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pH-Dependence of Manganese (II) Oxidation Reaction by Novel Wild-Type and Mutants Recombinant *Phlebia radiata* Manganese Peroxidase 3 (rPr-MnP3) Enzymes

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Abstract

The goal of this study was to determine whether mutation of the Mn-binding site of wild-type recombinant *Phlebia radiata* manganese peroxidase 3 affected the pH-dependence kinetic parameters. pH range investigated was 2.5 – 12.0. The catalytic efficiency of the mutant enzymes at high and low pH in comparison to the wild-type was investigated using standard rPr-MnP3 protocol. Wild-type recombinant *Phlebia radiata* MnP3 enzyme showed optimal activity with Mn (II) as substrate at pH 5.0 and remained moderately active (approximately 40%) in the pH range of 6.0 - 9.0. The rPr-MnP3 mutants' maximum activity ranged between 5.5 and 8.0. Wild-type and mutants rPr-MnP3 enzymes exhibited a similar pH profile with optimum pH of 3.0 for ABTS oxidation. Mutation has severely decreased the catalytic efficiency for Mn (II) oxidation at pH 5.0. The rPr-MnP3 enzymes showed enhanced affinity for Mn (II) at alkaline pH and a more alkaline range for catalysis than ever reported for any Manganese Peroxidase. This study reveals that at higher pH, rPr-MnP3 can function with alternative ligands in the Mn (II) site and does not have an absolutely obligate requirement for an all carboxylate ligand set. These results further strongly confirm that Mn^{2+} binding site is the only productive catalytic site for Mn (II) oxidation.

Keywords

pH-Dependence, *Phlebia radiata*, Manganese Peroxidase, Wild-Type, Mutants, Recombinant Enzyme

1. Introduction

Manganese peroxidase (MnP) (EC 1.11.1.13) is a class II extracellular fungal haem-containing peroxidase belonging to the plant peroxidase-like protein superfamily [1] [2]. Manganese peroxidase is still considered the most ubiquitous ligninolytic enzyme among white-rot fungi and was first discovered in *Phanerochaete chrysosporium* [3] [4] and thereafter in most of the wood lignin and litter-decaying basidiomycetes [5]. MnP has been isolated or expressed, purified and characterized from various white rot fungi [6]-[11].

In plants, MnP, a component of ligninolytic enzymes complex catalyzes lignin de-polymerization. The enzyme has been found to catalyze the breakdown of lignin and phenolic lignin model compounds, as well as the non-phenolic ones in the presence of certain compounds to polycyclic aromatic hydrocarbons by oxidation of Mn^{2+} to Mn^{3+} with H_2O_2 as an oxidant [1] [12]. Manganese peroxidase remains one of the most common lignin degradation enzymes and has great potential in the field of agriculture for degradation of some cellulose, hemicellulose, lignin, etc. In the environment, this enzyme has been widely employed in the degradation of some recalcitrant organic pollutants such as industrial dyes, polycyclic aromatic hydrocarbons, chlorophenols; and nitroaromatic compounds, known to be very harmful to human health [13]. Several researches have suggested that some azo dyes could be efficiently degraded by the purified MnPs, which were isolated from *P. chrysosporium*, *Lentinula edodes*, *Trametes versicolor*, *Dichomitus squalens*, *Stereum ostrea and Irpex lacteus* [13]-[18].

The key physiological role of MnP is the oxidation of Mn^{2+} to Mn^{3+} ions [4] [19] [20]. The MnP oxidizes the one-electron donor Mn^{2+} to Mn^{3+}. Manganese (III) ion manufactured enzymatically is assumed to form a diffusible oxidant complex with dicarboxylic acid chelators such as oxalate which is also secreted by the fungus [20] [21] [22] [23]. The Mn^{3+} organic acid complex then oxidizes phenolic substrates, including lignin substructure model compounds [24] and aromatic pollutants [4] [25] [26] [27] as well as possible mediator molecules [26] [27].

A unique characteristic of MnP is that the best reducing substrate for Compounds I and II is Mn (II), a metal ion naturally present in wood. However, compound II of MnP exhibits an absolute requirement for Mn (II) as an exclusive reductant essential for the completion of the catalytic cycle of the enzyme [22] [28]. Only Mn (II) is capable of very rapidly reducing MnP compound II to its original state, thereby allowing the enzyme to initiate new substrate oxidations [22] [29] [30]. Not only is Mn essential for the activity of MnP [31], but it also regulates the production of MnP [32] [33], laccase and Lignin peroxidase (LiP) in *P. chrysosporium* and in other white rot fungi [34]).

Ionic strength and pH are very important factors affecting enzyme activity. The protein surface is the point of contact with the solvent and media, thus the physical and chemical environment are sensed through the surface residues. However, changes in the ionization state of the polar residues of protein may interfere with enzymatic activity.

2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) is a well-known substrate for peroxidases [35] and has been shown to be rapidly oxidised by MnPs [36] [37] [38]. The chemical properties of ABTS and its oxidation by peroxidases, particularly HRPC; have been intensively studied [39] [40]. 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) undergoes a single electron oxidation in the presence of peroxidase and hydrogen peroxide to produce a long-lived cation radical. This radical product has an intense blue/green colour and thus its accumulation can be measured spectroscopically [35]. Note that MnP does not require a functional Mn (II) oxidation site for ABTS to be a good substrate as its oxidation is more non-specific in nature.

