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Alkali and halide-resistant catalysis by the multipotent oxidase from *Marinomonas mediterranea*

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Abstract

The incorporation of fungal laccases into novel applications has been delayed mainly due to their intrinsic sensitivity towards halides and alkaline conditions. In order to explore new sources of enzymes we evaluated the multipotent polyphenol oxidase PPO1 from the marine bacterium *Marinomonas mediterranea*. Here we report that, in contrast to its fungal counterparts, PPO1 remained functional above neutral pH presenting high specificity for phenolic compounds, in particular for methoxyl-substituted mono-phenols and catechols. These properties, in addition to its tolerance towards chloride (up to 1 M) and its elevated redox potential at neutral pH (0.9 V), suggest this enzyme may be an interesting candidate for specific applications such as the Amperometric determination of phenolic compounds and bio-fuel cells.
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Keywords: Laccase; Polyphenol oxidase; Amperometric biosensors; Fuel-cells; *Marinomonas mediterranea*

1. Introduction

Over the last two decades, the use of enzymes for a variety of technological applications has been explored (Aitken, 1993; Chen et al., 1999; Kirk et al., 2002; Liang et al., 2000). In general terms, enzymes are catalysts with high selectivity towards a given substrate, increasing the reaction rate by many orders of magnitude. Even further, their catalytic properties can be optimized with protein-design tools (Cherry and Fidantsef, 2003; Schmidt-Dannert and Arnold, 1999).

The use of redox enzymes in the construction of Amperometric devices has become a research field with considerable potential (Chen et al., 2001; Katz et al., 2001; Liang et al., 2000). In this regard, the natural affinity of polyphenol oxidases (PPOs) for envi-

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ronmentally and pharmaceutically important phenolic compounds has prompted their use in a variety of analytic applications, such as microelectrodes and biosensors (Dzyadevych et al., 2002; Ghindilis et al., 1992; Ghindilis, 2000; Kulys and Vidziunaite, 2003; Peter and Wollenberger, 1997). Briefly, PPOs efficiently oxidize phenolic compounds to quinones, which can then enter an amplification recycling process involving electrochemical reduction and enzymatic re-oxidation.

Members of the fungal laccase group of PPOs have been successfully employed as components of generic devices in the past (Ghindilis et al., 1992; Ghindilis, 2000; Peter and Wollenberger, 1997). Unfortunately, they are naturally sensitive to physiological concentrations of halides and hydroxyl ions, i.e. pH 7 and chloride concentrations near 100 mM (Solomon et al., 1996). For this purpose, alternative sources of PPOs have been explored, albeit with moderate success (Barton et al., 2002; Kulys and Vidziunaite, 2003; Skladal et al., 2002).

PPO activity has been observed in few bacterial groups (Claus and Filip, 1997; Diamantidis et al., 2000; Endo et al., 2003; Hullo et al., 2001; Sanchez-Amat and Solano, 1997; Shashirekha et al., 1997). To date, there is no information available regarding the potential application of bacterial PPOs for biotechnological purposes. Here we report our analysis of the multipotent PPO1 oxidase from the marine bacterium *Marinomonas mediterranea* (Sanchez-Amat and Solano, 1997), with emphasis in its potential use in miniature Amperometric devices and biosensors.

2. Materials and methods

2.1. Materials

All reagents used were analytical grade unless otherwise stated and were purchased from Sigma Chemical Co. (St. Louis MO). Solvents were HLPC grade from J.T. Baker Co. (Phillipsburg, NJ).

2.2. Culture conditions and extract preparation

M. mediterranea (CECT 4803) were cultured in 10 l stirred tank reactors with MMC medium (Fernandez et al., 1999) at 30 °C, 0.5 vvm and 300 rpm. Late exponential cultures were centrifuged at 26,000 × *g* for 20 min to collect the bacterial pellet, which was further washed with 1/10th of the volume of ice-cold 10 mM Tris–HCl pH 8.0, collected by centrifugation and frozen at −20 °C until further use. Twenty grams of the pellet were thawed and resuspended in 60 ml of 25% sucrose in 10 mM Tris–HCl pH 8 and 1× "Complete" protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cell wall was digested by incubation with 12 μg of lysozyme for 10 min at 4 °C. After the addition of 0.5 ml of 1 M $MgCl_2$, 0.4 ml of 0.2 M Phenylmethanesunfonylfluorid (PMSF) and 1.5 ml of 200 mg/ml Polyoxyethylene 23 lauryl ether (Brij 35) the mixture was incubated for 10 min at 37 °C and then transferred to ice. Protoplasts were disrupted by use of a Sonics and Materials Vibra-Cell sonicator (Danbury, CT). This material was centrifuged at 26,000 × *g* for 25 min and the supernatant was used as the crude cellular extract.

