Environmental and Experimental Biology (2016) 14: 83–90 *Original Paper*
DOI: 10.22364/eeb.14.12

Catalytic and kinetic properties of purified *Eichhornia crassipes* leaf peroxidase

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Abstract

Peroxidase from *Eichhornia crassipes* leaf was purified 23.58 fold with 18.58% yield by means of $(NH_4)_2SO_4$ precipitation, ion exchange and Sephadex G-75 gel filtration chromatography. Optimum temperature and substrate-dependent pH optimum for enzyme activity were 40 °C and pH 4.0, 9.0 and 6.0 for 2,2'-azino-bis-(3-ethylbenzthiazolin)-6-sulfonate (ABTS), guaiacol and pyrogallol, respectively. The enzyme had high pH stability and moderate thermal stability at temperatures up to 60 °C; the activation energy of inactivation of the enzyme was ~122.29 kJ mol^{-1}. Temperature-denaturing and spontaneous recovery were shown to be time-dependent while Ca^{2+}-enhanced recovery of the denatured enzyme was in a time-dependent manner. The enzyme showed preferential affinity for ABTS over guaiacol and pyrogallol with K_M values of 31.11, 21.91 and 6.45 mM respectively. It was reversibly inhibited by Pb^{2+}, Hg^{2+} and EDTA while urea only caused loss of ~30.40% activity after 60 min of incubation. *E. crassipes* leaf peroxidase has potential use for broad range of applications.

Key words: *Eichhornia crassipes*, peroxidase, pH and thermal stability, optimum temperature.
Abbreviations: ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonate.

Introduction

Peroxidases are common in nature. They catalyze the oxidation of several important substrates and play important roles in various physiological processes in plants (Passardi et al. 2005; Hamid, Khalil-ur-Rehman 2009). They are heme-containing proteins and are involved in the oxidation of broad range of substrates using hydrogen peroxide as the oxidant (Adam et al. 1999; Galati, O'Brien 2004). Peroxidases have been used in biotechnology, biomedicine, pharmacy and agriculture (Veitch, Smith 2001). Presently, horseradish peroxidase is the major commercial form of the enzyme. However, studies are still ongoing to identify peroxidases from other sources with properties suitable for biotechnological, biomedical and other industrial applications (Regalado et al. 2004). Some peroxidases from these sources have shown potential for applications as biocatalysts and bioelectrocatalysts (Adam et al. 1999; Torres et al. 2003; Carlos et al. 2005).

Several peroxidases have been identified as possessing important properties such as thermal stability, pH stability, and resistance to chemical denaturing, which make them suitable for use as biocatalysts. Purified peroxidases have been obtained from diverse plant sources (Deepa, Arumughan 2002), such as spring cabbage, soy bean and rice leaves (Ito et al. 1991), tea (Kvaratskhelia et al. 1997), okra (Yemenicioglu et al. 1998), *Ipomoea palmetto* (Srinivas et al. 1999) and *Copaifera longsdorffi* (Maciel et al. 2007). However, less attention has been directed toward sources that are naturally widely available and regarded as unwanted in the environment. *Eichhornia crassipes*, also widely known as water hyacinth, is an aquatic plant that is considered to be one of the world's persistent weeds. It is characterized by an aggressive growth pattern. Its abundance and persistence in many water bodies throughout the world favour its consideration as a pontential source of novel peroxidases (Fabricio et al. 2004). In this study, peroxidase was isolated from *E. crassipes* leaves and characterized for its kinetic properties and catalytic conditions. Improvement of the purification process could contribute to making it prospective for industrial applications.

Materials and methods

Materials

Fresh and healthy leaves of *Eichhornia crassipes* were collected from Asa Dam River in Ilorin, Kwara state, Nigeria. Ammonium sulphate and Sephadex G-75 were obtained from BDH Chemicals, England, guaiacol from Santa Cruz Biotech, USA. 2,2'-azino-bis-(3-ethylbenzthiazolin)-6-sulfonate (ABTS) and pyrogallol from Sigma-Aldrich, USA. All chemicals used in this study were of analytical grade and used without further purification.

Ammonium sulphate precipitation and dialysis

E. crassipes leaves were transported to the laboratory in a flask containing ice packs. Homogenate of *E. crassipes* leaves was obtained by homogenizing leaves (50 g) in 250 mL cold 100 mM Tris-HCl buffer, pH 7.5, in a blender, and filtering through a multilayer of cheese cloth. Homogenate was then centrifuged at 10 000 g for 15 min at 4 ˚C. The supernatant was then precipitated with $(NH_4)_2SO_4$ at 4 ˚C overnight for complete precipitation. Saturations of $(NH_4)_2SO_4$ between 0 and 50% as well as 50 and 90% were analyzed for peroxidase activity. Precipitate was collected by centrifuging the solution at 10 000 g for 15 min at 4 ˚C in a cold centrifuge.

