Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 44 (2007) 144–148
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Horseradish peroxidase in ionic liquids Reactions with water insoluble phenolic substrates

Simona Sgalla [a], Giancarlo Fabrizi [a], Sandro Cacchi [a], Alberto Macone [b], Alessandra Bonamore [b], Alberto Boffi [b,*]

[a] *Dipartimento di Chimica e Tecnologia delle Sostanze Biologicamente Attive, University "La Sapienza", P. Aldo Moro 5, 00185 Roma, Italy*
[b] *Dipartimento di Scienze Biochimiche, University "La Sapienza", P. Aldo Moro 5, 00185 Roma, Italy*

Received 17 March 2006; received in revised form 28 September 2006; accepted 3 October 2006
Available online 13 November 2006

Abstract

The reactivity of horseradish peroxidase (HRP) with water insoluble phenolic compounds has been studied in 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4])/water mixtures. The enzyme retained some catalytic activity up to 90% ionic liquid in water at 25 °C only at pH values higher than 9.0. Activity steadily decreased towards neutral and acidic conditions, as judged by 4-aminoantypirin/phenol activity tests. Inhibition of horseradish peroxidase under neutral acidic condition was due to the binding of fluoride anions released from tetrafluoroborate anion to the heme iron as demonstrated by the sharp UV–visible absorption transition diagnostic of the conversion from a five coordinated to a six coordinated high spin ferric heme iron. Thus, reactions with water insoluble phenols were carried out under alkaline reaction conditions and 75% [BMIM][BF_4]/water mixture. Under these conditions, the distribution of the reaction products was much narrower with respect to that observed in aqueous buffers or water/dimethylformamide or water/dimethylsulfoxide mixtures, and polymeric species other than dimers were not observed. Technical scale preparations of a novel 4-phenylphenol *ortho* dimer [2,2′-bi-(4-phenylphenol)] with a high yield of the desired product were obtained.

Keywords: Horseradish peroxidase; Phenolic coupling; Ionic liquids; Phenols oxidation; Heme ligand binding

1. Introduction

Peroxidase-mediated oxidative coupling of phenols is one of the most widely investigated redox reaction in biochemistry. In the last decades, the basic mechanisms that govern peroxide-driven heme activation, electron transfer between the protein and its substrates and pathways of radicalic coupling of phenol intermediates to yield oligomers or higher polymers has been progressively clarified [1–4]. The current view of peroxidase-driven phenols coupling entails the direct one electron oxidation of the substrate molecule in the presence of hydrogen peroxide followed by radical driven transformations that result in the formation of a wide array of polymeric products. The high stability, activity and relaxed substrate specificity of peroxidases have been exploited to develop oxidative biotransformations in the field of pulp bleaching and lignin degradation [5] and to set up peroxide sensing systems for diagnostic bioessays. Nevertheless, the potential usefulness of peroxidases for the catalysis of carbon–carbon and carbon–oxygen bond formation has not been fully developed for technical or bulk scale enzymatic synthesis. The major drawback that hampers the use of peroxidases in chemical synthesis obviously resides in the radicalic character of the reactions catalyzed by these enzymes that renders the reaction system difficult to control and not easily amenable to synthetic chemistry protocols.

Several attempts have been made in order to exert kinetic or thermodynamic control on the reactions catalyzed by peroxidases by exploiting the properties of mixtures of water and organic solvents that may help solubilization of hydrophobic substrates, reduce or quench the radicalic propagation or favour product extraction. In some cases, the peroxidase catalyzed reaction of phenolic compounds in organic solvents yielded interesting products' patterns, often characterized by the presence of more homogeneous polymeric products with respect to

* Corresponding author. Tel.: +39 06 4991 0990; fax: +39 06 4440 062.
E-mail address: alberto.boffi@uniroma1.it (A. Boffi).

1381-1177/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2006.10.002

the same reaction conducted in aqueous buffers [6,7]. However, most cosolvents hitherto used are endowed with intrinsic toxicity and low biodegradability thus rendering the catalytic process less convenient for large-scale synthesis.

