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Review Of Research

# BIOCHEMICAL CHARACTERIZATION OF PEROXIDASE FROM CALOTROPIS PROCERA LATEX

ABSTRACT:

Peroxidase from Calotropisproceralatex was purified by benzene treatment, DEAE-Sepharose and Sephacryl S-200 column. The purity was determined from the high specific activity (2200 units/mg protein), purification fold (7.0), and a single band in native PAGE, SDS-PAGE. Peroxidase had molecular mass of 45 kDa. Phenylenediamine had the highest peroxidase activity (140%), o-dianisidine had moderate activity (83%). p-Aminoantipyrine (42%) and pyrogallol (33%) had low affinity towards enzyme. The apparent Km value ofperoxidase from C. procera latex for H2O2 and guaiacol were 6.8 and 11.4 mM/ml, respectively. The enzyme showed a pH optimum at 5.5, whereasthe optimal temperature was 40°C. The enzyme activity was remained stable up to 40° C. Both Cu2+ and Fe2+ caused the highest activation at 2 and 5 mM (260, 115 and 290, 230%, respectively). Hg2+ caused the highest inhibitory effect. In conclusion, Calotropisproceralatex could be a new and potential source for peroxidase enzyme.

KEY WORDS:

Calotropisprocera, Latex, Peroxidase, Purification, Characterization.

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## INTRODUCTION

Peroxidases arewidelydistributed in plants, microbes, fungi and vertebrates. These enzymesact as an oxidoreductase that catalyzes a reaction, in which $H_2O_2$ actas the acceptor and another compound act as the donor of hydrogenatoms (Ikehata*et al*., 2005).Peroxidases could be broadly classified into threemajor classes. Plant ascorbate peroxidase, cytochrome c peroxidaseand bacterial peroxidase belong to same evolutionary branchand are designated as class I, whereas secretory fungal enzymes,ligninolytic peroxidase (Moreira *et al*., 2005),and manganese (Mn) peroxidase from fungi(Moreira *et al*., 2006)are classified as class II peroxidases. Secretory plant peroxidasesand vacuolar peroxidases such as horseradish peroxidases thatusually contain a ferriprotoporphyrin IX prosthetic group linkedto His residue are designated as class III peroxidases (Onsa*et al*., 2004). Class III peroxidaseshave been reported to play physiological roles such ascatabolizing phenolic compounds for biosynthetic and catabolic functions (Hamid and Khalil-ur-Rehman, 2009), cross linking of cell wall polysaccharide, cell elongation regulation, wound healing, abiotic stress, ethylene biosynthesis, scavenging of peroxides, oxidation of toxic compounds, defense mechanism towardspathogens, biodegradation reactions, metabolism of plant hormone, indole acetic acid (IAA) oxidation, lignification of cell wall (Manu and Rao, 2009) and anthocyanin degradation (Zhang *et al*., 2005).

Peroxidases were involved in production of alkaloid, biosensor construction, air pollution damage control (Lee, 2002), food processing, food storage, biotransformation of organic compounds, treatment of industrial waste water containing phenols and aromatic amines (Rudrappa*et al*., 2007), production of oxidants, lignin degradation in fuels, bio-bleaching process, site directedmutagenesis (Dubey*et al*., 2007), production of secondary antibodies for researchand medical diagnosis, production of dimeric alkaloids, preventingdeterioration of many frozen fruits Quality, biotransformation oforganic compounds, preventing deterioration of prepared foods (Dicko*et al*., 2006)and the combination of peroxidase with IAA was introduced as a novel cancer therapy(Kim et al., 2004).

*Calotropisprocera* was known to possess multifarious medicinal properties. The root of the plant was used as a carminative in the treatment of dyspepsia (Kumar and Arya, 2006). Furthermore, the root bark and leaves of *Calotropisprocera* were used by various tribes of central India as a curative agent for jaundice (Samvatsar and Diwanji, 2000). The chloroform extract of the root was shown to exhibit protective activity against carbon tetrachloride induced liver damage (Basu*et al.*, 1992). The milky white latex of this plant was reported to exhibit potent anti-inflammatory, analgesic and weak antipyretic activity in various experimental models (Kumar and Arya, 2006; Kumar and Basu, 1994; Dewan*et al.*, 2000a,b),besides, it was also demonstrated to possess antioxidant and anti-hyperglycemic property (Roy *et al*., 2005). The aim of present work is to study the biochemical properties of peroxidase as antioxidant enzyme from latex of *Calotropisprocerain* view of its applicationsin biotechnology and food industries.

