ROLE OF CALCIUM IN ENHANCING THE ACTIVITY AND THERMAL STABILITY OF A NEW CATIONIC PEROXIDASE PURIFIED FROM EUPHORBIA TIRUCALLI LATEX

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ABSTRACT

A new cationic peroxidase from Euphorbia tirucalli (pencil cactus) latex was purified to homogeneity using benzene fractionation, gel filtration and cation-exchange chromatography. The purified enzyme was found to be monomeric with a molecular weight of 44 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme had a broad specificity towards some phenolic substrates in the order of 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) (ABTS) > guaiacol > o-phenylenediamine > 4-aminoantipyrine, whereas no affinity towards ascorbic acid and o-dianisidine was recorded. The enzyme had pH and temperature optima at 7.0 and 40°C, respectively. Study of kinetic parameters demonstrated that ABTS had the highest affinity towards ELP, where $K_m$, $V_{max}$ and $V_{max}/K_m$ values were 0.503 mM, 500 U/assay and 994.04 U/mM, respectively. ELP was stable from 10°C up to 60°C and lost about 70% of its activity at 70°C. The thermal inactivation profile of ELP in absence of Ca$^{2+}$ is biphasic and characterized by a rapid decline in activity on exposure to heat, followed by a more gradual decrease in activity on continued exposure. However, the purified enzyme exhibited increased thermal stability in the presence of calcium ions. Furthermore, the activity of purified enzyme was enhanced by ~ 550% in the presence of 15 mM CaCl$_2$, suggesting a pivotal role for Ca$^{2+}$ in conferring structural stability to the heme environment and in retaining the active site of

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ELP. Most of the examined metal ions (except for Ca\(^{2+}\) and Mg\(^{2+}\)) and compounds had differential inhibitory effects on ELP activity. In conclusion, a locally available plant (*Euphorbia tirucalli*) could be a potential candidate source for peroxidase, the most widely used enzyme in industrial and biomedical applications. In addition, calcium was found to be essential for enhancing enzymatic activity and thermal stability of the purified *Euphorbia tirucalli* latex peroxidase.

**Keywords:** Peroxidase; *Euphorbia tirucalli*; Latex; Calcium; Thermal Activation.

**INTRODUCTION**

Peroxidases (PODs, EC 1.11.1.7) are enzymes that oxidize various hydrogen donors in the presence of H\(_2\)O\(_2\). They are widely existed in plants, animals and microorganisms. Based on sequence homology and location, non animal PODs have been classified into three classes: class I refers to intracellular POD (e.g., bacterial gene-duplicated catalase-peroxidase, yeast cytochrome C peroxidase and ascorbate peroxidase), class II includes secretory fungal PODs (e.g., lignin peroxidase and manganese peroxidase) and class III consists of the plant secretory PODs such as *Armoracia rusticana* peroxidase (HRP-C) and *Glycine max* peroxidase (SBP). The well known PODs are class III PODs, which share a heme prosthetic group, four conserved disulfide bridges, and two calcium binding sites (*Welinder, 1992; Cai et al., 2012*).

Plant PODs are implicated in several physiological processes, such as hydrogen peroxide detoxification, auxin metabolism, cell elongation, lignin and suberin formation, defense against pathogens, salt tolerance and oxidative stress. Peroxidases are present at every stage of plant growth, ranging from germination to senescence and, probably due to their implication in a broad range of physiological processes, higher plants show a wide array of peroxidase isoenzymes and a heterogeneous regulation of their expression (*Passardi et al., 2004*).

A large number of plant species may exude an often milky, variously colored sap known as latex which constitutes the cytoplasmic content of laticifers (*Hagel et al., 2008*), specialized elongated cells or vessel-like series of cells that permeate various aerial tissues of the plant, including sometimes the fruits, and also the
root system. Latex is an emulsion with a diversified composition that includes alkaloids, terpenoid compounds, resins, gums, starch, oils, and a large number of proteins and enzymatic activities (Ko et al., 2003).

Peroxidases have a great diversity of applications, partly owing to their wide substrate specificity and functional diversity. They are useful for industrial and analytic applications where they are used as catalysts for phenolic resin synthesis, as indicators for food processing, in the treatment of wastewater by removal of phenols and aromatic amines, in the bio-bleaching process, in lignin degradation, and in the biotransformation of organic compounds. Peroxidases are also used as markers in enzyme immunoassays, DNA probes, or clinical diagnosis, and represent the bases of numerous biosensors for the direct determination of many compounds (Marzouki et al., 2005).

Over the years, Armoracia rusticana roots (horseradish) has been the only commercial source of peroxidase (horseradish peroxidase; HRP) (Kamal and Behere, 2008). However, there is a great interest in finding more locally available alternative sources that exhibit properties similar to or better than those of horseradish enzyme. In the present study, we describe the purification and characterization of a new cationic peroxidase from Euphorbia tirucalli latex and report on the critical role for Ca\(^{2+}\) in increasing its specific activity and thermal stability.

