kinetics were observed and Hill coefficients in the 0.37–1.00 range were obtained. The catalytic constants and the Hill coefficients were found to be quantitatively correlated with two independent variables: the energy level of the highest-occupied molecular orbital (E_{HOMO}), which describes the intrinsic redox activity of the substrates and the pK_a-values, which are related to substrate ionization. Oxygen evolution in the presence of each phenol derivative was also measured, and good correlation between peroxidase-like and catalase-like activities of the protein was observed. It is also shown that bovine metHb, although less active than other peroxidases, may represent a good alternative from an economical point of view for phenol removal processes. The equations here obtained may serve as a basis to further explore the potential use of metHb-mediated reactions in the treatment of phenols in wastewaters and to predict which phenol will be removed most efficiently under this treatment with satisfactory reliability.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); 4AP, 4-amino-phenol; APAP, acetaminophen; *BDE*, bond dissociation energy; 4CNP, 4-cyanophenol; 4CP, 4-chlorophenol; *EA*, electron affinity; E_{HOMO}, energy of the highest-occupied molecular orbital; E_{LUMO}, energy of the lowest-unoccupied molecular orbital; *h*, Hill coefficient; 4HACP, 4'-hydroxy-acetophenone; Hb, hemoglobin; 4HBZ, 4-hydroxybenzaldehyde; 4HPA, 4-hydroxyphenethyl alcohol; HQ, hydroquinone; HRP, horseradish peroxidase; IC_{50}, concentration of the phenolic compound used to inhibit cell growth by 50%; *IP*, ionization potential; k_{cat}, catalytic constant; K_H, Hill constant; log *P*, octanol/water partition coefficient; metHb, methemoglobin; 4MP, 4-methoxyphenol; MPB, methyl paraben; 4NP, 4-nitrophenol; oxyHb, oxyhemoglobin; PHE, phenol; PPO, polyphenol oxidase; QSAR, quantitative structure–activity relationships; $[S]_0$, initial substrate concentration; $[S]_t$, substrate concentration at a time *t* after the start of the reaction; SBP, soybean peroxidase; 4TBP, 4-*tert*-butylphenol; TCA, trichloroacetic acid; V_M, molecular volume; $V_{max,p}$, maximum rate of the peroxidase-like activity (at short times); $V_{0,c}$, initial rate of O_2 evolution; $V_{0,p}$, initial rate of the peroxidase-like activity; ε, molar extinction coefficient; σ, Hammett constant; σ^+, Brown constant.

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1. Introduction

Phenolic compounds are a very significant group of chemicals of environmental concern, as they have extensive industrial applications. As a consequence of their widespread applications, their concentrations in waste streams typically range from 100 to 1000 mg/L, although it can become higher in certain cases [1]. Owing to their toxicity and persistence in the environment, both, the EPA and the EU have included some of them in their lists of priority pollutants [2,3].

Habitual methods for removal of these compounds from wastewaters use microorganisms, adsorption on polymeric resins or activated carbon, extraction with organic solvents, or chemical oxidation [4], although none of these methods is free from disadvantages, such as low efficiency or high cost. Treatment of wastewaters containing phenol derivatives with proteins such as HRP, SBP, PPO or laccase has been proposed to solve this environmental problem [5]. Hb represents one possible alternative to these enzymes because of its easy availability from animal blood at very low cost, a high stability under a wide range of pH and temperature conditions, as well as a known and documented structure. The protein is able to act on a wide variety of compounds in the presence of H_2O_2 and it has also been proposed as a possible alternative to the use of peroxidase enzymes for other industrial applications, including biosensors development [6] and conducting polyaniline synthesis [7].

Hb is not strictly an enzyme, but rather a heme protein which shows peroxidase– and catalase-like activities in its met form (metHb, Hb-Fe^{III}). The catalytic cycle is similar to that of HRP (Fig. 1), although it is worth noting some differences: (a) the second electron participating in H_2O_2 reduction (step 1) comes from the protein's globin, leaving it in the free radical state [10] and (b) the formation of oxyHb (equivalent to Compound III of HRP) has not been detected in the reaction of metHb with an excess of H_2O_2 [11]. This leads to a greater sensitivity of the protein against H_2O_2-induced inactivation, which means that more research is needed before this process can be implemented at the industrial scale. Therefore, the aim of the present work is to study the metHb-catalyzed oxidation of 13 phenol derivatives (see Table 1), which are mainly industrial chemicals and pharmaceuticals present as pollutants in wastewaters. Since the biocatalytic reaction involves electron transfer from the substrate to the heme group with simultaneous proton transfer (Fig. 1), electronic structures of the substrate molecules are expected to play a significant role in influencing reactivity. This led us to explore QSAR models that can explain the variability in reactivity. In addition, while many studies related to QSAR for the biocatalytic oxidation of phenols by other peroxidases like HRP have been performed [20–22], to our knowledge, a QSAR approach focused on correlating reactivities to molecular or electronic structures of phenol derivatives in its metHb-catalyzed oxidation has not been investigated. For this purpose, the following molecular descriptors have been calculated and correlated to the catalytic constant: electrochemical parameters including E_{LUMO}, E_{HOMO}, *EA* and *IP* as well as other related to the acidity of the substrate such as *BDE* of the O—H bond, σ and σ^+. Moreover V_M, polarizability, and log *P* have also been calculated. At last, the effect of the presence of each phenolic compound on O_2 evolution by the catalase-like activity of the protein has been measured.

