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Oxidation of 2,4-dichlorophenol catalyzed by horseradish peroxidase: characterization of the reaction mechanism by UV–visible spectroscopy and mass spectrometry

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Abstract

The hydrogen peroxide-oxidation of 2,4-dichlorophenol catalyzed by horseradish peroxidase has been studied by means of UV–visible spectroscopy and mass spectrometry in order to clarify the reaction mechanism. The dimerization of 2,4-dichlorophenol to 2,4-dichloro-6-(2,4-dichlorophenoxy)-phenol and its subsequent oxidation to 2-chloro-6-(2,4-dichlorophenoxy)-1,4-benzoquinone together with chloride release were observed. The reaction rate was found to be pH-dependent and to be influenced by the pK_a value of 2,4-dichlorophenol. The dissociation constants of the 2,4-dichlorophenol/horseradish peroxidase (HRP) adduct at pH 5.5 and 8.5 were also determined: their values indicate the unusual stability of the adduct at pH 5.5 with respect to several adducts of HRP with substituted phenols.

Keywords: Chlorophenol; Horseradish peroxidase; pH-dependence; Dissociation constants

1. Introduction

Chlorophenols are extensively used in pesticide and herbicide manufacture. One of the most important members of this family is 2,4-dichlorophenol (2,4-DCP), a key-intermediate in the synthesis of 2,4-D (2,4-dichlorophenoxyacetic acid), a herbicide found as a component of the infamous 'Agent Orange' used as a defoliant in the Vietnam war.

2,4-DCP is widespread in the environment and has even been detected in atmospheric emissions from the combustion of municipal solid waste, hazardous waste, coal, wood and herbicides [1,2]. Nevertheless, the main release of 2,4-DCP has been found in surface waters: it was detected in several different industrial waste discharges including some from sewage treatment plants and drinking water treatment plants [3]. Chlorophenol release in soil may also occur through accidental processes, uncontrolled discharges and incomplete biodegradation of both aromatic compounds and pesticide mixtures [3,4].

Chlorophenol toxicity has been proven both in vitro [5] and in vivo [6]. In particular, 2,4-DCP has been reported to cause lethargy, tremors and convulsions in mice [7], while workers who made pesticides or were exposed to chlorophenols developed acne and mild liver injuries [3]. Further, four cases of death have also been reported: a man who accidentally splashed pure 2,4-DCP on his right arm and leg [8] and three workers who were sprayed with 2,4-DCP-containing steam [9].

According to some studies, workers who had made pesticides for a long time had a slightly higher risk of cancer with respect to non-exposed people, although the fact that these workers had concurrently been exposed to high levels of other chemicals should be taken into account. So it is doubtful whether carcinogenicity is associated with chlorophenols or other compounds [3]. In fact, long-term treatment of rats and mice with high levels of 2,4-DCP in their diet did not cause cancer [3].

Physical, chemical and biological methods, including incineration, adsorption on activated carbon, chemical or enzymatic oxidation, solvent extraction, microbial degradation, incubation in the presence of microorganisms, etc., have been proposed for removing or degrading several chlorophenols from waste waters [10–14].

Specific studies on 2,4-DCP degradation report some good results obtained by using suspended and immobilized

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cultures of *Bacillus subtilis* [15], Ca-alginate/*Pseudomonas fluorescens* biocatalysts [16] or Nafion-Fe catalysts in the presence of hydrogen peroxide [17].

Another approach was based on the oxidation of 2,4-DCP by pure enzymes. This treatment has many advantages with respect to other biological or chemical/physical methods: handling and storage of isolated enzymes is easier than microorganism manipulation and enzyme concentration is not simply related to bacterial growth; moreover, conventional methods are not very selective, while the specificity of isolated enzymes is higher with respect to other catalysts [18].

Peroxidases from different sources are frequently used in this approach [18–21]. The most employed enzyme within this family, since the early studies of Klibanov et al. on the removal of phenols from coal-conversion waste waters [22], is horseradish peroxidase (HRP) due to its availability and well-known ability to catalyze the oxidation of a large number of aromatic substrates, including phenols and aromatic amines [23–25].

