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Journal of Inorganic Biochemistry 92 (2002) 75–81

Journal of Inorganic Biochemistry

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Enzymatic degradation of 2,6-dichlorophenol by horseradish peroxidase: UV–visible and mass spectrophotometric characterization of the reaction products

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Received 27 March 2002; received in revised form 12 June 2002; accepted 17 June 2002

Abstract

The reaction mechanism of the oxidation of 2,6-dichlorophenol (2,6-DCP) by horseradish peroxidase (HRP) and H_2O_2 has been investigated and the reaction products have been characterized by UV–visible and mass spectrometry. Evidence for the dimerization of 2,6-DCP to 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl and the subsequent fast oxidation of this product to the corresponding 3,3′,5,5′-tetrachlorodiphenoquinone have been collected. The reaction rate was found to decrease markedly as soon as the pH was raised, with a clear inflection point at pH ≅ 6.6–6.9; it also resulted independent from H_2O_2 concentration. Since the pK_a for 2,6-DCP is 6.80, the reaction rate might be influenced by the protonation state of the substrate.
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Keywords: Chlorophenol; Horseradish peroxidase; Enzymatic degradation

1. Introduction

Polychlorinated phenols belong to a family of highly toxic [1–4] and potentially carcinogenic compounds [5,6] that can be found in waste waters as persistent environmental pollutants; they result from several man-made activities such as coal conversion, water disinfection, uncontrolled use of herbicides, pesticides and fungicides, wood pulp and paper manufacturing [7,8]. Chlorophenols are widely distributed in the environment and they can be found also in water and soils of non-industrial regions.

Several different methods have been suggested in order to remove phenols and chlorophenols from industrial waste waters: adsorption on activated carbon, solvent extraction, microbial degradation, chemical oxidation, etc.; unfortunately, all of them suffer from shortcomings such as high cost, incomplete detoxification or conversion into even more hazardous products [9,10].

In most recent years, a new approach to this problem has been tried, based on enzymatic degradation. Compared to conventional biological methods, this strategy looks advantageous since handling and storage of isolated enzymes is easier than microorganism manipulation; besides, enzyme concentration is not affected by bacterial growth [11] and the specificity of isolated enzymes is higher if compared with that of intact organisms or even with chemical methods. Moreover, their employment in the selective removal of hazardous compounds makes subsequent treatments of waste waters easier, if required.

Due to their proven ability to oxidize a large number of substituted phenols [12–14], peroxidases are the most widely employed enzymes in the field. Many data concerning the oxidation of chlorophenols by lignin peroxidase [7], soybean peroxidase [11], lactoperoxidase [8] and some peroxidase-like models [15,16] can be found in the literature. Although, the most employed enzyme in the field is by far horseradish peroxidase (HRP, EC 1.11.1.7) due to its relatively high availability and low cost as well as its ability to oxidize a wide range of substrates [9,17–20].

The degradation of chlorophenols by peroxidases proceeds through the generation of phenoxyl radicals; these can diffuse out from the active site of the enzyme and react with other substrate molecules, giving rise to oligomers or polymers [9] that are much less water-soluble than the original monomers; as a consequence, they precipitate out of the solution and can be separated by

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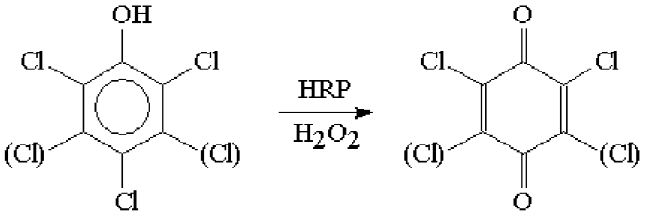
PII: S0162-0134(02)00488-9



Scheme 1.

filtration [10]. In some cases, precipitation can be enhanced by addition of a reactive cosubstrate [17], additives [9,21] or a complexing agent [18].