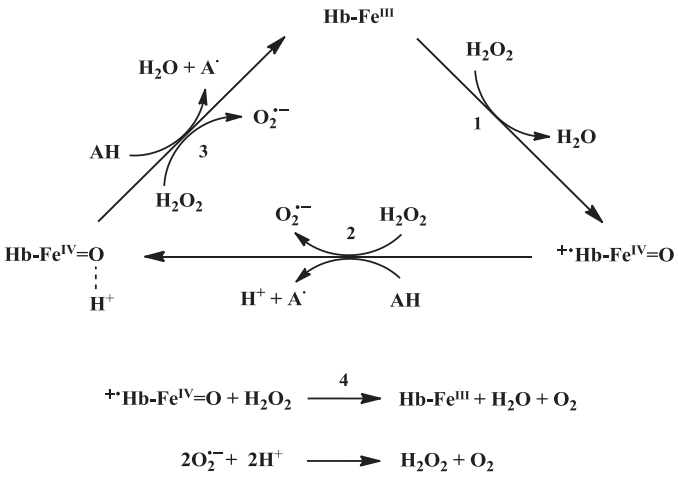


Fig. 1. Schematic depiction of the oxidation of phenolic substrates (AH) by the peroxidase-like activity of metHb.
Adapted from [8,9].

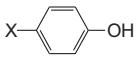
2. Experimental

2.1. Materials

Bovine metHb, HRP (Type II), phenolic compounds and TCA were obtained from Sigma–Aldrich (Madrid, Spain). H_2O_2 (30%) was from Fluka (Madrid, Spain). All the other reagents were of analytical grade and used without further purification. Solutions were prepared with demineralized water purified in a Milli-Q purification system (18.2 MΩ cm) (Millipore Corp., Bedford, MA).

The presence of catalase in commercial metHb was checked by means of gel filtration through Sephacryl S100HR, MW range

Table 1
Molar extinction coefficients of the *p*-substituted phenols here studied and their main uses in the chemical industry.

Substituent X 	Abbreviation	ε (M^{-1} cm^{-1})	λ (nm)	Reference	Main industrial applications
H	PHE	1.4×10^3	272	[12]	Resins and plastics production
—NH—CO—CH_3	APAP	9.7×10^3	240	[9]	Pharmaceutical industry
—NH_2	AP	2.15×10^3	296	This work	Intermediate in drugs production
—Cl	4CP	1.4×10^3	280	[13]	Manufacture of herbicides and bactericides
—CN	4CNP	1.9×10^4	247	[14]	Synthesis of pesticides and herbicides
—OH	HQ	2.62×10^3	290	[15]	Photographic industry and synthesis of antioxidants
—CH_2—CH_2—OH	4HPA	1.60×10^3	275	This work	Intermediate in medicine
—CO—CH_3	4HACP	7.71×10^3	294	[16]	Manufacture of pharmaceuticals
—CHO	4HBZ	1.46×10^4	280	[17]	Agricultural and food industry and synthesis of fragances
—CO_2CH_3	MPB	1.63×10^4	255	This work	Food preservative
—OCH_3	4MP	7.8×10^3	222	[18]	Pharmaceutical industry
—NO_2	4NP	1.7×10^3	405	[19]	Manufacture of fungicides and drugs
—$C(CH_3)_3$	4TBP	1.82×10^3	275	This work	Production of polymers and adhesives

1–100 kDa (Sigma–Aldrich, Madrid, Spain), as previously described [11]. MetHb concentration was calculated in the previously filtered (0.22 μm) stock solutions by using $\varepsilon = 9.48 \times 10^3\ M^{-1}\ cm^{-1}$ at 500 nm [23]. HRP concentration was calculated by using $\varepsilon = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$ at 403 nm [20]. H_2O_2 concentration in the stock solutions was spectrophotometrically determined at 240 nm by using $\varepsilon = 39.5\ M^{-1}\ cm^{-1}$ [24]. Phenols concentrations were calculated using the ε-values indicated in Table 1. Some of them were taken from the scientific literature and the corresponding reference has been included. ε-Values for the remaining substrates (4AP, 4HPA, MPB and 4TBP) were calculated by us in aqueous solution at the indicated wavelength. Measurements were performed by triplicate.

2.2. Experimental reaction conditions

A typical reaction mixture (total volume of 1 ml) contained metHb, phenolic substrate and H_2O_2 in a buffered media. Incubations were performed in a Thermomixer Comfort Eppendorf (Hamburg, Germany) at 25.0 °C with mild shaking (300 rpm). The reaction was started by the addition of H_2O_2. Aliquots were withdrawn from the reaction mixture at different times and stopped by the addition of TCA to give a final concentration of 4% (w/v). The precipitated protein was removed by centrifugation at 10,000 × *g* during 10 min. Fig. S1 of Supplementary Data shows a scheme of the protocol used to monitor the biocatalytic oxidation.

