



Original article

Kinetic studies and partial purification of peroxidase in wild pear

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ARTICLE INFO

Article history,

Received 07 February 2014

Accepted 19 February 2014

Available online 21 February 2014

Keywords,

Kinetics

Peroxidase

Wild pears

ABSTRACT

Peroxidase, extracted from wild pears was isolated by ammonium sulfate precipitation technique and purified by ion exchange chromatography. The crude enzyme having 19.5 U/mL activity and 1.3 U/mg specific activity was subjected to ammonium sulfate precipitation technique for partial purification and the resulted activity and specific activity were 16.5 U/mL and 3.17 U/mg respectively. After ion exchange chromatography through DEAE-cellulose, fraction between 35-42 exhibited maximum activity of 15 U/mL and specific activity of 7.14 U/mg. The enzyme under discussion was found to be quite active with optimum temperature of 45°C. Optimum pH for the enzyme was 6. Thermal treatment of crude extract of wild pears peroxidase was more stable at pH 6. It was found that enzyme followed the Michealis-Menton mechanism and 21 units/mg.protein and 70 mM were the calculated values for Vmax and Km in precence of various concentrations of guaiacol and constant concentration of H₂O₂. The results showed that wild pear peroxidase was a thermostable enzyme. After 30 min at 60°C, the remaining activity was 35%.

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1. Introduction

Peroxidases are iron-porphyrin ring containing enzymes, which belong to the class oxidoreductase. Peroxidase (E.C.1.11.1.7) is a ubiquitous enzyme which belongs to the oxidoreductase class of enzyme and

generally catalyzes a reaction between H₂O₂ as electron acceptor and many kinds of substrates by means of O₂ liberation from H₂O₂ (Zia et al, 2011). The enzyme occurs naturally in nearly all plants, animals and microorganisms (Burnette, 1977). It is found primarily in the roots and sprouts of higher plants (Tauber, 1949). The documented sources of peroxidase in plants are horseradish, turnip, potato, tomato, carrot, bananas and etc. Peroxidase has been reported to participate in late stages of lignin-forming process (Civello et al, 1995). Peroxidase is the most heat-stable enzyme having a wide range of application in health sciences as a diagnostic tool (Kwak et al, 1995). Autoantibodies directed against the thyroid peroxidase are widely used to diagnose human autoimmune thyroid disease (Nord et al, 1953). Several isoperoxidases, notably horseradish and turnip, have been studied in great detail during the past two decades (Young and Kim, 1994). Studies in our laboratories and by other workers on peroxidase isoenzymes from many different plants indicated that physical and kinetic properties and substrate preference of this isoperoxidase even from a single source might vary significantly (Converso and Fernandez, 1995; Hamed et al, 1998; Tabatabaie et al, 1998). The study of this enzyme in food has attracted interest because of its capacity to modify food in both desirable and undesirable ways. Peroxidase activity has been related to the existence of cationic and anionic isoenzymes (Van Huystee, 1987). Generally, the enzyme is found in glycosylated form and associated to membranes. The objectives of this work included the kinetic studies, partial purification and characterization through ion-exchange chromatography from wild pear.

2. Materials and methods

Preparation of Wild pear Extract, The peroxidase activity of wild pears was measured according to Jen et al. (1980). 500 gram of wild pears were added to 400 ml distilled water and thoroughly blended for 15 min. Sediments were discarded and supernatants were passed through filter paper. Total volume of prepared extract was 500 ml which was then heated at 65°C for 3 min in water bath to inactivate catalase present in the extract. The crude extract was subjected to partial purification of enzyme by using ammonium sulfate precipitation technique (Evans, 1968). The precipitates were recovered by centrifugation at 15000 g for 30 min at 4°C and was dissolved in a small volume of 0.1 M phosphate buffer (pH 6.8), dialyzed against the same buffer. The dialysis solution were applied to a DEAE-cellulose, and the protein was eluted with a linear salt gradient from 0 to 0.3 M NaCl in the above buffer. The fractions showing peroxidase activity were pooled, so 60 fractions were collected in about 7 hr by a constant drop rate. The protein with peroxidase activity was dialyzed again st distilled water for 48 h and was stored at -20°C until use. In order to correct for substrate autoxidation, the reaction mixture, was placed in the sample cuvette while the reference cuvette contained buffer and the substrate. Enzyme activity was calculated from the linear portion of the curve.

Enzyme Assay and Protein Estimation, Phosphate buffer of pH 6.5 containing 2 ml guaiacol was used for measuring at 420 nm for peroxidase assay in presence of H₂O₂ (Civello et al, 1995). Studies were conducted to determine the protein contents in enzyme extract before and after partial purification by lowry method (Lowry et al, 1951). Effect of Various Kinetic Parameters investigated on Partially Purified Peroxidase.

Application of Michealis-Menton equation was confirmed by using the partially purified extract of wild pears containing various concentrations of guaiacol. Crude extract reaction mixtures having pH 1-10 were prepared and analyzed on spectrophotometer at 420 nm after 1 min of reaction (Theorell, 1942). One millilitre of partial purified peroxidase was heated for 5 min at various temperatures and absorbance (OD) was recorded at 420 nm on spectrophotometer of 6305 JENWAY. Again 1ml of crude extract was subjected to a variable thermal treatment. The samples were heated at 60°C for 10 to 60 min and were then placed at 4°C for 5 min. The absorbance was noted at 420 nm.

