



The effect of pH, temperature, additives (CaCl₂ and EDTA) on the stability of pure recombinant *Phlebia radiata* wild-type and mutant (E40H/E44H) rPr-MnP3 enzymes

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Abstract

Thermal inactivation of a new recombinant *Phlebia radiata* manganese peroxidase (rPr-MnP3) in the presence and absence of additives (CaCl₂ and EDTA) is described for the first time. The influence of temperature and melting points (T_m) on the stability of rPr-MnP3 and its mutant (E40H/E44H) were determined. There was no significant inactivation at 25 – 40°C. However, we observed rapid inactivation of rPr-MnP3 at 50°C and above. Addition of CaCl₂ to the enzyme mixture resulted in a marked increase in the half-life (533 min) of the wild-type enzyme compared to E40H/E44H with the half-life of 92 min. Ethylenediaminetetraacetic acid (EDTA) increased the rate of rPr-MnP3 thermal inactivation as shown by the decay constant (k_d) of $0.070 \pm 0.007 \text{ min}^{-1}$ and half-life of 10 min. The decay constant (k_d) $0.029 \pm 0.002 \text{ min}^{-1}$ and half-life of 24 min were obtained for the control (untreated sample). Calcium ion had protective effect on the inactivation of the wild-type enzyme but not for mutant. The mutant (E40H/E44H) was observed to be more stable with a higher melting point of 58°C than the wild-type ($T_m = 54^\circ\text{C}$). The inactivation effect of EDTA on the E40H/E44H was lower than that of the wild-type. Calcium ions were found to be important structural elements responsible for the enzyme stability. Our findings showed that rPr-MnP3 is a highly stable enzyme and may be of significant industrial applications.

Keywords: Peroxidase, *Phlebia radiata*, thermal stability, thermal inactivation, reactivation, melting point, additives.

INTRODUCTION

The white-rot fungus, *Phlebia radiata* is a selective and effective degrader of lignin and a wide variety of recalcitrant

environmental pollutants (Vares and Hatakka, 1997) . *P. radiata* has been shown under certain conditions to be

a more effective lignin degrader than *Phanerochaete chrysosporium* (Hatakka and Uusi-Rauva, 1983). The biodegradative properties of this fungus are dependent on non-specific extracellular enzymes' system such as lignin peroxidase (LiP) and manganese peroxidase (MnP) (Kuwahara et al., 1984; Hatakka, 1994) found in *P. chrysosporium* and are also reported in other white-rot fungi (Hatakka, 1994). MnP functions in a rather indirect way by oxidizing Mn (II) to Mn (III) (Glenn et al., 1986). The kinetic mechanism of the oxidation of Mn^{2+} by the MnP enzymes has been well established (Kuan et al., 1983). Enzymatically produced Mn^{3+} is believed to form a diffusible oxidant complex with dicarboxylic acid chelators, tartrate, oxalate, malonate and lactate (Paszczynski et al., 1986).

Peroxidases are very stable enzymes and the mechanism of their stability is largely controlled by their structure, but the pH and thermostability of these enzymes are also associated with hydrophobic linkage (Daniel et al., 1996; Zelent et al., 2010). Two mol Ca^{2+} /mol enzyme have been found to be present in both LiP and MnP. Calcium is believed to have structural role in maintaining the haem configuration and activity of the peroxidases (Haschke and Freidhoff, 1978). Thermal inactivation of MnP is thought to be due to the loss of the more weakly bound distal calcium (Nie and Aust, 1997), which further result in the loss of activity and marked spectroscopic changes (Haschke and Freidhoff, 1978; Shiro et al., 1986; Boscolo et al., 2007). A similar situation has been reported for LiP (Nie and Aust, 1997). Excess calcium has also been found to decrease the rate of thermal inactivation and stabilise peroxidase enzyme at higher temperature (Timofeevski and Aust, 1997). The optimum temperature for peroxidase activity has been found to depend on the source of the enzyme (Lamikanra and Watson, 2000; Al-Senaïdy and Ismael, 2011).