In recent years, ionic liquids have gained a favourable impact as environmentally friendly solvents for organic synthesis [8]. Low vapour pressure, negligible toxicity and adjustable solvation properties are among the main features that characterize this vast class of compounds. Moreover, several ionic liquids have been demonstrated to be suitable solvents for enzyme catalyzed reactions. In particular, reactions catalyzed by lipases and hydrolases have been widely investigated in these solvents [8]. In contrast, little attention has been devoted to the examination of enzyme catalyzed redox processes or oxidative biotransformations in ionic liquids based mediums. So far, chloroperoxidase catalyzed stereoselective dioxygenation of 1,2-dihydroxynaphthalene in 30% 1-butyl-3-methylimidazolium methylsulphate [9] and oxidation of 3,4-dimethoxybenzyl alcohol by horseradish peroxidase in 25% [4-MBP] hexafluorophosphate [10] have been reported as promising examples of peroxidases based catalysis in ionic liquids. In this framework, a systematic investigation of the activity of horseradish peroxidase (HRP) in ionic liquids has been undertaken in the aim of establishing the operational procedures, the mechanism of action, the stability properties of the enzyme and the solvation properties of the substrates/products.

2. Experimental

Horseradish peroxidase type IA, 4-phenylphenol, 2,5-di-*tert*-butylphenol, 1-naphthol and hydrogen peroxide were from Sigma–Aldrich. 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) was synthesized according to ref. [11]. After the synthesis, the ionic liquid (100 ml) was diluted in CH_2Cl_2 (100 ml) and purified on chromatographic column charged with basic aluminum oxide, Brockmann II, (100 g) eluting with 500 ml of CH_2Cl_2. The eluate was then evaporated under reduced pressure, giving a colourless ionic liquid.

Spectrophotometric titrations were carried out on a HP8453 diode array spectrophotometer equipped with a peltier temperature controller (Agilent Technologies, Palo Alto, CA, USA). Titration of HRP (5 μM) with [BMIM][BF_4], $NaBF_4$ or NaF was carried out in a 1 cm quartz cuvette by adding small amounts of the reagents and allowing 10 min equilibration after each addition at 20 °C. Titrations were carried out in Bis–Tris (50 mM, pH 6.0), phosphate (50 mM pH 7.0), Tris–HCl (50 mM, pH 8.0) and Tris–glycine (50 mM, pH 9.0). Spectral changes were averaged along the 350–700 nm range, plotted against ligand concentrations and fitted to a simple ligand binding equilibrium at each pH value. The program Matlab 5.0 (The MathWorks, South Natick MA, USA) was used for data analysis and fitting procedures.

Ether or chloroform extracts at the end of the reaction were analyzed by gas chromatography–mass spectrometry using an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies). Chromatographic separations were carried out an Agilent HP5ms fused-silica capillary column (30 m × 0.25 mm i.d.) coated with polyethylene glycol (film thickness 0.25 μm) as stationary phase. Injection mode: splitless at a temperature of 260 °C. Column temperature program: 120 °C (1 min) then to 260 °C at a rate of 15 °C/min and held for 20 min. The carrier gas was helium at a constant flow of 1.0 ml/min. The spectra were obtained in the electron impact mode at 70 eV ionization energy and a mass range scan from *m*/*z* 50 to 500; ion source 280 °C; ion source vacuum 10^{-5} Torr. TMS-ethers derivatives were obtained by reaction with *N*-(trimethylsilyl)acetamide + trimethylchlorosilane + trimethylsilylimidazole (3:2:3) (Sylon BTZ, Supelco) for 30 min at 60 °C.

^{1}H NMR spectra (400.13 MHz) and ^{13}C NMR spectra (100.6 MHz) were recorded on a Bruker Avance 400 spectrometer, IR spectra were recorded on a Jasco FT-IR 430 spectrometer. Melting points were determined with a Buchi apparatus and are uncorrected.