The functions and applications of MnP enzyme stimulate interest in studying and understanding enzyme structure, biochemical characteristics, and genes. Therefore, searching for new MnP that is highly effective from widely distributed sources worldwide is crucial for its application in agricultural productions, medical, industrial and environmental protection. Recently, more and more attention has been paid to the value of bioremediation of this enzyme. The focus of this study was to determine the optimum conditions of pH to maintain recombinant *Phlebia radiata* MnP3 activities. Furthermore, the work also aimed at investigating if mutation of the Mn-binding site of wild-type recombinant *Phlebia radiata* manganese peroxidase 3 enzyme has affected the pH dependence kinetic parameters and to also ascertain the effectiveness of the mutant enzymes at high and low pH in comparison to the wild-type. This involved heterologous expression and purification of recombinant MnP3 from *Phlebia radiata*, followed by Manganese dependent peroxidase assays using the required substrates and reagents.

2. Materials and Methods

2.1. Materials

The complete MnP3 gene of *Phlebia radiata* strain 79 (ATCC 64658) was provided by Dr. Taina Lundell, Department of Food and Environmental Sciences, Division of Microbiology, University of Helsinki, Finland. The Gene Bank accession number for the cDNA encoding peroxidase Pr-MnP3 is AJ566200. The Pr-MnP3 cDNA was present in vector pCR2.1.TOPO. The *Escherichia coli* expression vector pFLAG1 was obtained from International Biotechnologies Inc, UK. Peroxidases, wild-type, E40H, E44H, E40H/E44H, DI86H and 186N (RZ = 5.6, 2.1, 4.5, 5.2, 4.4 and 5.0, respectively) were produced, activated and purified based on the procedure previously described [41]. All chemicals used in this study were obtained from Sigma–Aldrich, UK and Fisher Scientific, UK. Restriction enzymes were supplied by NEBiolabs, UK. All spectroscopic measurements in this study were carried out using UV/Vis spectrophotometer (UV-2401 PC, Shimadzu Scientific Instruments, Addison, IL).

2.2. Steady-State Kinetic Analysis of rPr-MnP3 Activity with Manganese (II)

Mn (II) is assumed to be the *in vivo* substrate for manganese peroxidase. A set of

apparent steady-state kinetic constants for Mn (II) to Mn (III) oxidation for each of the engineered *Phlebia radiata* MnP3 enzymes was obtained by measuring the initial rates of assays at 238 nm for varying $MnSO_4$ concentrations: 0.02 - 1.0 mM for wild-type, D186H and D186N mutants and 1 - 35 mM for E40H, E44H, and E40H/E44H mutants. The concentration of $MnSO_4$ was increased for E40H, E44H and E40H/E44H enzymes since the concentration used for the wild-type, D186H and D186N could not produce measurable activity. Enzyme concentrations of 0.8 nM and 0.2 μM and the assay buffer was 100 mM Na-tartrate, pH 5.0 and 8.0 for wild-type and mutants, respectively.

All assays were performed with a fixed 0.1 mM hydrogen peroxide at 25˚C using ultraviolet spectrophotometer (UV-2401 PC, Shimadzu Scientific Instruments, Addison, IL, USA). In this study, the product being detected was actually a Mn^{3+}-tartrate complex with ε238 = 6.5 mM^{-1} cm^{-1} [41]. To ensure quality control, best laboratory practices were adopted and all measurements were carried out in triplicate.

2.3. Steady-State Kinetic Analysis of Recombinant *Phlebia radiata* Manganese Peroxidase 3(rPr-MnP3) Activity with ABTS

2, 2'-azino-bis (3-ethylbenzthiozoline-6-sulphonic acid) (ABTS) is a commonly used peroxidase substrate with very high levels of activity as seen in plant peroxidases [35]. 2, 2'-azino-bis (3-ethylbenzthiozoline-6-sulphonic acid) assays for wild-type rPr-MnP3 were carried out in 100 mM Na-tartrate buffer, pH 3.0, with a fixed H_2O_2 concentration (0.1 mM), enzyme concentration (0.01 uM) and the ABTS final concentration of 3.5 mM for the wild-type enzyme while ABTS concentration range 1 - 6.5 mM was used for the mutants variants. Formation of the radical product as a function of time was measured at 25˚C by monitoring the increase in absorbance at 414 nm. The initial rates were determined using ε414 = 36.8 $mM^{-1}{\cdot}cm^{-1}$ [34] and all ABTS assays were in triplicate.

2.4. Determination of the pH Optima for Mn^{2+} and ABTS Oxidation by *rP. radiata* MnP3 Enzymes

The optimum pH values for the oxidation of Manganese (II) and 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) by wild-type rPr-MnP3 were determined by assaying enzyme activity in 100 mM tartrate buffers of varying pH. For Mn (II) oxidation, assay mixtures contained 1 mM $MnSO_4$, 0.1 mM H_2O_2, and 0.023 μM enzyme and a pH range from 3.5 to 10.0 was used. In ABTS oxidation, the assay mixtures were 1.6 mM ABTS, 0.1 mM H_2O_2, and 0.006 μM enzyme with a pH range of 3.0 to 5.5.