The stability of enzymes in a non-natural environment is a critical issue in biotechnology since their operational stability is of prime importance in bioprocessing. Poor environmental stability remains a major limitation to large-scale use of peroxidase catalysis (Zamorano et al., 2009). Enzyme stability at high pH and temperature is a key feature in evaluating the applicability of lignin degrading peroxidases in biorefinery-type applications. The identification of highly stable and active peroxidases remains a key step in the development of a catalyst with broad commercial utilizations. A good stability of any peroxidase at elevated temperatures would make it industrially more useful. Therefore, the thermal characterisation of recombinant *P. radiata* MnP3 under application-type conditions is crucial to understanding the stability properties of rPr-MnP3. Three distinct MnP iso-enzymes, Pr-MnP1, Pr-MnP2, and Pr-MnP3 have been isolated from *P. radiata* and biochemically characterised (Hilden et al., 2005). Recently, the Pr-MnP3 gene of *P. radiata* has been engineered, expressed, and the preliminary characterisation

of protein reported (Ufot, 2010; Ufot and Akpanabiatu, 2012). The potential application of rPr-MnP3 for a wide range of degradation conditions requires comprehensive knowledge of the regulation mechanisms. The present study was designed to gain a better understanding of the effect of pH, temperature, additives ($CaCl_2$ and EDTA) on the stability of pure recombinant *P. radiata* wild-type and mutant (E40H/E44H) rPr-MnP3 enzymes. Melting temperatures of the recombinant wild-type and E40H/44H Pr-MnP3 enzymes were also determined using circular dichroism (CD).

MATERIALS AND METHODS

Materials, reagents and equipment

All chemicals were obtained from Fisher or Sigma-Aldrich chemicals, Co, UK. Spectroscopic measurements were carried out using a Shimadzu-UV-2401 spectrophotometer. Restriction enzymes were purchased from NEBiolabs, UK. Circular Dichroism (CD) spectra were recorded with a JASCO J - 715 CD spectropolarimeter (Jasco Ltd, Japan). The complete MnP3 gene of *Phlebia radiata* strain 79 (ATCC 64658) was generously provided by Dr. Taina Lundell, Department of Food and Environmental Sciences, Division of Microbiology, University of Helsinki, Finland. The GeneBank accession number for the cDNA encoding peroxidase Pr-MnP3 is AJ566200. The Pr-MnP3 cDNA was present in vector pCR2.1.TOPO. The *E. coli* expression vector pFLAG1 was obtained from International Biotechnologies Inc, UK.

Enzyme production, activation and purification

The wild-type rPr-MnP3 and its site-directed mutagenesis variant (E40H/E44H) were produced in *E. coli* (W3110 strain) after transformation with a corresponding plasmid (Tam and Welinder, 1996). Cells were grown in Terrific Broth until the absorbance at 500 nm was 1.0 before induction with 1 mM Isopropyl- β -D-thiogalactopyranoside (IPTG) and further grown for 3.5 h. The apoenzyme accumulated in inclusion bodies, as shown by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was recovered using 6 M urea. *In vitro* folding was performed using 0.15 M urea, 5 mM $CaCl_2$, 0.5 mM oxidized-glutathione, 0.1 mM dithiothreitol ratio, 20 μ M hemin, 50 mM Tris-HCl, pH 9.5 and 200 μ g/ml rPr-MnP3. Active enzyme was purified using anion-exchange column (Mono-Q FPLC System; Pharmacia LKB Biotechnology Ltd), Sweden (Ogawa et al., 1979; Ufot and Akpanabiatu, 2012).

Thermal stability of recombinant wild-type and mutant (E40H/E44H) MnP3 from *P. radiata*

To determine the effect of temperature on the stability of *P. radiata* MnP3, the temperature-induced inactivation of the enzyme was monitored as follows: 2.5 μ M of enzyme solutions were incubated in 10 mM sodium succinate at pH 6.0 in the presence of EDTA (0.5 mM), in a water bath at 25, 40 and 50°C respectively. The kinetics of inactivation was measured directly using the conversion of Mn (II) to Mn (III). At intervals, aliquots of heat-treated enzyme mixture were taken and assayed for activity at 25°C. The assay mixture contained 100 mM Na-tartrate buffers (pH 5.0 or pH 8.0), 1mM $MnSO_4$ and 0.1 mM H_2O_2 .

Measurement of enzymes' activities

Direct oxidation of Mn^{2+} was estimated by the formation of a Mn^{3+} -tartrate complex ($\epsilon_{238} = 6.5 \text{ mM}^{-1} \text{ cm}^{-1}$) using 1 mM $MnSO_4$ for the wild-type and 3.5 mM $MnSO_4$ for E40H/E44H. Hydrogen peroxide (0.1 mM) was included in the assay buffer (100 mM sodium tartrate buffer pH 5.0 and 8.0). All enzymatic activities were measured at 25°C using a Shimadzu UV-2401 spectrophotometer at 238 nm.

