JBIC (1999) 4:232–237

ORIGINAL ARTICLE

Rosa Pia Ferrari · Enzo Laurenti · Francesco Trotta

Oxidative 4-dechlorination of 2,4,6-trichlorophenol catalyzed by horseradish peroxidase

Received: 20 November 1998 / Accepted: 29 January 1999

Abstract The well-known and easily available horseradish peroxidase (HRP) catalyzes the H_2O_2-dependent oxidative 4-dechlorination of the pollutant 2,4,6-trichlorophenol, which is recalcitrant to many organisms except those producing ligninases. UV-visible spectroscopy and gas chromatography-mass spectrometry identified the oxidized reaction product as 2,6-dichloro-1,4-benzoquinone. NMR and IR spectroscopic data further supported the above characterization. Experimental evidence for the elimination of HCl from the substrate was acquired by detecting the decrease in pH of the reaction mixture, and by observing the presence of the β-chlorocyclopentadienone cation fragment in the mass spectrum of 2,6-dichloro-1,4-benzoquinone. Consequently, nucleophilic attack by water on the 2,4,6-trichlorocyclohexadienone cation was proposed to give the final product. Our results indicate an oxidative dechlorination pathway catalyzed by HRP for 2,4,6-trichlorophenol, similar to that by extracellular lignin peroxidases. The relative catalytic efficiency of HRP seems higher than that of lignin peroxidases. The HRP-H_2O_2 catalytic system could be utilized in the degradation of polychlorinated phenols for industrial and biotechnological purposes.

Key words Horseradish peroxidase · Chlorophenols · Pollutant degradation · Metalloenzymes · Enzyme catalysis

Abbreviations *HRP* horseradish peroxidase · *LiP* lignin peroxidase · *TCP* 2,4,6-trichlorophenol

R. P. Ferrari (✉) · E. Laurenti · F. Trotta
Dipartimento di Chimica I.F.M., Università di Torino
Via P. Giuria 7, I-10125 Torino, Italy
e-mail: ferrari@ch.unito.it, Fax: +39-011-6707855

Introduction

Phenols and their derivatives are widely employed in industrial processes (i.e. coal conversion, petroleum refining, polymers, dyes and drug production). Some of them, and in particular polychlorinated phenols, show high toxicity and persistency in the environment. The US EPA priority list of dangerous pollutants includes 11 phenols and particularly 2,4,6-trichlorophenol (TCP) [1–3]. This compound is one of the major contaminants of Kraft paper mill effluents and it was included in directive 76/464/EEC (European Economic Community) about dangerous substances discharged into the aquatic environment [4].

Horseradish peroxidase (HRP, E.C.1.11.1.7), a glycoprotein containing the Fe(III)-heme protoporphyrin IX catalytic unit, is the archetypal enzyme for studying the biochemistry of peroxidases. The enzyme notably catalyzes the H_2O_2-dependent (Compound I and II) oxidation of phenols and aromatic amines [5, 6]. Phenoxy radicals and dimeric products are known to be the first intermediates in the phenols' oxidative mechanism.

Organohalides (potential peroxidase substrates) are usually considered as being recalcitrant to many organisms except those producing extracellular lignin peroxidases (ligninases) [7]. In particular, a well-detailed study has been performed elucidating the enzymatic oxidative 4-dechlorination of polychlorinated phenols by lignin peroxidase (LiP) [7]. Further, it was reported that not only peroxidases with ligninase activity [7, 8] but other fungal lignin peroxidases [9] and an enzyme from *Geotricum candidum* [10] seem active in the biodegradation of polychlorinated phenols and could catalyze their oxidative 4-dechlorination. The lactoperoxidase [11] and turnip peroxidase [12] catalyzed oxidation of 2,4,6-trichlorophenol was previously investigated but the authors were unable to isolate and identify any product. A few years ago, Pirzad et al. [13] studied the mechanism of HRP oxidative dehalogenation of

4-fluorophenol and characterized the reaction products. Recently we reported a preliminary study concerning the HRP-H_2O_2 dependent oxidation of 2,4,6-tribromophenol to 2,6-dibromo-1,4-benzoquinone [14].