Similarly, the pH optima for all rPr-MnP3 mutants were also determined for Mn (II) and ABTS as substrates. In the Mn (II) oxidation, the assay mixtures were enzyme concentration of 0.4 μM E40H/E44H, 0.34 μM E44H, 0.3 μM E40H, 0.3 μM D186H and 0.21 μM D186N, along with 0.1 mM hydrogen peroxide, 25 mM $MnSO_4$, and the pH ranged from 3.5 to 12.0. For ABTS oxidation by rPr-MnP3 mutants, the assay mixtures were 0.0022 μM E40H/E44H, 0.0094 μM

E44H, 0.0015 μM E40H, 0.0096 μM D186H and 0.0086 μM D186N, in addition to 1.6 mM ABTS, 0.1 mM H_2O_2 and the pH ranged from 2.5 to 5.5. The total volume of all assay mixtures was 1 ml and assays were performed at 25˚C. The reaction was initiated by the addition of hydrogen peroxide and the increase in absorbance at 238 nm or 414 nm, for Mn (II) and ABTS respectively, was measured using a UV/Vis spectrophotometer (Shimadzu UV-2401 PC). All measurements were in triplicate. To determine a *pKa* for Mn (II) oxidation, the mean rates for the various pHs were calculated; from the data log K (observed) was calculated and plotted against pH. The *pKa* was then extrapolated from each plot.

Data analysis, plotting and manipulations were carried out using the solver tools of Microsoft Excel and the statistical analysis software, SigmaPlot for Windows V4.01 (SPSS UK Ltd, Woking, UK). The Km (Michaelis-Menten constant) and Kcat obtained were used to calculate the specificity constants (kcat/Km). In addition, an activity assay was further conducted at pH 8.0 using the above steady-state conditions to see how rPr-MnP3 enzymes will behave catalytically at alkaline pH.

3. Results

3.1. pH Optimum for Mn (II) Oxidation by Wild-Type and Mutant rPr-MnP3 Enzymes

Figure 1 and Table 1 show the pH optima data for Mn (II) substrate oxidation by rPr-MnP3 enzymes. A sharp acidic pH optimum was observed in the wild-type rPr-MnP3 activity. As shown above, all mutants retained significant residual activity at pH values > 5.5 (up to 35% of wild-type).

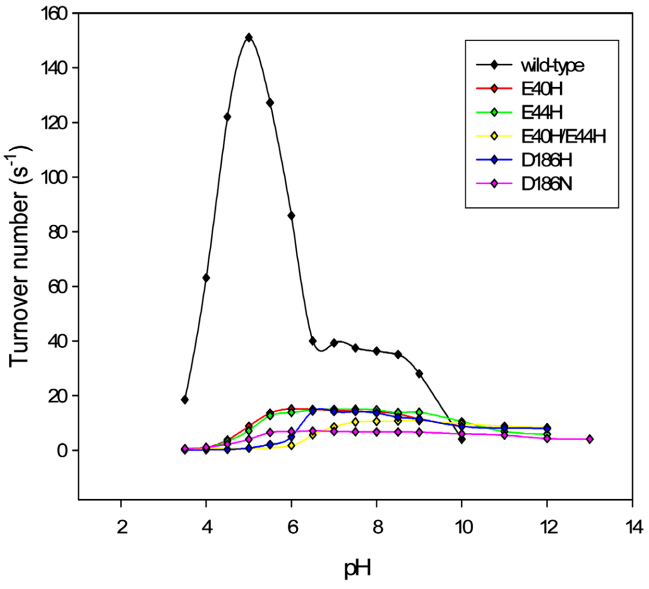

Figure 1. Plots of pH optima for Mn (II) oxidation by wild-type and mutants rPr-MnP3 enzymes. (—) WT, (—) E40H, (—) E44H, (—) E40H/44H, (—) 186H and (—) D186N. All assays were conducted at 25˚C.

Table 1. pH optima and apparent pKa's for Mn (II) oxidation by wild-type and mutants *rPr. radiata* MnP3 enzymes. The assays were conducted at 25˚C.

Enzyme	pH optima	Apparent *pKa*
Wild-type	~5.0/9.0	~4.5; 5.5; 6.5; ~9.0
E40H	6.0 - 8.0	~5.5
E44H	6.0 - 8.0	~5.5
E40H/E44H	7.5 - 10.0	~7.0
D186H	6.5 - 8.0	~6.5
D186N	5.5 - 12	~5.5

In **Figure 2(a)** the wild-type rPr-MnP3 pKa reflected at four points labeled 1, 2, 3, and 4. As presented in **Table 1**, the wild-type enzyme had its pKa at ~4.5, 5.5, ~6.5 and ~9.0. The variants E40H, E44H and D186N had their respective pKas at ~5.5 while that of the D186H mutant and E40H/E44H double variant were 6.5 and ~7.0 respectively.

3.2. pH Optimum for ABTS Oxidation by Wild-Type and Mutant rPr-MnP3 Enzymes

As shown in the **Figure 3** below, both the wild-type and the rPr-MnP3 mutant enzymes, E40H, E44H, E40H/E44H, D186H and D186N had their optimum pH at pH 3.0.

3.3. Mn (II) Oxidation by rPr-MnP3 Wild-Type and Mutant Variants at pH 5.0 and 8.0

The results of Mn (II) oxidation by rPr-MnP3 wild-type and mutant variants at pH 5.0 and 8.0 are shown in **Figure 4(a)** and **Figure 4(b)** for Mn (II) oxidation by rPr-MnP3 mutants. The kinetic parameters for Mn (II) oxidation by these enzymes are presented in **Table 2**.