2.3. Enzyme purification

All steps were performed at 4 °C unless otherwise stated. The homogenate was fractionated by precipitation with granular ammonium sulfate to 40% saturation. The precipitate was removed by centrifugation and the supernatant dialyzed overnight against 40 volumes of 10 mM Tris–HCl buffer pH 8. This mixture was applied onto a DEAE-Sepharose Fast Flow column (Amersham Biosciences, Amersham, UK) previously equilibrated with 10 mM Tris–HCl buffer pH 8. Elution was performed with a linear NaCl gradient (0–1 M). Fractions with 2,6-dimethoxyphenol (2,6-DMP) oxidation activity were collected, pooled and dialyzed overnight against 10 mM Tris–HCl buffer pH 8. This pool was applied to a *t*-butyl HIC cartridge (BIO-RAD, Hercules, CA) previously equilibrated with 2 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl buffer pH 8. Elution was performed with a descending linear $(NH_4)_2SO_4$ gradient (2–0 M). Fractions with 2,6-DMP oxidation activity were collected, pooled and dialyzed overnight against 10 mM Tris–HCl buffer pH 8. A concentration step was carried out by ultrafiltration using a cell fitted with an YM30 membrane (Amicon, Beverly, MA). Finally, this concentrate was loaded onto a Sephacryl S-200 column (Amersham Biosciences, Amersham, UK) pre-equilibrated with 10 mM Tris–HCl pH 8. Fractions presenting 2,6-DMP oxidation activity were collected, pooled and concentrated by ultrafiltration.

2.4. Enzymatic activity determinations

Polyphenol oxidase activity was determined spectroscopically by monitoring the oxidation of 2 mM 2,6-DMP to 3,3′,5,5′-tetramethoxy-diphenylquinone ($\varepsilon_{468\,nm} = 14{,}800\ M^{-1}\ cm^{-1}$). Tyrosinase activity was determined as the oxidation of 1 mM L-tyrosine and 50 μM L-dopa to L-dopachrome ($\varepsilon_{475\,nm} = 3700\ M^{-1}\ cm^{-1}$). Other substrates used were 2 mM L-dopamine ($\varepsilon_{475\,nm} = 2834.7\ M^{-1}\ cm^{-1}$), 5 mM ABTS ($\varepsilon_{436\,nm} = 29{,}300\ M^{-1}\ cm^{-1}$), 0.05 mM syringadalzine ($\varepsilon_{530\,nm} = 64{,}000\ M^{-1}\ cm^{-1}$), 2 mM L-DOPA ($\varepsilon_{475\,nm} = 3700\ M^{-1}\ cm^{-1}$), 2 mM catechol ($\varepsilon_{388\,nm} = 1300\ M^{-1}\ cm^{-1}$), and 20 mM $K_4Fe(CN)_6$ ($\varepsilon_{405\,nm} = 900\ M^{-1}\ cm^{-1}$). One unit was defined as the amount of enzyme that catalyzes the appearance of 1 μmol of product per minute. Catalytic constants were derived by fitting the experimental data unto the Michaelis–Menten model using the EnzFitter Software (BIOSOFT, Cambridge, UK).

2.5. pH profiles

Different enzymatic activities were spectroscopically monitored at specific pH values using a mixture of 300 U of enzyme and the respective substrate at the stated concentration. Reaction buffers were prepared as follows: 50 mM succinate buffer pH 3; 60 mM acetate buffer pH 4; 100 mM phosphate buffer pH 5, 6, or 7; 10 mM Tris–HCl buffer pH 8; and 50 mM borate buffer pH 9 or 10.

2.6. Tolerance towards halides

Samples containing 160 U of enzyme in 100 mM phosphate buffer pH 5 or pH 7 were mixed with the respective salt (NaCl, KBr, $(NH_4)_2SO_4$, NaH_2PO_4 or NaF) at different concentrations, incubated for 5 min or 1 h, and the residual 2,6-DMP oxidation activity evaluated without further dilution.