To determine k_{cat} for each phenolic derivative, the following experimental conditions were used: 3 μM metHb (for PHE, APAP, 4AP, 4CP, HQ, 4HPA, 4MP and 4TBP) or 20 μM metHb (for 4CNP, 4HACP, 4HBZ, MPB and 4NP) owing to the different reactivity of the substrates, 0.1–4.0 mM phenolic substrate (except 4TBP and MPB, which were used in the range 0.1–2.0 mM due to their lower solubility in aqueous medium) and 4 mM H_2O_2 in 50 mM sodium phosphate buffer pH 6.0. $V_{0,p}$ for each substrate was calculated using the next equation:

$$V_{0,p} = \frac{[S]_0 - [S]_t}{\Delta t} \quad (1)$$

The reaction time, Δt, was kept to 50 s since this was short enough to capture the linear portion of the progress curve of the reaction but still sufficiently long to ensure reliably handling and reproducible results. Calculated values for $V_{0,p}$ were plotted as a function of $[S]_0$ for each substrate, and each set of data fitted by nonlinear regression to the Hill equation:

$$V_{0,p} = \frac{V_{max,p}[S]_0^h}{[S]_0^h + K_H^h} \quad (2)$$

where h is a parameter which measures the degree of deviation from purely Michaelian behaviour. Strictly speaking, metHb concentration during the reaction time is not constant owing to the H_2O_2-induced suicide inactivation of the protein [11], but we assume that the reaction time (50 s) is sufficiently short, so that $[metHb]_t \approx [metHb]_0$. Then k_{cat} was calculated according to:

$$k_{cat} = \frac{V_{max,p}}{[metHb]_0} \quad (3)$$

Furthermore, it was also checked that the $V_{0,p}$ values for the biocatalytic oxidation of the different phenolic compounds exhibited a linear dependence on $[metHb]_0$ across the 1–30 μM range (data not shown).

2.3. Experimental techniques and apparatus

An ultraviolet/visible spectrophotometer Perkin-Elmer Lambda 35 coupled to a Water Peltier System PCB 150 (Perkin-Elmer, MA, USA) was used.

The progress of the reactions was followed in an Agilent (Waldbronn, Germany) HPLC 1200 series system equipped with a quaternary pump, automatic sample injection system, vacuum degasser, a column compartment and a diode array detector. Separations were performed on a reversed-phase 5 μm Discovery C_{18} (15 cm × 4.6 mm) from Supelco (Madrid, Spain). Samples were filtered through a 0.45 μm filter prior to injection.

Phenol concentration was quantified using calibration straight lines consisting of HPLC peak areas plotted against the concentration of the target compound in an injected series of stock solutions. Isocratic elution of methanol and water at a ratio that was optimized for each phenol (Supplementary data, Table S1) was used. The flow rate of the mobile phase was 1.0 mL/min. The elution conditions were as follows: injection volume, 20 μL and oven temperature, 20.0 °C. The solvents used were previously filtered through a 0.22 μm filter and degassed by sonication in a Selecta Ultrasons water bath (JP Selecta, Barcelona, Spain). An Agilent ChemStation B.03.02 revision was used to integrate the peak areas.

Oxygen production was continuously measured using a Hansatech oxygraph (Hansatech Instruments Ltd., Norfolk, England) equipped with an S1 oxygen electrode based on the Clark oxygen sensor, interfaced online with a PC-compatible computer. The electrode was calibrated using sodium hydrosulfite (Hansatech Instruments Ltd.). The temperature of the reaction chamber was controlled using a Selecta water Hetofrig circulating bath, and was checked using a Hanna Instruments pocket digital thermometer with a resolution of 0.1 °C. Reactions were started by the addition of H_2O_2, the final volume being 2 ml. The experiments were performed at pH 6.0, at which the catalase-like activity of bovine metHb in the absence of any phenolic substrate is maximum (data not shown). $V_{0,c}$ was defined as the slope of the initial linear portion of the O_2 evolution progress curve. Blanks in the absence of either H_2O_2 or metHb were performed and no oxygen evolution was found over a period of 5 h.

2.4. Computational details

Geometries were optimized and thermochemical properties were computed using the B3LYP hybrid functional with the 6-311+G (2df, pd) basis set. Equilibrium geometries were checked to be true minima by harmonic analysis. Aqueous solvent was taken into account using the Integral Equation Formalism Polarizable Continuum Model (IEF-PCM). The Gaussian 09 suite of programs was used for all calculations.

2.5. Fitting data

Data were fitted by using the SigmaPlot Scientific Graphing Software for Windows, version 12.0 (2011, SPSS Inc.).

3. Results and discussion

3.1. Time course of the reaction

The first part of this study tests the ability of metHb to oxidize a series of *p*-substituted phenol derivatives of industrial interest (Table 1) in the presence of excess H_2O_2. For that purpose, the time course of the biocatalytic oxidation of each phenol was monitored by HPLC analysis until no variation in substrate concentration was observed with time ($t \to \infty$; owing to the inactivation of the protein, since the addition of H_2O_2 to the reaction medium at this time did not produce any change in the remaining substrate concentration, but the addition of metHb did; data not shown). Exponential curves were thus obtained (see Fig. S1), which were then fitted by

Table 2
Percentage of oxidized phenolic compound, optimum pH-values of their biocatalytic oxidation at the indicated $[H_2O_2]_0/[S]_0$ ratios and pK_a (—OH) value for each compound. The reference for each pK_a value has also been included.