4. Discussion

4.1. Determination of pH Optimum and Apparent pKa for Mn (II) Oxidation by Wild-Type and Mutant Recombinant *Phlebia radiata* MnP3 Enzymes

The optimal conditions for the catalysis by different peroxidases are not identical [42]. Thus, the optimum pH for the oxidation of Mn (II) and ABTS by recombinant *P. radiata* MnP3 enzyme was determined by assaying enzyme activity in 100 mM tartrate buffers of varying pH ranges (from 3.5 to 12.0). **Figure 1** and **Table 1** showed the pH optima data for Mn (II) substrate oxidation by rPr-MnP3. pH is a determining factor in the expression of enzymatic activity as it changes the ionization states of amino acid side chains or ionization of the substrate [43]. The active site of enzymes is frequently composed of ionized groups and that must be in the proper ionic form in order to maintain the conformation of the active site. It is worthy of note that the behaviour of the enzyme

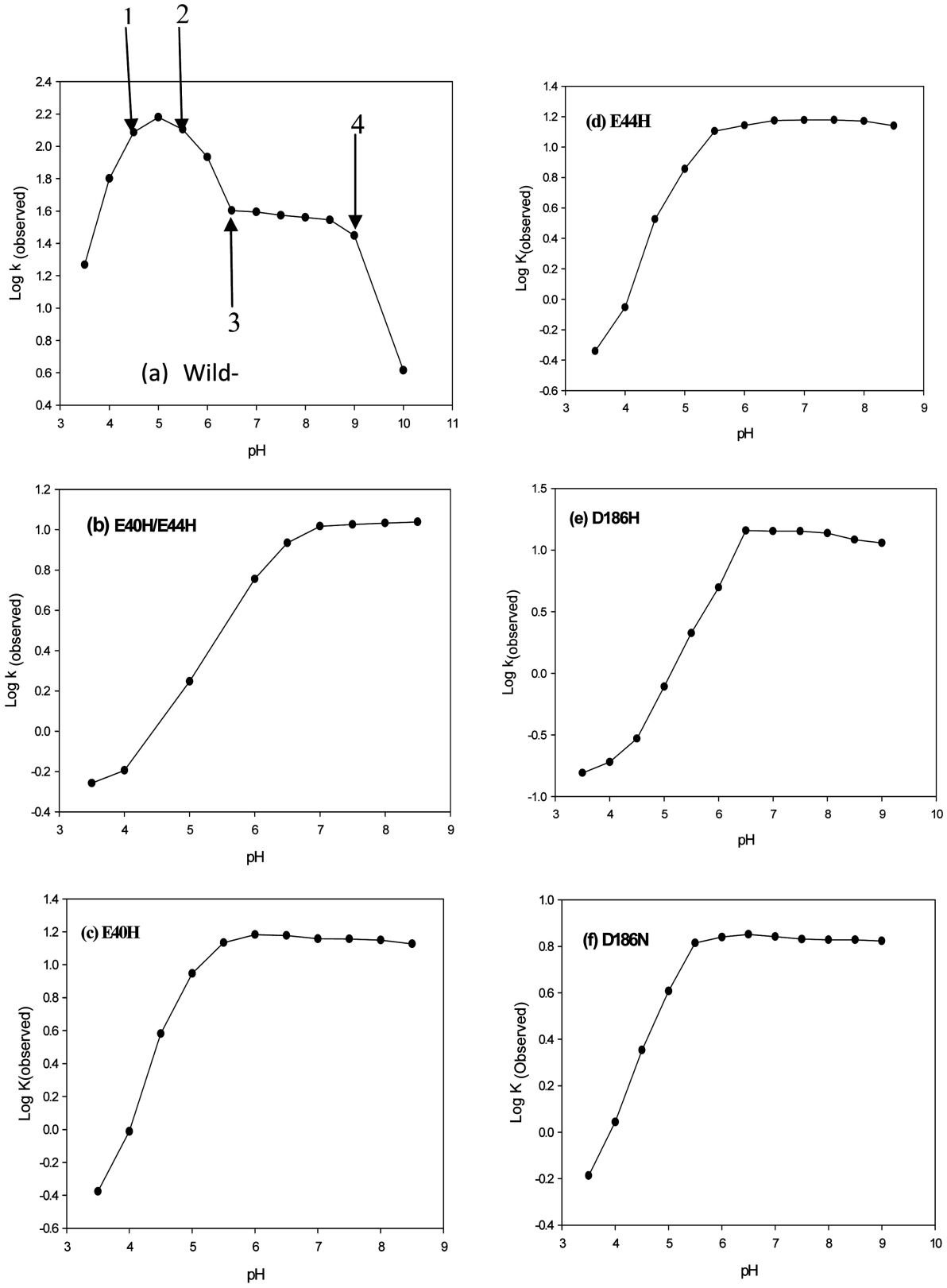

Figure 2. (a)-(f): Plots of log k (observed) against pH for the Mn (II) oxidation assay, for determination of pKa's for wild-type and mutant rPr-MnP3 enzymes. Apparent pKa's were determined from the intersection of the idealized slopes from the graphs. Assays were performed as for **Figure 1**. (Section 2.2.).