2.7. Enzyme concentration estimates

Total protein was determined throughout the purification procedure with the Bio-Rad reagent (Hercules, CA) using bovine serum albumin as reference (Bradford, 1976). The actual concentration of active sites was estimated by titration with the irreversible inhibitor sodium azide (0–1 μM). Mixtures containing 500–700 U of the purified enzyme per milliliter of 100 mM phosphate buffer pH 5 were incubated with different concentrations of the inhibitor for 30 min. The residual activity of the mixture was estimated spectroscopically in the presence of 2 mM 2,6-DMP. Measurements were performed in triplicates, the data obtained fitted into a lineal adjust and the *x*-axis intercept value extrapolated. This value indicates the amount of inhibitor required for the total abatement of the activity and, consequently, the actual concentration of active sites.

2.8. Redox potential

The redox potential of the purified enzyme was estimated in a mixture containing 0.015 mg of the pure enzyme in 50 ml of each buffer solution: 10 mM acetate buffer pH 4, 10 mM phosphate buffer pH 6, 10 mM phosphate buffer pH 7, 10 mM Tris–HCl buffer pH 8, or 10 mM borate buffer pH 9 with a platinum electrode at 500 mV/min against a standard solution composed of 100 mM KNO_3 and $K_3[Fe(CN)_6]$.

2.9. Protein electrophoresis

Denaturing PAGE was performed by conventional methods using 10% acrylamide and stained with Coomassie brilliant blue. Non-denaturing PAGE was modified by reducing the concentration of SDS in the separating gel and in the running buffer to 0.1% and dissolving protein samples at room temperature in a modified loading buffer without β-mercaptoethanol and only 0.1% SDS. Non-denatured molecular weight markers, chicken egg albumin (45 kDa) and bovine serum albumin (66 kDa), were from Sigma (Saint Louis, MO.)

3. Results and discussion

3.1. Enzyme purification

M. mediterranea strain MMB1 extracts were submitted to the purification procedure described in Section 2 which allowed the complete separation of the two polyphenol oxidase activities known in this strain, PPO1 and PPO2. Typical purifications of PPO1 yielded

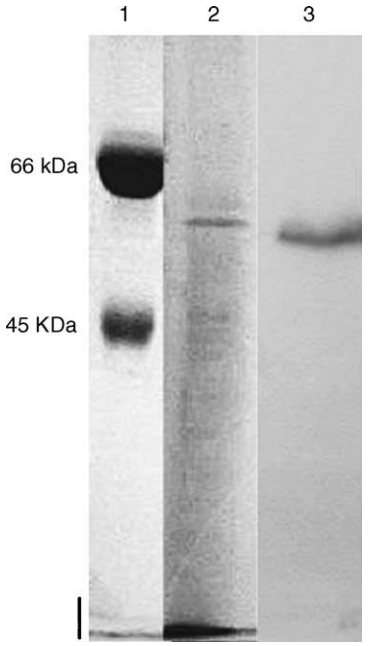

Fig. 1. Non-denaturing gel electrophoresis of purified *M. mediterranea* PPO1. Lane 1: non-denatured molecular weight markers: chicken egg albumin (45 kDa) and bovine serum albumin (66 kDa). Lane 2: purified PPO1 stained with Coomasie Brilliant Blue. Lane 3: purified PPO1 stained with 2,6-DMP.

12–15% of the initial activity with specific activities of 215–604 U/mg protein. The final preparations presented a single activity band of approximately 59 kDa as can be assessed in Fig. 1, suggesting a monomeric protein. The active PPO1 enzyme was 12 kDa smaller in size than expected from previous studies (Sanchez-Amat et al., 2001). This discrepancy might have possibly arisen from a different initiation codon usage in *M. mediterranea* versus *Escherichia coli*, or maybe from the proteolytic processing of a larger precursor. None of them can be ruled out.

3.2. pH dependence of activity

The pH dependence of the initial oxidation rate for four phenolic substrates and two non-phenolic substrates using PPO1 is shown in Fig. 2. These values were compared with those obtained using a laccase preparation from *Coriolopsis gallica* UAMH 8260 (Tinoco et al., 2001). PPO1 presented significant activity towards phenolic substrates from pH 4 to pH 9 (Fig. 2a–d). Optimal performance ranged from pH 5 for 2,6-DMP up to pH 8 for L-dopamine. These results were in clear contrast to the profile of the *C. gallica* laccase used as reference, which decreased sharply above pH 4.