Peroxidase activity was determined according to the 4-aminoantipyrine/phenol method [12] modified as follows. Fifteen millimolar H_2O_2 solutions in 50 mM buffers (phosphate, pH 7.0; Tris–HCl, pH 7.5 or borate, pH 9.2) were mixed with solutions containing 25 mM 4-aminoantypirine and 500 mM phenol (pH was adjusted to the desired value by addition of NaOH). The solution was then diluted 1:10 with distilled water containing variable amounts of [BMIM][BF_4] (up to pure ionic liquid) and placed in a 1 cm cuvette on a Jasco 570 spectrophotometer. A few microliter of a HRP stock solution in water were then added up to a final protein concentration of 0.1 μM. Absorbance increase at 510 nm was followed and initial velocity (1–15 min) was measured under each experimental conditions. pH values were checked again at the end of the reaction. The rate obtained at pH 7.0 (0.05 absorbance increase per minute) in the absence of ionic liquid was set as 100% activity. Rates obtained under different solvent and pH conditions were thus scaled to the rate obtained at pH 7.0 in water buffer.

Reactions with water insoluble phenolic compound were screened in 50–85% [BMIM][BF_4]/water solutions containing 1–10 μM HRP. The phenols were first dissolved in pure [BMIM][BF_4] liquid up to concentrations of 100–250 mM. pH was then adjusted to 10 by adding small amount of concentrated (1 M) NaOH solutions and checking the value with standard indicator substance or with a pH meter. A stock solution of water dissolved HRP was then added to the reaction mixture up to a concentration of 10 μM. Finally, 10–100 molar excess (with respect to the phenolic substrates) hydrogen peroxide (33% solutions) were added to the reaction mixture by slow feed with a mechanically driven syringe over 4–6 h at 20 °C. The reactions were monitored with TLC until the disappearance of the starting material and products were checked by GC/MS. The reaction was then quenched by adding distilled water at 4 °C with concomitant precipitation of the products. The same reactions were also screened with DMF or DMSO as cosolvents at pH 9.0, 0.1 M substrates concentrations, and 10 μM HRP. Five to ten percent DMF and 5–35% DMSO/water mixtures offered a suitable operational window for peroxidase catalyzed phenolic coupling at alkaline pH values as demonstrated by the complete disappearance of the substrates at the end of the reaction.

2.1. Synthesis, purification and characterization of 2,2′-bi-(4-phenylphenol)

4-Phenylphenol (100.0 mg, 0.588 mmol) was dissolved in 6 ml of a solution containing 75% [BMIM][BF_4] and 25% of 1 M NaOH (final pH 10.22). Fifteen microliters of a 0.5 mM stock solution of HRP in water were then added to the reaction mixture and 0.9 ml of H_2O_2 (30%, w/v) was added by slow feed (over 4 h) using a mechanically driven syringe. The solution was then cooled at 0 °C to allow the precipitation of the product, filtered on a sintered glass and washed with distilled water. The white residue was dissolved with ethyl acetate, washed (HCl 2N, saturated NaCl), dried over Na_2SO_4 and concentrated under reduced pressure. The residue, purified by chromatography (SiO_2, 40 g; *n*-hexane/ethylacetate 74/26 v/v), gave 67.7 mg of 2,2′-bi-(4-phenylphenol) with a final yield of 68%. Product properties were assessed as follows: mp: 186–187 °C; IR (KBr): 3479, 3375, 3058, 3028, 1477, 1229, 1180 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.63–7.55 (m, 4H), 7.43 (t, $J = 7.4$ Hz, 2H), 7.32 (t, $J = 7.4$ Hz, 1H), 7.14 (d, $J = 8.3$ Hz, 1H), 5.58 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 152.6, 140.3, 135.0, 130.1, 128.9, 128.7, 127.1, 126.9, 124.1, 117.2. GC/MS (*m/z*): 338 (30.0), 323 (4.6), 310 (9.3), 295 (4.2), 281 (3.6), 252 (3.8), 239 (3.7), 215 (3.6), 202 (1.8), 189 (3.3), 165 (3.2), 152 (3.4), 115 (5.2), 91 (3.2), 77 (7.0), 64 (3.3), 51 (2.1). Details of the ^1H NMR, ^{13}C NMR, FTIR and GC/MS spectra are given under Supplementary Material.