In previous studies, we investigated the H_2O_2-oxidation of 2,4,6-trichlorophenol (2,4,6-TCP) [26] and 2,6-dichlorophenol (2,6-DCP) [27] catalyzed by HRP, in order to clarify the mechanism and characterize the reaction products. We found that the molecular structure of substrates is a critical factor in driving the oxidation towards specific products. Within the same frame, we studied the HRP-catalyzed oxidation of 2,4-DCP, in order to get a more complete picture of the effect of this enzyme on the chlorophenol family. The results have been interpreted in the light of previously reported data, concerning a similar reaction catalyzed by lignin peroxidase [20].

2. Materials and methods

HRP was a Sigma product (type VIA, RZ=3.0); 2,4-DCP and hydrogen peroxide (30%, v/v) were purchased from Aldrich and found pure at gas chromatography–mass spectrometry analysis. All other chemicals were of the highest available grade.

UV–visible measurements were performed on a UVIKON 930 (Kontron Instruments) double-beam spectrophotometer.

Mass spectrometry analysis was executed with a liquid chromatograph HP1100 equipped with a 1100 binary pump, a UV–visible/diode array detector (HP1100 DAD) and a mass detector (HP1100 MS, negative ionization).

2.1. Product identification and characterization

2,4-DCP solutions were prepared by solubilizing the solute in doubly-distilled water. The concentration was checked spectroscopically at $\lambda_{max}=284$ nm, by using $\varepsilon_{284}=2050\ M^{-1}\ cm^{-1}$; this value was determined through a calibration curve.

The reaction was monitored by using both UV–visible absorption spectroscopy and mass spectrometry. In the former case, the spectral changes of an aqueous solution containing 2,4-DCP (3.0×10^{-4} M), HRP (1×10^{-9} M) and H_2O_2 (3.0×10^{-4} M) were followed in the 250–700-nm range. In the latter case, 20 μl of reaction mixture (prepared as described above) were withdrawn at different incubation times and injected directly into the mass spectrometer. The working conditions for the mass detector (APCI-negative) were: $T_{\text{drying gas}}$ 350 °C; $T_{\text{vaporiser}}$ 300 °C; flow-rate$_{\text{drying gas}}$ 7 l/min; capillary voltage 3000 V; corona current 30 μA; fragmentator 60 V.

2.2. Kinetic studies

The pH-dependence of 2,4-DCP reaction was studied at 25 °C in acetate/phosphate buffer 0.01 M according to the following procedure: 2.0 ml of solution containing 2,4-DCP 3.0×10^{-4} M and HRP 1×10^{-9} M at a fixed pH were added with H_2O_2, up to a final concentration of 3.0×10^{-4} M and the absorbance changes at 252 nm (which corresponds to the absorption peak of the reaction product) were recorded.

2.3. Quantitative determination of chloride

Production of chloride during 2,4-DCP oxidation was determined by the modified mercuric thiocyanate method [28] according to Hammel and Tardone [20].

The reaction was stopped by adding 0.3 ml of 5.25 M perchloric acid/0.375 ferric nitrate to 0.4-ml samples which were withdrawn periodically from the reaction vessel. Then 0.3 ml of ethanol saturated with mercuric thiocyanate was added to the sample and the absorbance at 460 nm was read after 5-min incubation time. Chloride concentration in each sample was determined by means of a calibration curve.

2.4. Binding studies

Binding measurements were done by optical titration according to a previously reported method [27,29,30]. A 2-ml amount of enzyme solution (6×10^{-6} M in acetate buffer, 0.01 M, pH 5.5) and 2.0 ml of buffer, in the sample and reference cell, respectively, underwent subsequent 200-μl additions of 2,4-DCP solution (1.0×10^{-2} M, dissolved in the same buffer as the enzyme) up to a final volume of 3.8 ml. The spectrum relative to the free enzyme was then subtracted from those of the enzyme-ligand adducts (corrected for dilution) and the absorbance changes at a fixed wavelength (ΔA) were correlated with 2,4-DCP concentrations by means of the following equation:

$$\Delta A = \frac{\Delta A_{\infty} \cdot [\text{2,4-DCP}]}{K_d + [\text{2,4-DCP}]}$$

where ΔA_∞ is the absorbance change at saturating substrate concentration. The dissociation constant K_d was obtained by means of a numerical fitting procedure.