Catalytic proficiency over such a wide range of pH is not a usual property for laccases. On the contrary, activity profiles for fungal laccases have been commonly observed to be narrow bell-shapes, with optimal pH values around 5, and no activity at pH 8 or above (Tinoco et al., 2001; Xu, 1997). To our knowledge, the only exceptions to this trend are the polyphenol oxidases from *Streptomyces griseus* (Endo et al., 2003), from *Pleurotus ostreatus* (Tinoco et al., 2001), and from *Myceliophtora thermophila* (Xu, 1997), which apparently present significant residual activity with some substrates at pH 8, albeit with some considerations. For the *S. griseus* PPO, the alkali-tolerant activity was only observed when *N*,*N*-dimethyl-*p*-phenylenediamine sulfate, an unusual substrate for laccases, was used as substrate (Endo et al., 2003). For the *Pleurotus* enzymes, up to 40% residual activity was observed when using syringaldazine as substrate but no other substrate was tested (Tinoco et al., 2001). In contrast, the *M. thermophila* laccase presented up to 75% activity at pH 8 only when substituted syringaldazine derivatives were used as substrates, but no with syringaldazine itself (Xu, 1997).

The currently accepted mechanism regarding the bell shapes of laccase activity when using phenolic substrates involves two opposing effects: while acidic inactivation is due to progressive substrate protonation, alkaline inactivation is the consequence of the accumulation of inhibitory hydroxyl ions (Xu, 1997). In contrast, for non-phenolic substrates, it has been previously observed that the activity profile of fungal laccases is monotonic, in which the rate decreased as the pH increased (Xu, 1997; Xu et al., 1999). Since the oxidation of non-phenolic substrates does not involve protons, this behavior has been interpreted as the sole effect of hydroxyl ion inhibition. In this regard, the monotonic shape of the pH dependence of the initial oxidation of non-ionizable substrates such as ABTS or $K_4Fe(CN)_6$ was consistent with the absence of ionizable residues involved (Fig. 2e and f). PPO1 was significantly active towards non-phenolic substrates at pH values above 6, albeit with a lower rate than when using phenolic

