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# Properties of a Cationic Peroxidase from *Citrus jambhiri* cv. Adalia

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**Abstract** The major pool of peroxidase activity is present in the peel of some Egyptian citrus species and cultivars compared to the juice and pulp. *Citrus jambhiri* cv. Adalia had the highest peroxidase activity among the examined species. Four anionic and one cationic peroxidase isoenzymes from *C. jambhiri* were detected using the purification procedure including ammonium sulfate precipitation, chromatography on diethylaminoethanol-cellulose, carboxymethyl-cellulose, and Sephacryl S-200 columns. Cationic peroxidase POII is proved to be pure, and its molecular weight was 56 kDa. A study of substrate specificity identified the physiological role of POII, which catalyzed the oxidation of some phenolic substrates in the order of *o*-phenylenediamine>guaiacol>*o*-dianisidine>pyrogallol>catechol. The kinetic parameters ($K_m$, $V_{max}$, and $V_{max}/K_m$) of POII for hydrolysis toward $H_2O_2$ and electron donor substrates were studied. The enzyme had pH and temperature optima at 5.5 and 40 °C, respectively. POII was stable at 10–40 °C and unstable above 50 °C. The thermal inactivation profile of POII is biphasic and characterized by a rapid decline in activity on exposure to heat. The most of POII activity (70–80%) was lost at 50, 60, and 70 °C after 15, 10, and 5 min of incubation, respectively. Most of the examined metal ions had a very slight effect on POII except of $Li^+$, $Zn^{2+}$, and $Hg^{2+}$, which had partial inhibitory effects. In the present study, the instability of peroxidase above 50 °C makes the high temperature short time treatment very efficient for the inactivation of peel peroxidase contaminated in orange juice to avoid the formation of off-flavors.

**Keywords** *Citrus jambhiri* cv. Adalia · Peroxidase · Properties · Thermal inactivation

## Introduction

Peroxidase (EC 1.11.1.7) catalyzes the oxidation of various electron donor substrates (e.g., phenols, aromatic amines) by hydrogen peroxide [1]. Plant peroxidase is widely distributed

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in higher plants [2]. Peroxidase is one of the key enzymes controlling plant differentiation and development. It is known that this enzyme participates in the construction, rigidification, and eventual lignification of cell walls, in the biosynthesis of $H_2O_2$, in the protection of plant tissues from damage and infection by pathogenic microorganisms, and in wound healing [1, 3]. In vitro, this enzyme is widely employed in microanalysis [4, 5]. Currently, peroxidases are used also in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals [6, 7].

On the other hand, extensive studies describe the action of peroxidase on substances that yield bright colors on oxidation, but peroxidase can promote a large variety of reactions and therefore can exhibit a degree of versatility unsurpassed by any other enzyme [8]. Commercial fruit juice production includes some albedo and peel of the fruit in the juice, which will make a large contribution to the total peroxidase activity. The quality of extracted citrus juices depends on enzymatic reactions that occur not only in the fruit during the development period but also in the juice during processing. The formation of off-flavors in canned fruit and vegetables has been associated with residual peroxidase activity following processing [9]. Peroxidase can contribute to deteriorating changes in flavor, texture, color, and nutrition in improperly processed fruits and vegetables [10]. In orange, the level of peroxidase in the juice is associated with loss of flavor quality [11]. In addition, the involvement of peroxidase as an active oxygen-detoxifying enzyme on postharvest rind staining occurring in citrus fruit in a nonchilling temperature has been investigated [12]. This disorder, manifested as extensive-collapsed and dry areas of the flavedo (outer colored part of the peel) and part of the albedo (inner part of the peel) that becomes dark with time, has been shown to be influenced by storage relative humidity (RH) and ethylene [13, 14]. These enzymes may play a role in the lower rind-staining incidence observed in “Navelina” fruit continuously exposed to low RH as compared with fruit held under high RH.

Citrus fruit is of high commercial value in the Egyptian market; therefore, we screened the peroxidase and promoted a large variety of reactions such as the loss of flavor quality of orange juice, in the peel of some species and cultivars of Egyptian citrus. Peroxidase from *Citrus jambhiri* cv. Adalia, with the highest activity, has been purified to homogeneity, and its kinetics for different substrates and thermal inactivation has been studied.