3. Results

The addition of HRP to a solution containing 2,4-DCP and hydrogen peroxide results in the appearance of a strong absorption band at 252 nm and a shoulder at 260–262 nm in the optical spectrum of the reaction mixture (Fig. 1, inset). Such absorption can be assigned to the reaction product, in agreement with previous studies on the oxidation of 2,4-DCP by H_2O_2 in the presence of lignin peroxidase [20]. In contrast with other polychlorophenols [26,27], the reaction does not bring about any absorbance change in the visible region and the reaction mixture remains colourless over the reaction time.

The absorbance at 252 nm increases quickly, until a plateau is reached in ~10 min (Fig. 1); further additions of HRP result in absorbance increases similar to that already observed. This recurrent pattern, associated with each HRP addition, is observed until complete consumption of 2,4-DCP and is typical of a process of enzyme inactivation.

Two distinct mechanisms are known to inactivate HRP: (i) the enzyme can be attacked by radical intermediates resulting from the oxidation of the donor substrate [31,32]; (ii) hydrogen peroxide, either in the absence of donor substrates or in large excess [32,33], can induce formation of inactive intermediates.

In our experimental conditions, an H_2O_2-mediated inactivation ($[H_2O_2]/[2,4\text{-DCP}]=1:1$) is not expected and product inhibition might well explain the effect described in Fig. 1. More experiments would be necessary to clarify the mechanism of inactivation, but this is beyond the aim of this work.

The pH dependence of the initial reaction rate has been

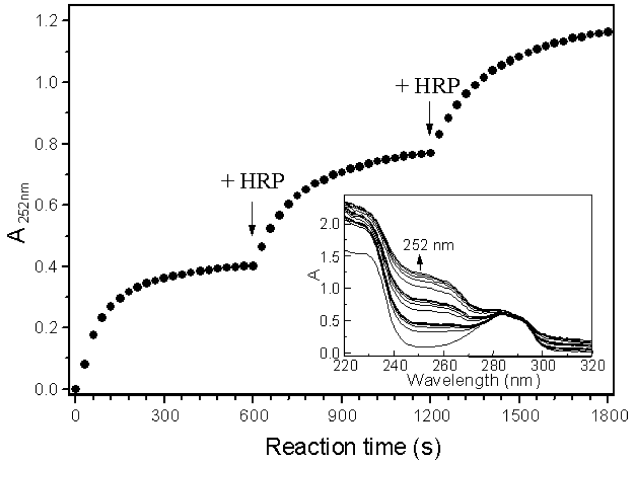


Fig. 1. Absorbance change at 252 nm versus reaction time; arrows indicate further additions of HRP at 600- and 1200-s reaction time. The concurrent evolution of the electronic spectrum is reported in the inset.

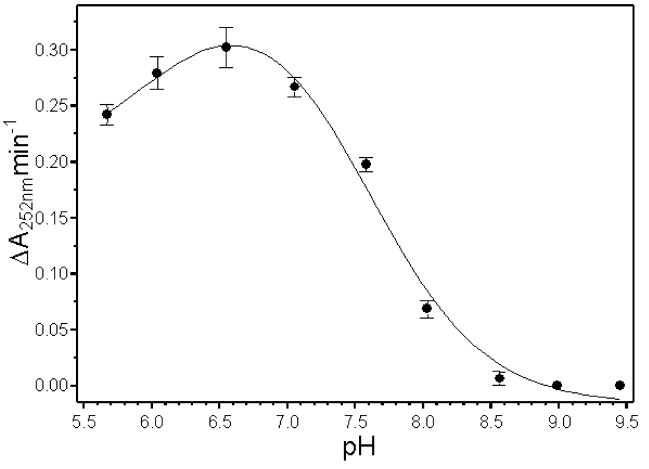


Fig. 2. pH-dependence of the initial rate of HRP-catalyzed oxidation of 2,4-DCP expressed as absorbance changes at 252 nm.

investigated in the pH range 5.5–9.5 by following the absorbance changes at 252 nm. The results are shown in Fig. 2. The maximum reaction rate is observed at pH 6.5; it decreases markedly when pH is raised and becomes negligible from pH 8.5 on.

Experimental data were fitted to the following model, which derives from the Henderson-Hasselbach equation [34] and describes a system undergoing a double ionization process:

$$v = \frac{[H^+]^2 \cdot b + [H^+] \cdot c + K_{a1} \cdot K_{a2} \cdot d}{K_{a1} \cdot K_{a2} + [H^+] \cdot K_{a1} + [H^+]^2}$$

The three parameters b, c, and d are correlated with different protonation states of two ionizable groups [24] with proteolytic dissociation constants K_{a1} and K_{a2}, respectively.