The fraction obtained by 50 to 90% saturation had higher peroxidase activity and was selected for further purification. The precipitate was dissolved in the isolating buffer and dialyzed against 100 mM Tris-HCl buffer, pH 7.5 over a period of 24 h with three changes of buffer at regular intervals. Protein concentration was estimated using bovine serum albumin as a standard protein as described by Lowry et al. (1951). The specific enzyme activity was calculated and the purification yield was determined (Rudrappa et al. 2007).

Ion exchange chromatography

The dialysed fraction was loaded on a DEAE cellulose column (1 × 30 cm) that was pre-equilibrated in 100 mM Tris-HCl buffer, pH 7.5. Bound enzyme was eluted stepwise with a 0 to 150 mM NaCl gradient in 100 mM Tris-HCl buffer, pH 7.5. The fractions were collected in volume 5 mL each. Each fraction was then assayed to determine enzyme activity using guaiacol as reducing substrate and the total protein concentration estimated. Fractions with high activity were pooled together and used in the next purification step.

Gel filtration chromatography

Fractions with high specific activity were subjected to molecular weight filtration using Sephadex G-75 in a 1 × 40 cm column that was pre-equilibrated with 100 mM Tris-HCl buffer, pH 7.5. The column was washed with eluting buffer (same as the equilibrating buffer) and fractions were collected in volume 5 mL each per fraction. The fractions were then assayed for their enzyme activity and the total protein estimated. Fractions with the highest specific activity were identified and used for characterization.

Assay of peroxidase activity

Standard peroxidase assay was performed at room temperature. Three assay methods were used to determine peroxidase activity with different reducing substrates. Peroxidase activity with guaiacol as a reducing substrate was carried out as described by Hu et al. (2012) with modifications. Briefly, 0.1 mL of enzyme source was added to 0.1 M phosphate buffer, pH 7.0. Then 0.1 mM guaiacol was added. The reaction was started with 150 mM H_2O_2. The total volume of the reaction mixture was 3.0 mL. Change in absorbance at 470 nm was estimated using a spectrophotometer (visible spectrophotometer 721D, Axiom Medical Ltd, UK) over a period of 3 min with 1 min interval.

The activity of peroxidase using ABTS as a reducing substrate was determined as described by Gray and Montgomery (2003) with slight modifications. The reaction mixture contained 1.67 mM ABTS, 0.1 µL enzyme, 150 mM H_2O_2, and 0.1 mM sodium acetate buffer, pH 4.0. Change in absorbance was measured at 415 nm over a period of 3 min at interval of 1 min. Assay for peroxidase using pyrogallol as a reducing substrate was carried out as described in the Sigma-Aldrich procedure manual. The reaction mixture contained 0.32 mL 5% pyrogallol, 0.16 mL 147 mM H_2O_2, 0.32 mL phosphate buffer pH 6.5, 2.1 mL H_2O and 0.1 mL enzyme. Change in absorbance was monitored at 590 nm using a spectrophotometer (visible spectrophotometer 721D, Axiom Medical Ltd, UK). All assays where read over a period of 3 min.

The following molar extinction coefficients were used in calculating peroxidase activity with different substrates; ABTS (36.0 mM cm^{-1}), guaiacol (26.6 mM cm^{-1}), pyrogallol (2.6 mM cm^{-1}). In all assays, the change in absorbance represented colour development as a result of the product formed and expressed as µmol of product formed per minute by *E. crassipes* leaf peroxidase. Except when otherwise stated, the reducing substrate used in this study was guaiacol.

Determination of pH optimum and stability of peroxidase

Optimum pH of *E. crassipes* leaf peroxidase was determined for three reducing substrates, ABTS, guaiacol and pyrogallol. Optimum pH for ABTS was determined using McIlvaine buffer prepared as described by Dawson et al. (1986). Peroxidase activity was tested over a pH range of 2.0 to 8.0. For guaiacol, peroxidase activity was determined using a buffer range as described by Khatun et al. (2012). Optimum pH of pyrogallol was determined using buffers as used in assay for peroxidase activity with guaiacol. pH stability of the enzyme was determined using guaiacol as a substrate and the same buffer range as previously described. The enzyme was incubated with specific buffers of known pH range for a period of 24 h at 4 ˚C, and peroxidase activity was determined after this time.