Since the molecular structure of the substrate (in particular, the number and position of chlorine atoms) is the driving force for these reactions, an investigation of the mechanism of polychlorophenol oxidation is needed and deserves interest.

Oxidation of pentachlorophenol and 2,4,6-trichlorophenol by lignin peroxidase [7] or HRP [19,20] is known to generate a dechlorinated p-benzoquinone as the main reaction product, according to Scheme 1.

The oxidation of 2,4,5-trichlorophenol [7] is thought to proceed via a similar mechanism and a dimer has been identified as the possible result of the reaction of 2,4-dichlorophenol with lignin peroxidase and H_2O_2 [7], while the enzymatically-generated oxidation products of 2,6-dichlorophenol (2,6-DCP) had never been identified. This paper describes the investigation of the 2,6-DCP oxidation mechanism by HRP and the characterization of the reaction products by the concerted use of UV-visible spectroscopy and mass spectrometry.

2. Materials and methods

HRP was purchased from Sigma (type VIA, RZ=3.0); 2,6-DCP and hydrogen peroxide (30% v/v) were purchased from Aldrich and found pure at gas chromatography–mass spectrometry analysis; 3,3′,5,5′-tetrachloro-4,4′-biphenyldiol was a Chem Service (West Chester, PA, USA) product. All other chemicals were of the highest available grade.

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

High-performance liquid chromatography (HPLC)/mass spectrometry (MS) 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

Water solubility of 2,6-DCP was measured by suspending an excess of solid product in 50 ml of doubly-distilled water and by keeping the suspension under stirring in a temperature-controlled bath set at 25 °C, for 24 h. The 2,6-DCP concentration was determined spectroscopically in the supernatant at $\lambda_{max} = 283$ nm ($\varepsilon_{283} = 1760$ $M^{-1}\,cm^{-1}$, previously determined by means of a calibration curve) after centrifugation.

The reaction was monitored by both UV-visible absorption and HPLC/MS. In the former case, spectrophotometrical changes of an aqueous solution containing 2,6-DCP (3.4×10^{-4} M), HRP (1×10^{-9} M) and H_2O_2 (3.4×10^{-4} M) were followed in the 250–700 nm range. The final pH was 5.5. In the latter case, 20 μl of reaction mixture (prepared as written above) were injected onto a RP-C18 column (Lichrospher, HP); the flow rate was 1 ml min^{-1} and a gradient elution with acetonitrile/acetic acid (0.09 M) was employed. The solvent programming was as follows: initially 1 min isocratic with 55% acetic acid, 18 min linear gradient to 100% acetic acid, 2 min 55% acetic acid to return the system to its initial conditions.

Subsequently, in order to overtake the solubility threshold of the reaction products and to get a precipitate, an excess of H_2O_2 (6×10^{-2} M) was added to a saturated solution of 2,6-DCP (1.5×10^{-2} M) containing HRP (1×10^{-6} M). After a 4-h incubation, the reaction mixture was centrifuged, the supernatant discarded and the precipitate washed twice with water and twice with acetone.

2.2. Kinetic studies

The pH-dependence of 2,6-DCP reaction was studied at 25 °C in acetate/phosphate buffer 0.01 M according to the following protocol: 2 ml of 2,6-DCP 3.4×10^{-4} M and HRP 1×10^{-9} M solution at a fixed pH were added with H_2O_2 up to a final concentration of 3.4×10^{-4} M, and the absorbance changes at 434 nm were recorded.

2.3. Binding studies

Binding measurements were done by optical titration as previously reported [22,23]. Two millilitres of enzyme solution (6×10^{-6} M in acetate/phosphate buffer 0.01 M, pH 5.5 or 8.0) and 2 ml of buffer, in the sample and reference cell, respectively, underwent subsequent additions of 2,6-DCP solution (1.4×10^{-2} M, dissolved in the same buffer as the enzyme) up to a final volume of 4 ml. The spectrum relative to the free enzyme was then subtracted from those of the enzyme–ligand adduct (corrected for dilution) and the dissociation constant K_d was obtained by correlating the absorbance changes with 2,6-DCP concentrations, by means of a numerical fitting procedure.