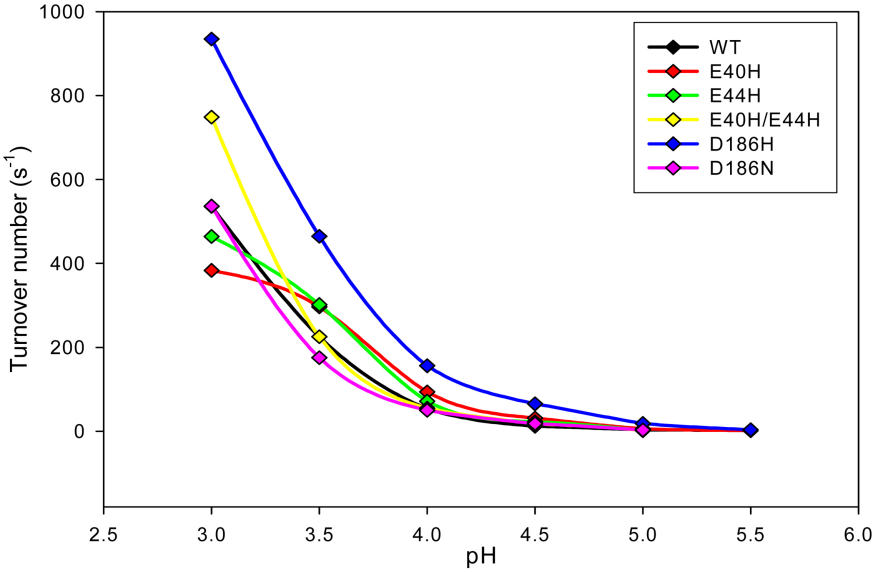

Figure 3. pH profiles for ABTS oxidation by wild-type and mutants, E40H, E44H, E40H/E44H, D186H and D186N) rPr-MnP3 enzymes. The reactions were performed in 100 mM sodium tartrate buffer, using 0.1 mM H_2O_2, 1.6 mM ABTS and enzymes of concentrations: 0.006 μM (WT) (—), 0.0015 μM (E40H) (—), 0.0094 μM (E44H) (—), 0.0022 μM (E40H/E44H) (—), 0.0096 μM (D186H) (—), and 0.0086 μM (D186N) (—) (Section 2.2.). The assay was conducted at temperature of 25˚C.

activity at different pH values gives information on the identities of the phototropic groups at the active site [44].

A sharp acidic pH optimum for wild-type rPr-MnP3 activity was observed in this study and the highest wild-type rPr-MnP3 turnover number was found to be at pH 5.0, while the enzyme was very active at the pH range of 4.5 to 5.5. Though the enzyme has it pH optimum in the acidic region, it was observed to be moderately active at alkaline pH, showing similar activity of approximately 18% of the maximum in the pH range of 6.5 to 8.5 with a sharp decrease towards no activity at pH 10.0. Wild-type *rPr-radiata* MnP3 with a pH optimum at 5.0 is consistent with the literature [45] [46]. The optimum pH for Mn (II) oxidation by MnP from *P. chrysosporium is* reported to be 4.5 [47] while those of other MnPs, *P. sordid* [20], *D. squaleus* [48], and *L. edodes* [49] are also in the acidic range. All mutant variants had substantially decreased activity at low pH, consistent with the loss reported to be 4.5 of one or more Mn ligands at the interaction site, but all retained significant residual activity at pH values > 5.5 (up to 35% of wild-type). The variants broadly fell into three groups (**Figure 1** and **Table 1**): those that were maximally active at pH 6.0 (E40H, E44H, E40H/E44H), D186H (pH values ≥ 6.5) and D186N (pH values > 7.5). It is important to note that several variants actually have a higher activity than wild-type at pH ~10.0 and approximately 40% of the wild-type activity in the mid alkaline range.

A plot of log k (observed) against pH (**Figure 1**) was used to determine the apparent pKa for Mn (II) dependent oxidation. **Figure 1** shows that the wild-type enzyme behaviour is complex and may involve as many as four critical