HRP is an easily available commercial product (relatively low price) and we are interested in the possibility that such a peroxidase might be involved in the degradation of polychlorinated phenols for industrial and biotechnological purposes. We have characterized the initial oxidative 4-dechlorination of the pollutant 2,4,6-trichlorophenol, and we have evaluated the enzyme-H_2O_2 catalytic turnover towards substrate.

Materials and methods

HRP was obtained from Sigma (type VI A, RZ = 3.0). TCP and 2,6-dichloro-1,4-benzoquinone (ACS grade) were purchased from Aldrich and judged pure by gas chromatography-mass spectrometry (GC-MS). Hydrogen peroxide (30%) and ultrafiltration devices were acquired from Merck. All other chemical were of the highest grade available.

UV-visible spectroscopic characterization and enzymatic activity measurements were performed on a UVIKON 930 (Kontron Instruments) spectrophotometer.

GC-MS analyses were done at 70 eV in a Hewlett-Packard HP5890 instrument fitted with a 30 m HP-5MS column. The gas chromatograph was programmed to rise from 120 to 260 °C at 10 °C min^{-1}.

FT IR and ^1H NMR spectra were obtained with a Bruker Equinox 55 and a Jeol EX400 instrument, respectively.

Product identification and characterization

Aqueous solutions (2.5 ml, pH 5.4) containing TCP (90 μM), HRP (0.1 μM) and H_2O_2 (45–135 μM) were preliminarily monitored spectrophotometrically. The reactions were initiated with H_2O_2 and followed in repetitive scans between 700 and 200 nm at 500 nm min^{-1}.

Reaction mixtures (4.5 ml, pH = 5.4) containing TCP (2.25×10^3 μM), HRP (0.1 μM) and H_2O_2 (2.85×10^3 μM) were used for GC-MS analysis. H_2O_2 was added as a last reagent. Aliquots (0.5 ml) of the aqueous solution, at different reaction times (10 s–24 h), were withdrawn from the reaction mixture; the organic phases were immediately extracted with four volumes of diethyl ether and analyzed by GC-MS.

At 30 min reaction time, one aliquot of the above reaction mixture, purged with argon, was treated with sodium dithionite. The organic phase was extracted two times with one volume of diethyl ether and was analyzed by GC-MS.

Another identical reaction mixture was purged with argon and completely extracted two times with two volumes of diethyl ether after 30 min from starting the reaction. The organic phases were separated and evaporated in order to obtain an orange powder.

^1H NMR and IR characterizations were performed after powder dissolution in 0.5 ml of $CDCl_3$ (99.9%), and after mixing the powder with 100 mg of KBr, respectively.

Catalytic reaction conditions

The relative turnover number of HRP for TCP oxidation was determined at room temperature in 0.1 M citrate or acetate buffer at pH 3.0 and 5.4. The ionic strength was kept constant with NaCl. Enzymatic reaction mixtures (2.0 ml) contained TCP (100 μM), HRP (0.1 μM) and H_2O_2 (400 μM) saturating. The HRP concentration was calculated by using an extinction coefficient of 103 $mM^{-1}cm^{-1}$ at 403 nm.

The reaction was initiated with H_2O_2 and quinone formation was monitored at 272 nm ($\varepsilon = 14\ mM^{-1}\ cm^{-1}$) [7]. The initial rates were determined from the linear part of the trace at 272 nm. To convert data from $\Delta A\ s^{-1}$ to $M\ s^{-1}$ the ε value of 2,6-dichloro-1,4-benzoquinone was used. The catalytic activity of HRP was calculated as relative turnover number in cycle s^{-1} [1 cycle $s^{-1} = 1$ mol of product (mol enzyme)$^{-1}$ s^{-1}).