Substrate[a]	$\left(\frac{[S]_0-[S]_\infty}{[S]_0}\right)\times 100$[b]	Optimum pH[b]	$[H_2O_2]_0/[S]_0$	pK_a (—OH)	Ref.
Donors					
4CP	50.36	7.5	8.91	9.41	[25]
HQ	58.45	5.0	5.00	9.85	[25]
APAP	98.20	6.0	4.62	9.86	[25]
PHE	38.78	6.5	4.87	9.99	[25]
4HPA	61.67	6.0	5.03	10.17	[26]
4MP	100	5.5	1.39	10.21	[25]
4TBP	60.52	7.0	5.12	10.23	[25]
4AP	100	5	1.00	10.30	[25]
Acceptors					
4NP	13.62	6.0	5.46	7.15	[25]
4HBZ	13.40	6.5	8.43	7.61	[25]
4CNP	7.93	6.0	4.89	7.97	[25]
4HACP	17.85	7.0	8.45	8.05	[25]
MPB	14.74	7.0	8.35	8.47	[27]

[a] $[metHb]_0 = 5.0\ \mu M$; $[H_2O_2]_0 = 2.5$ mM; $[S]_0 = 0.5$ mM; phosphate buffer 50 mM pH = 6.0.
[b] The buffers used were: 50 mM sodium citrate (pH 3.0–6.0), sodium phosphate (pH 6.0–8.0), and sodium pyrophosphate (pH 8.5–9.5).

non-linear regression to determine the remaining substrate concentration at $t\to\infty$ ($[S]_\infty$) and calculate the percentage of substrate oxidized in each case under the same experimental conditions (Table 2). Data obtained indicated that substrates with electron-withdrawing substituents like 4NP and 4CNP are oxidized by the peroxidase-like activity of metHb in much lower extension than those with electron-donating substituents like 4MP, 4AP and APAP. This fact is directly related to the mechanism of the biocatalytic process since it involves electron transfer from the reducing substrate to the ferrylHb intermediates in two steps of the mechanism (Fig. 1, steps 2 and 3). Therefore, two groups of phenolic derivatives were established as a function of the ability of the *p*-substituent to accept or give electrons from or to the aromatic ring: (a) The first group includes those phenols with an electron-donating substituent such as 4CP, HQ, APAP, PHE, 4HPA, 4MP, 4TBP and 4AP. They are more easily oxidized since electronic delocalization in the ring is favoured; (b) The second group of substrates comprises those phenol derivatives with an electron-withdrawing functional group such as 4NP, 4HBZ, 4CNP, 4HACP and MPB.

3.2. Effect of pH

Since the pH of industrial aqueous effluents may vary, we investigated the pH-dependence of the biocatalytic oxidation process of the different phenolic compounds here tested by measuring $[S]_\infty$ at pH ranging from 3.0 to 10.0. Table 2 shows the optimum pH-values obtained for each phenolic compound at the indicated initial $[H_2O_2]_0/[S]_0$ ratios compared to their respective values of pK_a. The $[H_2O_2]_0/[S]_0$ ratios were optimized for each phenolic substrate so that a bell-shaped curve was obtained. It was observed that at pH-values below 4.0 and above 9.0, metHb showed very weak removal efficiency. The optimum pH obtained for each compound was lower than its corresponding pK_a (hydroxyl group), indicating that the acid form of each compound is the active species able to transfer a H^+ and an electron to the protein intermediates $^{\bullet+}$Hb–Fe^{IV}=O and Hb–Fe^{IV}=O in the catalytic cycle (Fig. 1). The optimum pH-values obtained were in the 5.0–7.5 range, with 4AP and 4CP showing the lowest and the highest optimum pH values, respectively. These results indicate that metHb is active for removal of phenolic compounds in a wide range of pH values, adequate to the pH range between 6.0 and 9.0 recommended by the USEPA to protect microorganisms [28].

However, it is clear that additional factors should also influence the reactivity of metHb towards different compounds. For instance, since the reducing agent must access the heme pocket, the kinetics of the process are highly dependent, not only on the pH of the reaction medium, but also on the physical properties of the reducing substrate (i.e. size, hydrophobicity, etc.). In addition, the energy levels involved in the electron transfer process are known to have a very large effect on the electron transfer rates. So we decided to investigate this subject in depth.

3.3. Determination of kinetic parameters

Fig. 2 shows double reciprocal plots of $V_{0,p}$ against $[S]_0$, for greater clarity for only a selection of the phenols here studied. These plots showed a non-linear dependence, affording concave downward curves, which indicates a non-Michaelian behaviour of metHb in the expression of its peroxidase-like activity. Deviations of michaelian behaviour were observed for all of the substrates here assayed, except in the case of 4NP (Fig. 2B) and 4HBZ (data not shown), which contain an electron-withdrawing group and were scarcely oxidized by the protein.

This kinetic behaviour is very different to that observed for other peroxidase enzymes, for which michaelian behaviour has been reported [21,29,30]. In addition, it hinders the determination of the kinetic constants involved in the process, since it means an increase in complexity from the case of HRP. Moreover, this is not the first time that an unusual kinetic behaviour is observed in the expression of the peroxidase-like activity of metHb in the presence of H_2O_2. In fact, a similar behaviour also occurs in the expression of its catalase-like activity, although in this case concave upward curves were obtained [11]. Previously Ortiz de Montellano and Catalano [31] also observed non-linear double reciprocal plots in the epoxidation of styrene catalyzed by metHb, although this result was attributed to the protein inactivation. More recently, our group has reported a similar behaviour in the oxidation of APAP by human metHb [9], which was explained in mechanistic terms. However, other authors have reported michaelian behaviour for metHb, for example, in the oxidation of PAHs [32,33], phenothiazines [34], dopamine, L-dopa and ABTS [35] and ascorbate and urate [36].