MATERIALS AND METHODS

Plant materials: Euphorbia tirucalli latex was collected from the cut edges of the stem apices and branches of Euphorbia tirucalli plant (Orman Garden, Giza, Egypt).

Chemicals: O-Phenylenediamine (OPD), 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 4-aminoantipyrene (AAP), \(o\)-dianisidine, ascorbic acid, guaiacol and molecular weight markers for gel filtration were purchased from Sigma (MO, USA). Tris base [Tris-(hydroxymethyl) aminomethane], hydrogen peroxide and ethylenediamine tetraacetic acid (EDTA) were purchased from Fluka (Switzerland). Sephacryl S-200, carboxymethyl (CM)-Sepharose for chromatography and molecular weight markers for SDS-polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals (Sweden). Other chemicals were of high

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analytical grade.

**Purification of Euphorbia tirucalli latex peroxidase (ELP)**

1. **Collection and preparation of the crude extract:** The fresh latex sample obtained by cutting the stem apices and branches of *E. tirucalli* was allowed to drain into a glass tube and immediately diluted with an equal volume of 50 mM sodium acetate buffer (pH 5.5). The tubes were gently mixed and then transported to our lab in an icebox containing ice cubes.

2. **Benzene fractionation:** Different extraction methods of the crude latex were used, which include homogenization, ammonium sulfate precipitation and ultrasonication, as well as benzene fractionation. The best yield of ELP activity was found with benzene fractionation, which was used therefore in the present study. An equal volume of the diluted latex and benzene was mixed and the tube was centrifuged at 6,450 xg for 10 min to separate the mixture into three layers (the upper organic layer, the lower aqueous layer and the white interface layer). ELP activity of latex particles was examined in the three layers and was detected in the aqueous layer, which was collected and stored at -20°C for further purification steps.

3. **Gel filtration chromatography:** The aqueous layer was applied directly onto a Sephacryl S-200 column (90 x 1.6 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer (pH 5.5) at a flow rate of 30 ml/h. The column was eluted with the same buffer and 3-ml fractions were collected and assayed for protein content and peroxidase activity.

4. **CM-Sepharose column chromatography:** The pooled fractions from Sephacryl S-200 column containing ELP activity were concentrated through dialysis against solid sucrose and then applied to CM-Sepharose column (6 x 2 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer (pH 5.5). The adsorbed protein was eluted with a stepwise of NaCl ranging from 0.0 to 0.5 M prepared in the same buffer at a flow rate of 36 ml/h. Fractions of 3 ml were collected and assayed for protein content and peroxidase activity. Fractions exhibited peroxidase activity were pooled and stored at -20°C for characterization.

**Molecular weight determination**

1. **Gel filtration:** Molecular weight of ELP was determined by gel filtration technique using a Sephacryl S-200 column (90×1.6 cm i.d.) equilibrated with 50 mM sodium acetate buffer (pH 5.5). The column
was calibrated with cytochrome c (12.2 kDa), myoglobin (17 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Dextran blue (2,000 kDa) was used to determine the void volume (Vo). Protein solutions of 2.0 ml volume containing 4-6 mg protein/ml were applied to the same column and developed using the same equilibration buffer at a flow rate of 20 ml/h, and 3-ml fractions were collected. A calibration curve was constructed by plotting log molecular weight versus Ve/Vo, where Ve is the elution volume and Vo is the void volume.

2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): ELP molecular weight was determined by SDS-PAGE according to Laemmli (1970) using 12% separation gel and 5% stacking gel in a Mini-Protean apparatus (BIO-RAD, USA). The molecular weight of the ELP was determined by using the following protein standards: α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa) and phosphorylase b (97 kDa). Protein bands were visualized after staining the gel with Coomassie Brilliant Blue R-250. The electrophoretic mobility (Rf = Distance of protein migration/Distance of tracking dye migration) of each standard was measured and ELP molecular weight was determined from the curve.

Peroxidase assay: Estimation of peroxidase activity was carried out according to Miranda et al. (1995). The final reaction mixture in 1.0 ml contained 8 mM H₂O₂, 40 mM guaiacol, 50 mM sodium acetate buffer (pH 5.5) and an appropriate amount of enzyme preparation. The assay was carried out at room temperature and absorbance changes were monitored at 470 nm for 1 min at a 30-sec interval. One unit of peroxidase activity is defined as the amount of enzyme that increases the optical density by 1.0 per minute under standard assay conditions.

Protein Determination: Proteins were determined by measuring the absorbance at 280 nm (Warburg and Christian, 1942) for column fractions and by the method of Bradford (1976) for quantitative determination of protein in pooled fractions using bovine serum albumin (BSA) as standard.