3. Results and discussion

3.1. Horseradish peroxidase interactions with [BMIM][BF_4]

The UV–visible absorption spectra of horseradish peroxidase shown in Fig. 1 are highly diagnostic of a change in

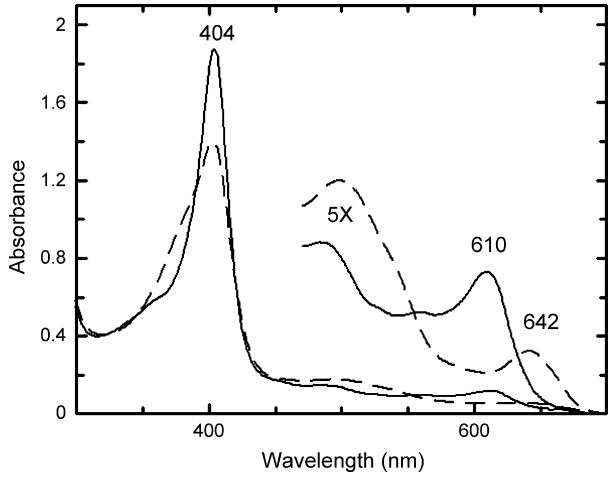

Fig. 1. UV–visible spectra of horseradish peroxidase in 75% [BMIM][BF_4]/water solutions. The spectrum of horseradish peroxidase (9 μM) at pH 7.0 in 10 mM phosphate buffer (continuous line) is compared to that obtained in a 75% [BMIM][BF_4]/buffer solution at pH 7.0 (10 mM phosphate buffer, dashed line) after baseline subtraction (the same solution in the absence of protein) in 1 cm cuvette and 20 °C.

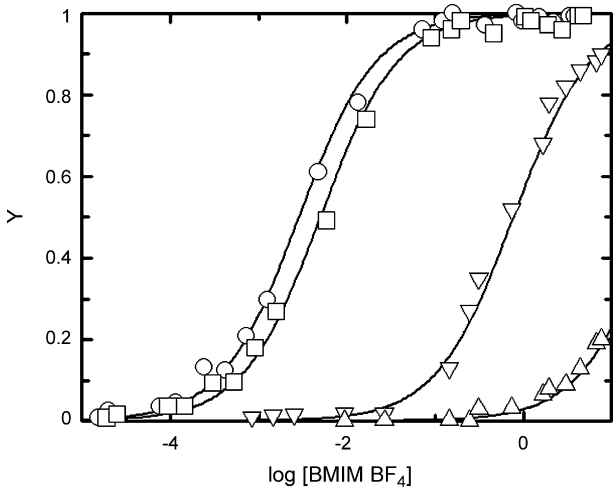

Fig. 2. Spectrophotometric titrations of horseradish peroxidase with [BMIM][BF_4] as a function of pH. Continuous lines represent the best fit to the experimental data assuming a simple ligand binding equilibrium. Data were obtained at pH 6.5 (◯), 7.0 (□), 8.0 (△) and 8.5 (▽) and 20 °C. Data fitting yielded binding equilibrium constants of 350, 198, 13 and 0.3 M^{-1}, respectively.

the coordination state of the ferric heme iron in the presence of [BMIM][BF_4] at pH 7.0. In particular, a clear transition between a ferric five coordinated to six coordinated, high spin iron is observed in the presence of 75% [BMIM][BF_4] at neutral pH values. The HRP spectrum in the presence of ionic liquid is almost superimposable to that of the typical HRP-fluoride adduct [13,14], as demonstrated by the presence of the high spin iron charge transfer band at 612 nm. The same spectral changes and similar pH dependent profiles (see below) could be observed by titrating HRP with $NaBF_4$ or NaF solutions confirming that the observed spectral changes involves uniquely the binding of the F^- anion (or HF, according to ref. [15]) to the heme iron and are independent of the type of cation (sodium or imidazolium) present in solution. Interestingly, the spectra of HRP at alkaline pH (20 mM Borate buffer 9.2) were identical both in water and 75% [BMIM][BF_4]. This observation prompted further investigation on the interplay betwen pH and ionic liquid. Thus, spectrophotometric titrations of HRP with [BMIM][BF_4] were carried out at pH values between 6.5 and 8.5 (Fig. 2). The titration profiles could be fitted according to a simple one site binding scheme with equilibrium association constants varying from 350 M^{-1} at pH 6.5 to 0.3 M^{-1} at pH 8.5. The apparent p*K* of the transition was estimated to be about 8.1. The observed transitions can be interpreted in the light of a more complex multiple equilibrium that entails the release of fluoride ions from BF_4^- and subsequent binding of fluoride to the heme iron of the peroxidase enzyme. The strong pH dependence of fluoride binding is in agreement with previous findings on cytochrome *C* peroxidase [13] which established that HF instead of fluoride anion is actually bound to the heme iron. Thus, although hydrolysis of BF_4^- to yield fluoride anions is favoured under alkaline conditions, HF binding to the heme iron may occur only at acidic pH. The observed pH dependent profiles thus contain thermodynamics contributions

