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Table of Contents: Volume 8 Number 6 August, 2014

ARTICLES

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Full Length Research Paper

Plasmodium berghei ANKA: Selection of pyronaridine resistance in mouse model

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Pyronaridine is a partner drug in, Pyramax®, a combination of artesunate (ASN)-pyronaridine (PRD) which was recently prequalified by WHO drug as a potential alternative for treatment of malaria in African setting. Pyronaridine is a mannich base, with a long half-life, thus predisposed to resistance. In this study, we selected pyronaridine resistance by submitting *Plasmodium berghei* ANKA line *in vivo* to increasing pyronaridine concentration for 20 successive passages over a period of six months. The effective doses that reduce parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) determined in the standard four-day suppressive test for the parent line were 1.83 and 4.79 $mgkg^{-1}$, respectively. After 20 drug pressure passages, the ED_{50} and ED_{90} increased by 66 and 40 fold, respectively. After dilution cloning, the parasites were grown in the absence of drug for five passages and cryo-preserving them at -80°C for at least one month, the resistance phenotypes remained stable. Thus, the resistant phenotype line could be used to explore genetic determinants associated with pyronaridine resistance; therefore, this strain represents a vital tool to study the mechanisms of resistance.

Key words: Malaria, pyronaridine, Pyramax®, resistance, *Plasmodium berghei* ANKA.

INTRODUCTION

Malaria is a global public health concern. The emergence of resistance, particularly in *Plasmodium falciparum*, has been a major contributor to the global resurgence of malaria in the last three decades (Marsh, 1998). In reality, *P.*

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Abbreviations: ASN, Artesunate; **GFP,** green fluorescent protein; PRDR; pyronaridine resistant clone.

falciparum has developed resistance to nearly every anti-malarial drug introduced to date, compromising its control (White, 2004). Resistance arises via the selection of parasites bearing specific mutations, and is decisive in determining the effective life-time of anti-malarial agents. In response to resistance, artemisinin-based combination therapies (ACTs), that combines a semi-synthetic derivative of artemisinin, with a partner drug of a distinct chemical class, has been adopted for the treatment of *falciparum* malaria to delay the development of resistance (Nosten and White, 2007; Eastman, et al., 2005; WHO, 2003). However, there is a considerable concern that this will otherwise not happen. Previous studies on antimalarial resistance mechanisms have shown that drug elimination profile is one of the key factors in the emergence and selection of resistant phenotypes (Nzila et al., 2000; Watkins and Mosobo, 1993).

When drugs are used in combination, a discrepancy between their half-lives can have a substantial impact on the evolution of drug resistance. If one drug is rapidly eliminated, the other drug persists alone and new infections are exposed to sub-therapeutic level of drugs, a fact that promotes the development of resistance (Hastings, 2004). For instance, a combination of artesunate (ASN)-pyronaridine (PRD), commercialized as Pyramax®, was recently prequalified by WHO as a potential alternative for the treatment of malaria in Africa (Ramharter et al., 2008; Vivas et al., 2008). All artemisinin derivatives are characterized by a short half-life (WHO, 2006); while, pyronaridine is a long acting drug with half-life of 16 to 17days (Sang and Pradeep, 2010). In this context, like any other antimalarial drug, the PRD is under intense selective pressure and resistance has, in the past, often developed rapidly.

Pyronaridine (Malaridine®) was first synthesized in China and introduced for the treatment of malaria as a single agent for over 30 years in certain malaria infested regions of China (Shao, 1990). Recently, interest has been renewed in pyronaridine as a possible partner for use in artemisinin-based combination therapy (ACT) for malaria treatment. Consequently, if strategy is to be devised to extend the expedient therapeutic lifetime of Pyramax®, there is a necessity to comprehend the molecular mechanisms of PRD resistance. However, to date, there is no well-established and characterized PRD-resistant *P. falciparum* strain, which could be used to study the mechanism of drug resistance.

In this study, we report the selection of pyronaridine resistance *in vivo* using piperaquine-resistant *P. berghei* ANKA clone sensitive to pyronaridine. We then established the stability of the selected pyronaridine-resistant parasite. We selected stable pyronaridine-resistant phenotypes in six months. This study underscores the necessity to understand the genetic and molecular basis of PRD resistance, which would allow surveillance efforts for the emergence of parasites resistant to the partner drugs as a key component of the effective utilization of ACTs.

MATERIALS AND METHODS

Parasites and experimental animals

To select pyronaridine-resistant parasites, we used a transgenic ANKA strain of *P. berghei* expressing green fluorescent protein (GFP), resistant to piperaquine but susceptible to pyronaridine, as described by Kiboi et al. (2009). The cryopreserved parasites were first thawed and maintained by serial passage of blood from mouse to mouse at KEMRI animal house, Nairobi, Kenya. Before establishment of PRD resistance, we first established that the starting parasite (piperaquine resistant clone) was sensitive to pyronaridine antimalarial drug by performing four suppressive day test and followed the development of the parasites for 15 days post infection.