Enzyme characterization

1. Substrate specificity: Substrate specificity for ELP was studied using H₂O₂ in combination with a number of potential natural electron
donor substrates, and enzyme activity was compared with guaiacol, which was regarded as 100% activity. The reaction mixture in 1.0 ml contained; 8 mM H₂O₂, 40 mM of each substrate, 2 units of ELP and 50 mM sodium acetate buffer (pH 5.5). Absorbance changes were monitored at 470 nm, 415 nm, 460 nm, 445 nm, 290 nm and 510 nm for guaiacol, ABTS, o-dianisidine, OPD, ascorbic acid and AAP, respectively.

2. **Optimum pH:** The pH profile for the purified ELP was performed using guaiacol as a substrate.

3. **Kinetic studies:** The Michaelis constant (Kₘ) and maximum catalytic rate (Vₘₐₓ) for ELP towards different substrates were calculated from Lineweaver-Burk plotting (Lineweaver and Burk, 1934).

4. **Optimum temperature, thermal stability and thermal inactivation in the absence and presence of calcium ions:** The optimum temperature was determined by measuring ELP activity under different temperatures ranging from 10 to 90°C. In order to determine the thermal stability of the purified ELP, the enzyme solution was incubated at different temperatures ranging from 0°C to 90°C for 15 min. After the incubation, the enzyme was cooled in an ice bath and the residual enzymatic activity was measured using the standard assay method. For thermal stability study, purified ELP (in 50 mM sodium acetate buffer, pH 5.5) was pre-incubated for 15, 30, 45 and 60 min in absence or presence of calcium ions (1 and 5 mM CaCl₂) at different temperatures (40, 50, 60 and 70°C). After incubation, the enzyme was cooled in an ice bath and immediately assayed for peroxidase activity.

5. **Effect of 5 mM CaCl₂ on ELP activity before and after incubation at 70°C:** The effect of CaCl₂ addition at the concentration of 5 mM before and after incubation of the enzyme at 70 °C (at which the enzyme is relatively thermally inactivated) was investigated. The enzyme (in 50 mM sodium acetate buffer, pH 5.5) was pre-incubated with 5 mM CaCl₂ for 30 min at 70°C then cooled in an ice bath and immediately assayed for peroxidase activity. Another test was done, in which the enzyme (in 50 mM sodium acetate buffer, pH 5.5) was pre-incubated in the absence of CaCl₂ for 30 min at 70°C then cooled in an ice bath and immediately assayed for peroxidase activity in the presence of 5 mM CaCl₂. The activity of ELP at the standard assay method (40°C) was considered as 100%.
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6. Effect of different Ca\(^{2+}\) concentrations on ELP activity: Purified ELP (in 50 mM sodium acetate buffer, pH 5.5) was pre-incubated at 40 °C with CaCl\(_2\) (1 to 20 mM) for 15 min and the enzymatic activity was measured using the standard assay method.

7. Effect of metal cations: Purified ELP was pre-incubated for 15 min at room temperature with various metal cations (Ca\(^{2+}\), Mg\(^{2+}\), Ba\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), Al\(^{3+}\), Mn\(^{2+}\), K\(^{+}\), Hg\(^{2+}\) and Cu\(^{2+}\)) at 1 and 5 mM concentrations followed by residual activity measurement. Activity in the absence of metal cations was taken as 100%.

8. Effect of various compounds: Purified ELP was pre-incubated for 15 min at room temperature with various metal cations (sodium dichromate, sodium azide, sodium sulfite, phenylmethylsulfonyl fluoride [PMSF], ethylenediamine tetraacetic acid [EDTA], citric acid, 1,10-phenanthroline, hydroxylamine, iodoacetic acid [IAA], dithiothreitol [DTT] and 5,5’-dithiobis-(2-nitrobenzoic acid) [DTNB]) at 1 and 5 mM concentrations following residual activity measurement. Activity in the absence of these compounds was taken as 100%.

RESULTS

*Purification and molecular weight determination of ELP*

A summary of each purification step for ELP is summarized in Table 1. Following benzene fractionation, ELP activity was recovered in the aqueous layer, with a specific activity of 1303 U/mg protein. Fractions of the Sephacryl S-200 column revealed a single peak of ELP activity (Fig. 1). The pooled fractions of the major peak from the Sephacryl S-200 column were applied onto a CM-Sepharose column. A cationic peroxidase was eluted into a single peak at about 0.1 M NaCl (Fig. 2).

The molecular weight of ELP was estimated to be 44 kDa by gel filtration chromatography. Similarly, SDS-PAGE of purified ELP showed a single band with a molecular weight of 44 kDa, indicating that the enzyme consists of a single polypeptide chain (Fig. 3).

*Determination of substrate specificity and optimum pH*

ELP showed highest affinity towards azino- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; 173%) as compared to guaiacol (regarded as 100% activity), while the affinity decreased in the order of o-phenylenediamine (OPD; 83%) and 4-aminoantipyrine (AAP; 73%).
In contrast, ELP had no affinity towards ascorbic acid and o-dianisidine (0%). On the other hand, ELP exhibited a pH optimum at 7.0 (Fig. 4).