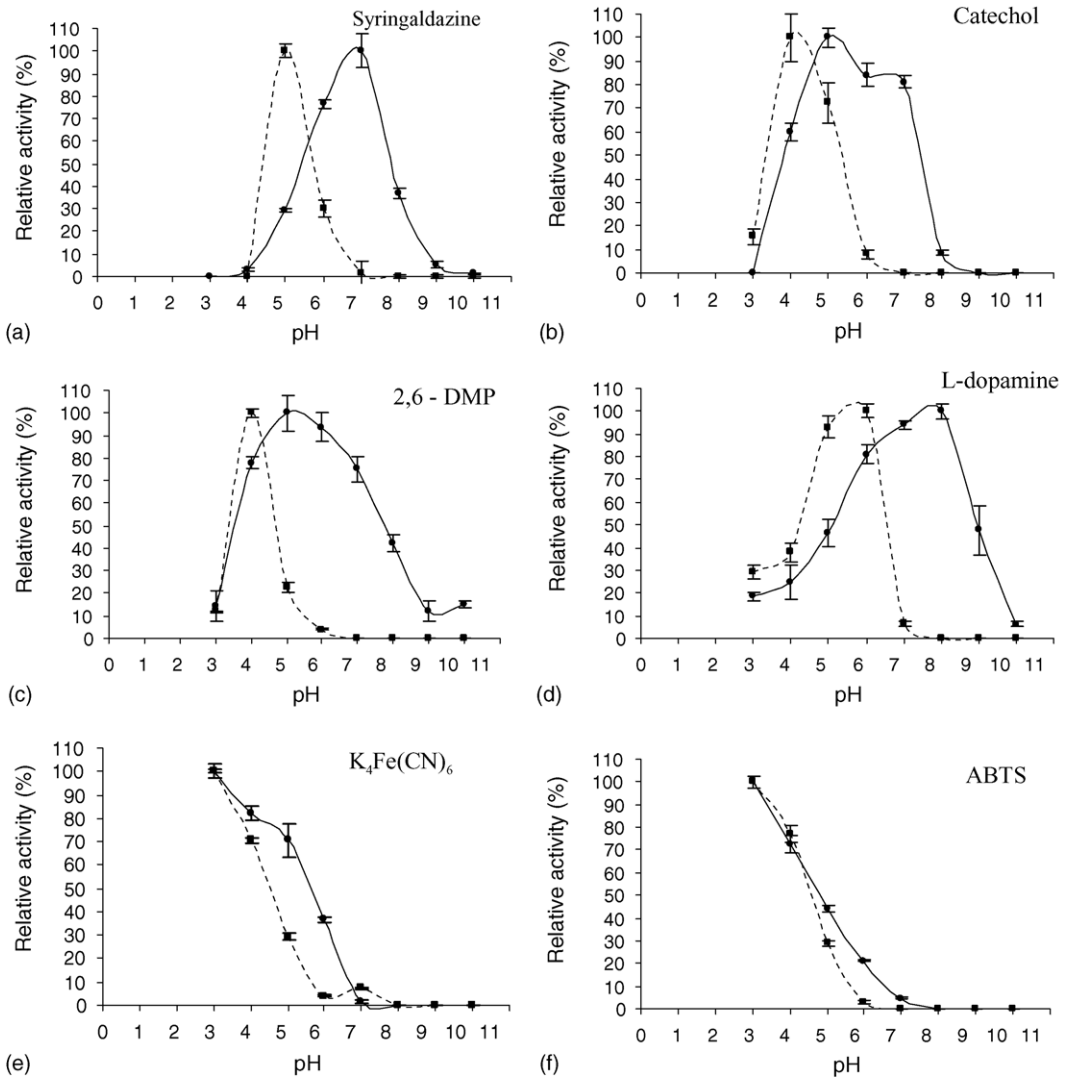

Fig. 2. pH dependence of the initial oxidation rate for different substrates by *M. mediterranea* PPO1 (solid line), and *C. gallica* laccase (dashed line).

substrates. This property will be discussed below in the context of a highly specific active pocket.

3.3. Tolerance towards halides

One of the major obstacles that have prevented rapid progress in the practical application of laccases in miniature implanted sensors or bio-fuel cells is the requirement for activity under physiological conditions, i.e. pH 7 and chloride concentrations near 100 mM (Chen et al., 2001). Most fungal laccases are inactive under these conditions because of their intrinsic sensitivity towards halides (Xu, 1996; Xu et al., 1998). Although the mechanism for chlorine and bromide ions inhibition has not been elucidated, it is known that fluoride ions bind to the T2 site of laccases with unusual high affinity, decreasing the thermodynamic driving force (ΔG°) for internal electron transfer from the T1 Cu to the trinuclear cluster (Bränden et al., 1973).

As can be seen in Fig. 3a and b, PPO1 is significantly tolerant towards all halides tested. At pH 5, PPO1 is up to 400 hundred-fold less sensitive towards

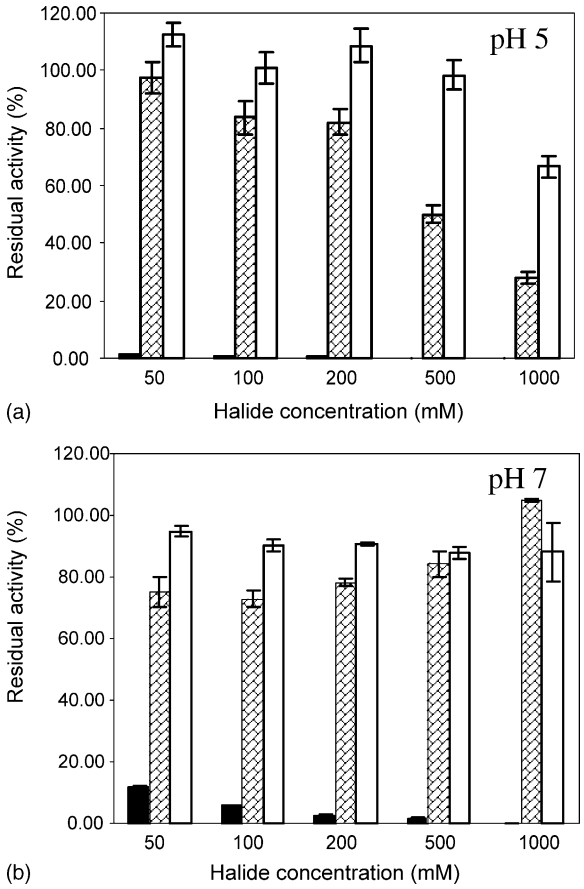

Fig. 3. Effect of different halides concentration upon the initial oxidation rate for 2,6-DMP by *M. mediterranea* PPO1 at pH 5 and pH 7. Filled bars: fluoride. Grey bars: chloride. Blank bars: bromide. All values are relative to the activity detected in the absence of salt.

fluoride ions [I_{50} = 8.1 mM] than some fungal laccases [I_{50} = 0.02–0.5 mM] (Xu, 1996). It has been previously observed that for fungal laccases, the tolerance towards fluoride ions increases at alkaline pH values (Xu et al., 1998). This behavior is explained by the competition between fluoride and hydroxyl ions for the T2 Cu atom. In this regard, the moderately enhanced tolerance of PPO1 towards fluoride we detect at pH 7 is consistent (I_{50} = 14 mM).