Parasitized red blood cells (PRBCs) were collected from the donor mice with a rising parasitaemia of 5-10% and according to the level of parasitaemia, blood was diluted with phosphate saline glucose (PSG) buffer to reach approximately 2×107 PRBCs per 200 µl of the inoculum. The animals used were six to eight weeks old, randombred, male Swiss albino mice weighing (20±2 g), housed in the experimental room in a standard Macrolon type II cages clearly labeled with investigational details at 22°C and 60 to 70% relative humidity and fed on commercial rodent feedstuff and water *ad libitum.*

Preparation of test compound

Pyronaridine tetraphospahate synthesized by Shin Poong Pharm Co. (Seoul, Korea) was a gift from Professor Steve Ward, Liverpool School of Tropical Medicine, and Liverpool, UK courtesy of Dr. Alexis Nzila, KEMRI. Kenya. On the day of administration, pyronaridine tetraphosphate was freshly prepared by solubilizing it in solution consisting 70% Tween-80 (density=1.08gml^{-1}) and 30% ethanol (density=0.81 gml^{-1}) and diluted 10 fold with double distilled water.

Determination of 50 and 90% effective doses level (ED_{50} and ED_{90})

The effective doses that reduce parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) were determined in the standard four-day suppressive test (4-DT) as described by Peters (1975). Random-bred, male Swiss albino mice (20±2 g), five mice per dose group in four different doses and five mice in the control group were inoculated intraperitoneally each with 2×10^7 PRBC in 200 µl inoculum on day zero (D_0). Drug was administered orally (p.o) at 4, 24, 48 and 72 h post infection. Thin blood films were prepared from tail snips on day 4 (D_4) post infection, fixed in methanol and stained for 10 min with freshly prepared in 10% (v/v) Giemsa solution.

Parasitaemia was determined by microscopic examination of Giemsa-stained blood films taken on day 4 (96 h post infection). Microscopic counts of blood films from each mouse were processed using MICROSOFT® EXCEL (Microsoft Corp.), then percentage (%) chemosuppression of each dose was determined as described elsewhere (Tona et al., 2001). 50 and 90% effective doses were estimated graphically using linear regression using version 5.5 of statistica 2000.

Procedures for exerting drug-selection pressure and assessing the level of resistance

In every passage (after inoculation with 2×10^7 parasitized erythrocytes contained in 200 µl inoculum), three mice were infected with *P. berghei* and after attainment of >2% parasitaemia, the mice were treated orally with drug pressure dose of PRD. Throughout the selection of resistance, the drug concentrations were increased gradually

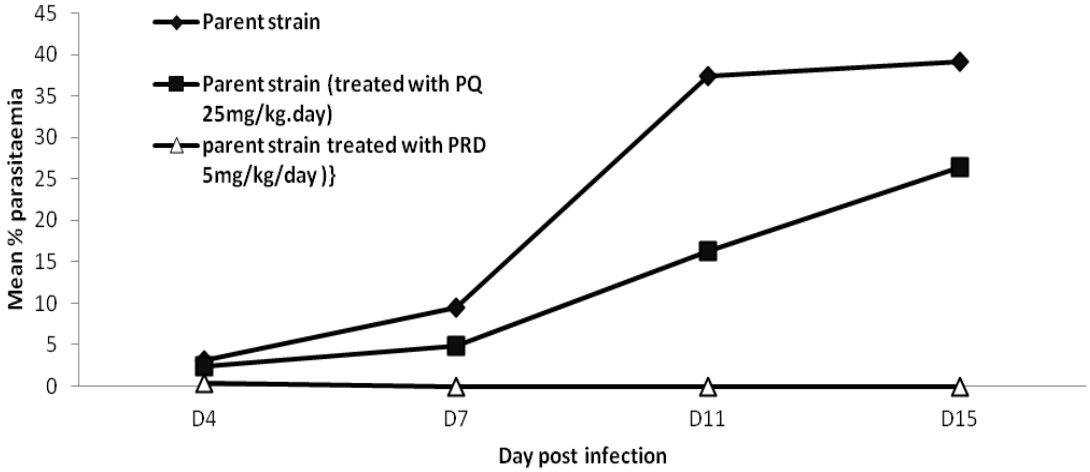

Figure 1A. The drug response of the parent strain (starting parasite) to piperaquine, pyronaridine and development of parasitaemia in untreated mice in a group of four mice taken on day 4, 7, 11 and 15 post infection before the start of the pyronaridine drug selection pressure. The starting parasites tolerated 25 mg/kg of piperaquine, in fact this depicted that the parasites show degree of resistance to the drug. Parasitaemias were assessed after four days post-infection (in both control and treated groups) and mice were treated using a 4-day test (4-DT).