**Kinetic studies**
The linear nature of Lineweaver and Burk plots (Fig. 5A-E) showed that ELP followed Michaelis-Menten kinetics towards H$_2$O$_2$ and different electron donor substrates including ABTS, guaiacol, AAP and o-phenylenediamine. ABTS showed the highest affinity towards ELP, where $K_m$, $V_{max}$ and $V_{max}/K_m$ values were 0.503, 500 and 994.04, respectively, whereas AAP and o-phenylenediamine showed the lowest affinity (Table 2).

**Temperature activity profiles**
The effect of temperature on purified ELP activity showed that the enzyme had an optimum temperature at 40°C, followed by an abrupt decrease in activity where it lost 40% at 60°C and a complete loss of activity at 70°C (Fig. 6A). Regarding the effect of temperature on enzyme stability, ELP was approximately stable up to 50°C. The activity of enzyme gradually decreased with increasing temperature, where the enzyme retained 87% of its activity at 60°C followed by a loss of 70% at 70°C and a complete loss of activity at 80°C (Fig. 6B).

**Thermal inactivation profiles of ELP in absence and presence of Ca$^{2+}$ ions**
In the absence of Ca$^{2+}$ ions, the profile is biphasic and characterized by a rapid decline in activity on exposure to heat, followed by a more gradual decrease in activity on continued exposure (Fig. 7A). However, in the presence of Ca$^{2+}$ ions, a rapid increase in ELP activity on exposure to heat for 15 min was observed, followed by a slight increase in activity at 40 and 50°C, and in contrast a more gradual decrease in activity on continued exposure at 60 and 70°C. Furthermore, Ca$^{2+}$ ions prevented the thermal inactivation of ELP in a manner directly dependent on the metal ion concentration (1 and 5 mM; Fig. 7B and C, respectively). On the other hand, a drastic activation before (224%) and after (115%) incubation of ELP at 70°C with 5 mM CaCl$_2$ was recorded, with respect to the activity measured at 70°C without CaCl$_2$ addition (3.7%).

**Effect of Ca$^{2+}$ on ELP activity**
It is noteworthy to mention that the activity of ELP was highly
induced following incubation with increased Ca^{2+} concentration up to 15 mM (550% activity increase), followed by a relative decrease in the activity at 20 mM (450% activity increase) (Fig. 8).

**Effect of metal cations and compounds on ELP activity**

Most of the examined metal ions had inhibitory effects on ELP activity, except for Ca^{2+} at 1 and 5 mM concentrations, as well as Mg^{2+} at 1 mM concentration. On the other hand, 1,10-phenanthroline, IAA and PMSF at both concentrations (1 and 5 mM) had weak to moderate inhibition on ELP activity, whereas the strongest inhibition was recorded for both concentrations of sodium azide and DTT (100% inhibition), as well as EDTA (98% inhibition) and at the 5 mM concentration of sodium sulfite (100% inhibition), hydroxylamine and citric acid (95% inhibition) (Table 3).

**Table 1:** Summary of each purification step of peroxidase from *Euphorbia tirucalli* latex.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Fold purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous layer of benzene extract</td>
<td>3.8</td>
<td>4950</td>
<td>1303</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>0.59</td>
<td>1000</td>
<td>1695</td>
<td>1.3</td>
<td>20</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>0.23</td>
<td>603</td>
<td>2621</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

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**Fig. 1:** A typical elution profile for the chromatography of ELP on Sephacryl S-200 column (90 x 1.6 cm i.d.) equilibrated with 50 mM sodium acetate buffer (pH 5.5) at a flow rate of 20 ml/h and 3-ml fractions.

**Fig. 2:** A typical elution profile for the chromatography of ELP on CM-Sepharose column (6 x 2 cm i.d.) equilibrated with 50 mM sodium acetate buffer (pH 5.5) and eluted with a stepwise of NaCl ranging from 0.0 to 0.5 M at a flow rate of 36 ml/h and 3-ml fractions.
Fig. 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified ELP. Exactly, 38 µg protein for the aqueous layer, 12 µg protein for purified ELP and the standard protein markers were mixed with the sample buffer (4:1, V/V) and incubated for 5 min in a boiling water bath. The samples were loaded, and the electrophoresis was performed at 50 volt for 15 min and then at 150 volt for approximately 2 h until the bromophenol blue marker reached the bottom of the gel. The separated proteins were stained for 2 h at room temperature in the staining solution (Coomassie Brilliant Blue R-250). The gel was diffusion-destained by repeated washing in destaining solution to visualize protein bands. Lane 1; standard proteins, Lane 2; purified ELP, Lane 3; aqueous layer of benzene extract.