The effects of chloride and bromide ions upon PPO1 activity at pH 5 (Fig. 3a) were better described by single-exponential decay kinetics yielding I_{50} values of 547 and 2000 mM, respectively. These values are unusual for laccases and are only matched by those from the laccase of *M. thermophila* (Xu, 1996). In contrast, the inhibition kinetics at pH 7 (Fig. 3b) was better described by a double-exponential decay model, with a sharp decrease at the initial phase and practically unresponsive above 50 mM halide concentration. In these cases, it was not possible to estimate the I_{50} values, since the activity did not drop below 60%. Furthermore, the ability of PPO1 to oxidize different substrates was entirely insensitive to the presence of 100 mM NaCl at pH 7, as can be seen in Table 1. We were unable to detect significant activity whatsoever when the laccase from *C. gallica* was assayed under these conditions.

Table 1
Pure PPO1 oxidative activity towards different substrates at pH 7 in the absence or presence of 100 mM NaCl (U mg prot^{-1})

	Without NaCl	With NaCl
Syringaldazine	0.166 ± 0.004	0.180 ± 0.014
2,6-DMP	0.169 ± 0.012	0.183 ± 0.014
L-Dopamine	0.145 ± 0.013	0.141 ± 0.004
L-Dopa	0.127 ± 0.007	0.124 ± 0.007
Catechol	0.284 ± 0.035	0.266 ± 0.042
ABTS	0.017 ± 0.001	0.016 ± 0.001

3.4. Substrate specificity

The ability of PPO1 from *M. mediterranea* to oxidize a variety of phenolic and non-phenolic substrates was investigated. PPO1 positively oxidized phenolic substrates such as syringaldazine, catechol, 2,6-DMP, L-dopamine and L-dopa, as well as non-phenolic substances like ABTS and $K_4Fe(CN)_6$. From the data presented in Table 2, it appears that the catalytic efficiency of PPO1 was governed by the affinity constant (∼3000-

Table 2
Substrate specificity of *M. mediterranea* PPO1[a]

	K_M (M)	k_{cat} (seg^{-1})	k_{cat}/K_M
Syringaldazine	1.5×10^{-6}	19.13	1.21×10^{7}
L-Dopamine	1.5×10^{-5}	0.89	5.96×10^{5}
Catechol	9.9×10^{-5}	50.26	5.07×10^{5}
2,6-DMP	1.7×10^{-4}	28.80	1.66×10^{5}
L-Dopa	2.0×10^{-4}	20.71	9.95×10^{4}
$K_4Fe(CN)_6$	1.9×10^{-3}	162.80	8.40×10^{4}
ABTS	4.8×10^{-3}	28.73	5.93×10^{3}

[a] Initial rate values were determined at the optimal pH for each substrate.

fold difference among K_M values), not by the reaction rate (only 8.5-fold among k_{cat} values). This observation suggests that for PPO1, the rate-limiting step is the productive access of the substrate to the T1 Cu site. Even further, the K_M values for all phenolic substrates tested were in the μM range, in contrast to the K_M values for ABTS and $K_4Fe(CN)_6$, which were in the mM range. In contrast, for the *Coriolus hirsutus* laccase, the reported k_{cat} values for a similar set of substrates ranged from 2.6×10^4 to 9.3×10^5 seg^{-1} (34.7-fold difference), whereas the K_M values were in the range of 40–142 μM (3.55-fold difference) (Shin and Lee, 2000).

For other laccases, K_M values in the mM range have been interpreted as evidence of outer-sphere interaction between the T1 Cu site and the substrate (Solomon et al., 1996; Xu, 1996). The catalytic efficiency (k_{cat}/K_M) of these enzymes correlates with the oxidation potential of the substrates, which is consistent with Marcus theory where the rate-determining step is substrate reduction, not specific binding (Solomon et al., 1996).

Taken together, this evidence suggests that fungal laccases lack a specific catalytic pocket. In contrast, other Cu-dependent oxidases such as ascorbate oxidase, which harbor specific substrate-binding pockets, present K_M values in the μM range and the catalytic efficiency is not related with the oxidation potential of the substrates (Solomon et al., 1996).