Results and discussion

Product identification and characterization

The HRP-H_2O_2 dependent oxidation of TCP was examined by UV-visible spectroscopy and GC-MS. Spectral changes at increasing reaction times (Fig. 1a) were the same as those reported before in the presence of the lignin peroxidases [7]; the presence of isosbestic points indicated that the substrate was oxidized to the end product (2,6-dichloro-1,4-benzoquinone) without accumulation of any intermediate species. The product was characterized by two absorption maxima at $\lambda_{max} = 272$ and 343 nm, respectively. GC-MS features at different reaction times showed the disappearance of TCP retention time (4 min) and molecular ion M^+ (196) peaks together with the appearance of the only oxidized product (TCP-ox) retention time (5 min) and molecular ion M^+ (176) relative peaks (Fig. 2 and Fig. 3a).

The catalytic reaction behavior at increasing TCP incubation time in the enzymatic mixture was followed with GC-MS by measuring the percentage area of the substrate and of its oxidized product retention peaks, and by monitoring spectrophotometrically the TCP-ox formation at 272 nm. Both methods showed that 50% and 90% of the substrate were converted to end product in 10 and 60 s, respectively (Fig. 1b and Fig. 2).

The catalytic conversion of TCP to oxidized product was very fast and practically completed in 1 min. The

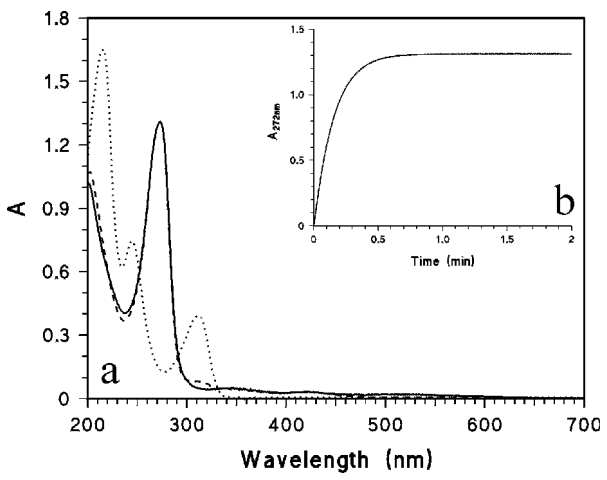

Fig. 1 UV-visible analysis of the HRP/ H_2O_2/TCP reaction (see experimental procedure): **a** spectral changes at different reaction times: ····· 0 min; --- 1 min; —— 2 min; **b** time course of TCP-ox formation monitored at 272 nm