depending on the growth of the parasites in the mice. During the first five passages, the drug pressure dose was increased by dose ranging from 5 to 10 mg/kg.day, while, in 5^{th} to 20^{th} passage, the PRD concentration was increased by 10 to 15 mg/kg.kg depending on the growth patterns of the parasites in mice. Acquisition of resistance was assessed after every five drug passages using standard 4-DT to confirm the response levels of the parasite to the pyronaridine compound. 4-DT permits the measurement of the ED_{50} and ED_{90}, as well as the index of resistance at the 50 and 90% levels (I_{50} and I_{90}), respectively. The indices of resistance (I_{50} and I_{90}) were defined as the ratio of the ED_{50} or ED_{90} of the resistant line to that of the parent strain (Merkli and Richle, 1980; Xiao et al., 2004). Resistance was classified into three categories based on earlier work (Merkli and Richle, 1980): I_{90} = 1.0, sensitive, (2) I_{90} = 1.01-10.0, slight resistance, (3) I_{90} = 10.01-100.0, moderate resistance and (4) I_{90} > 100.0, high resistance.

Stability tests

The stability of PRD resistant line was evaluated by: (i) measuring drug responses after making five drug-free passages and (ii) freeze-thawing of parasites from -80°C stored for a period of four weeks followed by the measurement of effective doses in the 4-Day suppressive test. Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least five passages in mice (Gervais et al., 1999).

Ethical consideration

This study reported here was conducted in accordance with KEMRI guidelines, as well as, internationally accepted principles for laboratory animal use and care. Permission to carry out the study was granted by KEMRI'S Scientific Steering Committee and the Ethical Review Committee. (Study SSC No. 2457, 2013).

RESULTS

Selection of resistance

Effective doses, ED_{50} and ED_{90} of PRD against the parent parasite were 1.83 and 4.79 mg/kg.day, respectively. The parasite density was followed microscopically for 15 days post infection, the parasite were suppressed or cleared by 5 mg/kg/day of PRD (Figure 1A). We thus concluded that piperaquine-resistant clone is sensitive to PRD. As a result, this parasite clone was used as the parent strain to select PRD resistance. After 20 passages under PRD selective pressure, the ED_{50} and ED_{90} increased to 122.49 and 195.98 mg/kg/day, respectively, yielding I_{50} of 66.93 and I_{90} of 40.91. Such value of $I_{50/90}$ depicts that the starting parasites acquired resistance (Table 1) after several drug pressure passages.

Pyronaridine pressured lines were subjected to further five passages in untreated mice (five drug-free passages), after which they were tested for the drug responses. The selected line retained resistance levels yielding ED_{50}=107.5 $mgkg^{-1}$ and ED_{90}=146.1 $mgkg^{-1}$. We then froze the parasite at -80°C, thawed after one month and inoculated into mice. The line retained the resistant-phenotype with marginal decrease in ED_{50} and ED_{90} of 73.48 and 107.50 $mgkg^{-1}$, respectively. Despite decrease in ED_{50} and ED_{90}, we concluded that the parasites retained resistance as I_{90} was over 40 times compared to I_{90} of parent strain. It is worthy to note that selection of resistance produced parasite populations with different susceptibility levels to the drug (Jiang et al., 2008) signifying different 90% index

Table 1. Changing response of the *P. berghei* GFP ANKA resistant clone to PRD during exposure of the parasites to continuous drug pressure in mice.

Passage no.	ED_{50} (mg/kg)	I_{50}	ED_{90} (mg/kg)	I_{90}
Parent parasite	1.83	1	4.79	1
5th	7.12	3.90	11.38	2.38
10th	25.82	14.11	69.43	14.49
15th	92.05	50.3	168.98	35.27
20th	122.49	66.93	195.98	40.91
20th after dilution cloning	145.51	79.51	193.1	40.31
Drug-free passages (20th passage)	107.5	58.74	146.1	30.5
20th passages(after one month cryopreservation)	73.48	40.15	107.10	22.36