Two pK_a values were obtained by means of non-linear least-squares fit: $pK_{a1}=6.04\pm0.66$ and $pK_{a2}=7.58\pm0.14$; the former is correlated with the protonation degree of a carboxylic residue on the enzyme moiety [24,35] and the latter can be associated either with the dissociation state of histal histidine ($pK_a=8.6$) [36] or with the pK_a value of the donor substrate [24]. pK_a values reported in the literature for 2,4-DCP fall in the range 7.6–8.0 [37], while imidazole of the distal histidine remains substantially protonated up to pH 7.6; this suggest that the reaction-rate determining-factor is deprotonation of the donor as previously observed with *p*-hydroxybenzaldehyde [24] and 2,6-DCP [27].

Since HRP reactivity is correlated with both the nature and strength of the enzyme-substrate interactions, dissociation constant (K_d) values of the HRP/2,4-DCP adduct were determined at two different pH, 5.5 and 8.5, corresponding to distinct protonation states of 2,4-DCP, the neutral and the negatively charged states, respectively. K_d values were obtained from the analysis of the optical difference spectra

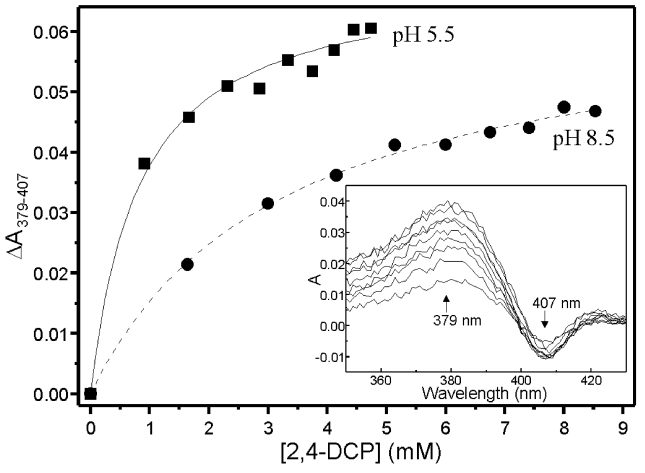


Fig. 3. Correlation plots of absorbance changes (ΔA) versus 2,4-DCP concentration at pH 5.5 and 8.5; the corresponding optical difference spectra are reported in the inset.

[38], a method largely used for studying the interactions between peroxidases and inorganic or organic ligands [29,30,39–41], including chlorophenols [27].

Experimentally determined K_d values (Fig. 3) were 0.84±0.11 mM at pH 5.5 and 3.32±0.40 mM at pH 8.5, respectively. The affinity of HRP is higher for the protonated (neutral) compared to the ionized phenol; this result is consistent with the hydrophobic character of the protein binding site [42].

In order to identify and characterize the reaction products, mass spectra of the reaction mixture at different times were analyzed. After 90-s incubation, the mass spectrum shows three multiplets (Fig. 4): the first one, which is already present before the beginning of the reaction, has the typical pattern of a molecular ion containing two

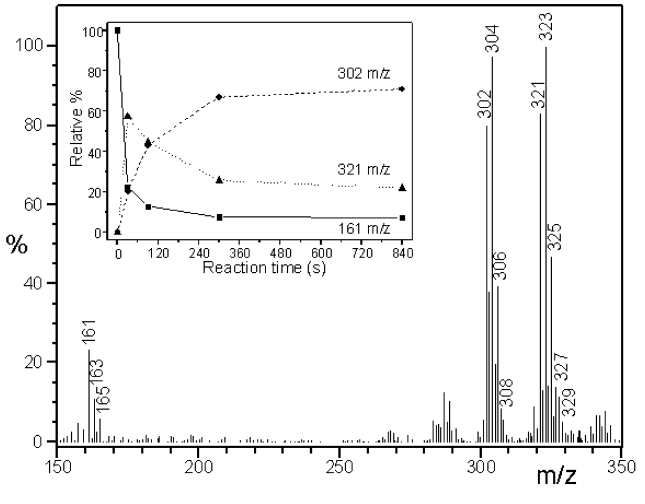


Fig. 4. Mass spectrum of the reaction mixture H_2O_2/HRP/2,4-DCP after 90-s incubation; the time-dependence of the relative intensity of 161, 302 and 321 m/z peaks is reported in the inset.

chlorine atoms (161, 163, 165 m/z, M-1 signal under negative ionization). It has been assigned to 2,4-DCP (M_r = 162).