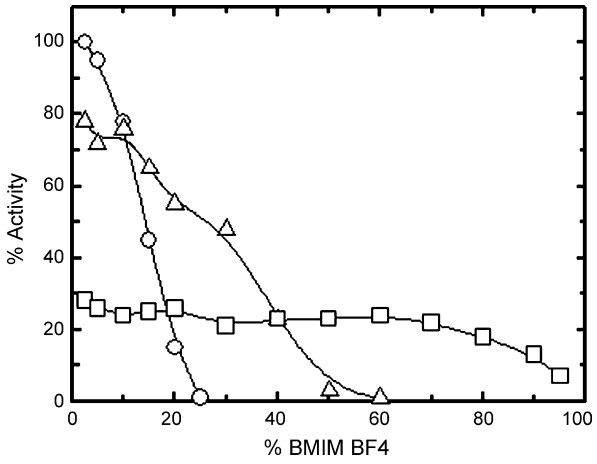

Fig. 3. Horseradish peroxidase activity in [BMIM][BF_4]/aqueous buffer mixtures. The activity of horseradish peroxidase is reported at pH 7.0 (◯) in 10 mM phosphate buffer, pH 8.0 (Δ) in 10 mM Tris–HCl buffer and pH 9.2 (□) in 10 mM borate buffer. The enzyme activity, as measured with the antypirine/phenol method, is normalized to that obtained in 10 mM phosphate buffer at pH 7.0 (1 μM H_2O_2 consumed per minute).

originating from BF_4^- hydrolysis and fluoride protonation, although other effects due to the pH dependent ionization of Arg^{38} and His^{42} residues in the distal heme pocket cannot be excluded.

3.2. Horseradish peroxidase activity in [BMIM][BF_4]/buffer mixture

The 4-aminoantipyrine/phenol chromogenic test was used to evaluate the catalytic activity of HRP in ionic liquid/water mixtures. The original reaction protocol [12] was modified to meet the requirements needed to operate at high ionic liquid concentrations. In particular, the concentration of aqueous buffers (phosphate and borate, in particular) has to be kept below 40 mM at 75% [BMIM][BF_4] due to the low anion solvation capability of the ionic liquid and consequent formation of salt precipitates at higher ionic strenght. As a consequence, the pH of phenol containing solutions had to be adjusted carefully to the desired pH by stepwise addition of NaOH solutions and the pH must be verified at the end of the reaction. The catalytic activity of HRP at pH 7.0 in buffer, corresponding to the consumption of 2 μM H_2O_2 per minute (0.04 absorbance units/min at 510 nm) was taken as a reference for measurements at different pH and ionic liquid mixtures. The profiles of the observed activities as a function of medium composition at different pH values are shown in Fig. 3. The link between pH and ionic liquid content is immediately apparent from inspection of the activity profiles. HRP activity decreases upon increase in [BMIM][BF_4] content at pH 7.0 and 8.0, whereas is relatively constant at pH 9.0 up to 80–90% ionic liquid. The observed trend is attributed to the inhibition of peroxidase activity due to the binding of F^- anion to the heme iron at pH 7.0 and 8.0, consistent with the ligand binding profiles described in the previous paragraph. Although the relative activity at pH 9.0 accounts for less than 25% of that measured at pH 7.0, the retention of the enzyme catalytic properties up to about 80% ionic liquid offers a convenient window for operating peroxidase-driven catalytic processes.