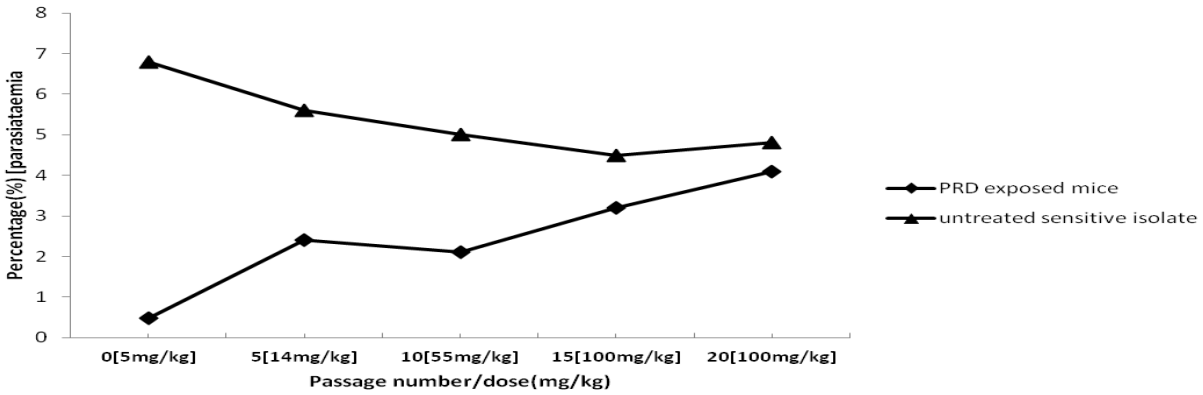

Figure 1B. Development of parasitaemia in the treated and untreated mice at different levels during the selection of PRD resistant *Plasmodium berghei* GFP in mice. Parasitaemias were assessed after four days post-infection (in both control and treated groups) and mice were treated using a 4-day test (4-DT).

of resistance (I90), one with higher value (minority population) and another with lower value (dominant population) (Nzila and Mwai, 2010). Therefore, we clone diluted the resistant parasites to generate a genetically homogenous parasite population and determined drug profiles as described by Rosario (1981). The clone retained resistance yielding the ED50 and ED90 of 145.5 and 193.1 mgkg-1, respectively.

Figure 1B shows the changing response of the *P. berghei* ANKA to PRD in the course of drug pressure. In the first five drug passages, the resistance rose rapidly as parasites in mice were able to tolerate quite low dose of PRD drug. At the 5th passage, a dose 14 mg/kg allowed a parasitaemia of 2.4% only (Figure 1B). Afterwards, PRD resistance arose quite slowly as drug pressure dose was increased to 55 mg/kg, indeed, the parasitaemia was determined to be 2.1% relatively lower compared to 2.4% observed in 5th passage when drug pressure was 14 mg/kg. This suggests that only few parasites grew when the drug concentration used was high (> 3 times the dose used in 5th passage). In the consecutive passages after 10th passage, the drug concentration was maintained at 100 mg/kg (>20 times the ED_{90}). PRD resistance evolved gradually at 15th passage to 20th passage. At 15th passage, the parasitaemia observed was 3.2% after treatment with 100 mg/kg. However, at the 20th passage, at the same dose (100 mg/kg), parasite grew and reached 4.1% parasitaemia, although the growth of PRD pressured parasites was slow compared to untreated group, this was indeed an indication that the parasites had acquired resistance. During all 20 passages, parasitaemias in the untreated controls remained steady, ranging between 4.8 and 6.8%. This resistant phenotype was stable after 5 drug-free passages and cryopreservation at -80°C and these parasite lines were recorded as PRD-resistant strains (scored as PRDR) (Table 1 and Figure 1C).

DISCUSSION

We have selected a stable pyronaridine resistant parasites using ST technique in 20 drug pressure passages over a period of six months. In this study, we used *P. berghei* to select pyronaridine resistance as preliminary step towards

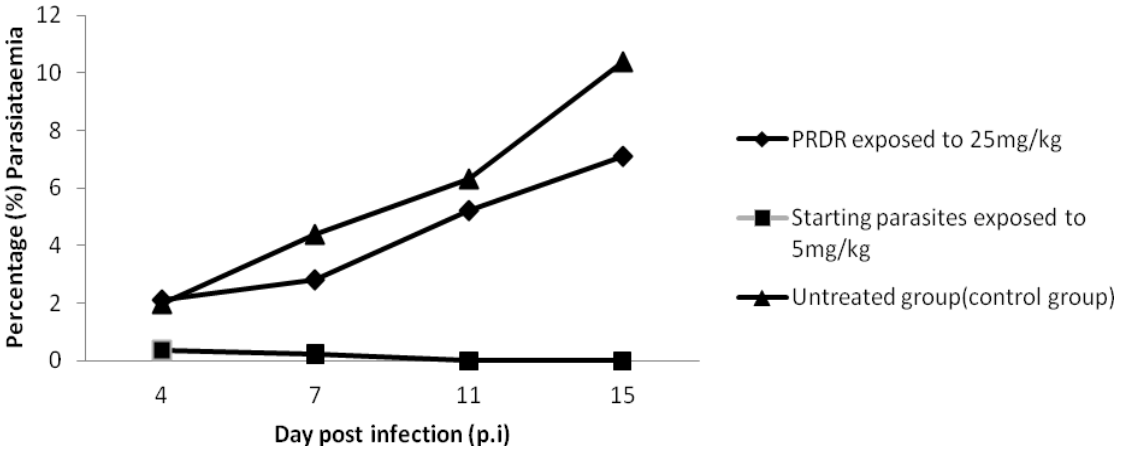

Figure 1C. Selected resistant parasites subjected to PRD in parallel with their sensitive parental clone which had never been exposed to PRD compared with untreated group of mice. As anticipated, sensitive parental clone (Previously shown to be resistant to Piperaquine in Figure 1A) was cleared by 5 mg/kg while PRDR parasites tolerated 25 mg/kg (five times the drug concentration used to treat parent strain parasites), an indication that the parasites had acquired resistance.