## MATERIALAND METHODS

### Plant materials:

The latex of *Calotropisprocera* was collected by breaking the stem.

### Purification of peroxidase from *Calotropisproceralatex*:

The latex was separated from rubber content by adding 0.5 ml of benzene to each 1.0 ml of latex followed by centrifugation at 16500 Xg for 15 min at room temperature, and two layers was obtained.The upper benzene layer designated as crude extract. The crude extract was loaded on a DEAE-Sepharose CL-6B column (10 x 1.6 cm i.d.) previously equilibrated with 20 mMTris-HCl buffer, pH 7.2. The enzyme was eluted with a stepwise gradient from 0.0 to 0.3 M NaCl in the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Protein fractions exhibiting peroxidase activity were pooled in one peak. DEAE-Sepharose peroxidase fraction was concentrated through dialysis against solid sucrose and loaded on Sephacryl S-200 column (90 x 1.6 cm i.d.) previously equilibrated with 20 mMTris-HCl buffer, pH 7.2 and developed at a flow rate of 30 ml/h and 3 ml fractions were collected.

### Molecular weight determination:

Enzyme molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (90 × 1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and ß-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for the calibration curve.

### Protein determination:

Protein was determined either by the method of Bradford (1976) using bovine serum albumin as a standard.

### Peroxidase assay:

Peroxidase activity was carried out according to Miranda et al. (1995). The reaction mixture contained in one ml, 8 mM $H_2O_2$, 40 mMguaiacol, 50 mM sodium acetate buffer, pH 6.5 and least amount of enzyme preparation. The change of absorbance at 470 nm due to guaiacol oxidation was followed at three

minutes intervals. One unit of peroxidase activity is defined as the amount of enzyme that increases the O.D. 1.0 per min under standard assay conditions.

**Characterization of peroxidase**

**Substrate specificity:**

The substrate specificity of the enzyme was determined using a number of potential natural electron donors are used under the standard assay conditions.

**Kinetic constant:**

Kinetic parameters of the peroxidase for hydrogen peroxide and guaiacol as substrates were determined at pH 5.5. The values of Michael's constants (Km) were determined from Lineweaver Burk plot.

**Optimum pH and temperature:**

The optimum pH for the peroxidase activity was determined by assaying the activity at different pH values, using the following buffers: 50 mM sodium acetate buffer (pH 4.5 to 6.5) and 50 mMTris-HCl buffer (pH 7.0 to 8.5). The optimum temperature for peroxidase activity was determined by assaying the enzyme at temperatures from 30 to 80°C at pH 5.5.

**Thermal stability:**

Heat stability was measured by incubating the enzyme at 30 to 80ºC for 15 min in 0.05 mM sodium acetate buffer, pH 5.5. After heat treatment, the enzyme solution was cooled and the residual activity was assayed under standard assay conditions.

**Metal ion effect:**

The effects of various metal ions on enzyme activity were determined by pre-incubating the enzyme with 2 and 5 mM metal ions for 15 min before assaying the enzyme activity. The activity in absence of metal ions was considered as 100%.

## RESULTS AND DISCUSSION

The purification procedure of peroxidase from *Calotropisprocera* latex included chromatography on DEAE-Sepharose column followed by Sephacryl S-200 column. The purification was summarized in Table (1). The elution profile of the chromatography on DEAE-Sepharose column showed that peroxidase activity was detected in one peak eluted by 0.0 M NaCl. The specific activity of peroxidase was 632 units/mg protein that represented 2.0 fold purification over the crude extract with 80 % recovery (Fig. 1). A Sephacryl S-200 column was used to obtain peroxidase with the highest possible specific activity of 2200 units/mg protein that represented 7.0 fold purification over the crude extract with 56 % recovery (Fig. 2). The *C. procera* latex peroxidase is proved to be pure after Sephacryl S-200 column as judged by SDS-polyacrylamide gel electrophoresis (Fig. 3).The molecular weight of peroxidase from *C. procera* latex was calculated from a Sephacryl S-200 calibration curve and estimated to be 45kDa. This was confirmed by SDS-PAGE (Fig. 3), and estimated to be 44 kDa, as single subunit.