Determination of optimum temperature and thermal stability of peroxidase

The optimum temperature of *E. crassipes* leaf peroxidase was determined by assaying peroxidase activity at temperatures between 20 and 80 ˚C at 10 ˚C intervals. Heat of inactivation was determined by measuring enzyme activity (A_o) at 40, 50, 60 and 70 ˚C. Briefly, an enzyme sample was heated at a specific temperature over a period of 60 min. Aliquot

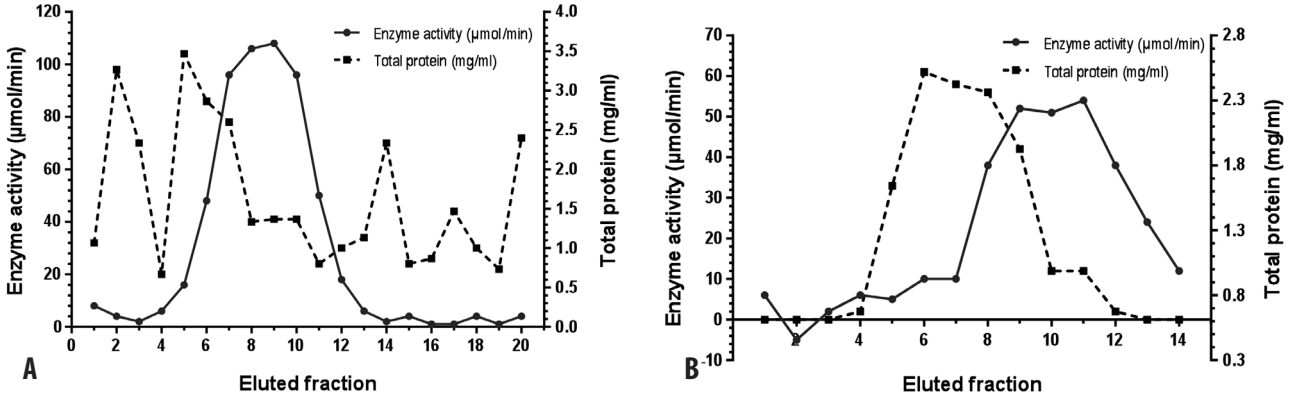

Fig. 1. Ion exchange (A) and gel chromatography (B) elution profiles of *Eichhornia crassipes* leaf peroxidase.

amounts of enzyme source were withdrawn at intervals of 10 min. The withdrawn samples were then cooled on an ice bath for a period of 30 min and incubated at 25 ˚C for another 15 min. The residual peroxidase activity (A) was then determined. The rate constant of inactivation (k) was obtained from the slope of a plot of the natural log of the residual activity (ln A/A_o) versus time (T) of heat exposure. The energy of inactivation of the enzyme was calculated from the slope of a straight line obtained using an Arrhenius plot of the natural logarithm of the first order rate constant for inactivation constant (k) versus the reciprocal of absolute temperature (K) (Thongsook, Barrett 2005).

Thermal recovery profile of peroxidase

The time-dependent thermal recovery profile of *E. crassipes* leaf peroxidase was determined by assaying peroxidase activity under different conditions. The enzyme source was heated in a water bath for 10, 40 and 60 min at 75 ˚C. At each time interval, samples of the enzyme were withdrawn, allowed to cool at 25 ˚C for periods of 20, 30, 40 and 50 min. Residual peroxidase activity was determined in the samples. The Ca^{2+}-induced spontaneous recovery was studied and compared with recovery in the absence of Ca^{2+}. Enzyme samples were heated in water bath for 1 h at 75 ˚C. Then the enzyme was incubated with 0.005 M Ca^{2+} in phosphate buffer at 25 ˚C for 5, 15, 30 and 60 min. The residual activity of the enzyme was then determined and expressed as a percentage, and compared with activity obtained in the absence of Ca^{2+}.

Substrate affinity of peroxidase and inhibition by Hg^{2+}, EDTA and Pb^{2+}

Substrate affinity of *E. crassipes* leaf peroxidase for ABTS, pyrogallol, guaiacol and ascorbic acid was determined. E. crassipes leaf peroxidase activity at various concentrations of the substrates and the double reciprocal plots were used to estimate K_M values for each of the substrates. The inhibition patterns of Hg^{2+}, Pb^{2+} and EDTA on *E. crassipes* leaf peroxidase catalysis were evaluated. Double reciprocal plots of both inhibited and non-inhibited enzyme catalysis were obtained.

Effect of urea on peroxidase

A *E. crassipes* leaf peroxidase sample was incubated with 8 M urea solution. The period of incubation spanned 60 min at intervals of 10 min. At each time the residual activity of peroxidase was determined.