Under this scenario we decided to fit experimental data to the Hill equation, as usual in enzyme kinetics, to obtain the kinetic parameters characterizing the oxidative reactions, k_{cat} and h (Table 3), h being a measure of the deviation degree of michaelian behaviour. Those substrates carrying an electron-donating group as *p*-substituent such as 4CP, HQ, APAP, 4HPA, 4MP, 4TBP, and 4AP showed in general higher k_{cat}-values than

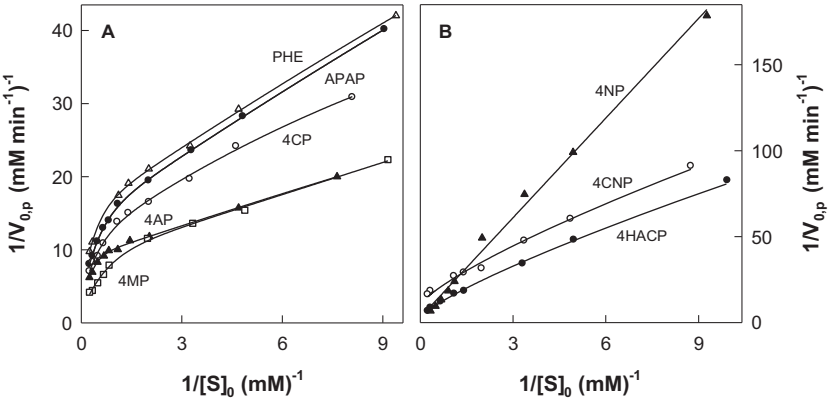


Fig. 2. Double reciprocal plots of $V_{0,p}$ versus $[S]_0$ for the metHb-catalyzed oxidation of (A) PHE (△), APAP (●), 4CP (○), 4AP (▲) and 4MP (□); (B) 4NP (▲), 4CNP (○) and 4HACP (●). Experimental conditions as indicated in Experimental section. The points correspond to experimental data, while the lines correspond to the data obtained by nonlinear regression analysis to Eq. (2).

those of the second group, such as 4NP or MPB, carrying an electron-withdrawing group. This implies that the electron-donating or electron-withdrawing effect of substituents on the aromatic ring greatly influences in determining the reactivity of phenolic compounds towards metHb. With respect to *h*, the first group of substrates showed values in the range 0.37–0.80, whereas in the second case the values of *h* were closer to unity (0.62–1.00).

3.4. Quantitative structure–activity relationship (QSAR)

To obtain a better understanding about factors affecting metHb-catalyzed oxidation of different phenolic compounds, a series of molecular descriptors were calculated (Table 4) to try to establish a correlation with k_{cat} and *h*. Fig. 3A shows a plot of $\log(k^X_{cat}/k^H_{cat})$ vs. σ and σ^+ for the phenolic compounds here tested, k^X_{cat} and k^H_{cat} being the catalytic constants for each *p*-substituted phenol and PHE, respectively. A good correlation was found in both cases, and a decrease in $\log(k^X_{cat}/k^H_{cat})$ as σ and σ^+ increase was observed. From the slope of the fit in Fig. 3A (σ) the value of ρ, the susceptibility factor in the Hammett equation (or reaction constant), was -1.15 ± 0.15. This value is less in absolute terms than that of -1.63 ± 0.30 calculated for HRP for a variety of *p*-substituted phenols [21], which indicates that metHb is less sensitive to substituents on the aromatic ring of the substrate. This could be related to the reactivity–selectivity principle, since it has been reported that both Compounds I and II of HRP are powerful oxidants, with redox potential estimated to be close to +1 V [40], whereas an even higher value (+1.6 V) has been reported for the redox potential of ferrylHb intermediates species [41], thereby explaining the smaller absolute value of its ρ parameter. To our knowledge, this is the first Hammett correlation obtained for the oxidation of phenols by metHb. In addition, and keeping in mind that the meaning of a negative value of ρ is that positive charge is built during the reaction, this might be related to the fact that the second electron participating in H_2O_2 reduction (step 1) comes from the protein's globin, i.e. the radical is more delocalized than in the case of HRP, in which it is located on the porphyrin ring of the heme [10] and so the charge cloud is more diffuse in metHb, supporting the smaller value of ρ.

Table 3
Kinetic parameters obtained for the different phenolic compounds.

Substrate	k^a_{cat} (s^{-1})	h^a
Donors		
4CP	1.83	0.56
HQ	2.70	0.46
APAP	2.20	0.53
PHE	1.63	0.48
4HPA	3.23	0.80
4MP	4.43	0.58
4TBP	2.17	0.66
4AP	4.32	0.37
Acceptors		
4NP	0.17	1.00
4HBZ	0.29	1.00
4CNP	0.12	0.62
4HACP	0.29	0.80
MPB	0.29	0.79

[a] $[H_2O_2]_0 = 4.0$ mM; $[metHb]_0$ and $[S]_0$ variable as a function of the substrate reactivity (see Section 2).

Fig. 3B shows the $\ln k_{cat}$ vs. *BDE* and pK_a plots for the studied *p*-substituted phenols. A better correlation was obtained with pK_a-values, which means that the oxidation rate of phenolic compounds catalyzed by metHb is also directly related to the ability of the —OH group of the phenolic derivatives to lose a H^+, in agreement with an electron transfer process accompanied by simultaneous proton transfer.