Determination of melting temperature (T_m) for recombinant *P. radiata* wild-type and mutant (E40H/E44H) rPr-MnP3

JASCO J-715 spectrophotometer fitted with an Electronic Temperature Control (ETC) unit was used in the circular dichroism method to determine the melting point of wild-type and mutant (E40H/E44H) rPr-MnP3 enzymes. Dry nitrogen was purged continuously into the instrument before and during the experiment with nitrogen purging rate of 25 L min^{-1} . Three sets (untreated, 5 mM $CaCl_2$ -treated and 0.5 mM EDTA-treated) of each rPr-MnP3 enzyme were used for this study. Recombinant *P. radiata* manganese peroxidase (1.6 μM) in 10 mM Sodium acetate buffer (pH 6.0) was placed in a 1 mm quartz cuvette and ellipticity values recorded at 222 nm as the temperature was raised from 5 to 90°C at a constant rate of 1°C/min. Equilibration of the machine was at 16 counts for each temperature. The temperature value at the midpoint of the denaturation curve (T_m) was determined assuming that during transition, two distinct states of protein were present (the native and the unfolded). The spectra were corrected for background and successfully smoothed with eight points Savitsky Golay smoothing procedure. The ellipticity values were obtained in millidegrees directly from the instrument and converted to ASCII files using the instrument software. The values obtained were plotted against temperature using SigmaPlot 8.0 software.

Reactivation of thermally inactivated EDTA-treated wild-type rPr-MnP3

The EDTA-treated wild-type rPr-MnP3 enzyme mixture (2.5 μM) in 10 mM Sodium succinate buffer, pH 6.0 that had been incubated at 50 °C for 5 h lost approximately 98% of the initial activity. The activity of the inactivated enzyme was measured in the Mn (II) assay and recorded as zero time. 5 mM $CaCl_2$ was added to this enzyme mixture, and the activity assayed conducted after correcting for dilution. The remaining sample was incubated at 4°C and assayed for activity at 30 min intervals for 6 h.

Reactivation of EDTA-treated wild-type enzyme was obtained by adding 5 mM $CaCl_2$ to the thermally inactivated enzyme incubated at 4°C and activity assayed at 25°C, pH 5.0 every 30 min for 6 h. The enzyme half-life was calculated using the equation:

$$t_{1/2} = 0.693/k$$

Where, k is the first-order deactivation rate coefficients (Mukhopadhyay, 1992).

RESULTS

The successful cloning, generation of mutant and expression of rPr-MnP3 has called for a detail characterization of these enzymes. The enzymatic activity of un-treated, $CaCl_2$ and EDTA-treated enzymes was monitored over time

at 25, 40 and 50°C to study the thermal stability of rPr-MnP3 enzymes. Investigation of enzymatic stability at 25°C showed that Pr-MnP3 was highly stable and active even after 21 h of incubation. In addition, incubation at 40°C indicated some inactivation of the enzyme with a half-life of 20 h. This implies that rPr-MnP3 was also stable at 40°C. Based on the above observations, the thermal inactivation experiments were conducted at 50°C.

Thermal inactivation of *P. radiata* MnP3 enzymes in the presence and absence of Ca^{2+} or EDTA at 50 °C at pH 5.0

The results of wild-type and mutant (E40H/E44H) rPr-MnP3 inactivation at 50°C are summarised in Figure 1a and b respectively and Table 1. The calculated half-life of rPr-MnP3 without any additive at 50°C was 24 min. The addition of 0.5 mM EDTA to the enzyme mixture resulted in the reduction of half-life to 10 min. However, at the 75 min incubation of the enzyme mixture, it was found that only 2 % activity of rPr-MnP3 was left. However, addition of $CaCl_2$ to the inactivated enzyme resulted in a marked increase in the half-life (533 min) for the wild-type rPr-MnP3 compared to the mutant which recorded the half-life of 92 min. On the other hand, the inactivation effect of EDTA on the E40H/E44H was lower than that of the wild-type. The decay constant (k_D) results for the two enzyme types are in agreement with the half-life results in this investigation (Table 1). The result shows that the presence of $CaCl_2$ in the wild-type rPr-MnP3 enzyme mixture impacts on it a very high stability with increased half-life of 533 minutes (22-fold increase). The effect of EDTA commonly used in the removal of bound Ca^{2+} ions from proteins was also investigated. EDTA resulted in almost complete loss of the activity of the enzyme.

Effect of pH of assay on thermally inactivated wild-type rPr-MnP3 enzymes

The effect of pH on thermally inactivated wild-type rPr-MnP3 as shown by the rate of decay of the enzyme is presented in Table 2. At 40°C and pH 5.0 the untreated wild-type rPr-MnP3 showed high activity, which correlate with high stability (half-life: 1155 min), while at pH 8.0 the thermal stability decreases appreciably with half-life of 433 min. This difference in the enzyme half-life may, at least in part, be explained by enzyme inactivation at pH 8.0. Peroxidases are most active at acidic pH and rPr-MnP3 act optimally at pH 5.0 during Mn (II) oxidation. Figure 2 shows that inactivated rPr-MnP3 is far unstable at pH 8.0 and at 40°C than at the same temperature at pH 5.0, which is the established optimum pH.