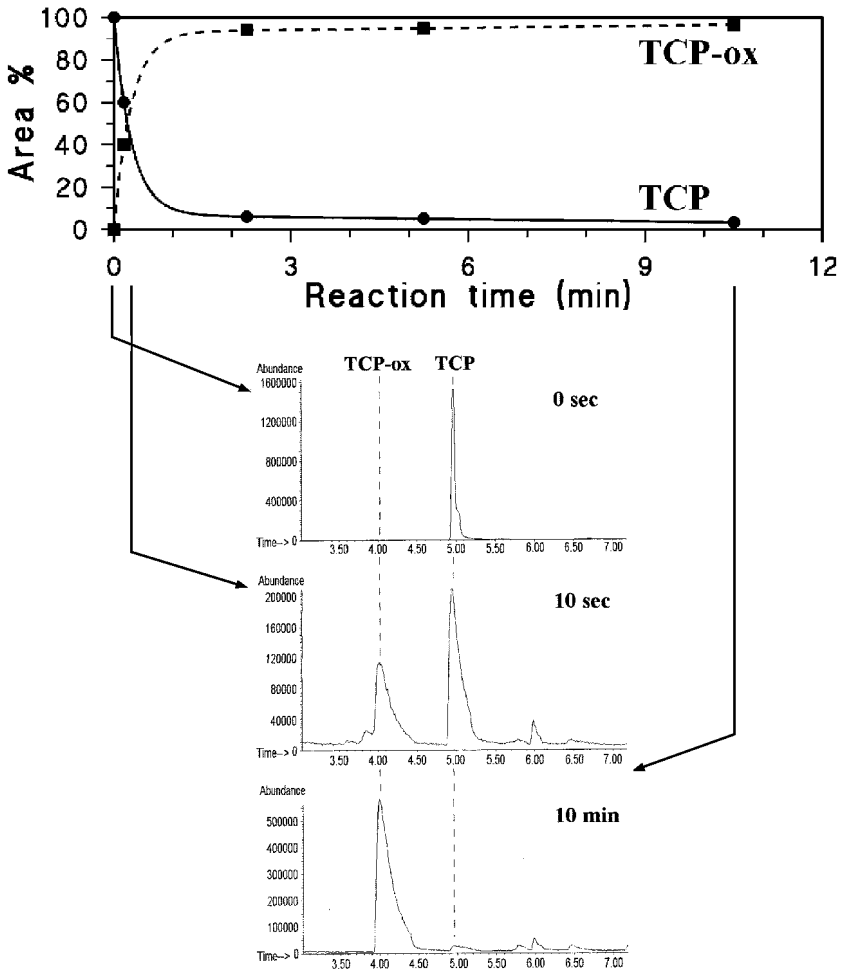

Fig. 2 GC retention time peaks of 2,4,6-trichlorophenol (TCP) and 2,6-dichloro-1,4-benzoquinone (TCP-ox). Relative retention time peak % areas at varying reaction times

percentage area of the above product retention peak in our experimental conditions remained constant for almost 24 h.

The UV-visible spectrum (Fig. 1a), the GC retention time (Fig. 2) and mass spectrum (Fig. 3a) of the TCP-ox product showed that the polychlorophenol parent molecule had been enzymatically dechlorinated by HRP to give 2,6-dichloro-1,4-benzoquinone. Further, we characterized the isolated quinone by NMR ($\delta = 7.04$ ppm, 2H) and IR [$\nu = 1703$ (vs), 1652 (vs), 1584 (s), 1322 (w), 1288 (m), 1278 (m), 1038 (vs) cm^{-1}]. All the spectral patterns matched those we obtained for authentic standard 2,6-dichloro-1,4-benzoquinone dissolved in an equivalent aqueous solution. Similarly, GC-MS of the reduced product, 2,6-dichlorohydroquinone (Fig. 3b), matched that of the corresponding commercial standard.

The stability of chloroquinone in water, pH 3, was also examined by measuring the percentage area of its GC retention peak which remained constant over ca. 24 h. This increase in stability at pH 3 is assignable to the high TCP molar concentration (2.25 mM) and to the TCP/H_2O_2 molar ratio (ca. 1).

The direct and indirect GC-MS characterization of TCP-ox as 2,6-dichloro-1,4-benzoquinone gives evidence for the 4-oxo substituent. Particularly in the mass spectrum of 2,6-dichlorohydroquinone, peaks at *m/z* 142 and 144 indicate the loss of HCl from the molecular ion whereas those at *m/z* 114 and 116 demonstrate a further loss of CO to give the monochlorinated cyclopentadienone radical cation [7]. The 4-oxo substituent present in this fragment was introduced during oxidative 4-dechlorination of TCP by HRP, as reported for the TCP oxidation by LiP [7]. NMR and IR characterizations of the isolated and identified product (98.2% yield) strongly support this finding.

Mechanism of oxidative dechlorination

In Fig. 4 a hypothetical scheme for the mechanism of TCP oxidation by the HRP-H_2O_2 system is reported, which is similar to that of TCP oxidation by the LiP-H_2O_2 system [7].

Phenoxy radicals are known to be intermediates in peroxidase-catalyzed phenol oxidation and a nucleophilic attack by water on the hypothetical 2,4,6-trichlorocyclohexadienone cation is quite possible. The mass spectrum of 2,6-dichloro-1,4-benzoquinone obtained in presence of $H_2{}^{18}O$ indicated this water attack was at position 4 [7]. The resulting 2,4,6-trichloro-4-hydroxy-