Fig. 4: Optimum pH of purified ELP. The reaction mixture contained in 1.0 ml; 8 mM H₂O₂, 40 mM guaiacol, 2 units of ELP enzyme and 50 mM sodium acetate buffer or Tris-HCl buffer. Each point represents the average of two experiments.
Fig. 5: Lineweaver-Burk plot showing $K_m$ values of ELP towards different substrates. The reaction mixture contained in 1.0 ml; 2 units of ELP enzyme, 50 mM sodium acetate buffer (pH 5.5) and different concentrations of ABTS (A), guaiacol (B), $H_2O_2$ (C), AAP (D) and OPD (E). Each point represents the average of two experiments.
**Table 2:** Kinetic parameters of purified *ELP*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (U/assay)</th>
<th>$V_{max}/K_m$ (U/mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>0.503</td>
<td>500</td>
<td>994.04</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>4.4</td>
<td>42</td>
<td>9.55</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>15</td>
<td>19</td>
<td>1.27</td>
</tr>
<tr>
<td>Aminoantipyrine</td>
<td>25</td>
<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td>O-Phenylenediamine</td>
<td>33.4</td>
<td>5</td>
<td>0.15</td>
</tr>
</tbody>
</table>

![Image A](image1)

![Image B](image2)

**Fig. 6:** Purified ELP was found to have an optimum temperature at 40˚C (A). The effect of temperature on ELP thermal stability (B) revealed that the enzyme was approximately stable up to 50˚C, whereas its activity decreased with increasing temperature, with a complete loss of activity at 80˚C. Each point represents the average of two experiments.
Fig. 7: Effect of temperature and incubation time on thermal stability of ELP in the absence (A) or presence of 1 mM (B) or 5mM (C) CaCl₂. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments.

Fig. 8: Percent increase in ELP activity at different concentrations of Ca²⁺. Activity in the absence of Ca²⁺ was taken as 100%. Each point represents the average of two experiments.
**Table 3:** Effect of metal cations and compounds on ELP. Activity in absence of cations or compounds was taken as 100%.

<table>
<thead>
<tr>
<th>Metal cations</th>
<th>% activity 1mM</th>
<th>% activity 5mM</th>
<th>Compounds</th>
<th>% activity 1mM</th>
<th>% activity 5mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>219</td>
<td>470</td>
<td>1,10-Phenanthroline</td>
<td>82</td>
<td>76</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>112</td>
<td>50</td>
<td>IAA</td>
<td>77</td>
<td>26</td>
</tr>
<tr>
<td>Ba^{2+}</td>
<td>57</td>
<td>27</td>
<td>PMSF</td>
<td>59</td>
<td>35</td>
</tr>
<tr>
<td>Ni^{2+}</td>
<td>33</td>
<td>26</td>
<td>Hydroxylami</td>
<td>53</td>
<td>5</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>29</td>
<td>21</td>
<td>Citric acid</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td>20</td>
<td>14</td>
<td>Sodium sulfite</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>Al^{3+}</td>
<td>20</td>
<td>5</td>
<td>DTNB</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>13</td>
<td>11</td>
<td>Sodium dichromate</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>K^{+}</td>
<td>7</td>
<td>7</td>
<td>EDTA</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Hg^{2+}</td>
<td>6</td>
<td>3</td>
<td>DTT</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>3</td>
<td>2</td>
<td>Sodium azide</td>
<td>0</td>
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</tbody>
</table>

**DISCUSSION**

In the present study, a simple reproducible method was adopted for the isolation and purification of a new cationic peroxidase from the latex of *Euphorbia tirucalli* plant (ELP). The specific activity of the purified ELP (2621 U/mg protein) is close to that of peroxidase purified from turnip roots (2760 U/mg protein) (Hamed *et al.*, 1998) and higher than that of *Euphorbia characias* latex (840 U/mg protein) (Floris *et al.*, 1984), as well as *Ipomoea carnea* latex (150.5 U/mg protein) (Patel *et al.*, 2008).

It was not possible to detect any protein bands by SDS-PAGE using directly the thick white latex, probably because of interference of its components. The analysis of isoform species was therefore made following benzene fractionation. The molecular weight of ELP was found to be 44 kDa using a plot of log molecular weight versus Ve/Vo.
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of standard proteins applied on a Sephacryl S-200 column. The electrophoretic behavior of the aqueous layer from benzene extract and purified preparation was examined. A denatured ELP protein migrated as a single band to a position corresponding to a molecular weight of 44 kDa by SDS-PAGE, which indicates that the ELP appears to be a monomer. The variability in the molecular weight of PODs (30-150 kDa; Hamid and Khalil-ur-Rehman, 2009) is attributed to the post-translational modifications and also the length and number of glycan moieties in the polypeptide chain (Van Huystee et al., 1992). Most of peroxidases reported to date are monomers, however, coconut peroxidase exists as a tetramer having a subunit molecular weight of 55 kDa (Mujer et al., 1983) and Leucaena leucocephala peroxidase, which exists as a heterotrimeric structure (consisting of two subunits of 66 kDa and one subunit of 58 kDa) (Pandey and Dwivedi, 2011).