The second and third multiplets appear later and are associated with the reaction products; their relative intensity changes during the reaction (Fig. 4, inset).

The second multiplet (302, 304, 306, 308 m/z) is typical of a species containing three chlorine atoms; it may be assigned to 2-chloro-6-(2,4-dichlorophenoxy)-1,4-benzoquinone (M_r = 302, without dissociable protons), in analogy with the mechanism of 2,4-DCP oxidation catalyzed by lignin peroxidase from *Phanerochaete chrisosporium* [20].

The pattern relative to the third multiplet (321, 323, 325, 327, 329 m/z) is consistent with the presence of four chlorine atoms. On the basis of the well-defined mechanism of phenol oxidation catalyzed by HRP [42], it can be assigned to a species deriving from the condensation of two 2,4-DCP molecules by means of radical intermediates formation (M_r = 322), its mass spectrum exhibiting a (M-1) signal due to the loss of a proton, under negative ionization.

2-Chloro-6-(2,4-dichlorophenoxy)-1,4-benzoquinone may derive from the oxidative dechlorination of a 2,6-disubstituted-4-chlorophenol, as reported both for lignin peroxidase-catalyzed 2,4-DCP oxidation [20] and in the reaction of 2,4,6-trichlorophenol with HRP [26]; on this basis, we assign the third multiplet to 2,4-dichloro-6-(2,4-dichlorophenoxy)-phenol.

The mass spectra recorded upon 10-min incubation (data not shown) show the presence of high molecular-weight species (presumably trimers, tetramers, etc.) deriving from parasite reactions, in analogy to that reported for 2,6-DCP oxidation [27]. These species have not been characterized in the present study.

The chloride loss associated with the mechanism proposed for the oxidative dechlorination has been confirmed and quantified by the optical method of mercuric thiocyanate. The analysis results are shown in Fig. 5: release of chloride proceeds together with 2-chloro-6-(2,4-dichlorophenoxy)-1,4-benzoquinone production (Fig. 4, inset). After 20-min incubation, the chloride concentration in the reaction batch approaches 25–30% of the initial concentration of 2,4-DCP: this percentage is similar to that observed during the oxidation of 2,4-DCP in the presence of lignin peroxidase from *Streptomyces viridosporus* [21].

4. Discussion

In analogy to what was observed with other phenols, the first step of the HRP-catalyzed oxidation of polychlorophenols implies the concerted transfer of one proton and one electron from the substrate to the protein and gives rise to a phenoxyl radical [42]. This species is subsequently converted into a more stable compound, whose structure is strictly related to that of the parent molecule.

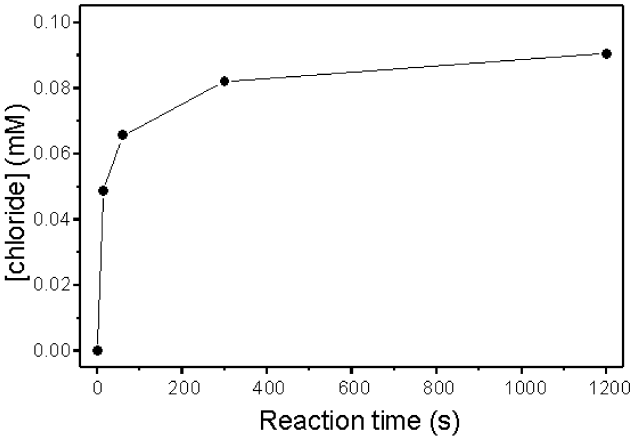


Fig. 5. Time-course of chloride release during the H_2O_2-oxidation of 2,4-DCP catalyzed by HRP. Chloride was detected as chloromercurate(II) complex ion with the indirect analytical method described in the Material and methods section.

The oxidation of 2,4,6-TCP has been shown to proceed via the substitution of a chlorine atom with an oxygen atom at position 4 of the aromatic ring and the consequent formation of *p*-quinone [20,26]. In contrast, a *p*-*p* dimer is generated by coupling of two 2,6-dihydroxyphenyl radicals when the substrate is 2,6-DCP [27].