3. Results and discussion

Addition of H_2O_2 to a 2,6-DCP water solution in the presence of HRP results in a rapid change of the reaction

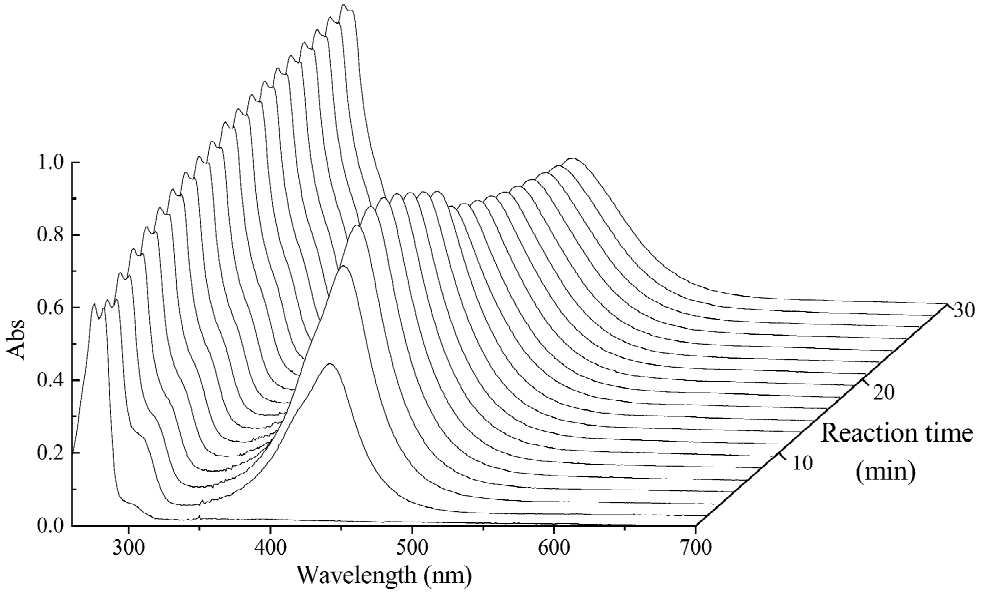


Fig. 1. Evolution of the UV-visible spectrum of the reaction mixture H_2O_2/HRP/2,6-DCP over a 30-min time length.

mixture from colorless to deep yellow; after a few minutes, the color intensity starts to decrease slowly. Fig. 1 shows the UV-visible spectral changes of this reaction mixture vs. time. A strong absorption band at $\lambda = 434$ nm appears upon peroxide addition and reaches its maximum intensity in 5–7 min; then it starts to decrease and broaden, undergoing a red-shift towards 437–438 nm. Such a time-dependence is consistent with formation of a transient species, characterized by low chemical stability, that slowly evolves towards a more stable product.

The reaction-rate dependence on peroxide concentration has been investigated. As shown in Fig. 2A, a limiting effect of peroxide concentration is observed for $[H_2O_2]/[2,6\text{-DCP}] \leq 0.4$, although at higher $[H_2O_2]/[2,6\text{-DCP}]$ ratio the generation rate of the yellow product is almost independent from $[H_2O_2]$ when this ratio falls in the range 0.4–4 (Fig. 2B–F). As well, the subsequent evolution of this reaction towards a secondary product (witnessed by the absorbance decrease at 434 nm) is not significantly affected by the $[H_2O_2]/[2,6\text{-DCP}]$ ratio. In the absence of HRP (Fig. 2G) the absorbance at 434 nm increases very slowly, indicating that 2,6-DCP does not actually react with hydrogen peroxide in these experimental conditions.