study of the molecular basis underlying PRD resistance. One early study found that resistance in *P. berghei* (ANKA) developed slowly to pyronaridine administered at 4 mg/kg, with no detectable high resistance within several passages (Shao and Xe, 1986). Peters and Robinson (1992) were able to derive pyronaridine-resistant *P. berghei* and *P. yoelii* strains by in *vivo* serial passage, applying drug at 3 or 10 mg/kg. However, resistance development was slow and was more difficult to achieve with the higher dose (Peters and Robinson, 1992). Interestingly, in our study we attained maximum level of resistance (>40 times the ED_{90} of parent strain) by applying higher doses ranging from 5 to 100 mg/kg for over a period of 180 days. In fact, our study shows that it is more effective to select PRD resistance by maintaining high continuous drug pressure. This may be as a result of a genetic potentiation of the parasites to generate mutations in response to drug treatment (called the accelerated resistance to multiple drugs phenotype (Rathod et al., 1997) which might have occurred during generation of pyronaridine-resistant line. After making 5-drug free passages and upon revival of the parasites after one month of cryopreservation at -80°C, as shown in our data, there was a marginal degree in resistance. Observation from previous PRD stability studies on *P. berghei* (RP) and *P. berghei* (ANKA) have shown that the sensitivity started to return after making a number drug-free passages, after which the resistance remained stable (Xiao et al., 2004; Peters and Robinson, 1999).

Our study shows that PRD resistance may be selected within six months with a starting parasite line which is resistant to PQ. This study suggests that selective pressure for resistance to antimalarial combinations is exerted by the longer acting antimalarials, which persist in the body below effective concentrations long after treatment, promoting the selection of tolerance and ultimately resistance. Furthermore, studies suggest that even when true clinical resistance is not apparent, drug tolerance might be associated with specific biological processes in the parasite (Price et al., 2006; Nosten and White, 2007; Sisowath et al., 2007). If the concept of the existence of selective pressure because of long elimination half-life applies to all anti malarial drugs, it is expected that selective pressure to the ACTs, for instance, ASN-PRD would be exerted by PRD which is the partner drug with longer half-life (compared to the short acting artemisinin components; Artesunate). In the laboratories, two methods have been used to select resistant murine malaria parasites: the 2% relapse technique (2%RT) in which a single and high drug dose is administered at the time of each passage (Li et al., 1985) and the serial technique (ST), in which drug dose is gradually increased after each passage (Li, 1985; Li et al., 1985). Overall, the ST approach has proven to be more efficient to select for stable resistant strains than 2% RT (Peters and Robinson, 1999; Peters, 1999; Afonso et al., 2006). Using the ST technique, we have successfully established stable PRD-resistant *P. berghei* strains over a spell of six months of drug pressure.

In our study, we used rodent malaria parasite *P. berghei* as a surrogate for *P. falciparum* to study pyronaridine resistance. However, the mechanism of resistance in *P. falciparum* and murine *Plasmodium* species may be different. For instance, the mechanisms of resistance to chloroquine are different in *P. falciparum* and in murine malaria, and there is still a debate whether those of

artemisinin derivatives will be similar (Cravo et al., 2003; Puri and Chandra, 2006). However, for drugs such as mefloquine, antifolates and atovaquone, similar mechanisms of resistance have been reported (Afonso et al., 2006; Carlton et al., 2001; Hunt et al., 2004a, b, 2007). Thus, this motivated the use of murine malaria in this study.

Structurally related compounds to pyronaridine, such as amodiaquine, demonstrated slight resistance against PQ-resistant line (Kiboi et al., 2009), additionally, the AQ resistant line shown resistance to pyronaridine (Kiboi et al., unpublished data). These studies suggest that the PRD resistance may share similar mechanism of resistance.

Conclusion

Selection of Pyronaridine resistance in *Plasmodium berghei* GFP ANKA strain using serial technique, suggests that PRD resistance develops rapidly as long as the selection pressure is maintained. From these results, we concluded that stable pyronaridine resistant *P. berghei* lines were selected. This stable pyronaridine resistance line could be used to facilitate molecular surveillance/monitoring and aid the development of strategies for the reversal of pyronaridine resistance.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Influence of $CaCl_2$ and EDTA on reversible thermal inactivation of recombinant wild-type and mutant (E40H/E44H) *Phlebia radiata* manganese peroxidase 3 (rPr-MnP3)

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Thermal inactivation of a new recombinant *Phlebia radiata* manganese peroxidase (rPr-MnP3) in the presence and absence of additives ($CaCl_2$ 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 $CaCl_2$ 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 ± 0.007 min^{-1} and half-life of 10 min. The decay constant (k_d) 0.029 ± 0.002 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 (Tm =54°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.