Results

Isolation and purification of E. crassipes *leaf peroxidase*

E. crassipes is an aquatic weed with invasive growth and in some countries it has raised environmental concerns because of the absence of natural predators. Several attempts have been made to control the spread of these plants in many parts of the world (Ghosheh 2005; Tellez et al. 2008). This study represents an attempt to convert these wastes to potential wealth by identifying and isolating an important commercial enzyme from *E. crassipes* leaves. Peroxidase was purified by the combination of ammonium sulphate precipitation and column chromatography on DEAE anion

Table 1. Summary of the purification steps of *Eichhornia crassipes* leaf peroxidase

Purification step	Enzyme activity (µmol min^{-1})	Total protein (mg mL^{-1})	Specific enzyme activity (µmol min^{-1} mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme extract	507.52	25.44	19.94	1.00	100.0
$(NH_4)_2SO_4$ precipitation	384.06	5.21	73.77	3.67	75.67
Ion exchange chromatography	130.10	1.43	91.07	4.57	25.63
Gel filtration	94.30	0.20	470.32	23.58	18.58

exchange resin and Sephadex G-75 (Fig. 1, Table 1). DEAE chromatography (Fig. 1A) showed that fraction 9 exhibited the highest specific activity using guaiacol as substrate, with purification 4.57 fold. For Sephadex G-75 chromatography (Fig. 1B), fraction 6 showed the highest specific activity using guaiacol as a substrate, with purification 23.58 fold. The fractions were pooled together for characterization.

Optimum pH of E. crassipes *leaf peroxidase using different substrates*

Fig. 2 shows the effect of pH on *E. crassipes* leaf peroxidase activity using guaiacol, pyrogallol and ABTS as reducing substrate. The optimum pH for peroxidase for guaiacol was 8.9, with steep decline at higher pH. Using pyrogallol as a reducing substrate, the pH optimum for peroxidase was 5.5. There was much lower enzyme activity in the acidic range compared to basic values. *E. crassipes* leaf peroxidase activity had optimum pH 4.0 using ABTS, with a sharp decrease in the basic range.

pH stability of E. crassipes *leaf peroxidase*

Fig. 3 shows the effect of pH on the stability of *E. crassipes* leaf peroxidase after 24 h exposure. The profile characteristics obtained for the enzyme are similar, with a peak in enzyme activity at pH 7.0. This indicates that neutral pH is optimum for peroxidase activity during prolonged exposure.

Optimum temperature and thermal stability of E. crassipes *leaf peroxidase*

Fig. 4 shows effect of temperature on *E. crassipes* leaf peroxidase activity. The activity was in optimum at temperature 40 ˚C with a wide optimum temperature range, with a 60% residual enzyme activity maintained in approximately 70% of the temperature range.

The rate constant of inactivation (k) was obtained from the slope of a plot of the natural log of the residual activity ($\ln A/A_o$) versus time (T) of heat exposure (Fig. 5A). The energy of inactivation of the enzyme was calculated from the slope of the straight line obtained using an Arrhenius plot of natural logarithm of the first order rate constant for inactivation constant (k) versus the reciprocal of absolute temperature (K). In this study, the value of activation energy of heat inactivation (E_a) for *E. crassipes* leaf peroxidase as obtained from Fig. 5B was 122 kJ mol^{-1}.

Thermal recovery profile of E. crassipes *leaf peroxidase*

Fig. 6 shows the time-dependent recovery profile of *E. crassipes* leaf peroxidase after thermal treatment at 75 ˚C for various duration. Recovery for the three treatments occurred at a fixed constant rate. Only slight recovery of enzyme activity was observed for the treatments. The thermal stability profile showed that loss of peroxidase activity at a particular temperature was time-dependent, while recovery increased during the first 20 min, after which recovery became insignificant.

Fig. 7 shows the profile of *E. crassipes* leaf peroxidase when the temperature-denatured enzyme was allowed to spontaneously refold, compared to folding in the presence of Ca^{2+} in reaction mixture. The results showed that Ca^{2+} improved enzyme activity. Presence of Ca^{2+} resulted in two times higher peroxidase activity. The presence of Ca^{2+} also affected the reactivation of the denatured enzyme in a time-dependent manner.

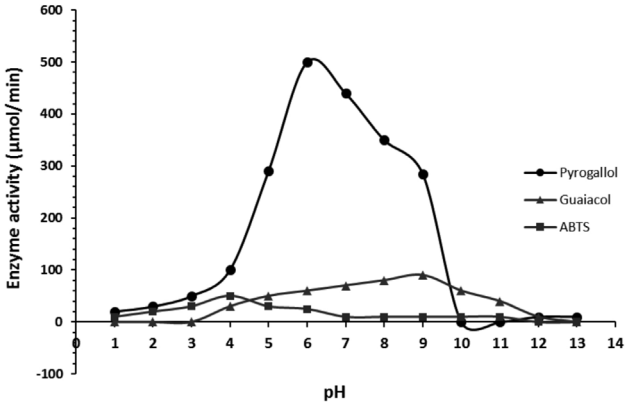

Fig. 2. Optimum pH of *Eichhornia crassipes* leaf peroxidase using guaiacol, pyrogallol and ABTS as substrates.