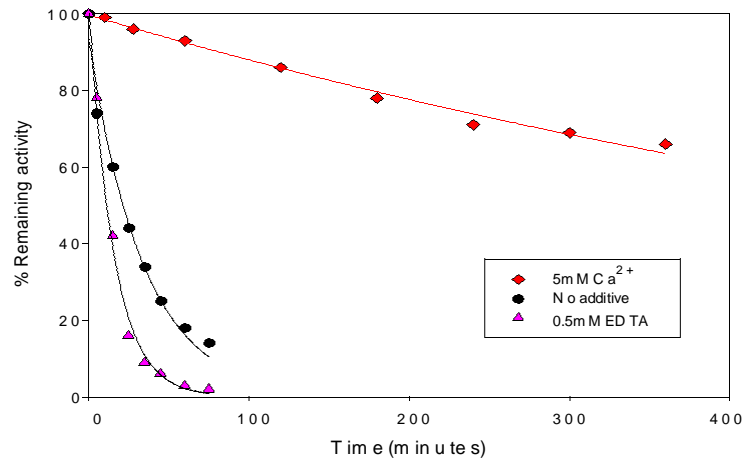


Figure 1a. Thermal stability of recombinant *P. radiata* MnP3 at 50°C. Plots represent no additive, 5 mM CaCl₂ added and 0.5 mM EDTA added, to MnP3 during incubation. Enzyme concentration was 2.5 μM in 10 mM sodium succinate buffer, pH 6.0. At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 μM enzyme, 0.2 mM MnSO₄, 0.1 mM H₂O₂ and 100 mM sodium tartrate, pH 5.0.

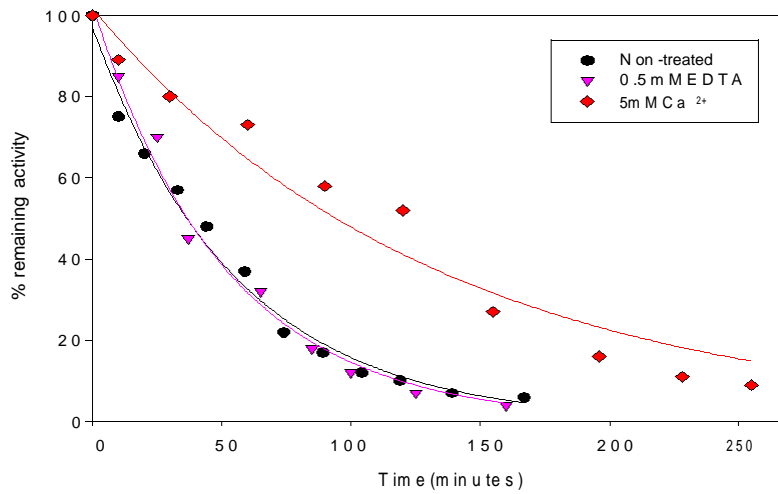


Figure 1b. Thermal stability of recombinant *P. radiata* mutant (E40H/E44H) MnP3 at 50°C. Plots represent no additive, 5 mM CaCl₂ added and 0.5 mM EDTA added, to MnP3 during incubation. Enzyme concentration was 10 μM in 10 mM sodium succinate buffer, pH 6.0. At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 μM enzyme, 40 mM MnSO₄, 0.1 mM H₂O₂ and 100 mM sodium tartrate, pH 8.0.

Table 1. Decay constants (k_d) and half-life for wild-type rPr-MnP3 in the presence or absence of additive (5 mM CaCl₂ and 0.5 mM EDTA) at 50°C.

| Treatment | Wildtype rPr-MnP3 | | | Mutant rPr-MnP3 (E40H/E44H) | | | |
|----------------------------|--|-------------------------------|-----|-----------------------------|--|-------------------------------|-----|
| | Decay constants (k_d) (min ⁻¹) | Half-life ($t_{1/2}$) (min) | pH | Treatment | Decay constants (k_d) (min ⁻¹) | Half-life ($t_{1/2}$) (min) | pH |
| Untreated | 0.029 ± 0.002 | 24 | 5.0 | Untreated | 0.018 ± 0.001 | 39 | 8.0 |
| CaCl ₂ -treated | 0.0013 ± .0001 | 533 | 5.0 | CaCl ₂ -treated | 0.0075 ± 0.001 | 92 | 8.0 |
| EDTA-treated | 0.070 ± 0.007 | 10 | 5.0 | EDTA-treated | 0.020 ± 0.0012 | 35 | 8.0 |

Table 2. Effect of pH of assay on the thermal denaturation of rPr-MnP3.