Key words: 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

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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-Senaidy 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 prelimminary 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, tem-perature, 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 Shimazdu-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 it 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-thiogalacto-pyranoside (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. *Invitro* folding was performed using 0.15 M urea, 5 mM $CaCl_2$, 0.5 mM oxidized-glutathione, 0.1 mM dithiothreitol ratio, 20 µM hemin, 50 mM Tris-HCl, pH 9.5 and 200 µg/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 µM 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 (pH5.0 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 (ε_{238} = 6.5 $mM^{-1}cm^{-1}$) 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 (Tm) 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-MnP3enzymes. Dry nitrogen was purged continuously into the instrument before and during the experiment with nitrogen purging rate of 25$Lmin^{-1}$. 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 (Tm) 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 Savisky 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 ºC 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-MnP3has called for a detail characteri-zation 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. radiate* 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 to10 min. However, at the 75 minincubation 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.

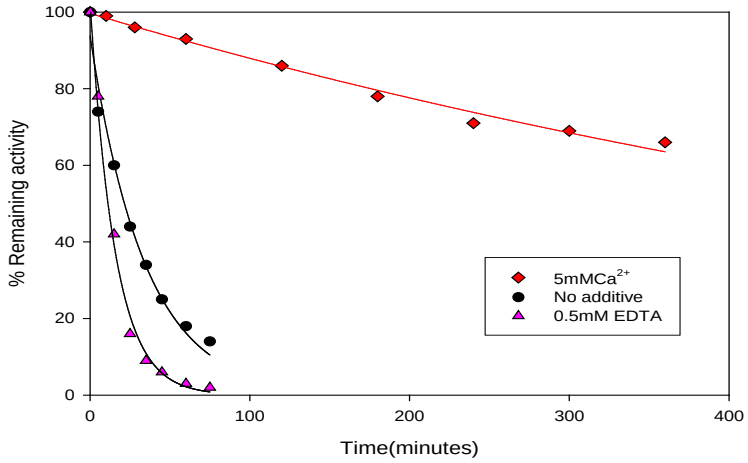


Figure 1a. Thermal stability of recombinant *P. radiata* MnP3 at 50°C. Plots represent no additive, 5 mM $CaCl_2$ added and 0.5 mM EDTA added, to MnP3 during incubation. Enzyme concentration was 2.5 µM in 10 mM sodium succinate buffer, pH 6.0. At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 µM enzyme, 0.2 mM $MnSO_4$, 0.1 mM H_2O_2 and 100 mM sodium tartrate, pH 5.0.

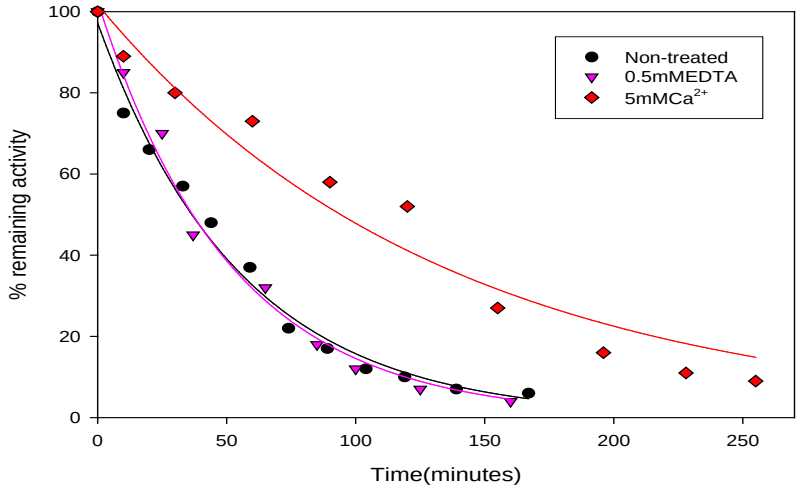


Figure 1b. Thermal stability of recombinant *P. radiata* mutant (E40H/E44H) MnP3 at 50°C. Plots represent no additive, 5 mM $CaCl_2$ added and 0.5 mM EDTA added, to MnP3 during incubation. Enzyme concentration was 10 µM in 10 mM sodium succinate buffer, pH 6.0. At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 µM enzyme, 40 mM $MnSO_4$, 0.1 mM H_2O_2 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 $CaCl_2$ 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