The pH-dependence of the conversion rate of 2,6-DCP into the first oxidation product (the transient one) was investigated as well, by using the absorbance change at 434 nm as a marker. The reaction rate decreases markedly as soon as the pH is raised and an inflection point at

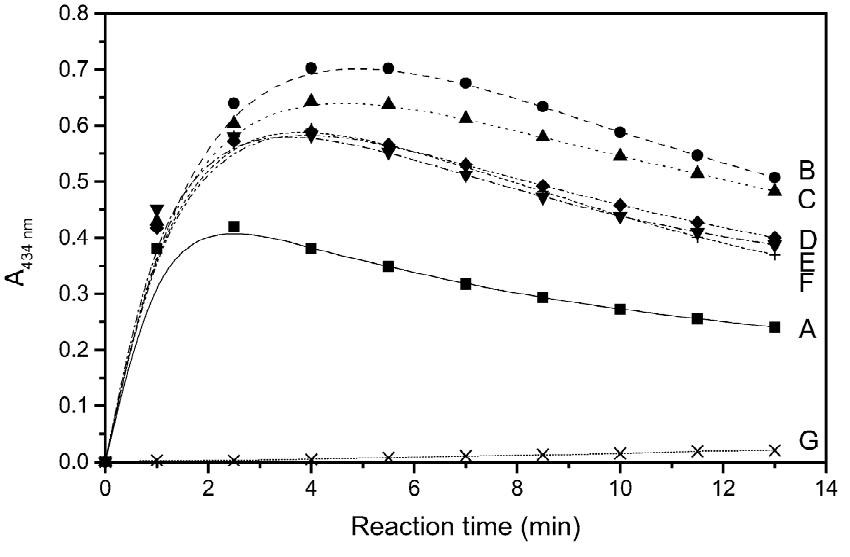


Fig. 2. Absorbance change at 434 nm vs. time for different $[H_2O_2]/[2,6\text{-DCP}]$ ratio: A = 0.1; B = 0.4; C = 0.8; D = 1.2; E = 1.6; F = 4.0; G = 0.8 in the absence of HRP.

pH ≅ 6.6–6.9 becomes evident, as shown in Fig. 3. Since 2,6-DCP is slightly acidic (its pK_a being 6.80 [24]), the reaction rate might be influenced by the protonation state of the substrate as previously observed for the oxidation of 3-methyl-2-benzothiazolone hydrazone [25] (pK_a = 5.8 [26]).

In order to check the possible correlation between the affinity of 2,6-DCP for HRP and its protonation state, the dissociation constant K_d of the HRP/2,6-DCP adduct was determined spectrophotometrically at two different pH values, according to a previously published method [22,23]. K_d values of 7.8±1.2 mM at pH 5.5 and 17.4±3.1 mM at pH 8.0 were obtained. These results show that HRP displays a higher affinity for the protonated (neutral) 2,6-DCP compared to its ionized form.

In order to clarify its mechanism, the reaction was monitored by mass spectrometry. In the mass spectrum of the reaction mixture after 2 min of incubation (data not shown), four groups of peaks are evident: the first one shows the characteristic pattern of a molecular ion containing two chlorine atoms (161, 163, 165 m/z, under negative ionization) and corresponds to unreacted 2,6-DCP; the assignment of the second and the third ones, falling at 285–295 m/z and 300–306 m/z, respectively, is not straightforward; the fourth one falls at 320–330 m/z and might be associated with a few different 2,6-DCP dimers.

Since, in these conditions, a deeper interpretation of the experimental data is not possible, the oxidation was monitored by sampling the reaction mixture in a fixed time range (between 0.5 and 45 min) and injecting the collected samples in a HPLC–MS machine; the results are reported in Figs. 4 and 5. The chromatogram reported in Fig. 4 (incubation time = 6 min) still shows a peak assignable to unreacted 2,6-DCP (this assignment was checked by injecting 2,6-DCP alone in the LC–MS and by measuring its retention time). Two partially overlapped peaks (marked as A and B, respectively) are visible in the same chromatogram at higher retention times. These peaks are not found in the absence of HRP (data not shown).