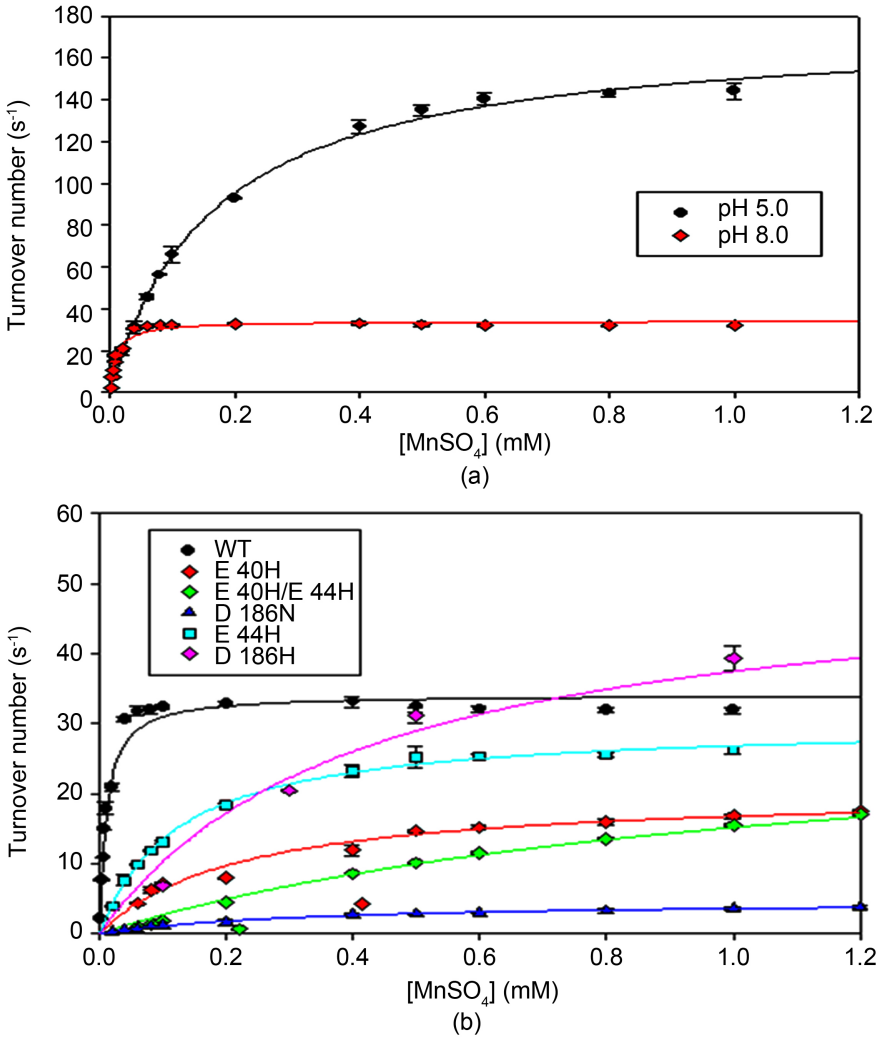

Figure 4. (a). Plots depicting pH dependence of Mn (II) oxidation by rPr-MnP3 at pH 5.0 (—) and 8.0 (—). A data point represents the mean of three independent determinations with standard errors indicated. Assays were carried out using standard MnP3 protocol as described in Section 2.2. Data were fitted to the Michaelis-Menten equation using SigmaPlot 8.0. (b) Mn (II) oxidation by wild-type (—) and mutant rPr-MnP3 enzymes at pH 8.0 (—) E4OH, (—) E44H, (—) E40H/E44H, (—) D186H, (—) D186N. A data point represents the mean of three independent determinations with standard errors indicated. Assays were carried out using standard MnP3 protocol as described in Section 2.2. Data were fitted to the Michaelis-Menten equation using SigmaPlot 8.0. The kinetic parameters are presented in **Table 2**.

ionizations, labelled 1,2,3,4 in **Figure 2(a)**. These presumably reflect deprotonation of one or more carboxylate ligands at the Mn site, starting at pKa ~4.5, and followed at higher pH by pKa's at ~5.5, ~6.5 and ~9.0 respectively. The origins of these are unclear at present, although the complex dissociation and ionization equilibrium of the Mn tartrate complexes relevant as substrates are being considered. An acidic pKa at ~5.5 is conserved in the E40H, E44H and D186N mutants, but is shifted to 6.5 in the D186H mutant, implying that for this variant deprotonation of the new H186 residue is essential to allow Mn oxidation at

Table 2. A table showing kinetic parameters during the pH dependence of Mn (II) oxidation by wild-type and mutants recombinant *Phlebia radiata* manganese peroxidase 3 at pH 5.0 and 8.0.

Enzymes	pH	Km (mM)	Kcat (s^{-1})	Kcat/Km (s^{-1}·mM^{-1})	pH	Km (mM)	Kcat (s^{-1})	Kcat/Km (s^{-1}·mM^{-1})
MnP III (WT)	5.0	0.17 ± 10	175 ± 3.0	1029.41	8.0	0.011 ± 0.0001	34.2 ± 0.8	3109.1
E40H	5.0	11.1 ± 0.4	12.0 ± 0.2	1.01	8.0	0.222 ± 0.03	20.5 ± 0.7	92.3
E44H	5.0	8.5 ± 0.5	12.1 ± 0.3	1.49	8.0	0.124 ± 0.01	30.2 ± 0.4	243.6
E40H/E44H	5.0	19.9 ± 2.5	1. 7 ± 0.1	0.08	8.0	1.086 ± 0.10	31.7 ± 1.5	29.2
D189H	5.0	23.1 ± 3.9	0.84 ± 0.1	0.04	8.0	0.42 ± 0.05	53.0 ± 1.2	128.0
D189N	5.0	30.3 ± 3.7	8.0 ± 0.5	0.30	8.0	0.33 ± 0.02	5.0 ± 0.1	15.2

A data point represents the mean of three independent determinations with standard errors indicated. Assays were carried out using standard rPr-MnP3 protocol as described in Section 2.2. Data were fitted to the Michaelis-Menten equation using SigmaPlot8.0.

higher pH. Similarly the apparent pKa for the E40H/E44H double variant is shifted to ~7.0 perhaps because both His residues must be deprotonated to allow activity. Mn oxidation in mid-alkaline range with up to two of the classical carboxylate ligands replaced is still quite significant (~40% of wild-type). Moreover, the results for D186N and D186H variants imply that histidine is better tolerated in this respect than asparagine. The loss of carboxylate ligands clearly impacts the low pH end of the activity profile more than the alkaline end. It would appear that at higher pH Mn oxidation is less dependent on the precise ionization of the Mn binding site, particularly if histidine is the substituting residue.