## Materials and Methods

### Plant Materials

All of the studied 13 different citrus species and cultivars were obtained from the Citrus Department, Horticulture Institute Research, Agriculture Research Centre, Cairo, Egypt.

### Peroxidase Assay

Peroxidase activity was carried out according to Miranda et al. [15]. The reaction mixture contained in 1 ml: 8 mM $H_2O_2$, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5, and the least amount of enzyme preparation. Assays were carried out at room temperature. The change of absorbance at 470 nm due to guaiacol oxidation was followed at 30-s intervals. One unit of peroxidase activity is defined as the amount of enzyme which increases the optical density by 1.0 per minute under standard assay conditions.

## Purification of Peroxidase from *Citrus jambhiri* cv. Adalia

Unless otherwise stated, all preparation steps were carried out at 4–7 °C. Twenty grams of freshly chopped orange peel were blended at 4 °C with 0.05 mM sodium acetate buffer, pH 5.5, containing 1 M sodium chloride. The extract was filtered, and the filtrate was designated as the crude extract. The crude extract was precipitated by solid ammonium sulfate up to 75% saturation. The precipitate was collected by centrifugation at 12,000×*g* for 20 min and dissolved in a least volume of 0.05 mM sodium phosphate buffer, pH 7.2, and dialyzed against successive changes of the same buffer. The dialyzate was applied directly to a diethylaminoethanol (DEAE)-cellulose column (4×1.6 cm inner diameter [i.d.]) pre-equilibrated with the same buffer. The adsorbed material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.5 M prepared in the same buffer at a flow rate of 30 ml/h, and 3-ml fractions were collected. Fractions exhibiting peroxidase activity were eluted at 0.0, 0.05, 0.1, 0.2, and 0.3 M NaCl, where most of the peroxidase activity was eluted at 0.0 M NaCl. The pooled nonadsorbed fraction (0.0 M NaCl) was dialyzed against 50 mM sodium acetate buffer, pH 5.5, overnight and concentrated through dialysis against solid sucrose then analyzed on a carboxymethyl (CM)-cellulose column (6×1 cm i.d.) previously equilibrated with the same buffer. The exchanged material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.5 M prepared in the same buffer at a flow rate of 40 ml/h, and 3-ml fractions were collected. Fractions exhibiting peroxidase activity were eluted at 0.0 and 0.05 M NaCl and designated POI and POII, respectively, according to their elution order. POII with the highest activity was concentrated through dialysis against solid sucrose and applied on a Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with the same buffer and developed at a flow rate of 20 ml/h, and 3-ml fractions were collected. The POII was eluted with the same buffer.

## Protein Determination

Protein was quantified by the method of Bradford [16]. Bovine serum albumin was used as the protein standard.

**Table 1** Screening of orange peel peroxidase in the crude extract of Egyptian citrus species and cultivars.

| Citrus species | U/g peel | Specific activity (U/mg protein) |
|---|---|---|
| *Citrus sinensis* | | |
| cv. Abosora | 29.6 | 220 |
| cv. Khalely Ahmer | 73.4 | 174 |
| cv. Balady Faker | 66.8 | 225 |
| cv. Sifi | 76.5 | 130 |
| cv. Balady Aswani | 35.0 | 113 |
| cv. Banati Balady | 82.3 | 182 |
| cv. Balady Daem Elhaml | 125 | 155 |
| *Citrus jambhiri* cv. Adalia | 638 | 778 |
| *Citrus reticulata* | | |
| cv. Balady Cleopatra | 45.7 | 97.0 |
| cv. Balady | 31.5 | 142 |
| *Citrus mitis* | 44.8 | 69.5 |
| *Citrus paradisi* | 148 | 347 |
| *Citrus aurantium* | 69.0 | 171 |

**Table 2** Purification scheme of peroxidase from *C. jambhiri* peel.

| Purification step | Total protein (mg) | Total activity (U[a]) | Specific activity (U/mg protein) | Fold purification | Recovery percent |
|---|---|---|---|---|---|
| Crude extract | 17 | 12,768 | 751 | 1 | 100 |
| Ammonium sulfate precipitation | 2.1 | 6,143 | 2,925 | 4.8 | 48 |
| Chromatography on DEAE-cellulose 0.0 M NaCl | 0.310 | 4,682 | 15,103 | 20.1 | 36 |
| Chromatography of 0.0 M NaCl DEAE-cellulose on CM-cellulose | | | | | |
| 0.0 M NaCl (POI) | 0.126 | 1,235 | 9,802 | 13.10 | 9.7 |
| 0.05 M NaCl (POII) | 0.119 | 2,459 | 20,664 | 27.50 | 19.3 |
| Gel filtration on Sephacryl S-200 POII | 0.069 | 1,950 | 28,261 | 37.6 | 15.2 |