| rPr-MnP3 | Decay constants(k_d) (min^{-1}) | Half-life($t_{1/2}$) (min) | pH |
|--------------|--|------------------------------|-----|
| Untreated | 0.0006 ± 0.0000 | 1155 | 5.0 |
| | 0.0016 ± 0.0002 | 433 | 8.0 |
| EDTA-treated | 0.0024 ± 0.0001 | 289 | 5.0 |
| | 0.0039 ± 0.0002 | 178 | 8.0 |

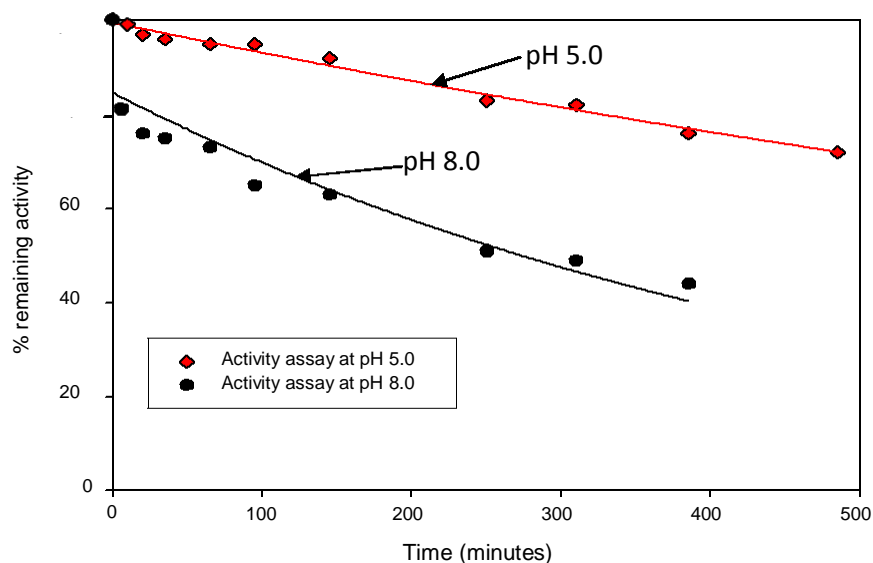


Figure 2. Thermal stability of wild-type recombinant *P. radiata* MnP3 at 40°C, pH 5.0 and 8.0. Enzyme concentration was 2.5 μM in 10 mM sodium succinate (pH 6.0). At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 μM enzyme, 0.2 mM MnSO_4 , 0.1 mM H_2O_2 , 100 mM sodium tartrate buffer (pH 5.0 or pH 8.0).

The effect of Ca^{2+} on the reactivation of *P. radiata* MnP3

The effect of calcium on the reactivation of *P. radiata* MnP3 was determined after EDTA-treatment and thermal inactivation at 50°C for 55 min. The EDTA-treated wild-type rPr-MnP3 in 10 mM Na succinate, pH 6.0 incubated at 50°C was observed to have lost approximately 98% of the initial activity, implying that only 2% of its initial activity was left (Figure 1a and b). A regain of peroxidase activity occurred in the sample, when later treated with 5mM CaCl_2 at 4°C. A total of 28% of the original activity was restored after 185 min, with a reactivation constant of $0.026 \pm 0.01 \text{ min}^{-1}$, and half-life of 27 min (Figure 3.).

At 40°C and pH 5.0 the untreated wild-type rPr-MnP3 showed higher activity, which correlate with high stability and half-life of 1155 min, while at pH 8.0 the thermal stability of the enzyme decreases appreciably (half-life of about 433 min) (Table 2). The findings in this study also

indicate that rPr-MnP3 is more stable at 40°C than 50°C when exposed to the same pH. A remarkable loss of activity was observed at 75 min ($t_{1/2} = 24$ min) but in the presence of EDTA, the inactivation was faster, occurring in 55 min with $t_{1/2} = 10$ min (Table 1). These results suggest that the rPr-MnP3 experimental conditions: pH 5.0; 25°C for the determination of the initial enzymatic activity are not the most useful with regard to thermal stability. Thus, the nature of the additive present in enzyme solution and the time of exposure of rPr-MnP3 at reactor temperatures are critically important to maximizing its thermal stability.