With guaiacol as a substrate, the pH optimum for ELP was found to be 7.0 in Tris-HCl buffer and the enzyme retained about 70% of its activity at pH 5.5 (sodium acetate buffer). Different pH optima were reported for peroxidases; e.g. from strawberry (pH 6.0) (Civello et al., 1995), Raphanus sativus (pH 7.0) (Kim and Lee, 2005), whereas buckwheat seed PODs and Lycopersicon esculentum POD had alkaline pH optima at 9 (Suzuki et al., 2006). Many other reported plant PODs have their highest activity in acidic condition. For example, Vigna mungo POD and Momordica charantia POD share a same optimum pH of 5.5 (Ajila and Prasada Rao, 2009). Nicotiana tabacum POD and V. planifolia POD have their highest activity at 4.5 and 3.8, respectively (Márquez et al., 2008; Gazaryan and Lagrimini, 1996), and wheat bran peroxidase at pH 4.8 (Manu and Prasada Rao, 2009).

We found that ELP lost most of its activity at a pH lower than 4.5 and higher than 8.5. It has been reported that many class III PODs lose their activities under extreme pH conditions and the loss of activity might be due to the detachment of the heme group (Kim and Lee, 2005). In contrast, the loss of ELP activity at a high pH may be due to protein denaturation or chemical changes in the heme moieties (Adams, 1997).

ELP catalyzed the oxidation of some phenolic electron donor substrates in the order of ABTS > guaiacol > o-phenylenediamine > 4-aminoantipyrine, whereas it has no affinity towards ascorbic acid and
ROLE OF CALCIUM IN ENHANCING THE ACTIVITY

... o-dianisidine. These results reveal a significant similarity of this cationic enzyme to class III peroxidases (extracellular) and it does not belong to the family of ascorbate peroxidases (class I; intracellular) (Johri et al., 2005).

The lower $K_m$ value and higher $V_{\text{max}}$ of the enzyme indicates its high affinity towards the substrate. ELP was found to have a relatively higher $K_m$ for $\text{H}_2\text{O}_2$ (15 mM) than that reported for pear (1.5 mM) (Richard-Forget and Gauillard, 1997), Brassica oleracea isoenzymes (11.4 and 6.2 mM) (Regalado et al., 1999), marula fruit (1.77 mM) (Mdluli, 2005), Euphorbia characias latex (2.8 mM) (Mura et al., 2005), and Leucaena leucocephala (5.6 mM) (Pandey and Dwivedi, 2011). ABTS and guaiacol showed high affinity towards ELP, whereas AAP and OPD manifested lower affinity. Various $K_m$ values using different electron donor substrates were reported for peroxidases from different plant sources such as Elaeis guineensis leaf (3.96 mM guaiacol and 1mM ABTS) (Deepa and Arumughan, 2002), Euphorbia characias latex (1.3 mM ABTS) (Mura et al., 2005), buckwheat seeds (0.288 mM guaiacol and 0.229 mM o-dianisidine) (Suzuki et al., 2006), and Leucaena leucocephala (3.8 mM guaiacol) (Pandey and Dwivedi, 2011).

The optimum temperature for ELP activity was found to be 40˚C. Similar results were reported for peroxidases from Citrus jambhiri peel (40˚C) (Mohamed et al., 2008) and Solanum melongena (35˚C) (Vernwal et al., 2006). A wide variability concerning the optimum temperature for peroxidase activity has been reported from various sources including Allium sativum (84˚C) (Marzouki et al., 2005), buckwheat seed (10-30˚C) (Suzuki et al., 2006) and vanilla bean (16˚C) (Márquez et al., 2008).

ELP was reported in the present study to be relatively stable at 10 - 60˚C and unstable above 70˚C, with a complete inactivation at 80˚C. Plant peroxidases from different sources exhibit differential thermolability. The thermal stabilities of two buckwheat seed isoenzymes (POD I and POD II) were reported by Suzuki et al. (2006). POD I was stable at 0-30˚C and unstable above 40˚C, whereas POD II was stable at 20˚C and unstable above 30˚C. Ipomoea carnea POD had a reasonable activity up to 75˚C followed by an abrupt loss of activity at higher temperatures (Patel et al., 2008). A peroxidase from Australian carrot was completely inactivated by heating at 80˚C for 4 min, whereas a turnip anionic peroxidase retained good activity...
(~21%) even after 25 min heating at 80°C (Manu and Prasada Rao, 2009). The variability in the heat stability of POD can be largely attributed to the particular enzyme structure. Non-covalent, electrostatic and hydrophobic interactions of individual isoenzymes determine enzyme folding and stability, as well as, hydrogen bonds and the degree of glycosylation, which has also been found to play a role in enzyme stability (Adams, 1991). It has also been shown that the thermal stability of POD is mainly due to the presence of a large number of cysteine residues in the polypeptide chain (Deepa and Arumughan, 2002; Johri et al., 2005).