[a] 1 U of peroxidase activity is defined as the amount of enzyme that increases the optical density 1.0 per min under standard assay conditions.

## Polyacrylamide Gel Electrophoresis

Electrophoresis under nondenaturing conditions was performed in 10% (*w*/*v*) acrylamide slab gel according to the method of Davis [17] using a Tris–glycine buffer, pH 8.3. Protein bands were located by staining with silver nitrate.

## Molecular Weight Determination

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 β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume ($V_o$). Subunit molecular weight was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) [18]. 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.

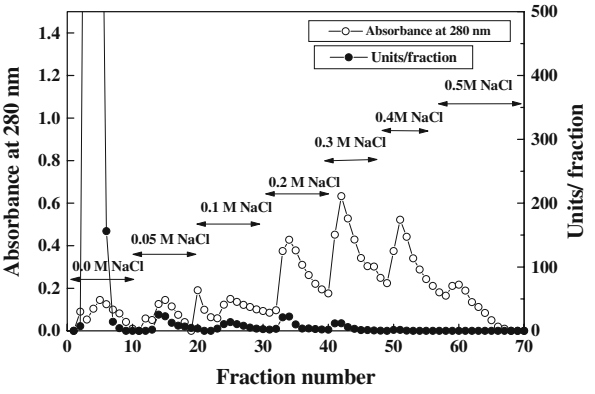


**Fig. 1** A typical elution profile for the chromatography of *C. jambhiri* peel peroxidase on DEAE-cellulose column (4× 1.6 cm i.d.) previously equilibrated with 20 mM sodium phosphate buffer, pH 7.2, at a flow rate of 30 ml/h and in 3-ml fractions

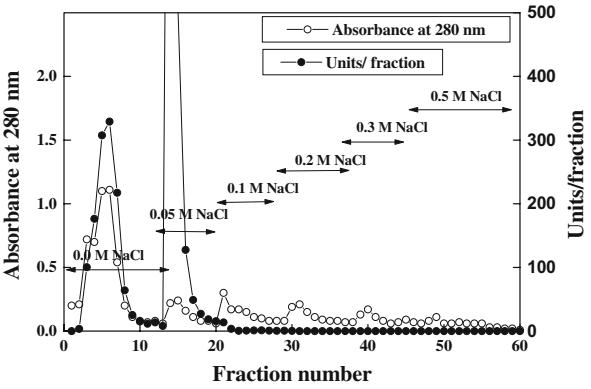


**Fig. 2** A typical elution profile for the chromatography of *C. jambhiri* peel peroxidase DEAE-celluose fraction 0.0 M NaCl on CM-cellulose column (6×0.8 cm i.d.) previously equilibrated with 50 mM acetate buffer, pH 5.5, at a flow rate of 40 ml/h and in 3-ml fractions

## Results and Discussion

In the present paper, we demonstrate that the major pool of peroxidase activity is present in the peel of some Egyptian citrus species and cultivars compared to the juice and pulp. Generally, the greatest peroxidase activity was detected in peels of orange [8] and apple [19]. Such localization indicates that citrus peroxidase may participate in suberization by facilitating the formation of polyphenolic matrix [20].The quantitative screening of peel peroxidase activity in the 13 species and cultivars of Egyptian citrus is shown in Table 1. The enzyme activities were detected in all examined species and ranged from 29.6 to 638 U/g peel, and their specific activities ranged from 69.5 to 778 U/mg protein. The species *C. jambhiri* cv. Adalia was found to be the most suitable source among the 13 examined species for the purification and characterization of peroxidase as it had the highest enzyme activity (638 U/g peel) and specific activity (778 U/mg protein).