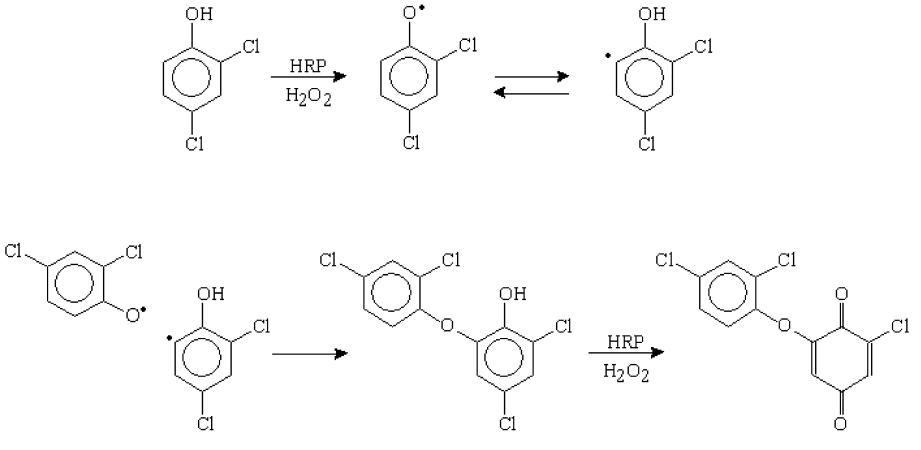
The reaction mechanism of 2,4-DCP oxidation is somehow intermediate between the two pathways described above. According to Scheme 1, the first reaction step results in the formation of 2,4-dichlorophenoxyl radical, which is partially converted into the correspondent phenyl radical. The first reaction product (2,4-dichloro-6-(2,4-dichlorophenoxy)-phenol) is generated by the coupling of these two species; since this product is still oxidizable, the reaction proceeds further and a second product appears: 2-chloro-6-(2,4-dichlorophenoxy)-1,4-benzoquinone. The mechanism is likely the same as that reported for 2,4,6-TCP oxidation [20,26]: a radical-cation is formed by the action of HRP on 2,4-dichloro-6-(2,4-dichlorophenoxy)-phenol and undergoes nucleophilic attack by a water molecule, on position 4 of the aromatic ring. HCl is eliminated and a quinone is generated, after rearrangement.

Experimental data on chloride release during the reaction support this hypothesis; it is also in agreement with literature data on the oxidation of 2,4-DCP catalyzed by lignin peroxidase from *P. chrisosporium* [20], although, in that case, only the final product was analyzed and identified.

Further useful information on the reaction mechanism is provided by the enzyme-substrate binding data. First of all, they prove that the interaction between HRP and 2,4-DCP is strong, since the adduct ($K_d = 0.84 \pm 0.11$ mM at pH 5.5) is ~9-fold more stable than the 2,6-DCP/HRP complex ($K_d = 7.8$ mM at pH 5.5 [27]). On average, the HRP/2,4-DCP adduct stability is higher with respect to many other phenols (K_d reported in the literature fall within the range 3–11 mM [24,37,38,43]). Moreover, the increased K_d value at pH 8.5 with respect to pH 5.5 suggests that HRP interacts mainly with the protonated (neutral) form of 2,4-DCP and this agrees with the highly hydrophobic character of the substrate binding site [42].

pH affects HRP reactivity, as well: the reaction rate of 2,4-DCP oxidation increases in slightly acidic conditions. This is likely related to the increased degree of protonation of a carboxylic residue of the enzyme moiety, with $pK_a \sim 5.4$ [24,44]. The experimental value $pK_{a1} = 6.0$, extrapolated from Fig. 2, might be affected by the lack of data below pH 5.5. At higher pH values, the reaction rate dependence from pH shows an inflection point at $pK_{a2} = 7.6$. This value agrees with the pK_a value of 2,4-DCP in aqueous solution reported in the literature [37].

The agreement between kinetic and binding data suggests that the 2,4-DCP protonation state affects the reaction rate of the oxidation reaction, as previously observed both in the case of 2,6-DCP [27] and *p*-hydroxybenzaldehyde [24] oxidation catalyzed by HRP.



Scheme 1.

5. Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DCP	2,4-dichlorophenol
2,6-DCP	2,6-dichlorophenol
2,4,6-TCP	2,4,6-trichlorophenol
HRP	horseradish peroxidase

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