Fig. 4 (see the inset) reports the measured areas of these three chromatographic peaks (A, B and 2,6-DCP): the progressive decrease of the 2,6-DCP amount gets along with the appearance of peak A, whose intensity reaches a steady state after 6 min; at the same time, peak B becomes evident and progressively increases, without reaching a plateau. Fig. 5 reports the UV-visible and mass spectra of the reaction products A and B. The optical spectrum of species A shows an absorption maximum at 270 nm, while species B absorbs at both 270 nm and 430–435 nm. This latter absorption is much more intense than the former and is almost identical to that reported in Fig. 1.

As for the mass spectra, a zoom of the spectral pattern between 310 and 340 m/z is reported in Fig. 5. A closer analysis of these data reveals that the two products are similar, both containing four chloride atoms and differing for 1 mass unit.

The multiplet in Fig. 5A (321, 323, 325, 327, 329 m/z) can be assigned to the dimer deriving from the condensation of two 2,6-DCP molecules: 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl (TCDHBP, M_r = 322 Da); under negative ionization conditions, the spectrum exhibits a (M-1) signal due to the loss of a proton. The second multiplet (320, 322, 324, 326, 328 m/z, Fig. 5B) corresponds to the molecular ion of 3,3′,5,5′-tetrachlorodiphenoquinone (TCDPQ, M_r = 320 Da), which comes from the oxidation of TCDHBP and does not contain dissociable protons.

A mass spectrum of TCDHBP alone, as a standard sample, was run and confirmed this interpretation. TCDHBP mass spectrum is almost coincident with that reported in Fig. 5A; furthermore, upon reaction with HRP and hydrogen peroxide, TCDHBP gives rise to a yellow soluble product, whose color is the same as the one obtained after oxidation of 2,6-DCP and whose UV-visible

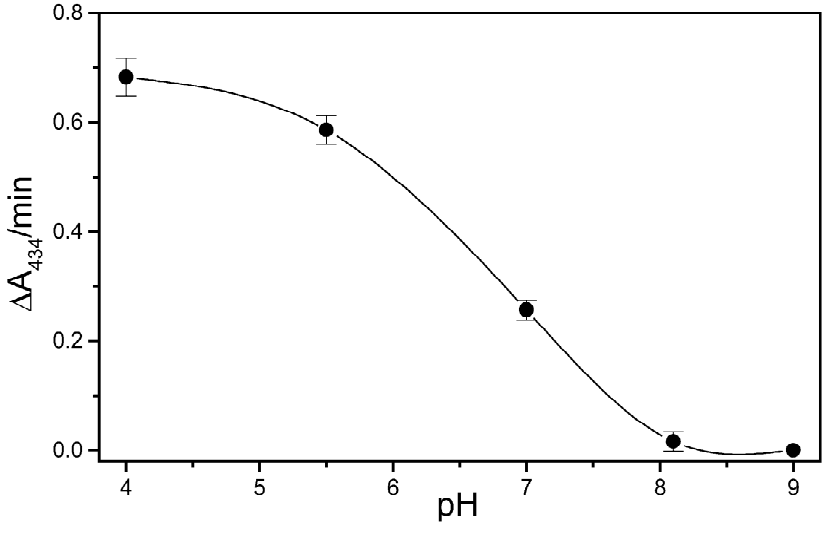


Fig. 3. pH-dependence of the initial rate of 2,6-DCP oxidation by HRP/H_2O_2.