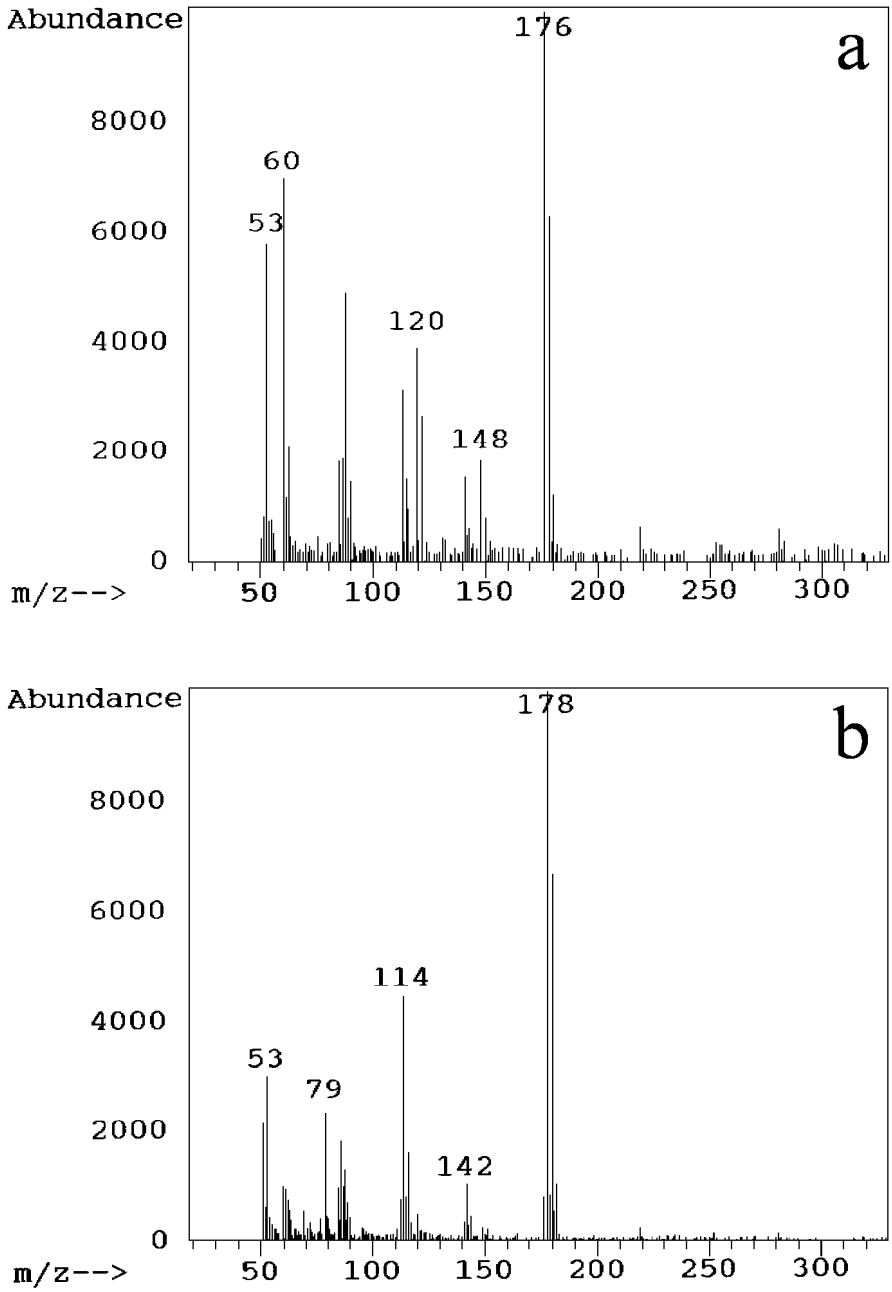

Fig. 3 Mass spectra of **a** 2,6-dichloro-1,4-benzoquinone and **b** 2,6-dichlorohydroquinone monitored after 30 min of enzymatic mixture incubation time

cyclohexadienone would eliminate HCl to give the final 1,4-benzoquinone, in agreement with a decrease of aqueous solution pH to 3.0 and a 1:1 quinone/Cl^- stoichiometry [7]. In addition, oxidation of TCP by HRP-H_2O_2, performed under nitrogen, did not limit quinone formation. This suggested that the phenoxy radical did not interact at its *para* position with molecular oxygen to yield peroxy radicals, which would further react to give hydroquinones or quinones (autooxidation) [15].