As it is well known that the peroxidases in plant tissues are present as both soluble and membrane-bound forms [21], the effect of ionic strength on the yield of citrus peel peroxidase extraction was studied (data not shown). A NaCl concentration of 1 M was optimal for the extraction with sodium acetate buffer, pH 5.5. The purification of peroxidase from *C. jambhiri* is summarized in Table 2. The purification step of ammonium sulfate precipitation increased the fold of purification 4.8 times. The elution profile of the chromatography on DEAE-cellulose (Fig. 1) showed that peroxidase activity was detected in five peaks, a nonadsorbed peak and four anionic peroxidases. The nonadsorbed peak

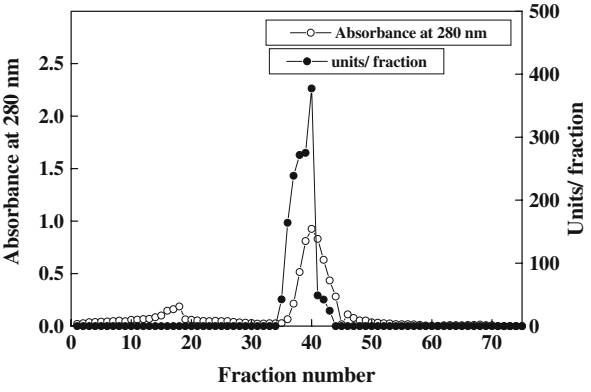


**Fig. 3** A typical elution profile for the chromatography of *C. jambhiri* peel POII CM-cellulose fraction on a Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with 50 mM sodium acetate buffer, pH 5.5, at a flow rate of 20 ml/h and in 3-ml fractions

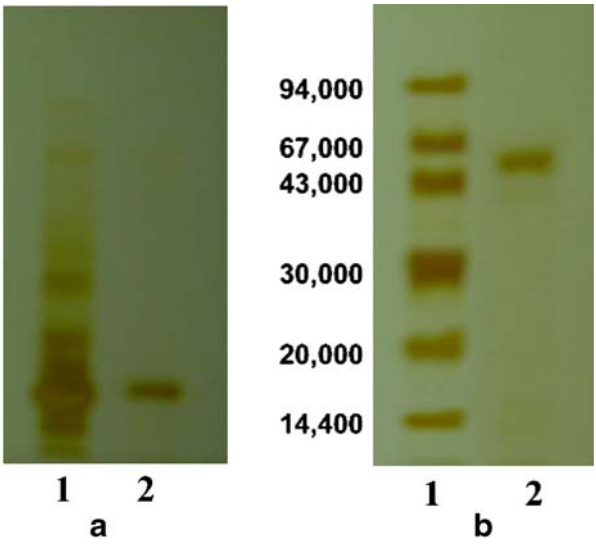


**Fig. 4** **a** PAGE for *C. jambhiri* peel peroxidase during purification steps. *1* Crude extract, *2* Sephacryl S-200 POII. **b** SDS-PAGE for molecular weight determination of orange peel peroxidase. *1* Standard proteins, *2* Sephacryl S-200 POII

with the highest peroxidase activity was applied on a CM-cellulose column, where it separated into two peaks of peroxidase POI (nonadsorbed peroxidase) and POII (cationic peroxidase; Fig. 2). POII with the highest peroxidase activity was applied on a Sephacryl S-200 (Fig. 3). The cationic peroxidase POII is proved to be pure after Sephacryl S-200 column analysis as judged by PAGE (Fig. 4a). The specific activity of Sephacryl S-200 POII was 28,261 U/mg protein, which represented 37.6-purification fold increase over the crude extract. A number of cationic and anionic isoperoxidases from *C. sinenis* [8] and apple [19] were detected. It has been suggested that the number of peroxidase isoenzymes from the same kind of fruit may differ depending on ecological and environmental differences, as well as differences in variety and also differences in stage of maturity and detection techniques [22].