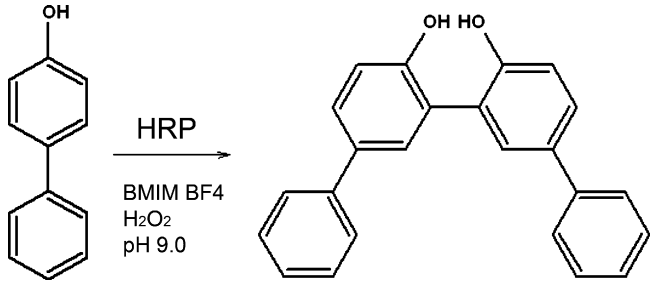

Fig. 4. Reaction scheme for horseradish peroxidase catalyzed 4-phenylphenol coupling.

3.3. Horseradish peroxidase catalyzed oxidations of substituted phenols in [BMIM][BF_4]/buffer mixtures

The medium composition and reaction conditions identified in the previous paragraph served as a basis for investigating the potential capabilities of HRP in the oxidative transformation of phenolic substrates endowed with poor solubility in aqueous buffers. Three substituted phenols were analyzed, namely 1-naphthol, 4-phenylphenol and 2,5-di-*tert*-butylphenol. The peroxidase-mediated oxidative coupling in water buffers has been previously studied for all three compounds under high dilution conditions. In all species, heterogenous polymeric adducts were obtained [15,16]. The use of DMF or DMSO as cosolvents to increase substrates concentrations (>0.1 M) for convenient technical scale reactions was first analyzed according to the procedures outlined in refs. [6,7] (see also experimental procedures). With both solvents, even in proximity of the highest cosolvent/water ratio (10% DMF and 35% DMSO), a wide distribution of coloured polymeric products were formed, as judged by TLC analysis (data not shown). Moreover, in the case of 4-phenylphenol, several polyhydroxylated products were also identified by GC/MS spectrometry. The three compounds were thus screened as outlined under experimental procedure in order to optimize HRP, [BMIM][BF_4], phenolic substrates and hydrogen peroxide concentrations. Polymeric species were never obtained at [BMIM][BF_4]/water > 50% with all three substrates, as judged by TLC and GC/MS analysis at the end of the reaction. GC/MS profiles (data not shown), demonstrated that high yields of dimeric species were obtained in the case of 4-phenylphenol (85% dimers) with 10 H_2O_2/substrate molar excess and in the case of 2,5-di-*tert*-butylphenol (35% dimers) with 80 H_2O_2/substrate molar excess in the presence of 5 μM HRP. In contrast, several hydroxylated products but only small amounts of dimeric adducts (3–5%) were obtained in the case of 1-naphthol even at >100 H_2O_2/substrate molar excess. On the basis of this preliminary screening, a protocol was developed for the synthesis and characterization of the 4-phenylphenol dimeric species. The synthesis of 2,2′-bi-(4-phenylphenol) (Fig. 4) under the conditions described in the experimental section afforded a remarkable product yield.

4. Conclusions

The data obtained in the present work indicate that [BMIM][BF_4]/water mixtures provide a suitable reaction medium for peroxidase-mediated oxidative coupling of water insoluble phenolic substrates. As a general comment, the overall enzyme activity is lower within [BMIM][BF_4]/water mixtures with respect to the activity measured under standard water buffers. On the basis of the present data and of recent reports that demonstrated regain of peroxidase activity promoted by enzyme resuspension in [BMIM][BF_4] [17] and decreased heme iron redox potential on the same solvent [18], it can be inferred that the lower peroxidase activity is not correlated to impaired enzyme stability in [BMIM][BF_4]. The quenching effect of [BMIM][BF_4] on the peroxidase turnover can be thus intrerpreted according to three different mechanisms that entails, respectively, (i) the binding of F^- anion (or HF) to the peroxidase active site at neutral and acidic pH values, (ii) the dielectric constant induced lowering of the heme iron redox potential and (iii) a solvent shielding effect that impairs productive encounters of radicalic species. As a result of these effects, products distribution is drastically affected and polymeric species formation is inhibited thus favouring the formation of discrete dimers. Thus, although the narrow operational window determined by the interplay between pH (higher than 9.0) and [BMIM][BF_4] concentration (50–75%, v/v) may appear limiting for most "traditional" peroxidase chemistry applications, the high product homogeneity and the ease of raw product recovery (precipitation after water dilution) pave the way to novel possible synthetic strategies based on the use of peroxidases for selective C–C bond formation.