Determination of melting temperature of recombinant wild type and mutant (E40H/E44H) Pr-MnP3 enzymes using circular dichroism (CD)

Measurement of melting temperature (T_m) as an index of

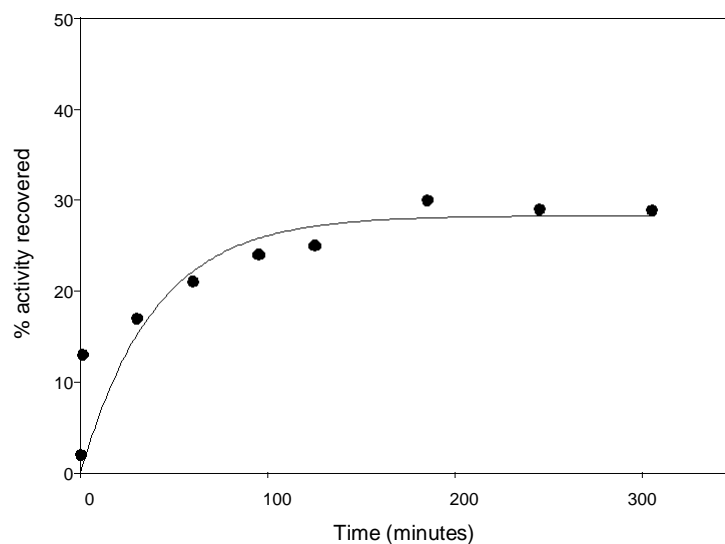


Figure 3. Calcium ion reactivation of thermally inactivated (EDTA treated) wild type MnP3. Reactivation of thermally inactivated enzyme (55 min at 50°C with 0.5 mM EDTA) was obtained by adding 5 mM CaCl₂ to 2.5 μM enzyme solution at 4°C for up to 5 h. At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 μM enzyme, 0.2 mM MnSO₄, 0.1 mM H₂O₂ and 100 mM sodium tartrate, pH 5.0.

thermodynamic stability of rPr-MnP3 was conducted for the wild-type and E40H/E44H rPr-MnP3 enzymes using Circular dichroism (CD) technique. Also assessed were the effects of CaCl₂ and EDTA on the melting point of these enzymes. Figures 4a and b show thermal melts for wild-type and mutant (E40H/E44H) of rPr-MnP3 enzyme, both in the presence and absence of 5 mM CaCl₂ and 0.5 mM EDTA. Figure 4c compared the results of thermal denaturation of the wild-type enzyme without additive and mutant proteins. No aggregate or precipitate was seen at the end of the process, suggesting enzyme reversibility. The reversibility of the unfolding process makes the melting temperature directly related to conformational stability. Figure 4c and Table 3 show that the E40H/E44H mutant enzyme is slightly more thermostable than the wild-type enzyme, with a higher T_m (58°C) compared to 54°C for the wild-type. It was also observed that for both the wild-type and mutant enzymes, EDTA has no significant effect on the T_m (Figure 4a).

DISCUSSION

The results of this investigation are detailed in the model shown in Scheme 1. The influence of additives (CaCl₂ and EDTA) in reversible thermal inactivation of recombinant wild-type and mutant (E40H/E44H) *P.radiata* manganese peroxidase 3 (rPr-MnP3) are discussed. Figures 1a, b and Table 1 show that at high temperature (50°C), EDTA, a divalent cation chelating

agent increased the rate of rPr-MnP3 inactivation. We observed that treatment of the enzyme with EDTA resulted in almost complete loss of the enzyme activity. The plausible mechanism of this inactivation is that of calcium removal from the enzyme by EDTA (Nie and Aust, 1997). However, addition of CaCl₂ to the inactivated enzyme resulted in a marked increase in the half-life, for the wild-type rPr-MnP3 compared to the mutant which recorded a lower half-life value. Our observations imply that calcium addition is necessary to ensure the correct haem conformation. The role of Calcium ions as an important factor in the stabilization of this enzyme has been reported (Tam and Welinder, 1996; Nie and Aust, 1997; Davies et al., 2008). The high concentration of calcium relative to enzyme is therefore to keep the distal calcium site fully occupied. Also the inactivation effect of EDTA on the E40H/E44H was lower than that of the wild-type in this experiment, indicating that the E40H/E44H mutant of rPr-MnP3 was more stable than that of the wild-type.

Many plant peroxidases are highly susceptible to acidic pH (Sakharov et al., 2002) and in this study we determined pH involvement in the activity of thermally inactivated rPr-MnP3. As shown in Figure 3, inactivated rPr-MnP3 is far unstable at pH 8.0 than pH 5.0 at 40°C. A much lower activity at pH 8.0 than pH 5.0 with pronounced immediate decrease in activity at start recorded here demonstrated the stability of rPr-MnP3 at pH 4.0 and 40°C. At 40°C and pH 5.0 the untreated wild-type rPr-MnP3 showed high activity, which correlate with