Similarly, we might exclude the formation of hydroxyl radicals by metal-mediated homolysis of the O-O bond of H_2O_2 [15, 16]. The affinity of H_2O_2 for iron-heme is very high, and we monitored quantitative stable quinone formation at low pH for a substrate/H_2O_2 ratio of 1:0.5 or less. These results indicate that TCP oxidative dechlorination was mediated by high-valent metal-oxo species (Compounds I and II), generated by the primary substrate.

Evaluation of HRP catalytic activity towards 2,4,6-trichlorophenol

The oxidative dechlorination of TCP to quinone was practically quantitative in aqueous solution at the starting pH of 5.4, with a substrate/peroxide molar ratio around 1:1 and an enzyme/substrate ratio around 1:18000 (Fig. 1b and Fig. 2). There was no evidence of enzyme saturation by the substrate at concentrations up to 2.25 mM.

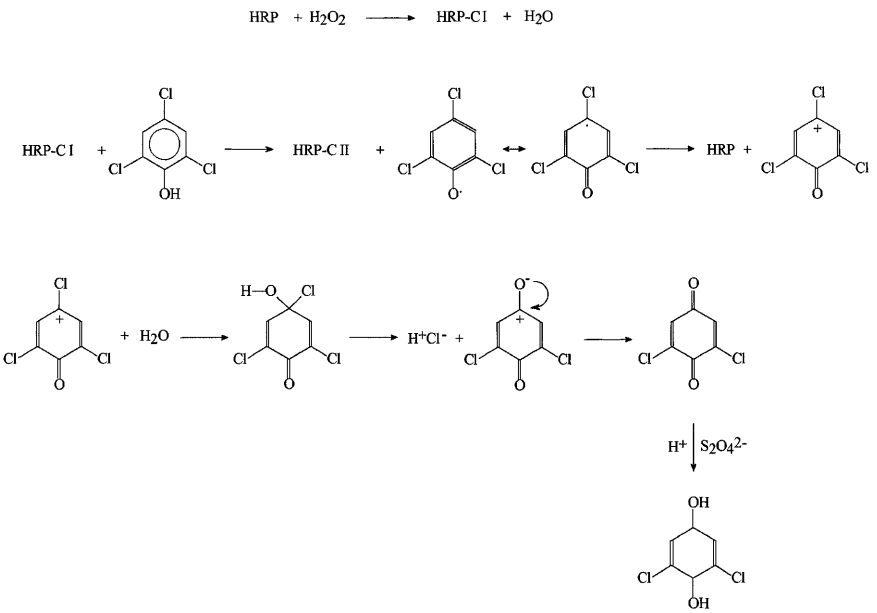

Fig. 4 Hypothetical scheme for the catalytic mechanism of TCP oxidative dechlorination by the HRP-H_2O_2 system

To correctly evaluate the HRP-H_2O_2 catalytic efficiency, the reaction was performed in buffered aqueous solutions at, pH 5.4 and 3.0 (optimal quinone stability), with saturating H_2O_2 concentration and an enzyme/substrate ratio of 1:1000. HRP was seen to oxidize the substrate (0.1 mM) at turnover numbers of 68 and 55 s^{-1} at pH 5.4 and 3.0, respectively. In these experimental conditions, HRP-H_2O_2 seems to be more efficient than LiP-H_2O_2 (1–5 cycles s^{-1}) [7] and comparable with a catalytic system such as metalloporphyrin-$KHSO_5$ (20 cycles s^{-1}) [17], both at pH = 3.

Conclusions

Peroxidases-H_2O_2 dependent activity towards polychlorinated phenols was noticed many years ago by Saunders and Stark [12] (turnip peroxidase) and Öberg and Paul [11] (HRP A2, C2 and lactoperoxidase), who were unable to detect and characterize the oxidation products. Recently, we reported some preliminary results on the HRP-H_2O_2 oxidative 4-debromination of 2,4,6-tribromophenol to 2,6-dibromo-1,4-benzoquinone [14]. Ligninases of *Phanerochaete chrysosporium* were shown to catalyze the oxidative 4-dechlorination of polychlorinated phenols [7]. The authors identified the final products, chloroquinones, through the characterization of their reduced and acetylated hydrochloroquinones.