The first product of the biocatalytic oxidation is the free radical corresponding to the substrate. Moreover, it has been shown that the relative ability of a phenolic compound to give the corresponding phenoxyl radical is related to its toxicity [42], and so *BDE* is a parameter which correlates very well to the toxicity of phenol derivatives containing electron donating groups [43]. Therefore it is to be expected that the k_{cat} for the metHb-catalyzed oxidation of phenolic compounds, which is a good indicator of the radical production rate, should correlate to the toxicity. A plot of $\log k_{cat}$ vs. $\log(1/IC_{50})$ (data not shown) showed two well defined regions corresponding to the electron donor and acceptor-substituents, respectively, and only the k_{cat}-values for the first type of substrates correlated well with toxicity. Therefore, metHb shows higher ability to catalyze the oxidation of those phenols with an electron donating substituent, which are the most toxic. This is a great advantage of enzymatic methods in phenol removal processes.

Fig. 4A shows the QSAR obtained when $\ln k_{cat}$ was plotted against the calculated values of E_{HOMO} for the phenolic derivatives indicated in Table 1. A better correlation with E_{HOMO} than with E_{LUMO} (data not shown) was found. Since $-E_{LUMO} \approx EA$ and $-E_{HOMO} \approx IP$ [44] the relationships with *EA* (data not shown) and *IP* (Fig. 4A, inset) were also tested and the results obtained showed the same trend. Therefore, a clear dependence of k_{cat} upon the ionization potentials of the HOMO of each phenolic compound, that is, the energy required to take an electron from the molecule, was found.

Table 4
Chemical parameters of the studied *p*-substituted phenols.

Substrate	*BDE* (eV)	E_{LUMO} (eV)	E_{HOMO} (eV)	*EA* (eV)	*IP* (eV)	V_M ($cm^3 mol^{-1}$)	Polarizability (10^{14}·C m^2 V^{-1})	log *P*[a]
Donors								
4CP	3.85	−0.90	−6.46	1.42	6.39	99.3	2.4	2.26
HQ	3.59	−0.72	−5.96	1.16	5.94	73.8	2.1	0.96
APAP	3.64	−0.73	−6.01	1.12	6.05	126.4	3.1	0.68
PHE	3.87	−0.63	−6.49	1.13	6.40	68.8	2.0	1.53
4HPA	3.58	−0.69	−5.92	1.10	5.88	129.6	2.9	1.05
4MP	3.57	−0.69	−5.89	1.12	5.85	82.1	2.5	1.45
4TBP	3.77	−0.55	−6.24	1.01	6.22	138.2	3.3	3.23
4AP	3.34	−0.61	−5.54	1.08	5.51	79.1	2.3	0.54
Acceptors								
4NP	4.16	−2.88	−7.14	3.02	7.07	105.3	2.8	1.68
4HBZ	4.04	−2.01	−6.85	2.28	6.75	82.2	2.7	1.29
4CNP	4.05	−1.48	−6.87	1.83	6.78	88.5	2.6	1.47
4HACP	4.00	−1.81	−6.75	2.06	6.68	114.9	2.9	1.43
MPB	4.01	−1.51	−6.77	1.76	6.72	121.8	3.0	1.75

[a]Obtained from [37].

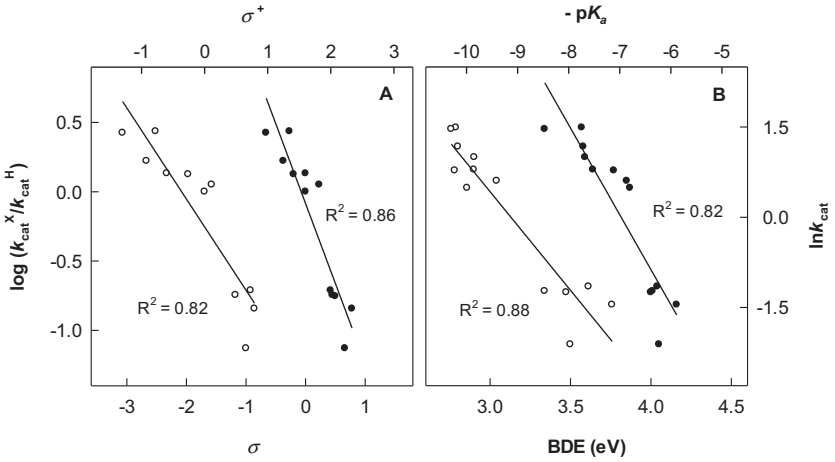


Fig. 3. (A) Hammett plot for the different phenols here tested using the σ (●) and σ^+ (○) values [38,39]. The equations obtained were: $\log(k_{cat}^X/k_{cat}^H) = -0.09 - 1.15\sigma$ and $\log(k_{cat}^X/k_{cat}^H) = -0.25 - 0.70\sigma^+$. (B) Relationship between $\ln k_{cat}$ and *BDE* (●), and pK_a (○) values. The equations obtained were: $\ln k_{cat} = 17.96 - 4.71$ *BDE* and $\ln k_{cat} = -9.51 + 1.04\ pK_a$. The points correspond to experimental data, while the lines correspond to the data obtained by linear regression analysis.

These results are consistent with previous QSAR studies on HRP-mediated oxidation of other phenolic compounds [21,22], although the k_{cat}-values here obtained are lower, according to the smaller catalytic activity of metHb.