The native molecular weight of POII was estimated to be 56 kDa using the Sephacryl S-200 column. This value was confirmed by SDS-PAGE (Fig. 4b), where the molecular weight of POII had the same value. This value is similar to the molecular weight value of buck wheat peroxidase (56,100 kDa) [23] and higher than those reported for peroxidases

**Table 3** Relative activities of *C. jambhiri* peel POII toward substrates.

| Substrate | Percent relative activity |
|---|---|
| Guaiacol | 100 |
| *o*-Phenylenediamine | 171.7 |
| *o*-Dianisidine | 93.7 |
| Pyrogallol | 6.2 |
| Catechol | 4.0 |
| Tyrosine | 0.0 |
| Ascorbic acid | 0.0 |
| $\alpha$-Naphthylamine | 0.0 |
| *p*-Aminoantipyrine | 0.0 |
| Potassium ferrocyanide | 0.0 |
| Potassium iodide | 0.0 |
| NADH | 0.0 |

The reaction mixture contained in 1 ml: 8 mM $H_2O_2$, 40 mM substrate, 50 mM sodium acetate buffer, pH 5.5, and suitable amount of enzyme. The activity with guaiacol was taken as 100% activity. Each value represents the average of two experiments.

**Table 4** Kinetic parameters of *C. jambhiri* peel POII.

| Substrate | $K_m$ (mM substrate) | $V_{max}$ (units/assay) | $V_{max}/K_m$ (units/mM sustrate) |
|---|---|---|---|
| $H_2O_2$ | 0.54 | 100 | 185 |
| *o*-Phenylenediamine | 2.85 | 23.25 | 8.14 |
| Guaiacol | 5 | 18 | 3.6 |
| *o*-Dianisidine | 11 | 3.125 | 0.28 |
| Pyrogallol | 23 | 0.176 | 0.0076 |
| Catechol | 125 | 0.36 | 0.0028 |

from papaya fruit (41–54 kDa) [24], oranges (22 to 44 kDa) [8], kiwi fruit (40–42 kDa) [25], pea nuts (40–42 kDa) [26], horse radish (44 kDa) [27] and *Withania somnifera* (34–48 kDa) [28]. The highest molecular weight was detected for marula fruit peroxidase (71 kDa) [29].

The substrate specificity of POII has been examined using a number of potential natural electron donor substrates (Table 3). The activity with guaiacol is regarded as 100% activity. POII had the highest activity level toward *o*-phenylenediamine compared to guaiacol, while *o*-dianisidine had an activity level similar to guaiacol. Pyrogallol and catechol had very low affinity toward POII. These results identified the physiological role of POII, which catalyzed the oxidation of some phenolic substrates and revealed a significant similarity of this enzyme with class III peroxidases. Therefore, POII may change the color of orange because several reports have shown that peroxidase plays an important role in enzymatic browning together with phenolic compounds [30]. POII had no peroxidase activity toward the other examined substrates especially ascorbic acid indicating that this enzyme does not belong to the family of ascorbate peroxidases. Similarly, *W. somnifera* peroxidases readily catalyzed the oxidation of phynolic substrates like guaiacol and *o*-dianisidine and did not catalyze ascorbic acid [28]. However, *Cucumis sativus* peroxidases catalyzed the oxidation of pyrogallol and guaiacol than that of ascorbate [31].

The kinetic parameters of POII for hydrolysis toward $H_2O_2$ and electron donor substrates, guaiacol, *o*-phenylenediamine, *o*-dianisidine, pyrogallol, and catechol, were obtained by a typical double reciprocal Lineweaver–Burk plots (Table 4). The apparent $K_m$, $V_{max}$, and $V_{max}/K_m$ for the catalysis of $H_2O_2$ in the presence of 40 mM guaiacol were 0.54 mM, 100 and 185, respectively. The lower $K_m$ value and higher $V_{max}$ of the enzyme indicated its higher affinity toward $H_2O_2$. Other reported $K_m$ values for hydrogen peroxide include 0.85 mM for one turnip isoenzyme [32], 1.77 mM for marula fruit [29], 11.4 and

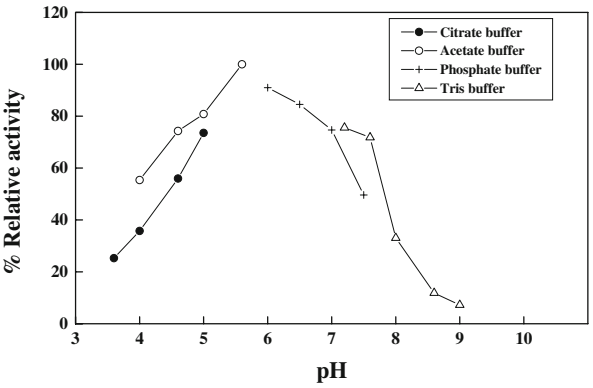


**Fig. 5** pH optimum of *C. jambhiri* peel POII. The reaction mixture contained in 1.0 ml: 8 mM $H_2O_2$, 40 mM guaiacol, a suitable amount of enzyme, and 50 mM sodium citrate buffer (pH 3.6–5.0), sodium acetate buffer (pH 4.0–5.5), sodium phosphate buffer (pH 6.0–7.5), and Tris–HCl buffer (pH 7.2–9.0). Each point represents the average of two experiments