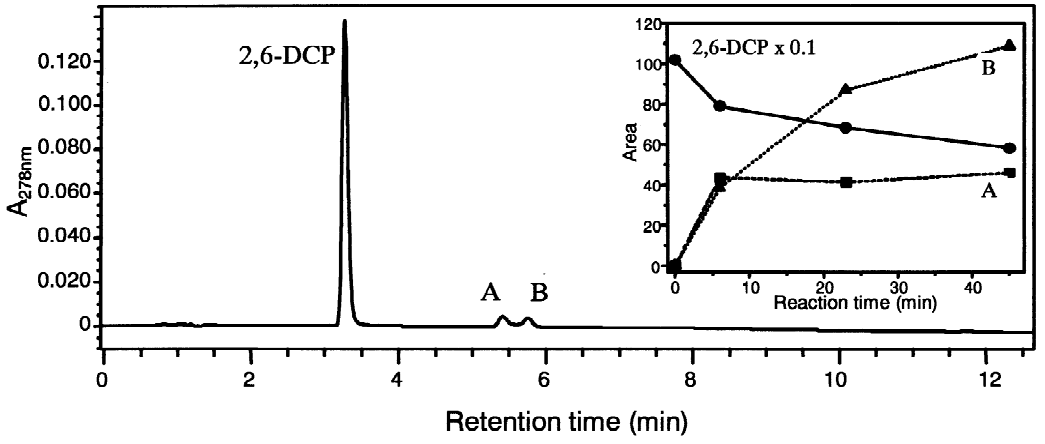


Fig. 4. Chromatogram obtained from HPLC/MS analysis of the reaction mixture H_2O_2/HRP/2,6-DCP after 6 min incubation. The trend of peak areas vs. reaction time are reported in the inset.

spectrum coincides with the one showed in Fig. 5B as well as with that previously reported for TCDPQ [27].

Finally, the MS analysis of a sample drawn out from the reaction mixture after 1 h of incubation (Fig. 6) shows the presence of several high molecular weight species. These likely derive from parasites reactions of the 2,6-dihydroxyphenyl radical with primary reaction products.

The presence of high molecular weight species, generated through a radical mechanism, was confirmed by the analysis of the red-orange precipitate produced by mixing saturated solutions of 2,6-DCP with a large peroxide excess, in the presence of relatively high enzyme concentrations (1×10^{-6} M). Thin layer chromatography shows that at least seven different reaction products are present in the precipitate and desorption chemical ionization–MS analysis suggests that the reaction mixture contains several oligomers with molecular weight varying from 322 (dimer) up to to 1800 m/z (data not shown).

4. Conclusions

HRP is known to give rise to stable adducts with different substituted phenols; K_d values for such adducts falls into the range 3–11 mM when phenols are in the