In particular, the unprecendented high yield of 4-phenylphenol *ortho* dimer species emerges as a most relevant result in that this compound represents a member of an important class of synthons. It is worth noting that properly substituted 2,2′-*ortho*-phenol dimers can be employed as useful precursors for the synthesis of chiral bidentate metal ligands [19] and to generate novel classes of molecular templates. Still, further investigation will be necessary to clarify the mechanistic aspect of *ortho*-phenolic coupling in ionic liquids and understand the reasons for the widely different yields observed, for instance, with 1-naphthol with respect to 4-phenyl phenol. Moreover, the present investigation is limited to the use of [BMIM][BF_4], a "first generation" ionic liquid that has demonstrated its usefulness in several bio-synthetic applications. The whole field of ionic liquids is however in rapid development. New generations of functionalized, toxicity tested, ionic liquids are in fact becoming readily available and are expected to foster future research in the field.

Acknowledgment

Grant FIRB 2003 from MIUR (Ministero dell'Università e della Ricerca) to A.B. and S.C. is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2006.10.002.

References

[1] A.M. Azevedo, V.C. Martins, D.M. Prazeres, V. Vojinovic, J.M. Cabral, L.P. Fonseca, Biotechnol. Annu. Rev. 9 (2003) 199.
[2] G. Ward, Y. Hadar, I. Bilkis, L. Konstantinovsky, C.G. Dosoretz, J. Biol. Chem. 276 (2001) 18734.
[3] N.C. Veitch, Phytochemistry 65 (2004) 249.
[4] A.T. Smith, N.C. Veitch, Substrate binding and catalysis in heme peroxidases, Curr. Opin. Chem. Biol. 2 (1998) 269.
[5] K. Piontek, A.T. Smith, W. Blodig, Biochem. Soc. Trans. 29 (2001) 111.
[6] S. Jonathan, M.A. Dordick, A.M. Marletta, A.M. Klibanov, Biotechnol. Bioeng. 30 (1987) 31.
[7] P.A. Adams, D.A. Baldwin, G.S. Collier, J.M. Pratt, Biochem. J. 179 (1979) 273.
[8] F. van Rantwijk, R. Madeira Lau, R.A. Sheldon, Trends Biotechnol. 21 (2003) 131.
[9] C. Sanfilippo, N. D'Antona, G. Nicolosi, Biotechnol. Lett. 26 (2004) 1815.
[10] G. Hinckley, V.V. Mozaev, C. Budde, Y.L. Khmelnitsky, Biotechnol. Lett. 24 (2002) 2083.
[11] J. Dupont, C.S. Consorti, P.A.Z. Suarez, R.F. De Souza, Org. Synth. Coll. 10 (2004) 184.
[12] P. Trinder, Ann. Clin. Biochem. 6 (1966) 24.
[13] F. Xu, D.E. Koch, I.C. Kong, R.P. Hunter, A. Bhandari, Water Res. 39 (2005) 2358.
[14] F. Neri, D. Kok, M.A. Miller, G. Smulevich, Biochemistry 36 (1997) 8947.
[15] F. Cui, D. Dolphin, Bioorg. Med. Chem. 2 (1994) 735.
[16] S.F. DeLauder, J.M. Mauro, T.L. Poulos, J.C. Williams, F.P. Schwarz, Biochem. J. 302 (1994) 437–442.
[17] M.F. Machado, J.M. Saraiva, Biotechnol. Lett. 27 (2005) 1233.
[18] S.F. Wang, T. Chen, Z.L. Zhang, X.C. Shen, Z.X. Lu, D.W. Pang, K.Y. Wong, Direct electrochemistry and electrocatalysis of heme proteins entrapped in agarose hydrogel films in room-temperature ionic liquids, Langmuir 21 (2005) 9260.
[19] M. Berthod, G. Mignani, G. Woodward, M. Lemaire, Chem. Rev. 105 (2005) 1801.