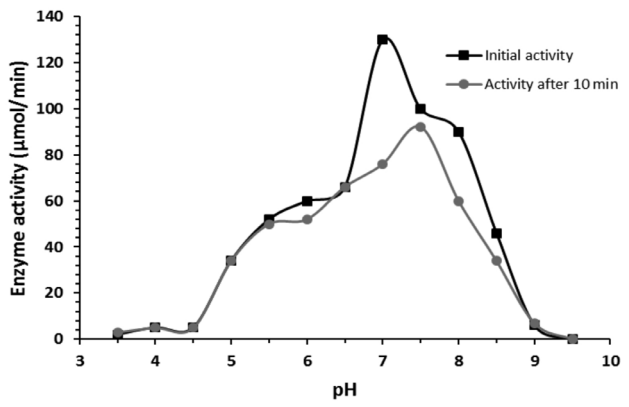

Fig. 3. pH stability of *Eichhornia crassipes* leaf peroxidase using guaiacol as substrate.

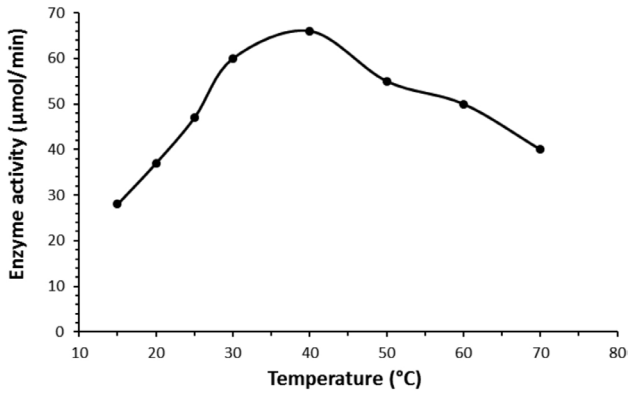

Fig. 4. Optimum temperature of *Eichhornia crassipes* leaf peroxidase.

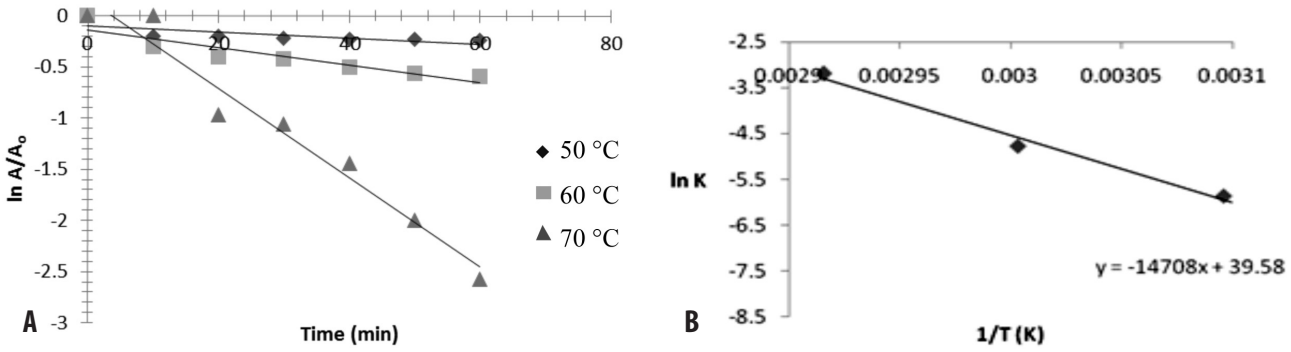

Fig. 5. (A) Rate of heat inactivation of *Eichhornia crassipes* leaf peroxidase. The ordinate represents the natural log of relative activity, the ratio of the activity (A) to the original activity (A_o) before heat treatment. (B) Arrhenius plot showing the effect of temperature on the rate constant for the thermal inactivation of peroxidase.

Substrate specificity of E. crassipes *leaf peroxidase*

K_M and V_{max} values obtained for each of the three reducing substrates are given in Table 2. ABTS had the lowest K_M value followed by pyrogallol. Despite its relatively high K_M value, pyrogallol still gave the highest V_{max} value followed by ABTS. *E. crassipes* peroxidase showed no activity using ascorbic acid as a substrate.