This discovery that *P. radiata* wild-type recombinant Pr-MnP3 enzyme is able to oxidize Mn (II) under both acidic (faster) and alkaline (slower) conditions, and the relatively good performance of the E40H/E44H and D186H variants at alkaline pH values, is particularly interesting in the light of potential industrial applications. Pulping and pulp bleaching are routinely performed under alkaline conditions and thus any enzyme used in the pulp and paper industry is required to react under alkaline conditions. Most MnP's from wood-colonizing white-rot fungi have been found to have highly acidic pH optima [50], so in previous studies on lignin degrading enzymes [47] [51] [52], the enzymes tried were active only under acidic pH conditions. Wild-type recombinant *P. radiata* MnP3 enzyme being moderately active at alkaline pH range therefore makes it unique in comparison with other MnP's of white-rot fungi studied to date. The ability of recombinant MnP3 enzymes from *P. radiata* to be active under alkaline conditions might make them useful in the pulp and paper industry for treatment of alkaline black liquor and bleaching effluent and/or to bleach the pulp previously cooked under alkaline conditions.

4.2. Determination of pH Optimum ABTS Oxidation by Wild-Type and Mutant Recombinant *Phlebia radiata* MnP3 Enzymes

Figure 4 illustrates the pH dependence of ABTS turnover for the wild-type and

mutants (E40H, E44H, E40H/E44H, D186H and D186N) rPr-MnP3 enzymes. As observed, all the six enzymes showed a strong acidic pH preference. The activity for ABTS oxidation by wild-type rPr-MnP3 was highest at pH 3.0 and decreased to zero at pH 5.5. All the rPr-MnP3 mutant enzymes exhibited a similar profile with pH optimum at pH 3.0. A non-specific interaction site for ABTS in the general region of the Mn (II) binding site cannot be excluded since there are significant effects for all variants. It implies that protonation events are required for ABTS oxidation. The general effect is probably related to charge neutralization of sulphonate groups on ABTS.

4.3. pH Dependence of Mn (II) Oxidation Michaelis-Menten Kinetic Parameters

Given earlier results (**Figure 1**) showing significant Mn (II) oxidation activity at both high and low pH for wild-type rPr-MnP3, Mn (II) oxidation activity was measured at pH 5.0 and pH 8.0 in the presence of 100 mM tartrate. The pH variations of Mn^{2+}-MnP3 reactions may provide uniquely useful information to assess relative importance of ionisable functional groups on the enzyme as well as the substrates.

The pH dependence of Mn (II) oxidation activity was measured to determine whether mutation of the Mn-binding site has affected the pH dependence kinetic parameters of the wild-type rPr-MnP3 and the mutant variants. The results as presented in **Figure 4(a)** for wild-type Mn (II) oxidation at pH 5.0 and 8.0, and in **Figure 4(b)** for Mn (II) oxidation by mutant enzymes at pH 8.0 illustrate the pH dependence of Mn (II) steady-state kinetic parameters, particularly, Km, Kcat and catalytic efficiency (Kcat/Km) for wild-type recombinant *Phlebia radiata* MnP3 and variants in which the anionic charge at position 40, 44 and 186 has been removed (E40H, E44H, E40H/E44H, D186H, and D186N). The complete results for these reactions are presented in **Table 2**. The catalytic activity of most peroxidase enzymes is strongly dependent on pH. Manganese peroxidase is known to have a low pH optimum, achieving the highest at pH 4.5 (4). However, in this study the pH optimum for Mn (II) by wild-type *Phlebia radiata* MnP3 is at pH 5.0 and the mutants showed greater effectiveness at different pH values ranging from about neutral to alkaline.

In wild-type and mutants rPr-MnP3 enzymes, the apparent K_m value for Mn (II) oxidation decreased as the pH was increased. Activity assay at pH 5.0 yielded Km of 0.17 ± 10 mM for the wild-type rPr-MnP3. This Km values was observed to dramatically drop by approximately 94% (0.011 ± 0.0001 mM) when the reaction was conducted at pH 8.0. Likewise, the MnP3 mutant variants show a similar trend in Km at pH 5.0 and 8.0 as the Km for E40H, E44H, E40H/E44H, D179H, and D179N mutant variants at pH 8.0 decreased by 98%, 98.5%, 94.5%, 98.2% and 98.9% respectively relative to the value at pH 5.0. In this case, alkaline pH (8.0) extremely enhanced recombinant *Phlebia radiata* MnP3 enzymes affinity for Mn^{2+}. The wild-type distinctly shows acidic pH dependence with the Kcat

value of 175 ± 3.0 s^{-1} at the enzyme's pH optimum (5.0) but at pH 8.0, the Kcat drops to 34.2 ± 0.8 s^{-1} (81% decrease). Despite the huge drop in Kcat, the catalytic efficiency at pH 8.0 was approximately 3-times higher relative to the value at pH 5.0 It therefore implies that at pH 8.0 all r*P. radiata* MnP3 enzymes have enhanced affinity for Mn (II), showing that deprotonation of the Mn site results in progressively higher affinity.