**Table 1: Purification scheme of peroxidase from *C. procera* latex.**

| Step | Total protein (mg) | Total activity (units*) | S.A. (units/ mg protein) | Fold purification | Recovery % |
|---|---|---|---|---|---|
| Crude extract | 3.1 | 980 | 316 | 1 | 100 |
| Chromatography on DEAE- Sepharose 0.0 M NaCl | 1.25 | 790 | 632 | 2.0 | 80 |
| Gel filtration on Sephacryl S-200 Peroxidase | 0.25 | 550 | 2200 | 7.0 | 56 |

*One unit of peroxidase activity is defined as the amount of enzyme that increases the O.D. 1.0 per min under standard assay conditions.

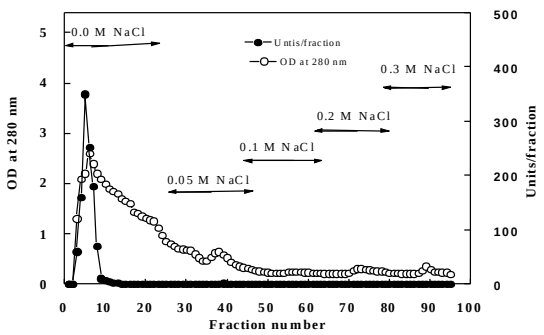

**Fig. 1: A typical elution profile for the chromatography ofperoxidase from *C. procera* latex on DEAE-Sepharose column (10 x 1.6 cm i.d.) previously equilibrated with 20 mMTris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.**

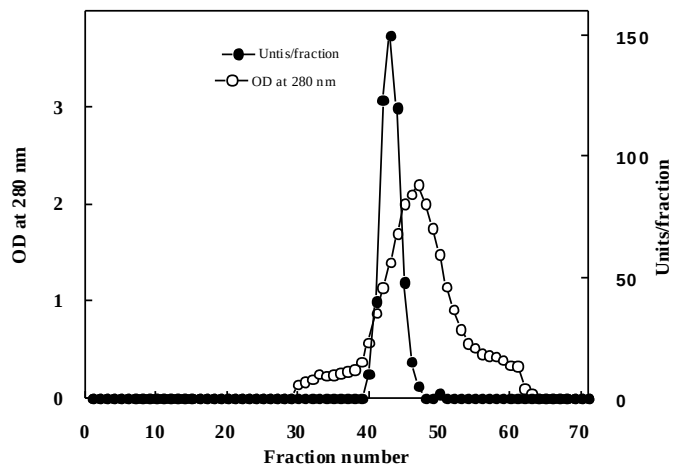


**Fig. 2: Gel filtration ofperoxidase from *C. procera* latex from DEAE-Sepharose fraction on Sephacryl S-200 column (90 x 1.6 cm i.d.). The column was equilibrated with 20 mMTris-HCl buffer, pH 7.2 at a flow rate of 30 ml/h and 3 ml fractions.**

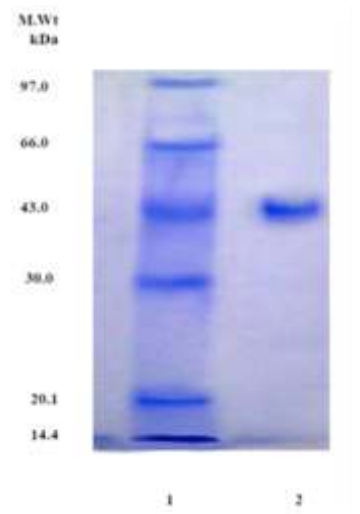


**Fig. 3: SDS-PAGE for homogeneity and molecular weight determination of peroxidase from *C. proceralatex* .1- Protein markers; 2- Sephacryl S-200 peroxidase.**

This molecular weight was similar to that reported for peroxidases from *Ficussycomoruslatex* (43 kDa) (Mohamed et al., 2011a), *Raphanussativus* (44 kDa) (Kim et al., 2005)and *Withaniasomnifera*(34-48 kDa) (Johri*et al*., 2005).Most of the peroxidases reported to date are monomers. Some exceptionswere reported for peroxidases from coconut (tetramer subunit molecular weight of 55 kDa) (Mujer*et al*., 1983), oil palm leaf (native and tetramer subunit molecular weights of 200 and 48 kDa, respectively) (Deepa and Arumughan 2002) and vanilla bean (native and tetramer subunit molecular weights of 186 and 46.5 kDa, respectively) (Marqueza*et al.*, 2008).