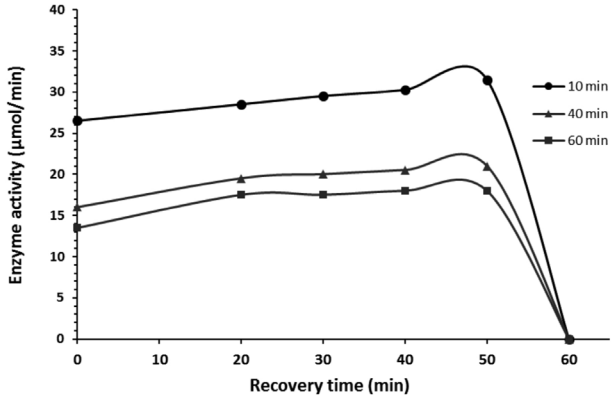

Fig. 6. Time-dependent spontaneous recovery of *Eichhornia crassipes* leaf peroxidase after thermal treatment for 10, 40 and 60 minutes at 75 °C.

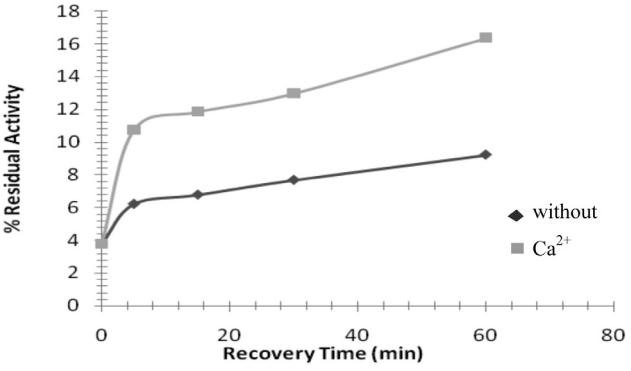

Fig. 7. Ca^{2+}-induced recovery of *Eichhornia crassipes* leaf peroxidase after heating at 75 °C for 1 h.

Kinetics of inhibition of E. crassipes *leaf peroxidase by Pb^{2+}, Hg^{2+} and EDTA*

Fig. 8A, B and C show double reciprocal plots of the inhibitory effects of EDTA, Pb^{2+} and Hg^{2+} on activity of *E. crassipes* leaf peroxidase. For the two concentrations of Hg^{2+} studied, a mixed form of competitive inhibition was observed. The same pattern was also observed for EDTA and Pb^{2+} inhibition of the enzyme.

However, the effects of the studied inhibitors on the kinetic parameters of *E. crassipes* leaf peroxidase differed (Table 3).

Effect of urea

Urea is a common compound with ability to denature proteins. It acts by interfering with protein folding into its native conformation. *E. crassipes* leaf peroxidase maintained its stability under urea treatment, with about 70% of its activity in the presence of urea over a time range of 60 min.

Discussion

The degree of purity (23.58 fold) of peroxidase attained in this study was higher than that (14.08 fold) obtained by Rehman et al. (1999). The specific activity (470 µmol min^{-1} mg^{-1}) of *E. crassipes* leaf peroxidase achieved after the last step of the three-step purification in this study was considerably higher than levels obtained previously for different sources of the enzyme: 15.21 units mg^{-1} (Rehman et al. 1999), 86 units mg^{-1} (Regalado et al. 1996), 135.44 units mg^{-1} (Maciel et al. 2007), 346.43 units mg^{-1} (Khatun et al. 2012) and 349.8 units mg^{-1} (Srinivas et al. 1999).

Table 2. Kinetic parameters of *Eichhornia crassipes* leaf peroxidase for different substrates

Substrate	K_M (mM)	V_{max} (µmol min^{-1})
Guaiacol	31.11	47.62
Pyrogallol	21.91	755.36
ABTS	6.45	161.29
Ascorbic acid	–	–