PPO1 was unable to oxidize guaiacol, industrial dyes, 1-hydroxyl benzotriazol (HBT), flavonoids (catechin, epicatechin, or naringenin), phenolic acids or esters (gallic acid, ferullic acid, vanyllic acid, syringic acid or *n*-propyl gallate), nor lignin precursors such as ellagic acid. Compared to fungal laccases, which are known to oxidize a vast diversity of compounds as well as some inorganic ions (Thurston, 1994), PPO1 showed remarkable specificity, not only for phenolic substrates, but also for a particular type of structures, excluding acids or esters, as well as bulky molecules. This evidence, along with the elevated affinity values, strongly suggests the existence of a highly restrictive substrate-binding pocket in PPO1 with specificity towards phenolic substances. In accordance, PPO1 was able to bind the pharmaceutically important substrate L-dopamine with 10,000 times higher affinity than the polyphenol oxidase from *Anona muricata* L. [1.45×10^{-2} M] which is currently used in the construction of Amperometric biosensors (Bezerra et al., 2003).

3.5. Redox potential

The T1 Cu site of fungal laccases is an unusual blue Cu site because it lacks an axial ligand, rendering the site trigonal planar. Previous spectroscopy studies on the T1 Cu site from three different wild-type fungal laccases revealed that this T1 Cu site is characterized by increased covalency of the Cu–S(Cys)π bond and increased strength of the ligand field as compared to the classic T1 Cu site in plastocyanin, which has an axial Met ligand. It has been proposed that the nature of the axial ligand affects E°; stronger axial ligands help stabilize Cu(II), thereby lowering the E°, whereas weaker axial ligands tend to destabilize Cu(II), thus raising the E°. Several site-directed mutagenesis studies of simple blue (T1) Cu proteins have shown that detectable effects [including −30 to +100 mV changes in E°] could be induced when the T1 Cu axial ligand (Met) was mutated to various other amino acids (Chang et al., 1991; Farver et al., 1993; Hart et al., 1996; Karlsson et al., 1997; Messerschmidt et al., 1998; Murphy et al., 1995; van Pouderoyen et al., 1996). In *Trametes villosa* laccase, an artificial T1 Cu axial ligand was created by the site-directed substitution F463M which led to a significant change in the electronic structure of the T1 Cu, as was assessed by EPR and absorption spectra (Xu et al., 1999). While this mutant protein presented a significantly different catalytic behavior, the E° of its T1 Cu was only slightly reduced [from 0.79 to 0.68 V]. Thus, the nature of this axial amino acid residue has been shown to tune the covalency, geometry, and electronic structure of the site. The mononuclear T1 Cu in other bacterial Cu-binding proteins, such as CotA and CueO, has the typical geometry found for this site in simple blue (T1) Cu proteins, with two His, one Cys, and a Met displaying a distorted bipyramidal geometry, plus an additional vacant axial position for the binding of the reducing substrate (Enguita et al., 2003; Roberts et al., 2002). The deduced protein sequence for PPO1 presents significant similarity with other bacterial PPOs around the Cu binding residues, including the axial Met ligand (Table 3).

The T1 Cu ion of PPO1 has a higher redox potential at pH 7 [+900 mV versus NHE] than any laccase from fungal origins described to date [+450 to +780 mV versus NHE] (Xu et al., 1996, 1999; Xu, 1997). Despite the fact that several lines of evidence indicate that the role of the weak axial interaction of the T1 Cu with a

Table 3
Sequence alignment among different PPOs (ligands to T1 Cu are indicated in bold)

GI number or reference	Source	E° (V)	T1 Cu ligands											
8572537	*M. mediterranea* PPO1	0.9	^{647}H	**C**	H	I	L	D	**H**	E	D	Q	G	**M**^{658}
2506227	*E. coli* CueO	–	^{499}H	**C**	H	L	L	E	**H**	E	D	T	G	**M**^{510}
7404344	*B. subtilis* CotA	–	^{491}H	**C**	H	I	L	E	**H**	E	D	Y	D	**M**^{502}
7432984	Zucchini Ascorbate oxidase	0.34	^{506}H	**C**	H	I	E	P	**H**	L	H	M	G	**M**^{517}
Xu et al. (1999)	*M. thermophila*	0.47	^{502}H	**C**	H	I	A	W	**H**	V	S	G	G	L^{513}
Xu et al. (1996)	*Scytalidium thermophilum*	0.51	^{506}H	**C**	H	I	A	W	**H**	V	S	G	G	L^{517}
2833191	*Rhizoctonia solani* (4)	0.71	^{459}H	**C**	H	I	D	W	**H**	L	E	A	G	L^{470}
1100244	*T. villosa*	0.78	^{452}H	**C**	H	I	D	F	**H**	L	E	A	G	F^{463}
1172163	*Trametes versicolor*	0.79	^{451}H	**C**	H	I	D	F	**H**	L	E	A	G	F^{462}
46578391	*C. hirsutus*	–	^{471}H	**C**	H	I	D	F	**H**	L	E	G	G	F^{482}