Regarding the other calculated parameters such as V_M, polarizability and log *P*, a 2D-relationship with $\ln k_{cat}$ could not be found (data not shown). However, 3D-correlations with E_{HOMO} as independent variable led to the next equations:

$$\ln\ k_{cat} = 15.43 + 2.41 \times E_{HOMO} - 4.6 \times 10^{-5} \times V_M, \qquad R^2 = 0.84 \tag{4}$$

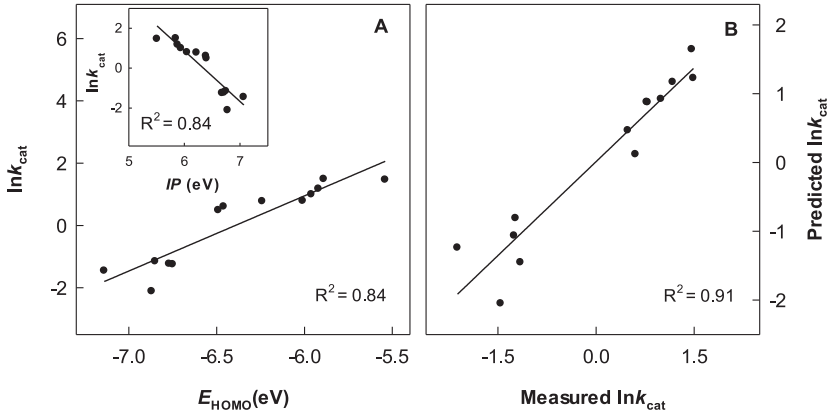


Fig. 4. (A) Relationship between $\ln k_{cat}$ for the oxidation of *p*-substituted phenols and their respective E_{HOMO} and *IP* (inset). The obtained equations were: $\ln k_{cat} = 15.43 + 2.41 \times E_{HOMO}$ and $\ln k_{cat} = 16.12 - 2.54 \times IP$. (B) Correlations between measured and predicted values of $\ln k_{cat}$ using Eq. (7). The points correspond to experimental data, while the lines correspond to the data obtained by linear regression analysis.

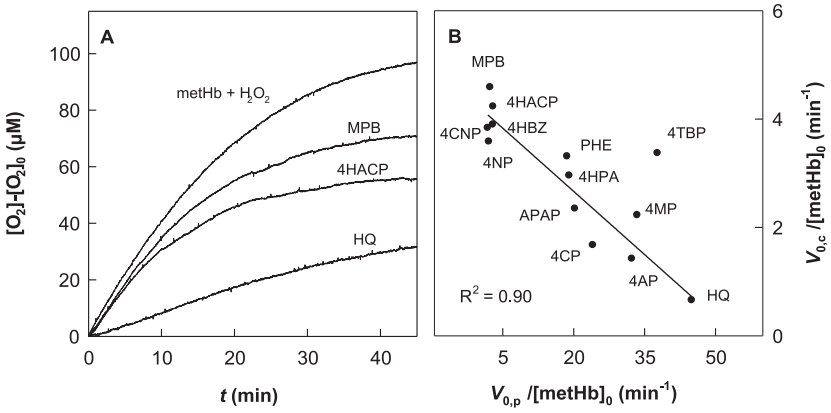


Fig. 5. (A) Progress curves of O_2 evolution in absence and presence of MPB, 4HACP and HQ. The experimental conditions were: $[S]_0$ = 0.5 mM, $[metHb]_0$ = 2.0 μM, and $[H_2O_2]_0$ = 1.5 mM. (B) Relationship between the relative initial rates of oxidation of each phenolic derivative, $V_{0,p}$, and O_2 evolution, $V_{0,c}$, in its presence. The initial concentrations here used to measure the two activities of metHb under the same experimental conditions were: $[S]_0$ = 0.9 mM, $[metHb]_0$ = 3.0 or 20.0 μM depending on the substrate reactivity (see Section 2), and $[H_2O_2]_0$ = 4 mM. Rate values have been divided by $[metHb]_0$ to be able to compare the results obtained. The points correspond to experimental data, while the lines correspond to the data obtained by linear regression analysis.

$$\ln\ k_{cat} = 15.76 + 2.38 \times E_{HOMO} - 0.21 \times \text{Polarizability}, \quad R^2 = 0.85 \tag{5}$$

$$\ln\ k_{cat} = 16.18 + 2.63 \times E_{HOMO} + 0.41 \times \log\ P, \qquad R^2 = 0.89 \tag{6}$$

As can be seen, log P is the parameter that yields the best optimization of the $\ln k_{cat}/E_{HOMO}$ trend (Fig. 4A), which means that the hydrophobicity of the phenolic substrate also seems to play an important role on the mechanism of the biocatalytic oxidation. This could be related to the fact that the phenol molecule must diffuse into a hydrophobic pocket in the protein before the oxidation takes place.

Furthermore, it was also found that the QSAR approach previously obtained (Fig. 4A) could be optimized when the pK_a-values of the phenol derivatives are included (Fig. 4B). From the linear regression using E_{HOMO} and $(pK_a^H - pK_a^X)$ -values as independent variables, where pK_a^H and pK_a^X are the pK_a-values for PHE and the p-substituted phenol, respectively, and $\ln k_{cat}$ as dependent variable, Eq. (7) was derived:

$$\ln\ k_{cat} = 7.15 + 1.03 \times E_{HOMO} - 0.65 \times (pK_a^H - pK_a^X), \qquad R^2 = 0.91 \tag{7}$$

The higher correlation coefficient obtained with respect to the corresponding 2D-correlations (Figs. 3B and 4A), indicates that these two parameters, E_{HOMO} and $(pK_a^H - pK_a^X)$ should be considered together to establish a good relationship with $\ln k_{cat}$, since the biocatalytic process involves both electron and proton transfer from the substrate.