In this work the HRP-H_2O_2 oxidation of TCP was demonstrated through a direct and more exhaustive characterization of the isolated reaction product 2,6-dichloro-1,4-benzoquinone. Chloroquinones are reported to be unstable in water (pH 6 or more) [12, 17, 18], and to develop a purple color; 0.1 mM phenol solution (pH 3) degraded within ca. 1 h [7]. Our success in stabilizing (ca. 24 h) and directly identifying quinone in water was naturally assignable to the low working pH, and thus to the low H_2O_2 concentration and high phenol concentration.

The HRP oxidative 4-dechlorination mechanism bears some resemblance with that of lignin peroxidase [7] and metalloporphyrin complexes [17].

High-valent metal-oxo species induce the reaction pathway to yield 1,4-benzoquinone in one-electron (phenoxy radical) or two-electron steps (cyclohexadienone cation).

In addition, it is noteworthy that another extracellular peroxidase such as HRP (probably the most studied member of plant peroxidases) accomplishes an oxidative dechlorination of the ring in one enzymatic step. This is at variance with the well-known bio-oxidative pathway for aromatic dechlorination which requires hydroxylation and then ring cleavage [19, 20].

We would like to underline that the HRP-H_2O_2 system appears to be more efficient in 1,4-quinone production than LiP-H_2O_2 [7] and metalloporphyrin-H_2O_2 [17] and competitive with metalloporphyrin-$KHSO_5$ [17]. This assertion might be crucial if we correlated the activity and good efficiency of HRP towards TCP ($E_{1/2}$ = 1.7 V) only with the oxidizing power of its catalytic intermediates. Indeed, HRP was indicated to be able to oxidize only substrates with $E_{1/2}$ values up to 1.4 V [17], although the redox potential of HRP-Compound I is evaluated at 1.1 V; the $E_{1/2}$ value for iron-oxo unit in cytochrome P-450 was estimated at 1.7–2.0 V [21] and a value of 1.6 V was reported for manganese-oxoporphyrin [22].

Recently, the X-ray structure of recombinant HRP isoenzyme C was resolved [23] and a crystallographic study on the HRP-benzhydroxamic acid adduct was reported [24]. Benzhydroxamic acid is tightly bound in a specific site by means of hydrogen bonds, with the amino acid residues and a water molecule in the distal re-

gion of Fe(III)-heme and hydrophobic interactions with the aromatic residues localized on the outer zone of the substrate channel.

Concerning TCP, the flexibility of Phe68, as reported in [24], appears to allow its access to an equivalent catalytic site in spite of its greater steric hindrance. The presence of chlorine atoms should weaken the hydrophobic interactions of TCP with the aromatic binding pocket but, on the other hand, to favor hydrogen bond formation with polar amino acids present in the catalytic site (Arg38, His42, Pro139). These protein-substrate interactions can promote electron transfer from Phe179 and therefore make substrate oxidation easier.

For these reasons it is important to note that HRP catalytic activity is more related to the favorable access and disposition of the substrate in its active site, also in agreement with previous peroxidase-substrate binding studies [25–28].

Further investigations (binding and docking studies) are in progress in our laboratory in order to confirm the above hypothesis.

We further note that HRP is a commonly stable and easily available peroxidase, while ligninases are not available commercially and are rather fragile enzymes in the presence of an excess of H_2O_2 [29].

In the bioremediation perspective we think that the present investigation might be a good starting point for studying the environment compatible quinone evolution (quinone stability is pH and TCP/H_2O_2 molar ratio dependent). Indeed, at the moment, only the basidiomycete *Phanerochaete chrisosporium* with ligninase activity [30] and some iron sulfophthalocyanine complexes (H_2O_2 or $KHSO_5$) [31] are found to be active in performing the oxidative mineralization of TCP.