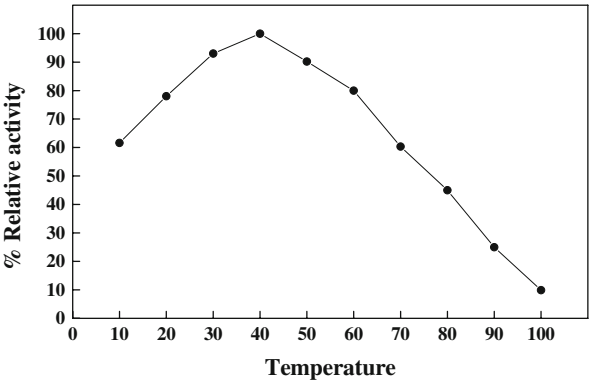


**Fig. 6** Optimum temperature of *C. jambhiri* peel POII. The enzyme activity was measured at various temperatures using the standard assay method as previously described. Each point represents the average of two experiments

6.2 mM for Brussels sprouts isoenzymes [33], and 1.5 mM for pear [34]. Guaiacol, *o*-phenylenediamine, and *o*-dianisidine showed higher affinity toward POII, where their $K_m$ values are 5, 2.85, and 11 mM, $V_{max}$ are 18, 23.2, and 3.12, and $V_{max}/K_m$ are 3.6, 8.14, and 0.28, respectively. The other electron donor substrates showed lower affinity toward POII. Various $K_m$ values, using different electron donor substrates, were reported for peroxidases from buckwheat seeds (0.202–0.288 mM guaiacol, 0.137–0.229 mM *o*-dianisidine) [23], *Pleargonium graveolense* (7.3 mM guaiacol, 0.31 mM *o*-dianisidine) [35], and *Brassica napus* (3.7 mM guaiacol) [36].

The apparent pH optimum for POII is shown in Fig. 5. The enzyme has a pH optimum of 5.5, in sodium acetate buffer, with guaiacol as substrate. *Withania sommifera* peroxidases were optimally active at pH 5.0 in sodium acetate buffer with guaiacol as substrate [28]. Both *C. sativus* peroxidases show a bell-shaped pH dependence on catalytic activity, which reaches a maximum between 5.5 and 7.5 and sharply decreases outside the interval [31]. More acidic and alkaline pH optima were detected for peroxidases from marula (pH 4.0) [29] and buck wheat (pH 9.0) [23].

The optimal temperature and thermal stability of POII activity were investigated using guaiacol as substrate (Figs. 6 and 7). The optimal temperature for POII was 40 °C. More than 50% was retained in the temperature range of 10–50 °C. POII was stable at 10–40 °C and unstable above 50 °C, where it incubated for 15 min at different temperatures prior to substrate addition. The enzyme was inactivated at 90 °C. The thermal inactivation profile of POII is shown in Fig. 8. The profile is biphasic and characterized by a rapid decline in

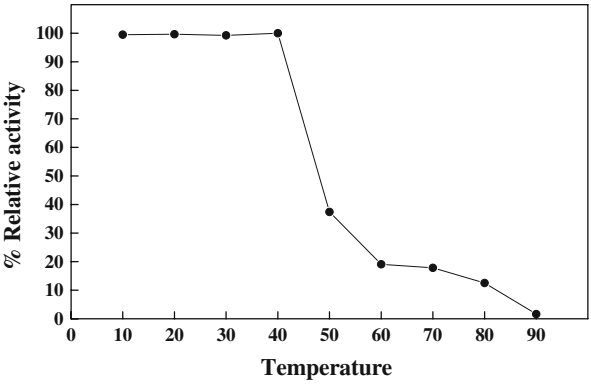


**Fig. 7** Effect of temperature on the thermal stability of *C. jambhiri* peel POII. The reaction mixture contained in 1.0 ml: 8 mM $H_2O_2$, 40 mM substrate, 50 mM sodium acetate buffer, pH 5.5, and a suitable amount of enzyme. The reaction mixture was preincubated at various temperatures for 15 min prior to substrate addition, followed by cooling in an ice bath. Activity at zero time was taken as 100% activity. Each point represents the average of two experiments