The substrate specificity of peroxidase from *C. procera* latex was examined using a number of potential natural electron donor substrates (Table 2). The activity with the guaiacol is regarded as 100% activity. o-Phenylenediamine had the highest peroxidase activity (140%), while o-dianisidine had moderate activity (83%). p-Aminoantipyrine (42%) and pyrogallol (33%) had lower affinity towards the enzyme.Similarly, o-phenylenediamine was found to be the best substrate for peroxidase from chewing stick miswak followed by guaiacol, o-dianisidine, pyrogallol and p-aminoantipyrine (Mohamed *et al*., 2012). Horseradish peroxidase exhibited high activity toward o-phenylenediamine and guaiacol, while o-dianisidine had moderate peroxidase activity. Pyrogallol and p-aminoantipyrine had low affinity toward horseradish peroxidase (Mohamed et al., 2011b). Substrates commonly used in immunodiagnostic kits as 2,2-azino-bis [3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS), 4-chloro-1- naphthol (4C-1N), o-phenylenediamine and 3,3`,5,5`- tetramethylbenzidine (TMB) were found to be the best substrates for peroxidase from *Ficussycomoruslatex* (Mohamed *et al*., 2011a). *Withaniasomnifera* peroxidases readily catalyzed the oxidation of phynolic substrates like guaiacol and o-diansidinebut did not catalyze ascorbic acid (Johriet al., 2005). Kim et al. (2005) reported that *Raphanussativus* peroxidase had higher affinity

towardso-dianisidine than guaiacol and o-phenylenediamine. o-Dianisidine was also found to be the best substrate for black gram husk peroxidase followed by guaiacol and pyrogallol (Ajila and Rao, 2009).

**Table 2: Relative activities of peroxidase from *C. procera* latex toward different substrates.**

| Substrate | Relative activity (%) |
|---|---|
| Guaiacol | 100 |
| *o*-Phenylenediamine | 140 |
| *o*-Dianisidine | 83 |
| *p*-Aminoantipyrine | 42 |
| Pyrogallol | 33 |

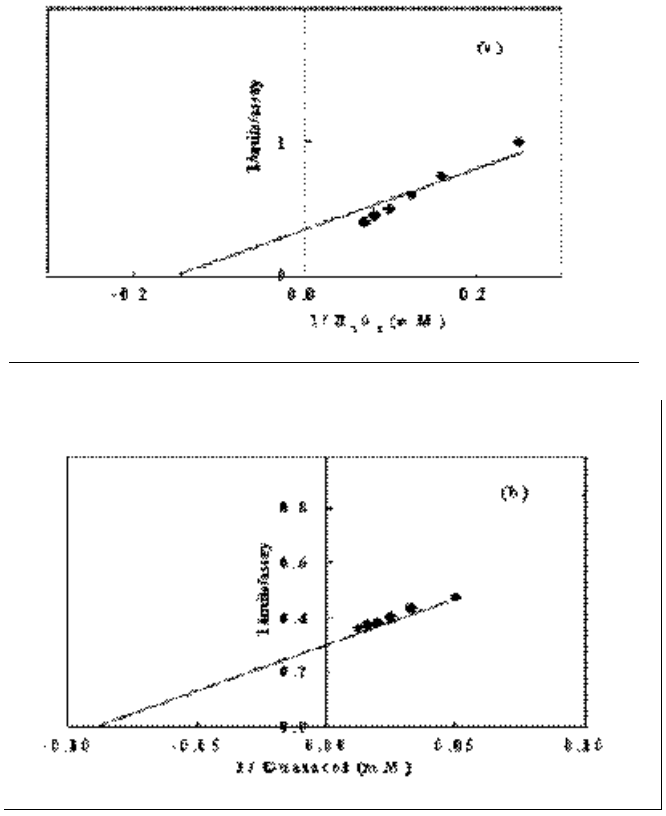

**Fig. 4: Lineweaver-Burk plot relating ofperoxidase from *C. procera* latex reaction velocities to $H_2O_2$ (a) and guaiacol (b) as substrate concentration.**