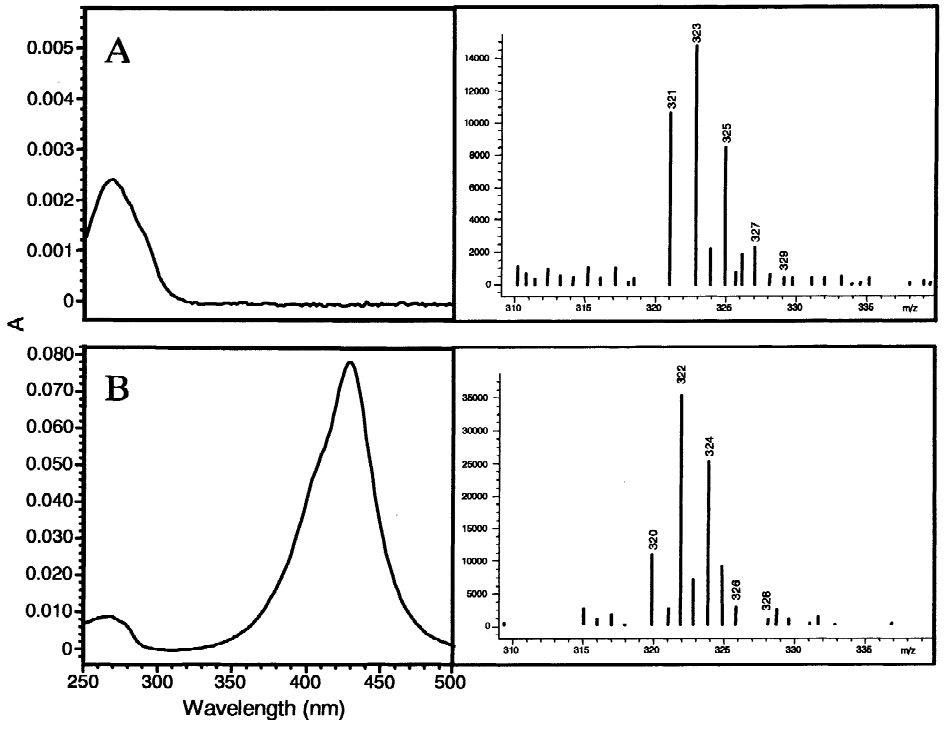


Fig. 5. UV-visible and mass spectra of the reaction products A and B (see Fig. 4).

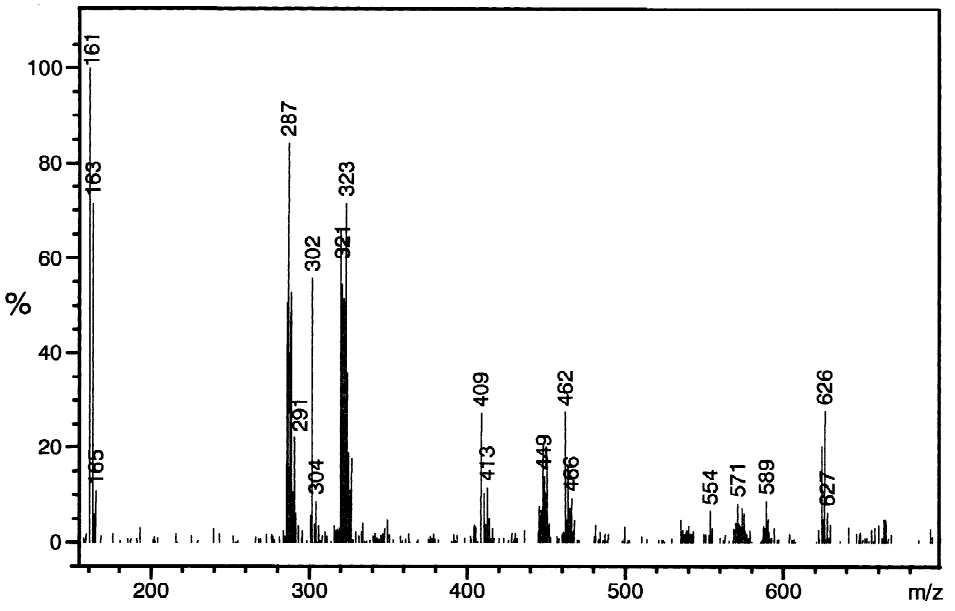


Fig. 6. Mass spectrum of the reaction mixture H_2O_2/HRP/2,6-DCP after 1 h of incubation.

undissociated (neutral) form [14,28–31]. The stability of the 2,6-DCP–HRP adduct at pH 5.5 (when the substrate is in its neutral form) falls within this range ($K_d = 7.8$ mM) while it is much lower at pH 8.0 ($K_d = 17.4$ mM); such a behaviour could be explained by the ionization of the ligand at pH 8.0 and also by considering that the substrate binding site is highly hydrophobic, according to what was reported by Henriksen et al. in their paper on the HRPC/benzhydroxamic acid adduct [32]. Moreover, this behaviour is not likely to depend from the deprotonation state of a residue on the protein scaffold, since HRP is known to give rise to an adduct with benzhydroxamic acid ($pK_a = 8.8$), whose stability does not show any pH dependence in the 4–8.5 range [33].

The oxidation of 2,6-DCP in the presence of HRP and hydrogen peroxide is a complex process that generates several products. An hypothesis for the starting reaction steps, based on the above-reported data and on the well-established properties of HRP, is reported in Scheme 2.