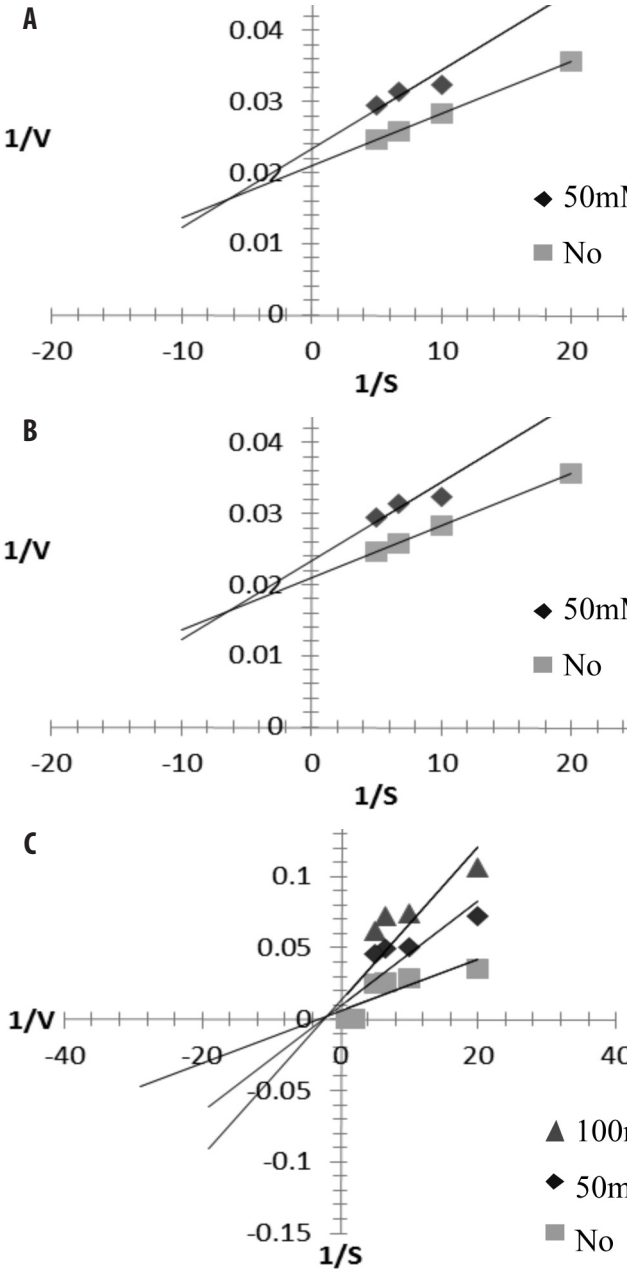

Fig. 8. Inhibition of *Eichhornia crassipes* leaf peroxidase by (A) EDTA, (B) Pb^{2+} and (C) Hg^{2+}.

Peroxidases from different sources have been reported to have pH optima mostly in the range of 4.5 to 6.5 (Thongsook, Barrett 2005). The *E. crassipes* leaf peroxidase pH optima for pyrogallol and guaiacol are similar, with overlapping range. The range for pH optimum (5.2 to 6.8) using pyrogallol as a substrate was similar to that obtained by Gray and Montgomery (2003). However, peroxidases from some sources show higher pH optimum (Dubey et al. 2007). The differences in optimum pH for ABTS compared to the other two substrates may be a result of change in the ionization of substrates. This pattern was similar to those obtained for some previous studies (Melo et al. 1995; Gray, Montgomery 2003).

Table 3. K_M values of *Eichhornia crassipes* leaf peroxidase with selected inhibitors

Inhibitor	K_M (mM)
None	31.11
Pb^{2+}	47.21
EDTA	74.56
Hg^{2+} (50 mM)	50.56
Hg^{2+} (100 mM)	56.68

E. crassipes leaf peroxidase activity was high at acidic, alkaline, as well as neutral pH, depending on the substrate type. In addition, the wide pH stability showed that the enzyme displayed an uncommonly high level of stability, which may favourably enhance its suitability for diverse industrial processes. The enzyme activity obtained over the range of temperature shows that *E. crassipes* leaf peroxidase has a rather large optimum temperature range, with 60% residual enzyme activity maintained within approximately 70% of the temperature range. Other studies have shown peroxidase activity to have a similar optimum temperature range. Bhatti et al. (2006) showed that activity of peroxidase from lettuce stem had an optimum temperature of 45 °C, while activity of peroxidase from cauliflower had optimum temperature of 30 °C (Koksal, Gulcin 2008).

The activation energy of heat inactivation (E_a) value (122 kJ mol^{-1}) for *E. crassipes* leaf peroxidase obtained in this study is comparable to that of neutral broccoli isoperoxidase (189 kJ mol^{-1}) reported by Thongsook and Barrett (2005), horesradish peroxidase (114k kJ mol^{-1}; Lu, Whitaker 1974), asparagus (140 kJ mol^{-1}; Ganthavorn et al. 1991), and cauliflower (193 kJ mol^{-1}; Lee, Pennesi 1984). Thonsook and Barett (2005) argued that higher purity can negatively affect the stability of a purified enzyme and thus resulting in a lower E_a value. However, the stability can also be affected by the isoenzyme isolated. Other studies have shown that time range of thermal exposure can result in