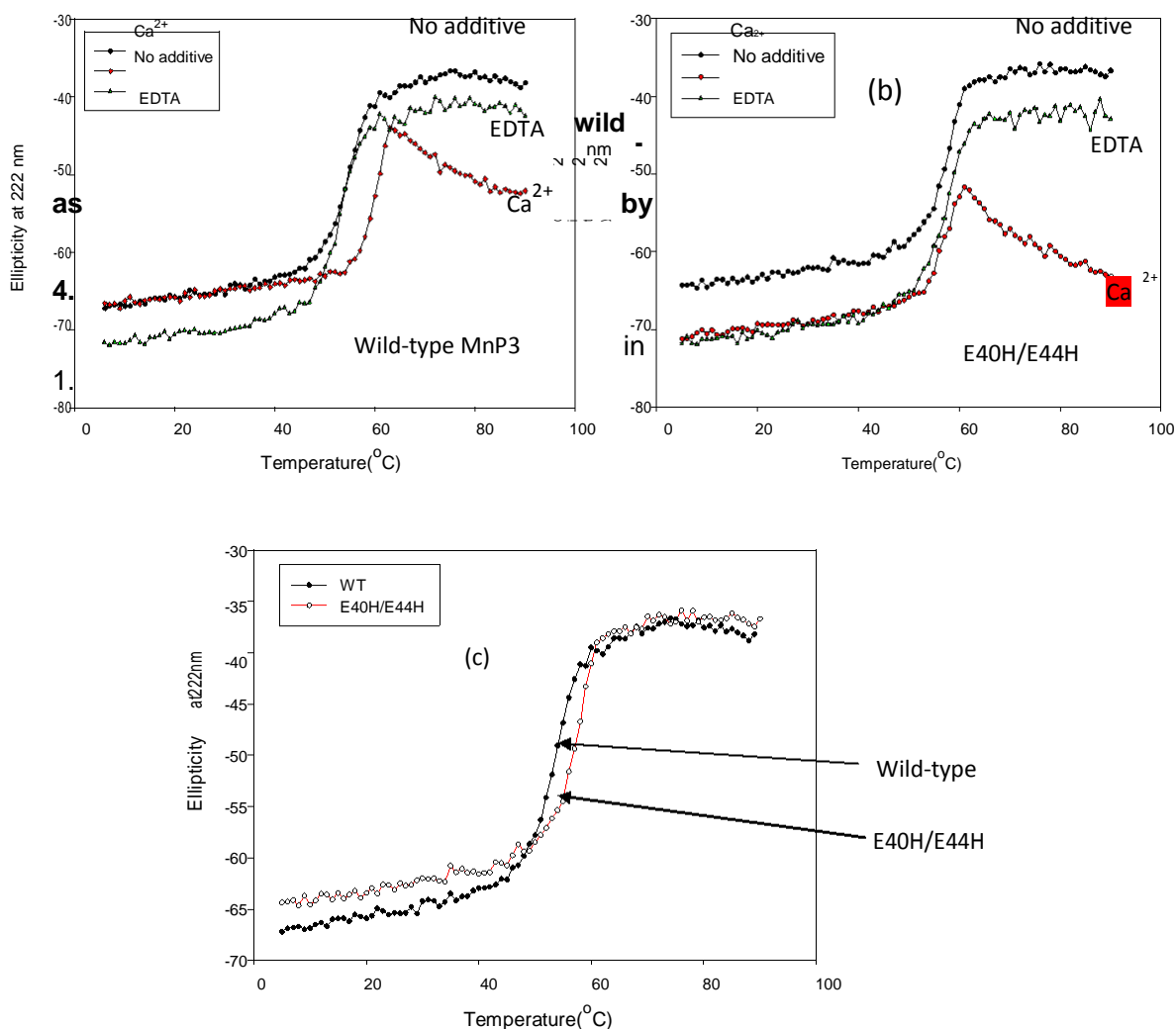


Figure 4. Thermal denaturation plot of wild type and mutant, E40H/E44H, Pr-MnP3 as a function of temperature as measured by Circular dichroism. Plots (a) and (b) represent no additive, 5 mM CaCl₂ added and 0.5 mM EDTA added, to wild-type or E40H/E44H MnP3 during incubation. (c) Overlay of wild-type and E40H/E44H mutant under no additive conditions. Enzyme concentration was 1.6 μM each enzyme in 10 mM sodium acetate, pH 6.0. Readings were taken at 222 nm as the temperature was raised from 5 to 90°C at a constant rate of 1°C / min, with 16 s of equilibration at each temperature.