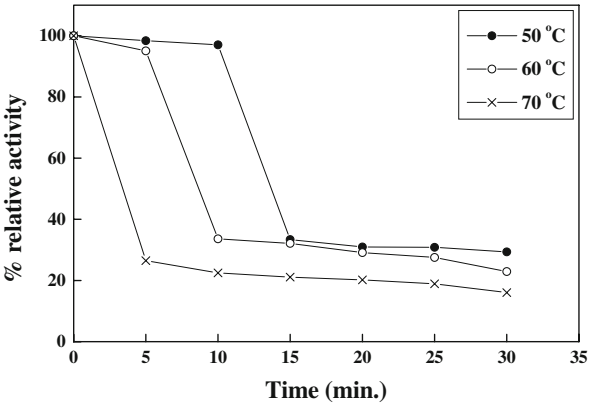


**Fig. 8** Heat inactivation profile for *C. jambhiri* peel POII. The enzyme was incubated for different times at different temperatures, and the residual peroxidase activity was assayed under standard assay conditions

activity on exposure to heat, followed by a more gradual decrease in activity on continued exposure. The most of POII activity (70–80%) was lost at 50, 60, and 70 °C after 15, 10, and 5 min, respectively. Similarly, the thermal treatment of peroxidases from apple [19], orange [8], mango [37], *W. somnifera* [28], and marula fruit [29] proved to be nonlinear and biphasic in relation to the factors time and temperature. For apple peroxidases, the thermal treatment between 65 and 80 °C showed that the residual activity of the cationic isoenzyme is at least 80% (a maximum inactivation of only 20%), where the residual activity of anionic isoenzyme is at least 30% [19]. In addition, the purified isoperoxidases from orange juice lost about from 15% to 80% of their original activity when exposed to 70 °C for 50 s compared with a loss of about 85% when present as a mixture in the soluble crude extract [8]. Johri et al. [28] reported that the thermal stability of peroxidases has been attributed to the presence of a large number of cysteine residues in the polypeptide chain.

Most of the examined metal ions had very slightly effect on POII (Table 5). The moderate inhibitory effect on enzyme was determined for $Li^{+}$ and $Zn^{2+}$, while $Hg^{2+}$ had a strong inhibitory effect. Very little articles had been studied the effect of metals on the activity of peroxidase. Lobarzewski et al. [38] studied the effect of metal ions on the immobilized and solubilized cabbage peroxidase. The results showed that the ions of Mg, Mn, Cu, Ca, and Zn stimulate the activity of the immobilized peroxidase more effectively

**Table 5** Effect of metal ions on *C. jambhiri* peel POII.

| Metal | Percent relative activity |
|---|---|
| None | 100 |
| $Ba^{+2}$ | 108 |
| $Co^{+2}$ | 100 |
| $Ca^{+2}$ | 100 |
| $Mn^{+2}$ | 99 |
| $Fe^{+3}$ | 99 |
| $Cu^{+2}$ | 96 |
| $Ni^{+2}$ | 90 |
| $Zn^{+2}$ | 87 |
| $Li^{+}$ | 78 |
| $Hg^{+2}$ | 20 |

Enzyme was preincubated for 15 min at 37 °C with 5 mM of listed ions as a final concentration prior to substrate addition. Each value represents the average of two experiments.

than the preparation of soluble proteins. Although the Hg and Pb ions caused some decrease in peroxidase activity, immobilization of the proteins partially protected them from the harmful effect of these ions. In conclusion, the major pool of peroxidase activity is present in the peel of some Egyptian citrus species and cultivars compared to the juice and pulp. However, the instability of peroxidase above 50 °C makes the high temperature short time treatments used commercially in fruit and vegetable processing very efficient for inactivation of peel peroxidase contaminated in orange juice to avoid the formation of off-flavors.

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