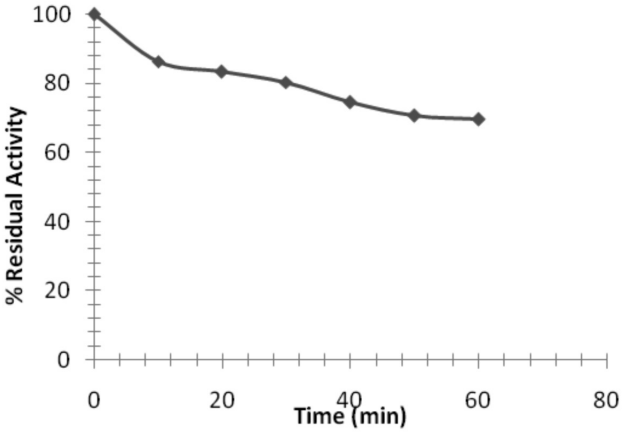

Fig. 9. Time-dependent effect of urea on the activity of *Eichhornia crassipes* leaf peroxidase.

conflicting values (Duarte-Vazquez et al. 2003; Thongsook, Barrett 2005; Manu, Rao 2009; Nouren et al. 2013). A shorter time exposure has lower denaturing effect but higher E_a.

Thongsook and Barrett (2005) reported that broccoli peroxidase showed some ability to recover after heat treatment in a way that was time- and tempetrature-dependent. However, since this study only considered treatment at 75 °C, it is assumed that the reactivation of peroxidase was strictly time-dependent (especially in the first 20 min). Studies have shown the ability of different peroxidases to recover after heat treatment (Halpin et al. 1989). Information on the reactivation of HRP after heat treatment are however conflicting (Thongsook, Barrett 2005).

Calcium is important for both activity and maintenance of the protein structure around the heme pocket (Barber et al. 1995; Thongsook, Barrett 2005). It has been reported that broccoli peroxidase has the ability to recover in the presence of Ca^{2+} after heat treatment (Thongsook, Barrett 2005). Gray and Montgomery (2003) reported a similar effect in corn steep water peroxidase, although greater recovery was observed. This study however, showed that a longer period of time was needed for Ca^{2+}-induced recovery of *E. crassipes* leaf peroxidase. Possibly pH might have caused the low rate recorded.

The low K_M value obtained for ABTS shows that *E. crassipes* leaf peroxidase had highest affinity for ABTS over both pyrogallol and guaiacol. The values obtained for pyrogallol oxidation by *E. crassipes* leaf peroxidase are however debatable. This is because autooxidation of pyrogallol was observed under the selected assay conditions even in the absence of *E. crassipes* leaf peroxidase. Absence of peroxidatic activity in the presence of ascorbic acid as a reducing substrate by peroxidase from different sources has been reported (Khatun et al. 2012).

Adsorption of specific heavy metals by some biomolecules results in structural and functional changes (Einollahi et al. 2006). Various studies have shown that peroxidase activity is susceptible to modulation by certain metal ions (Marzouki et al. 2005; Bhatti et al. 2006). The mixed type inhibition pattern obtained for each of the inhibitors indicates that they are able to inhibit *E. crassipes* leaf peroxidase by binding to either the free enzyme or its enzyme-substrate complex. The inhibition profile of *E. crassipes* leaf peroxidase by Hg^{2+} agrees with results obtained by Einollahi et al. (2006) on inhibitory effect of Hg^{2+} on horseradish peroxidase. Other studies have shown that Pb^{2+} and EDTA have inhibitory effects on peroxidase from different sources (Hu et al. 2012; Khatunet al. 2012). The inhibitory effect of EDTA on *E. crassipes* leaf peroxidase was probably due to the sequestering effect of EDTA on Fe^{3+} and Ca^{2+}, which are necessary for structural and functional integrity of the enzyme.

Time-dependent effect of urea showed that *E. crassipes* leaf peroxidase maintained its stability (70% optimum activity). Nouren et al. (2013) showed that *Citrus reticulata* peroxidase retained 84.22% of its activity after 60 min incubation with urea. Thus, *E. crassipes* leaf peroxidase has moderate stability against chemical destabilization of urea. This characteristic increases its potential use in certain applications, where conformational stability of the enzyme is of great value.

In conclusion, peroxidases are becoming more useful in clinical, environmental and industrial applications, but these enzymes require stability over a broad range of physical and chemical conditions. *E. crassipes* leaves are available in large quantities in water bodies in Nigeria. The partially purified *E. crassipes* leaf peroxidase showed moderate stability under some of these conditions. Thus, *E. crassipes* leaf peroxidase may have great potential for industrial applications.

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Received 21 February 2016; received in revised form 14 April 2016; accepted 2 May 2016