The kinetic parameters of peroxidase from *C. procera* latex for $H_2O_2$ and guaiacol were obtained (Fig. 4). The apparent Km value ofperoxidase from *C. procera* latex for $H_2O_2$ and guaiacol were 6.8 and 11.4mM/ml, respectively. Different Km values of $H_2O_2$were reported for peroxidases from *C. jambhiri* (0.5 mM) (Mohamed *et al*., 2008), *Ficussycomoruslatex* (1.2 mM) (Mohamed *et al*., 2011a), chewing stick miswak(0.9 mM) (Mohamed et al., 2012), turnip (0.85 mM) (Duarte-Vazquez et al., 2001), marula fruit (1.77 mM) (Mdluli, 2005), *Raphanussativus* (1.27 mM) (Kim *et al*., 2005). The apparent $K_m$ value of peroxidase from *C. procera* latex for guaiacol was 11.2 mM/ml. Different $K_m$ values of guaiacolwere reported for peroxidases from buckwheat seeds (0.202-0.288 mMguaiacol) (Suzuki *et al*., 2006), *Pleargoniumgraveolense* (7.3 mMguaiacol) (Seok*et al*., 2001), *Brassica napus*(3.7 mMguaiacol) (Duarte-Vazquez *et al*., 2001), *chewing stickmiswak*(17.33 mMguaiacol) (Mohamed *et al*., 2012) and C. jambhiri (5 mMguaiacol) (Mohamed *et al*., 2008).

The pH profile for the *purifiedperoxidase* from *C. proceralatex* was carried out. *Peroxidase* activity exhibited a pH optimum at 5.5 (Fig. 5). pHOptimum of 5.5 was detected for *peroxidases* from horseradish (Mohamed *et al*., 2011b),chewing stick miswak (Mohamed *et al*., 2012), black gram husk (Ajila and Rao, 2009), date palm leaves (Al-Senaidy and Ismael, 2011) and Citrus jambhiricv. Adalia (Mohamed *et al.*, 2008).

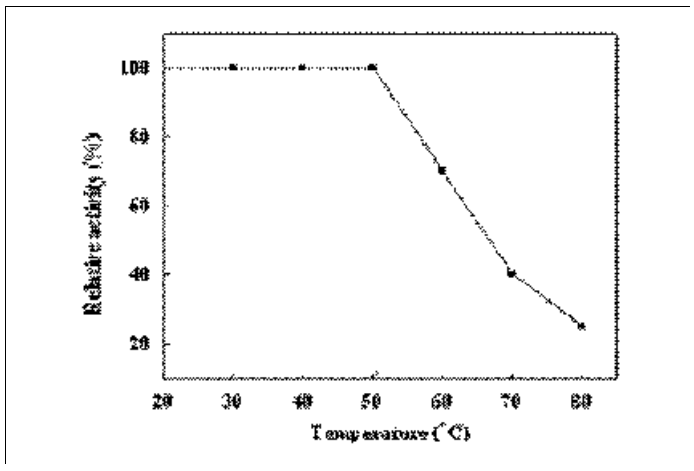


**Fig. 7: Thermal stability of *peroxidase* from *C. procera* latex.**

The effect of different metal ions on *peroxidase* from *C. procera* latex was examined (Table 3). $Cu^{2+}$,$Fe^{2+}$ and $Co^{2+}$were found to enhanced activity of peroxidase, while both $Cu^{2+}$ and $Fe^{2+}$ caused the highest activation at 2 and 5 mM (260, 115 and 290, 230%, respectively). The other metals had slight inhibitory effect at 2 mM, while at 5 mM moderate inhibitory effect on enzyme activity was detected. $Hg^{2+}$ caused the highest inhibitory effect compared to the other tested metals. The same result was detected for peroxidase from horseradish except $Hg^{2+}$ had strong inhibitory effect (Mohamed *et al*., 2011b). Mohamed *et al*.(2008) reported that most of examined metal ions ($Ba^{2+}$, $Co^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Fe^{3+}$, $Cu^{2+}$ and $Ni^{2+}$) had very little effect on Citrus jambhiriperoxidase. The moderate inhibitory effect on enzyme was found for $Li^{+}$ and $Zn^{2+}$, while $Hg^{2+}$ had strong inhibitory effect. It was reported that $Zn^{2+}$, $Li^{+}$, $Mg^{2+}$, $Ba^{2+}$, $Ca^{2+}$ and $Fe^{3+}$ stimulated the activity of black gram husk peroxidase at 5 mM, whereas$Mn^{2+}$, $Cd^{2+}$, $Al^{3+,}$ $Na^{+}$ and $K^{+}$ moderately inhibited the enzyme at the same concentration (Ajila and Rao, 2009). Most of the metal ions tested had partially inhibitory effects on peroxidase from chewing stick miswak, while $Co^{2+}$, $Fe^{3+}$ and $Ca^{2+}$ were greatly able to enhance the activity (Mohamed *et al*., 2012). The activities of *Metroxylonsaguperoxidases* were highly enhanced by $Al^{3+}$, $Ca^{2+}$ and $Fe^{3+}$ but they were moderately inhibited by $Zn^{2+}$ (Onsa*et al*., 2004). However, vanilla bean peroxidase was strongly inhibited by $Fe^{2+}$ and $Hg^{2+}$ (Marqueza*et al*. 2008). In conclusion, *Calotropisproceralatex* could be a new and potential source for *peroxidase* which might be used in several applications.