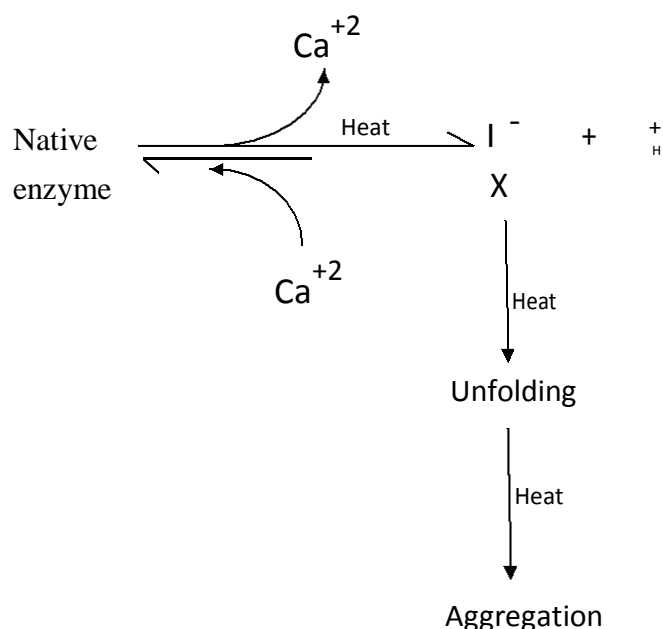
Table 3. Melting temperature of recombinant wild-type and mutant (E40H/E44H) Pr-MnP3 enzymes.

| Sample | Wild-type rPr-MnP3 Melting temperature (T _m) °C | E40H/E44H rPr-MnP3 melting temperature (T _m) °C |
|------------------------|---|---|
| No additive | 54 | 58 |
| 5 mM CaCl ₂ | 60 | 58 |
| 0.5 mM EDTA | 52 | 57 |

high stability, indicated by the half-life of 1155 min, while at pH 8.0 the thermal stability decreases appreciably (half-life: 433 min). This difference in the enzyme half-life may, at least in part, be explained by enzyme inactivation

at pH 8.0. Peroxidases are most active at acidic pH and rPr-MnP3 acts optimally at pH 5.0 during Mn (II) oxidation (Ufot, 2010; Ufot and Akpanabiatu, 2012).

Heat-treated peroxidases from several plant sources



Scheme 1. A scheme describing the temperature-dependent changes in the resting state of *P. radiata* MnP3 enzymes. Reactions shown by solid arrows indicate changes taking place when the enzyme was heated. The I⁻ and X are possible intermediates that may form when Ca²⁺ is removed.

have shown the ability to recover after being stored at ambient temperature (Lu and Whitaker, 1974). However, only partial regeneration of peroxidase activity following heat treatment and cooling has been reported (Tamura and Morita, 1975). The structural calcium ions present in peroxidases are released during unfolding and can be efficiently bound by EDTA, leading to irreversible conditions. In view of this, the decay rate constant (k_D) and the refolding rate constant (k_f) were measured independently. EDTA-treated enzyme was thermally inactivated but regained peroxidase activity when later treated with 5 mM CaCl₂ at 4°C. Our finding is in agreement with previous reports (Rodrigo et al., 1997) and the extent of reactivation depends on enzyme, heating conditions, temperature and time (Thong and Barrett, 2005).

Heat-denaturation of untreated wild-type rPr-MnP3 at 50°C resulted in a first-order kinetics. The plot in Figures 4 depicts two distinct exponential decays that could best be described using a model involving a single enzyme form undergoing parallel denaturation steps to different forms. The rPr-MnP3 incubated at 50°C was observed to be more stable in the presence of excess exogenous calcium. Scheme 1 shows the formation of multiple intermediates upon loss of Ca²⁺ during exposure to high temperature. If no calcium is available to the intermediate, it will eventually undergo denaturation and subsequently, aggregation (irreversible inactivation). However, if calcium is available to the intermediate before irreversible denatu-

ration occurs, the enzyme reverses to its active form (reversible inactivation).

The melting temperature of recombinant wild-type and mutant (E40H/E44H) Pr-MnP3 enzymes using circular dichroism (CD) (Figure 4a, b, c) showed that calcium ions was protective of the unfolding of wild-type rPr-MnP3, however, the E0H/E44H mutant responded differently to Ca²⁺ in this study. Presumably calcium loss from the wild-type enzyme was lowered by the presence of excess CaCl₂ which was added to the experimental mixture, resulting in protein tertiary structure stabilisation. The observed melting temperature of the untreated wild-type MnP3 protein was lower than that reported by other researchers (Tamura and Morita, 1975). However, CaCl₂ has been observed to increase the T_m value of wild-type rPr-MnP3, thereby demonstrating the thermo-stabilizing effect of Ca²⁺ on the enzyme. It was also observed that for both the wild-type and mutant enzymes, EDTA has no significant effect on the T_m (Figure 4a), suggesting that removal of calcium by EDTA does not affect the general stability of the protein tertiary structure that is EDTA is not able to remove calcium from the protein under the experimental conditions.

Conclusion

We observed that the presence of excess calcium or EDTA did not really influence the melting point of E40H/E44H

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