Use of pure immobilized enzymes (in our case the commercially available HRP) to improve their large-scale handling should have the advantage of dealing with highly biocompatible catalytic systems which may possess some substrate specificity [32]. On the other hand, the complete comprehension of the enzymatic mechanisms will allow the development of highly efficient and selective enzyme-like models.

Acknowledgements This work was supported by grants from the Italian "Ministero per l'Università e la Ricerca Scientifica e Tecnologica (MURST)" and by "Comitato Nazionale Scienze e Tecnologie Ambiente e Habitat" at "Consiglio Nazionale delle Ricerche (CNR)", to whom the authors' gratitude is addressed.

References

1. Ruzgas T, Emneus J, Gorton L, Marko-Varga G (1995) Anal Chim Acta 311:245–253
2. EPA Method 604 (1984) Phenols in Federal Register. Environmental Protection Agency, Washington, Part VIII, 40 CFR Part 136, pp 58–66
3. EPA Method 625 (1984) Base/neutrals and acids in Federal Register. Environmental Protection Agency, Washington, Part VIII, 40 CFR Part 136, pp 153–174
4. Vincent G (1991) In: Angeletti G, Bjorseth A (eds) Organic micropollutants in the aquatic environment. Kluwer, Dordrecht, p 285
5. Job D, Dunford HB (1976) Eur J Biochem 66:607–614
6. Berry DF, Boys SA (1984) Soil Sci Am Soc J 48:565–569
7. Hammel KE, Tardone PJ (1988) Biochemistry 27:6563–6568
8. Male KB, Brown RS, Luong JHT (1995) Enzyme Microb Technol 17:607–614
9. Kirk TK, Croan S, Tien M, Murtagh KE, Farrell R (1986) Enzyme Microb Technol 8:27–32
10. Bordeleau LM, Bartha R (1972) Can J Microbiol 18:1865–1871
11. Öberg LG, Paul KG (1985) Biochim Biophys Acta 842:30–58
12. Saunders BC, Stark BP (1967) Tetrahedron 23:1867–1872
13. Pirzad R, Newman JD, Dowman AA, Cowell DC (1994) Analyst 119:213–218
14. Trotta F, Ferrari RP, Laurenti E, Moraglio G, Trossi A (1996) J Inclus Phenomena 25:225–228
15. Ortiz de Montellano PR, Grab LA (1987) Biochemistry 26:5310–5314
16. Sorokin A, Meunier B (1996) Chem Eur J 2:1308–1317
17. Labat G, Seris JL, Meunier B (1990) Angew Chem Int Ed Engl 29:1471–1473
18. Hancock JW, Morrell CE, Rhum D (1962) Tetrahedron Lett 22:987–989
19. Schmidt E, Knackmuss HJ (1980) Biochem J 192:339–347
20. Kersten PJ, Chapman PJ, Dagley S (1985) J Bacteriol 162:693–697
21. Macdonald TL, Gutheim NG, Martin RB, Guengerich FP (1989) Biochemistry 28:2071–2077
22. Bortolini O, Ricci M, Meunier B, Friant P, Ascone I, Goulon J (1986) New J Chem 10:39
23. Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL (1997) Nat Struct Biol 4:1032–1038
24. Henriksen A, Schuller DJ, Meno K, Welinder KG, Smith TA, Gajhede M (1998) Biochemistry 37:8054–8060
25. Casella L, Poli S, Gullotti M, Selvaggini C, Beringhelli T, Marchesini A (1994) Biochemistry 33:6377–6386
26. Banci L, Carloni P, Gori Savellini G (1994) Biochemistry 33:12356–12366
27. Smith AT, Veitch NC (1998) Curr Opin Chem Biol 2:269–278
28. Ferrari RP, Traversa S, De Gioia L, Fantucci P, Suriano G, Ghibaudi EM (1999) JBIC 4:12–20
29. Wariishi H, Gold MH (1989) FEBS Lett 243:165
30. Reddy GV, Gelpke MD, Gold MH (1998) J Bacteriol 180:5159–5164
31. Sorokin A, Seris JL, Meunier B (1995) Science 268:1163–1166
32. Chaplin MF, Bucke C (1990) Enzyme technology. Cambridge University Press, Cambridge