The optimum pH of almost all ligninolytic enzymes, including MnP, reported to date lies in the acidic range. However, some industry activities, such as pulping and bleaching are mainly performed under highly alkaline conditions and the waste generated is also alkaline, this is an exciting finding. Ligninolytic enzymes having activity only in the acidic conditions cannot be used under alkaline conditions [53]. The ability of rPr-MnP3 to have a high catalytic efficiency over a wide pH range makes this enzyme unique. There is dearth of comparative data for *P. chrysosporium* MnP and other lignolytic peroxidases at high pH. Therefore, whether this property has simply been overlooked or not is unclear. In contrast to the wild-type enzyme, the k_{cat} values at pH 8.0 for four of the rPr-MnP3 mutant enzymes, E40H, E44H, E40H/E44H and D186H, were significantly higher by approximately 42%, 60%, 95% and 98% respectively than the corresponding values at pH 5.0 (**Table 2**). This, along with the already mentioned dramatic decreases in K_m leads to an increase effectiveness ratio for all variants. However, none of the mutant enzymes reach the efficiency of the wild-type enzyme, even at pH 8.0. This implies that the mutation of the Mn^{2+}-binding site of MnP3 has generated enzyme with reasonable effectiveness at alkaline pH region. With the significant reduction in the Km for rPr-MnP3 enzymes at pH 8.0 and the enzyme's corresponding increase in affinity for Mn^{2+}, it is strongly believed that binding of Mn (II) to MnP3 will be highly favoured under alkaline conditions. Interestingly, like in MnP, the catalytic activity of wild-type *Phlebia radiata* MnP3 increases with decreasing pH. This trend may reflect the increasing redox potential of reaction intermediates with decreasing pH observed for numerous peroxidases [54] [55] [56].

Mutation of the Mn^{2+} binding ligands had profound effects on the catalytic properties of the enzyme. The removal of a carboxylate ligand at the Mn (II)-binding site was found to severely depress the catalytic efficiency of E40H (1.0 $mM^{-1}{\cdot}s^{-1}$), E44H (1.5 $mM^{-1}{\cdot}s^{-1}$), E40H/E44H (0.08 $mM^{-1}{\cdot}s^{-11}$), D186 (0.04 $mM^{-1}{\cdot}s^{-1}$) and D186N (0.30 $mM^{-1}{\cdot}s^{-1}$) for Mn (II) compared to wild-type (1029 $mM^{-1}{\cdot}s^{-1}$) at lower pH. Mutation specific preferences were also created with the rPr-MnP3 DI86N variant being the kinetically poorer than D186H at higher pH. Clearly His is a more acceptable alternative for catalysis than Asn. These changes in k_{cat} and the specificity constants for Mn (II) oxidation by rPr-MnP3 mutants were to some extent expected from the nature of the mutants involved and prior work on the *Phanerochaete chrysosporium* MnP enzymes [57].

Excitingly, it was observed that histidine substitutions at the Mn (II) binding site lead to a mutant with a similar effectiveness ratio to the wild-type enzyme at

higher pH, when the His ligands were in a deprotonated state. Activity towards Mn (II) at lower pH was greatly decreased, presumably due to the presence of the positively charged histidine. In contrast to expectation, the enzyme can therefore function at higher pH with alternative ligands in the Mn^{2+} site and does not have an absolutely obligate requirement for an all carboxylate ligand set. The results obtained for wild-type rPr-MnP3 for Mn (II) oxidation at pH 5.0 and 8.0 also showed enhanced affinity for Mn (II) at alkaline pH. Progressive deprotonation of Mn ligands results in a progressively higher affinity for Mn, which is evident in a relatively good catalytic efficiency at higher pH, even for the mutants, E40H (92 $mM^{-1}{\cdot}s^{-1}$), E44H (244 $mM^{-1}{\cdot}s^{-1}$), E40H/E44H (29 $mM^{-1}{\cdot}s^{-1}$), D186H (128 $mM^{-1}{\cdot}s^{-1}$) and D186N (15 $mM^{-1}{\cdot}s^{-1}$) But none of the catalytic efficiencies of the mutants was in any way near the of the wild-type (3109 $mM^{-1}{\cdot}s^{-1}$) at alkaline pH. The rPr-MnP3 may be used as an alternative catalytic agent in some industrial applications since it was heat stable up to ~60°C [58] and in this study its activity was retained over a wide range of pH values (5.0 - 9.0).

5. Conclusion

The wild-type recombinant *Phlebia radiata* MnP3 enzyme showed optimal activity with Mn (II) at pH 5.0 and remained moderately active (approximately 40%) in the pH range 6.0 - 8.5; the rPr-MnP3 mutants maximum activity ranged between the pH of 5.5 and 8.0. The activity of manganese peroxidase at higher pH is not common and a unique characteristic that has not been reported for any MnP enzyme before and therefore would have considerable interest for commercial uses. Wild-type rPr-MnP3 seemed to have a substantially higher pH optimum than the more classical *Phanerochaete* MnP enzyme. The rPr-MnP3 mutants exhibited a similar pH profile to wild-type, with an optimum at pH 3.0, for ABTS oxidation. This strongly suggests that ABTS is oxidised at the Mn (II) binding site, as charge neutralisation mutations decreased K_m for ABTS and improved effectiveness. Our findings offer a new insight into MnPs with novel properties that will find applications in the paper and pulp industries. The results offered new insight into MnPs with novel properties for industrial applications.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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