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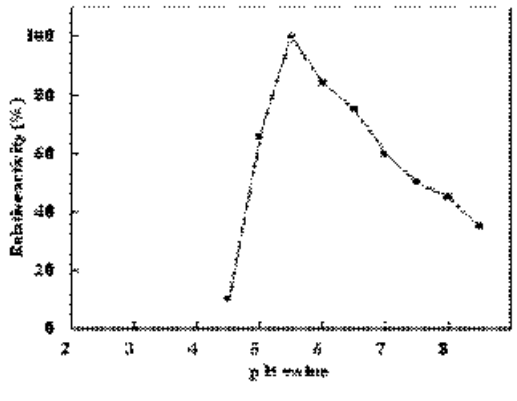

**Fig. 5: pH Optimum ofperoxidase from *C. procera* latex.**

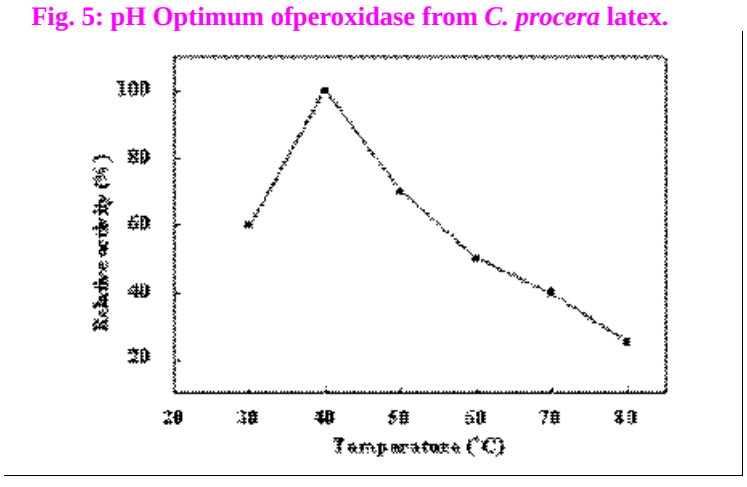


**Fig. 6: Optimum temperature ofperoxidase from *C. procera* latex.**

Peroxidasefrom *C. procera* latexwas found to have temperature optimum at 40℃ (Fig. 6).The same optimum temperature was detected for peroxidases from horseradish (Mohamed *et al*., 2011b) and *Citrus jambhiri* (Mohamed *et al*., 2008). The optimum temperature for *Ficussycomoruslatex* peroxidase was 35 to 40°C (Mohamed *et al*., 2011a). The lowest temperature optima were detected for *peroxidases* from miswak (30℃) (Mohamed *et al*., 2012), buckwheat seed (10-30℃) (Suzuki *et al*., 2006) and vanilla bean (16℃) (Marqueza*et al*., 2008). The effect of temperature on the thermal stability of *peroxidase* from *C. procera* latex wasobtained (Fig. 7). The enzyme activity remained stable up to 40℃. The enzymerestored 70 and 50% of its activity at 50 and 60℃, respectively. Similarly, the thermal stability of *peroxidases* from horseradish (Mohamed *et al*., 2011b), Citrus jambhiri (Mohamed *et al*., 2008), apple (Valderrama and Clemente, 2004), orange (Clemente, 1998), mango (Khan and Robinson, 1993), Withaniasomnifera(Johri*et al*., 2005) and marula fruit (Mdluli, 2005) was reported to remain stable up to 40℃. *Peroxidases* from buckwheat seeds exhibited low thermal stability (20-30℃) (Suzuki *et al*., 2006).

**Table 3: Effect of metal ions on activity of peroxidase from *C. procera* latex.**

| MetalIon | Relative activity (%) | |
|---|---|---|
| | 2 mM | 5 mM |
| $Cu^{2+}$ | 260 | 290 |
| $Fe^{2+}$ | 115 | 230 |
| $Co^{2+}$ | 105 | 130 |
| $Zn^{2+}$ | 95 | 60 |
| $Ca^{2+}$ | 84 | 73 |
| $Ni^{2+}$ | 86 | 57 |
| $Fe^{3+}$ | 81 | 48 |
| $Pb^{2+}$ | 80 | 43 |
| $Hg^{2+}$ | 54 | 33 |

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