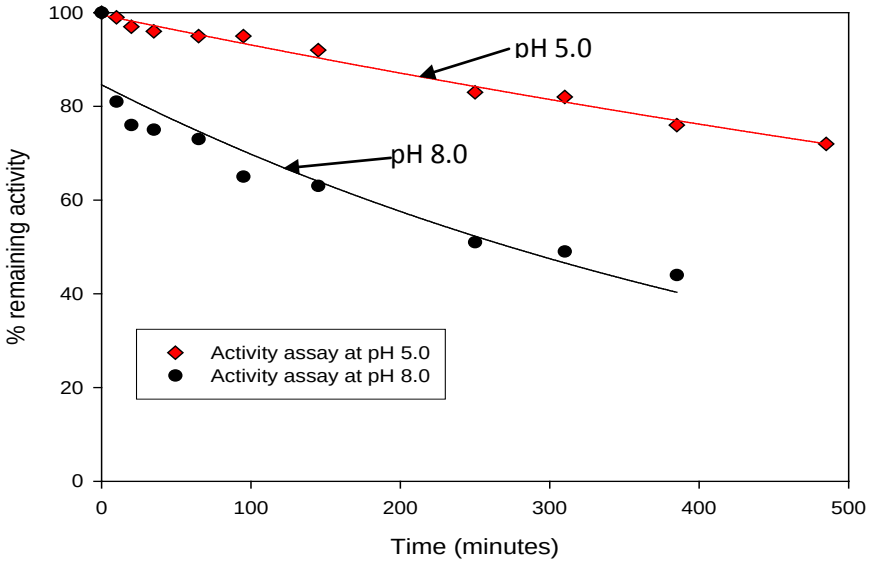


Figure 2. Thermal stability of wild-type recombinant *P. radiata* MnP3 at 40°C, pH 5.0 and 8.0. Enzyme concentration was 2.5 µM in 10 mM sodium succinate (pH 6.0). At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 µM enzyme, 0.2 mM $MnSO_4$, 0.1 mM 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 50ºC 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 ± 0.01 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

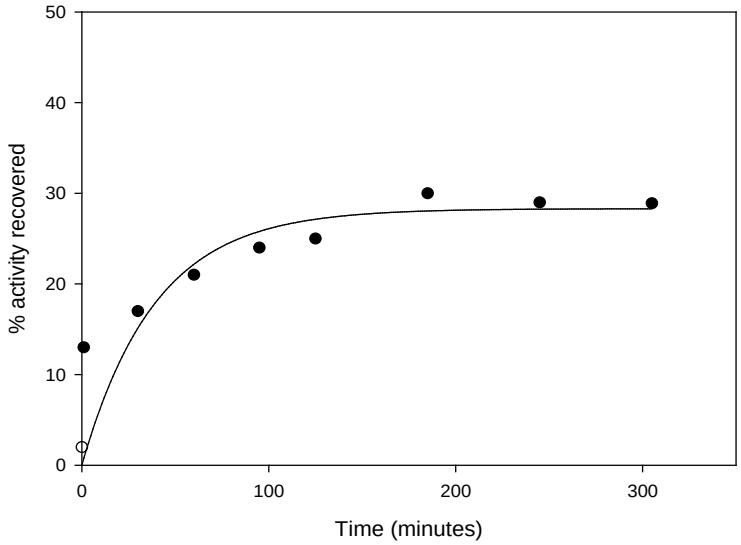


Figure 3. Calcium ion reactivation of thermally inactivated (EDTA treated) wild type MnP3. Reactivation of thermally inactivated enzyme (55 min at 50°C with 0.5 mM EDTA) was obtained by adding 5 mM $CaCl_2$ to 2.5 µM enzyme solution at 4°C for up to 5 h. At specified times aliquots were measured for Mn (II) oxidation activity. Assay mixtures contained 0.4 µM enzyme, 0.2 mM $MnSO_4$, 0.1 mM H_2O_2 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 $CaCl_2$ 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 $CaCl_2$ 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 ($CaCl_2$ 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 $CaCl_2$ 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