In agreement with our results concerning the thermal inactivation profile of ELP in the absence of Ca\(^{2+}\) ions, thermal treatment profiles of peroxidases from mango (Khan and Robinson, 1993), apple (Valderrama and Clemente, 2004), Withania somnifera (Johri et al., 2005) and marula fruit (Mdluli, 2005) proved to be nonlinear and biphasic in relation to the factor of time and temperature. On the other hand, Ca\(^{2+}\) improved the thermal stability of ELP in a manner directly dependent on the metal ion concentration (1 and 5 mM), pointing out to the protective role of Ca\(^{2+}\) ions in thermal inactivation of ELP. Heating of wheat bran peroxidase at 60 °C for 25 min destroyed almost all of the enzyme’s activity, whereas in the presence of 1 mM Ca\(^{2+}\), the enzyme retained about 50% of activity even after heating for 25 min at 60 °C (Manu and Prasada Rao, 2009). In addition, the differential enhancing effect following addition of 5 mM CaCl\(_2\) before and after incubation of ELP at 70 °C indicates that Ca\(^{2+}\) ions reactivate the thermally inactivated enzyme, suggesting that Ca\(^{2+}\) acts as a stabilizer and activator for ELP. Manu and Prasada Rao (2009) reported that wheat bran peroxidase is the first example of a peroxidase, which exhibits both Ca\(^{2+}\)-dependent enhancement in activity and thermal stability.

Peroxidases generally contain two binding sites for Ca\(^{2+}\) (distal and proximal sites), which are very essential for the structural and thermal stabilities of the enzyme (Maranon and Van Huystee, 1994). Although Euphorbia characias latex peroxidase has two calcium binding sites (proximal and distal), the purified protein contains 1 mol of endogenous Ca\(^{2+}\)/mol enzyme, and it is strongly bound to the proximal site. This calcium ion plays a critical role in retaining the active site geometry of the enzyme (Pintus et al., 2011). The addition of a second Ca\(^{2+}\) ion to the low affinity binding distal site of the native
**Euphorbia characias** latex peroxidase is necessary for expression of the full activity of the enzyme. The drastic enhancement of $K_{cat}/K_{m}$ that this Ca$^{2+}$ produces, together with the ease of its removal, suggests that the enzyme activity is regulated strictly by the presence of calcium. The loss of the proximal Ca$^{2+}$ ion induced by temperature causes distinct modifications in the heme environment, without gross changes in the protein secondary and tertiary structure, which lead to essentially a reversible inactivation. Considering that Ca$^{2+}$ has a second messenger action in plants, this activation could be a control mechanism of *Euphorbia characias* latex peroxidase activity of physiological relevance (Medda et al., 2003). Calcium-dependent thermal stability is important in industrial applications of peroxidases such as wastewater treatment, food processing and phenolic resin synthesis, among others, which involve high temperatures to achieve better economic viability of the process (Manu and Prasada Rao, 2009).

In the present study, ELP activity was highly increased with increasing Ca$^{2+}$ ion concentration up to 15 mM (550% activity increase). Similarly, a 1100-fold activation was observed in *Euphorbia characias* latex peroxidase activity at 10 mM Ca$^{2+}$ ion concentration (Medda et al., 2003), as well as wheat bran peroxidase that showed maximal activity (444%) at 3 mM Ca$^{2+}$ ion concentration (Manu and Prasada Rao, 2009). *Euphorbia* peroxidase is a protein, which carries out several specific functions only finely tuned by calcium (Pintus et al., 2011).

The literature implies controversial results concerning the inhibitory effect of metal ions on peroxidases. We reported a 370% increase in ELP activity at 5 mM CaCl$_2$ concentration, whereas the tested metal ions at the same concentration inhibits ELP in the order of Mg$^{2+}$<Ba$^{2+}$<Ni$^{2+}$<Co$^{2+}$<Fe$^{2+}$<Al$^{3+}$<Mn$^{2+}$<K$^+$<Hg$^{2+}$ and <Cu$^{2+}$, which means that such inhibition depends upon the chemical structure of the metal ions used. Marzouki et al. (2005) reported that *Allium sativum* peroxidase was inhibited by all of the tested metallic ions (at 5 mM concentration) including Fe$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$. Mohamed et al. (2008) documented that most of the examined metal ions (Ba$^{2+}$, Co$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Fe$^{3+}$, Cu$^{2+}$ and Ni$^{2+}$) had very slightly effect on *Citrus jambhiri* peel peroxidase (POII), whereas a moderate inhibitory effect was recorded for Li$^+$ and Zn$^{2+}$, and in contrast Hg$^{2+}$ had a strong inhibitory effect. The activity of *Jatropha curcas* leaves peroxidase
was slightly increased by 1 mM Cu$^{2+}$ and 5 mM Pb$^{2+}$ & Zn$^{2+}$, an low inhibition was recorded with Mn$^{2+}$ and Mg$^{2+}$ (10 mM), whereas Al$^{3+}$, Ni$^{2+}$, Co$^{2+}$, Li$^+$ and Cd$^{2+}$ had no significant effect on POD activity. About 30% activity increase was observed with 1 mM Ba$^{2+}$ while 40% inhibition was caused by 10 mM Fe$^{2+}$ (Cai et al., 2012). Sodium azide, hydroxylamine, sodium sulfite, and sodium dichromate (typical inhibitors of heme proteins), PMSF (a serine inhibitor), 1,10-phenanthroline and citric acid (metal chelators), IAA and DTNB (blockers of catalytic SH residues), and EDTA (chelator of Ca$^{2+}$) were found to inhibit ELP activity with different degrees in the present study, suggesting the presence of serine and cysteine amino acids and metal residues in the enzyme’s active site, which corresponds with the structure characterization of class III peroxidase. In addition, the inhibitory effect of DTT suggests that disulfide bonds within the structure of ELP were responsible for its peroxidase activity (Cai et al., 2012).