Met residue may lead to a significant decrease of the E° of the site, we cannot rule out that the high redox potential of PPO1 might due to the presence of this ligand in a rather unusual geometry. Interestingly, the axial Met ligand is essential for the catalytic activity of *Bacillus subtilis* CotA (Hullo et al., 2001). Furthermore, and in contrast to the observed behavior of the *M. thermophila* and *T. villosa* laccases (Xu et al., 1998, 1999), the observed E° of PPO1 decreases with increasing pH (Fig. 4). The alkaline PPO1 E° inflection occurred between pH values of 6 and 7 which is the threshold for the oxidation of non-phenolic substrates for PPO1 and for all substrates by the control *C. gallica* and the wild type *M. thermophila* laccases (Xu et al., 1999).

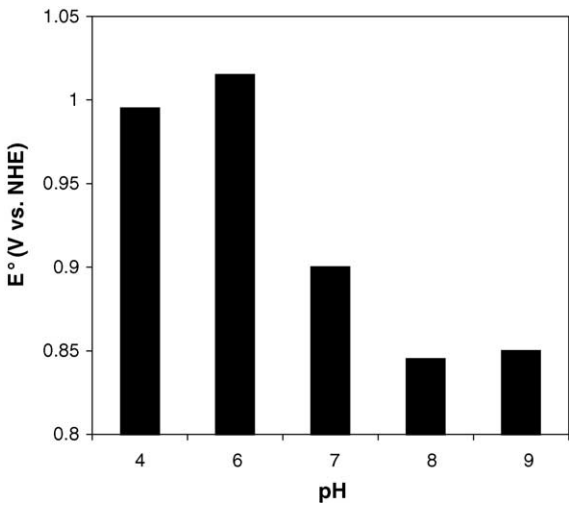

Fig. 4. Redox potential determination of a preparation of pure *M. mediterranea* PPO1 at different pH values.

This phenomenon is reminiscent of alkaline transitions studied in other metal-based enzymes, such as *iso*-cytochrome *c*, which is the consequence of a deprotonation event associated with ligand exchange (Wilson and Greenwood, 1996). Although in need to be tested, the only residue side-chain with pK_a values around 6–7 is the imidazol group of histidine. We thus suspect that one of these residues might be involved in the transition, either directly as a ligand or by reorganizing the T1 site surroundings.

4. Conclusions

M. mediterranea PPO1 may outperform fungal laccases as a more promising resource for the construction of phenol-specific miniature implanted sensors or biofuel cells. These applications require significant oxidative activity under physiological conditions, i.e. pH 7 and chloride concentrations near 100 mM. Most fungal laccases are inactive under these conditions because of their intrinsic sensitivity towards halides (Xu, 1996; Xu et al., 1998). In contrast, PPO1 presented significant activity towards phenolic substrates from pH 4 to pH 9, with optimal performance at pH 5 for 2,6-DMP, at pH 6 for catechol, pH 7 for syringaldazine, and pH 8 for L-dopamine. Furthermore, PPO1 was significantly tolerant towards halides. At pH 7, PPO1 was insensitive towards chloride and bromide ions in the range of 50 mM to 1 M, and tolerant towards fluoride ions (I_{50} = 14 mM). Compared to fungal laccases, which are known to oxidize a vast diversity of compounds as well as some inorganic ions (Thurston, 1994), PPO1 was able to utilize only a fairly restricted number of

substrates, demonstrating high specificity. Even further, PPO1 K_M values for phenolic substrates are all in the μM range, in contrast to the K_M values for ABTS and $K_4Fe(CN)_6$, which are in the mM range, suggesting the existence of a binding pocket highly specific towards phenolic substances. The sum of all these properties suggests that the multipotent PPO1 from *M. marinomonas* may outperform fungal laccases in the construction of enzyme-based analytical Amperometric devices.

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