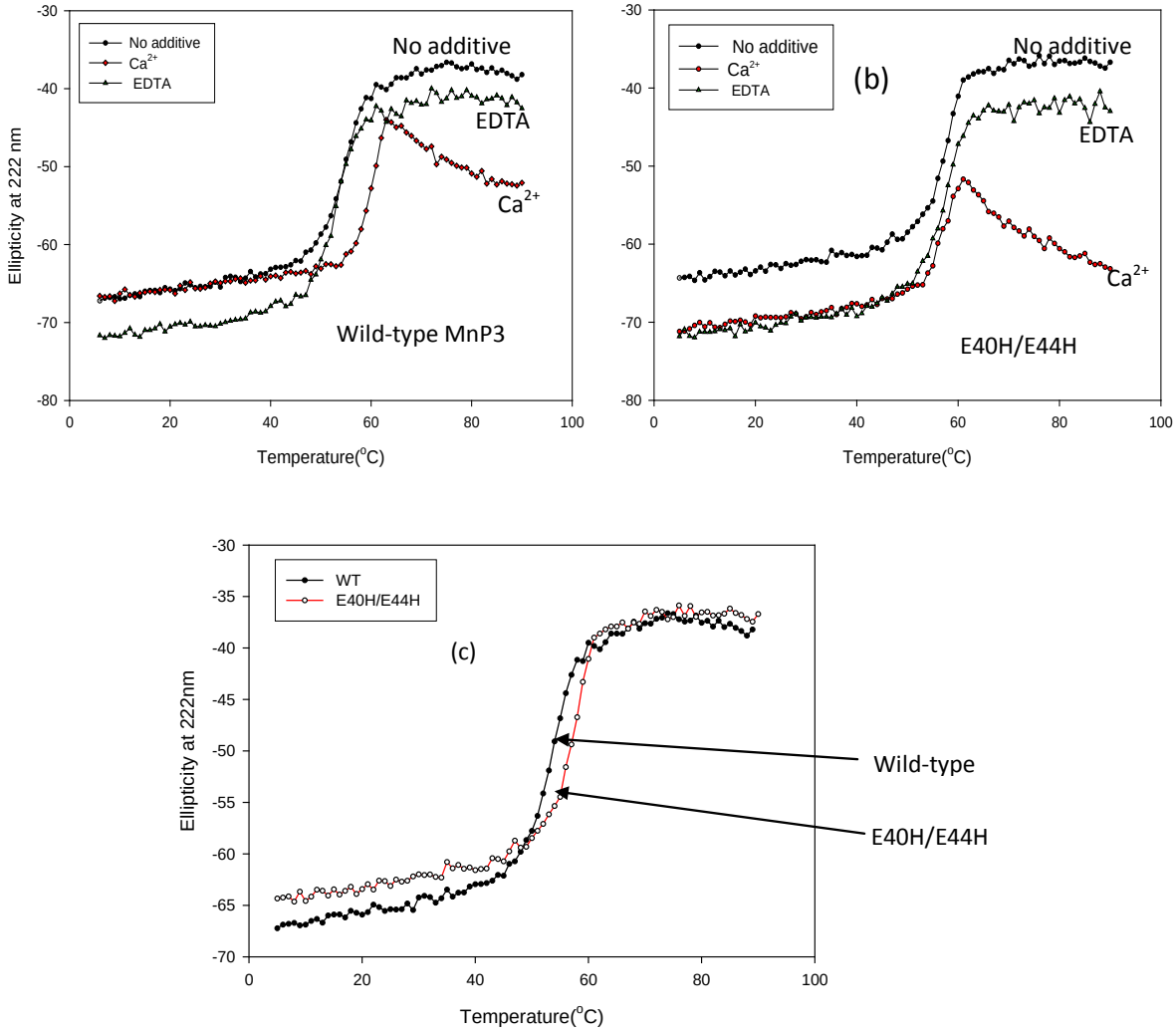


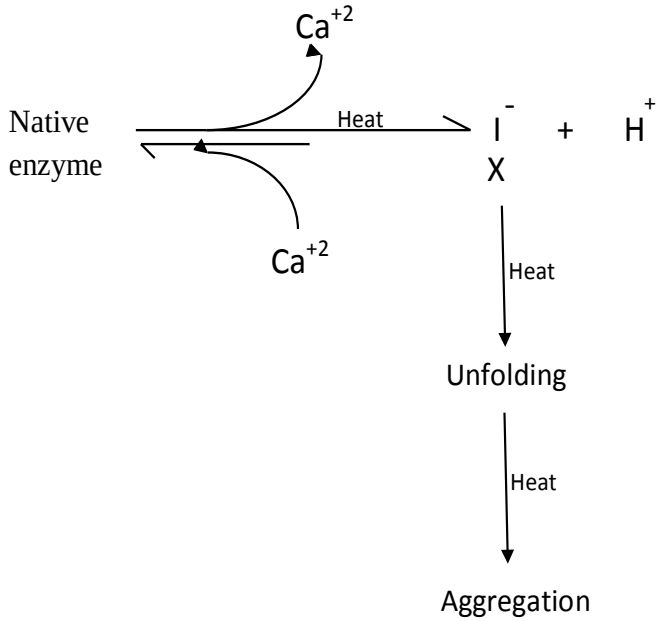
Figure 4. Thermal denaturation plot of wild type and mutant, E40H/E44H, Pr-MnP3 as a function of temperature as measured by Circular dichroism. . Plots **(a)** and **(b)** represent no additive, 5 mM $CaCl_2$ added and 0.5 mM EDTA added, to wild-type or E40H/E44H MnP3 during incubation. **(c)** Overlay 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 Ca^{2+} 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 $CaCl_2$ 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 Ca^{2+} 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 denaturation 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 Ca^{2+} in this study. Presumably calcium loss from the wild-type enzyme was lowered by the presence of excess $CaCl_2$ 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, $CaCl_2$ has been observed to increase the T_m value of wild-type rPr-MnP3, thereby demonstrating the thermo-stabilizing effect of Ca^{2+} 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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