In conclusion, the properties of the purified *E. tirucalli* latex peroxidase in the current study meet the prerequisites needed for extensive potential analytical, biomedical, industrial and food processing applications especially its calcium-enhanced thermal stability and high affinity towards some phenolic substrates.

REFERENCES


**Civello, PM, Martinez, GA, Chaves, AR and Anon, MC (1995):** Peroxidase from strawberry fruit (*Fragaria ananassa* Duch): Partial
purification and determination of some properties. J. Agric. Food Chem. 43: 2596-2601.


Patel, AK, Singh, VK, Moir, AJ and Jagannadham, MV (2008): Biochemical and spectroscopic characterization of morning glory peroxidase from an invasive and hallucinogenic plant weed Ipomoea 266

**Pintus, F, Spanò, D, Medda, R and Floris, G (2011):** Calcium ions and a secreted peroxidase in *Euphorbia characias* latex are made for each other. Protein J. 30: 115-123.


دور الكالسيوم في زيادة نشاط الأيونات في النباتات الحارية

المختصر العربي

تم تطبيق إنزيم البروكسيديز الكاثيوتي من العصارة اللبنية لنباتات الإليوفروبية تريكايلي على درجة التجانس باستخدام الصلب بالبوزنون و أعدم الجل التربة الكروموجرافي (السيكاريلإس 400 و البروكسيسي ميليف-سفاراز). وقد تم التأكد من أن الإنزيم يتحرك في حزمة درجة التحليض بأي جل البولي أكريليميد كبريتيد دودسل الصوديوم في جهاز الفصل، بما يدل على أنه ينتمي من حزمة واحدة بوزن جزيئي 44 كيلو دالتنون. ومن خلال دراسة خواص الإنزيم الففي، تم قدرته على أكسة بعض المواد الفيتولوجية وألله أس هيدروجيني (pH) أمتلك عند 7 و درجة حرارة مثلى عند 400 م. وقد أوضحت الدراسة الحيوية أن أنيرب الأيونيابين (ABTS) أعلق قابلية تجاج ELP، حيث أن قيمتين ثابتة مكيفية (Km) و أقصى معدل تحميي (Vmax) لما 0.503 mM و 0.3400 U/ASSAY. ووجد أن الإنزيم يتحمل درجات الحرارة من 10 إلى 60 م. ثم فقد 70% من نشاطه عند 20 م. وقد وصف النتائج الحرارية لنشاط الإنزيم في عم ووجود أيونات الكالسيوم بالبوزنون الرمزي ثم المندرج عند التعرض للحرارة. وعلى الرغم من ذلك، تم تسجيل زيادة الثبات الحراري للإنزيم في وجود أيونات الكالسيوم. وتمت دراسة تأثير الأيونات المختلفة من أيونات الكالسيوم على ELP حيث زاد نشاط الإنزيم المفق ليوم 15 مل مللي مول في وقود كلربايد الكالسيوم، مما يوجه بالدور بالدائم للإيبروبة تريكايلي. الأيونات في النباتات الحارية ميلينة للهيم وفي الحفاظ على الموقع النشط للإنزيم. وقد وجد أن معظم الأيونات الفيزيائية (فمثلا أيونات معدني الكالسيوم والمغنيسيوم) ومركبات التي أظهرت لها تأثير مبطن جزيئي على الإنزيم. و تأشيس الدراسة إلى إمكانية استخدام بئة الإليوفروبة تريكايلي المنتج محليا في مصر كصدر الإنزيم البروكسيديز الذي يستخدم في العديد من التطبيقات المصنعة والطبية الحيوية. و أثبتت الدراسة أيضا أهمية أيونات الكالسيوم في تحرير نشاط و زيادة الثبات الحراري للإنزيم النفي المقصود من العصارة اللبنية لنباتات الإليوفروبة تريكايلي.