Reaction starts with the concerted transfer of a proton and an electron from the substrate moiety towards the protein, according with the typical oxidation mechanism of phenolic substrates by HRP: as a result, a phenoxyl radical is produced [34]. This is subsequently converted into the unstable 2,6-dihydroxyphenyl radical that further dimerizes (through a radical mechanism) and gives rise to the first oxidation product: 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl (TCDHBP). A similar reaction pattern has been reported for the enzymatic oxidation of tyrosine [35] and ferulic acid [34] by HRP.

In turn, TCDHBP is unstable in such reaction medium, since it can act as a substrate for HRP and be converted into a second oxidation product, 3,3′,5,5′-tetrachlorodiphenoquinone (TCDPQ), the species responsible for the deep yellow colour of the solution.

Kinetic data recorded at different pH values agree with such a mechanism and suggest the presence of a correlation between oxidation rate and pH, just like the one observed for K_d: in other words, the protonation degree of 2,6-DCP is likely to influence the reaction rate.

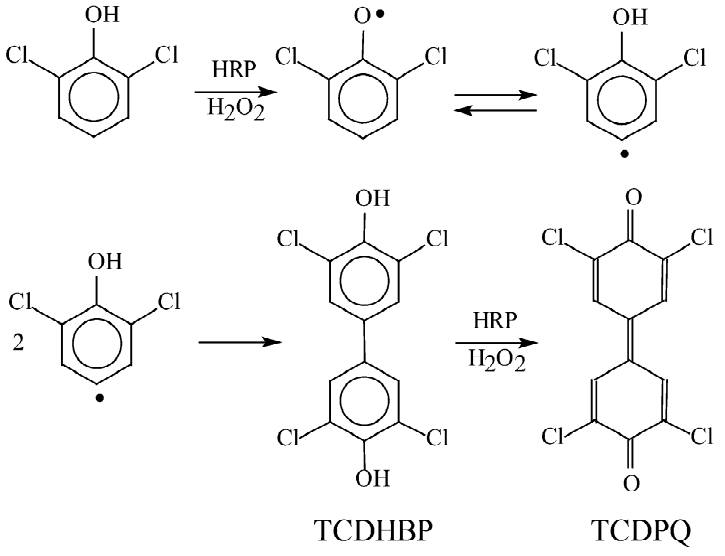
Actually, it is well established [14,31,36] that the ionization of distal histidine ($pK_a = 8.6$) in HRP strongly influences the phenols oxidation rate; on the other hand, at lower pH the ionization degree of substrates seems to be the discriminating factor [9,25].

The longer the reaction, the most complex the reaction mixture becomes. In fact, as long as the reaction goes on, new species rise from the interaction of both primary oxidation products with 2,6-dihydroxyphenyl or more complex radicals, according to the typical ‘cascade’ mechanism of radically processes. As a result, trimers and tetramers are generated (and identified by HPLC–MS), as well as higher molecular weight products, observed in the presence of higher reagent concentrations. These heavier species, being much less soluble than the primary products of this reaction, tend to precipitate and can be easily separated from the liquid reaction phase.

This suggests that the oxidation reaction brought about by HRP in the presence of 2,6-DCP and high concentrations of peroxide could be used as a tool for detoxifying waste waters from 2,6-DCP through formation of an easily removable solid phase, as has already been suggested for other chlorophenols [9,10,17].

5. Abbreviations

HRP	horseradish peroxidase
2,6-DCP	2,6-dichlorophenol



Scheme 2.

TCDHBP 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl
TCDPQ 3,3′,5,5′-tetrachlorodiphenoquinone

Acknowledgements

The authors wish to thank Dr. R. Gamberini and Prof. A. Vanni for helping in HPLC–MS analysis and for their helpful comments and remarks. This work was supported by the Italian 'Ministero dell'Istruzione, dell'Università e della Ricerca' (MIUR) (PRIN 2000 - prot. MM03185591_005).

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