Interesting relationships were also obtained with the kinetic parameter h with E_{HOMO} and $(pK_a^H - pK_a^X)$ -values as follows:

$$h = -0.19 - 0.12 \times E_{HOMO} + 0.13 \times (pK_a^H - pK_a^X), \qquad R^2 = 0.85 \tag{8}$$

In general, all of the studied phenol derivatives fit the trend and an acceptable regression coefficient was obtained (R^2 = 0.85). This equation is helpful since it allows predicting the kinetic behaviour of metHb towards a target p-substituted phenolic compound. As far as we know, this is the first time that h is correlated to structural parameters from a group of substrates.

3.5. Influence of the different phenolic compounds on the catalase-like activity of bovine metHb: O_2 evolution as a competitive process to the biocatalytic oxidation

The oxidation of phenol derivatives by the peroxidase-like activity of metHb is accompanied by oxygen evolution (catalase-like activity) in agreement to the catalytic cycle of the protein (Fig. 1, step 4). Since both activities run in parallel, the oxidation of the substrate and that of H_2O_2 being competitive processes in steps 2 and 3 of the mechanism, the amount of O_2 released should depend on the type of compound to be oxidized. To address this issue, different experiments were carried out by measuring O_2 evolution in the absence and presence of each phenolic substrate. Fig. 5A shows a selection of the O_2 evolution progress curves thus obtained. A decrease in O_2 evolution with respect to the control measurement in the absence of any phenolic compound took place for all of the phenolic substrates here tested.

The O_2 evolution progress curves showed an initial linear phase in which the protein inactivation by H_2O_2 can be considered negligible and it is known as pseudo steady-state of the reaction. The initial steady-state rate of O_2 production in the presence of a phenolic compound is a useful parameter to evaluate the influence of that compound on the catalase-like activity of metHb. In fact, it was observed that, in general, the higher k_{cat}, the lower was the initial rate of O_2 evolution and so the amount of O_2 evolved (data not shown). Therefore it is to be expected that a relationship between both catalytic activities of metHb, peroxidase and catalase, could be established. Fig. 5B shows that indeed a good correlation exists between the rates of both catalytic processes under the same experimental conditions. As can be seen all of the studied p-substituted phenols followed the trend, with the only exception of 4TBP. Probably, the marked differences observed in the V_M, polarizability and log P values for 4TBP with respect to the other phenol derivatives (Table 4) could explain this behaviour. The equation obtained by fitting these data by linear regression was:

$$\left(\frac{V_{0,c}}{[metHb]_0}\right) = 4.22 - 0.08 \times \left(\frac{V_{0,p}}{[metHb]_0}\right), \qquad R^2 = 0.90 \tag{9}$$

From this relationship the effect of a phenol derivative on the O_2 evolution by the catalase-like activity of metHb can be obtained; the higher amount of O_2 was produced in the absence of p-susbtituted phenols ($V_{0,p}$ = 0) and the minimum value of $V_{0,c}$ was observed in the presence of substrates with donor p-substituents (higher $V_{0,p}$) such as HQ, 4AP and 4MP. This fact indicates that the amount of O_2

released in a medium containing phenolic compounds as pollutants might be predicted. The oxygen demand of a wastewater is one of the most important parameters and this type of biocatalytic processes may contribute to increase the amount of dissolved oxygen in the aqueous medium.

Finally, from the results obtained in this work it can be concluded that the use of bovine metHb instead other peroxidase enzymes like HRP might represent a good alternative at economical level, despite its less comparatively decreased activity. To confirm this suitability in a simple way, we performed an experiment consisting of oxidizing a substrate to the same extent (50%) by the two proteins under the same experimental conditions ($[H_2O_2]_0$ = 2.5 mM and $[4CP]_0$ = 0.5 mM). The required amount of metHb and HRP was 5.0 and 0.02 μM, respectively, which implies a cost of 0.18 and 0.33 €/ml of assay, respectively (prices updated from Sigma). Therefore, metHb from animal sources, which can easily be obtained from blood, a residue of the meat industry, could be used effectively for phenol removal. These prices may seem expensive when extrapolated to industrial scale, but they may be drastically reduced by immobilizing the protein and searching of new materials to protect it from H_2O_2-induced inactivation and possibly QSAR-assisted design of a model protein with enhanced reactivity.

4. Conclusions

The values of k_{cat} for the metHb-catalyzed oxidation in the presence of an excess of H_2O_2 of a series of *p*-substituted phenol derivatives have been determined for the first time. Kinetic studies here performed indicated that the protein shows non-michaelian behaviour in its catalytic action on phenolic compounds, and a good QSAR correlation between k_{cat}-values and the Hill coefficients with E_{HOMO} and the pK_a of each phenol derivative could be established. A good correlation was also found between the peroxidase-like and the catalase-like activities of metHb. The QSAR equations here obtained allow us predicting the kinetic parameters for other phenol derivatives and may serve as a basis for the optimization of biocatalytic processes. Overall, the results of this study indicate that bovine metHb, although less active than other peroxidases in agreement with the k_{cat}-values here obtained, is a suitable protein which may, using appropriate technology, be competitive from an economical point of view.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2012.09.028.

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