3. Results and discussion

The partial purification of peroxidase of wild pear was done by using ammonium sulfate precipitation (Evans, 1968) and DEAE-cellulose chromatography. To purify the enzyme from wild pears, firstly it was salted out with (NH₄)₂SO₄. The enzyme of interest was earned at precipitation from 30 to 80% saturation. Degree of purification after ammonium sulfate precipitation was found 2.4. Rehman et al. (1999) reported the degree of purification as 1.93 in horseradish peroxidase, whereas Civello et al (1995) reported 2.37 degree of purification from strawberry fruit using the same technique. The most often used cellulosic anion exchanger is DEAE-cellulose (Halpin et al, 1989). Degree of purification of wild pears peroxidase was 5.49 fold with DEAE-cellulose chromatography. Specific

activity of wild pears peroxidase in crude extract was 1.3 unit/mg.protein and it increased during the process of purification to 7.14 (Table I). Protein contents were estimated by lowry method. The absorbance values of crude and partially purified extracts were recorded at specific wavelength of 420 nm after 3 min reaction period. The OD values with respect to time interval were noted (Fig. 1). Guaiacol was used as a substrate in partially purified extract (Fig. 2). In crude and partially purified extract, the optimum pH was 6 at a range 3-10. It was observed that activity of enzyme increased gradually with increasing pH with Its peak at pH 6 with guaiacol. The optimum pH depends upon H⁺ donor. It may be changed according to the substrate used (Halpin et al, 1989). Jen et al. (1980) also found pH 5.5 as optimum with guaiacol while purifying tomato peroxidase. The purification procedure and results by crude extract, precipitation with ammonium sulfate and ionexchange chromatography are summarized in Table 1. Table I shows that the specific activity of wild pear peroxidase was 7.14 U/mg protein and the degree of purification was 5.49. Specific activity of wild pear peroxidase was 1.3, 1.7, 3.17 and 7.14 U/mg protein for enzyme in stage of crude extract, (NH₄)₂SO₄ 30% , (NH₄)₂SO₄ 80% and after DEAE-cellulose chromatography, respectively. Activity of wild pear peroxidase increased in presence of various concentrations of guaiacol and constant concentration of H₂O₂ (10mM). So with increase of guaiacol, activity of enzyme increased until reached to maximum of rate at 21 unit/mg.protein (Figure 4). More increase in concentrations of guaiacol accompanied with decrease of activity and showed substrate inhibition of peroxidase. Km of peroxidase calculated 70 mM and catalytic efficiency is 0.3 unit/mg.protein per mM. When different concentrations of H₂O₂ were added to wild pear peroxidase solution at pH 6, it was found that the peroxidase activity gradually increased (Fig. 5). So its apparent V_{max} and Km were 3.5 U/mg protein and 4 mM respectively in presence of constant concentration of guaiacol(150 mM). Wild pear peroxidase in presence of guaiacol and H₂O₂ showed fluctuations in activity with increasing temperature even as high as 90 °C. The plot for temperature demonstrated that the enzyme was very thermostable between 30 and 60 °C. Our results showed optimum temperature is 45 °C for enzyme. Like most chemical reactions, with increase of temperature from 27 °C, gradually, activity of peroxidase increased so; we reached to maximum of activity at 45 °C (130%) (Fig. 6). With more increase in temperature from 80% to 60 °C and deactivated at 85 °C. The drop in percentage residual activity at high temperatures can actually be due to the unfolding of the tertiary structure of the enzyme to form the secondary structure. Variations in reaction temperature as small as 5 degrees from 55 to 60 °C introduce decrease of 20% in the activity. We measured the relative activity of enzymes at constant temperature (60°C) at a fixed pH 6 and at a concentration of H₂O₂ (10 mM) and guaiacol 100 mM after incubating for 5-60 min. The results showed that wild pear peroxidase was a thermostable enzyme. After 30 min at 60°C, the remaining activity was 35% (Fig. 7).

4. Conclusion

Peroxidase, extracted from wild pears was isolated by ammonium sulfate precipitation technique and purified by ion exchange chromatography having 19.5 U/mL activity and 1.3 U/mg specific activity was subjected to ammonium sulfate precipitation technique for partial purification and the resulted activity and specific activity were 16.5 U/mL and 3.17 U/mg respectively. After ion exchange chromatography through DEAE-cellulose, fraction between 35-42 exhibited maximum activity of 15 U/mL and specific activity of 7.14 U/mg. The enzyme under discussion was found to be quite active with optimum temperature of 45°C. Optimum pH for the enzyme was 6. The results showed that wild pear peroxidase was a thermostable enzyme. After 30 min at 60°C, the remaining activity was 35%.

Table1
Summary of wild pear peroxidase purification.

	Protein (mg/ml)	Activity (u/ml)	Specific activity	Degree of purification
Crude extract	15	19.5	1.3	1
(NH ₄) ₂ SO ₄ 30%	11	18.7	1.7	1.3
(NH ₄) ₂ SO ₄ 80%	5.2	16.5	3.17	2.4
DEAE- cellulose	2.1	15	7.14	5.49

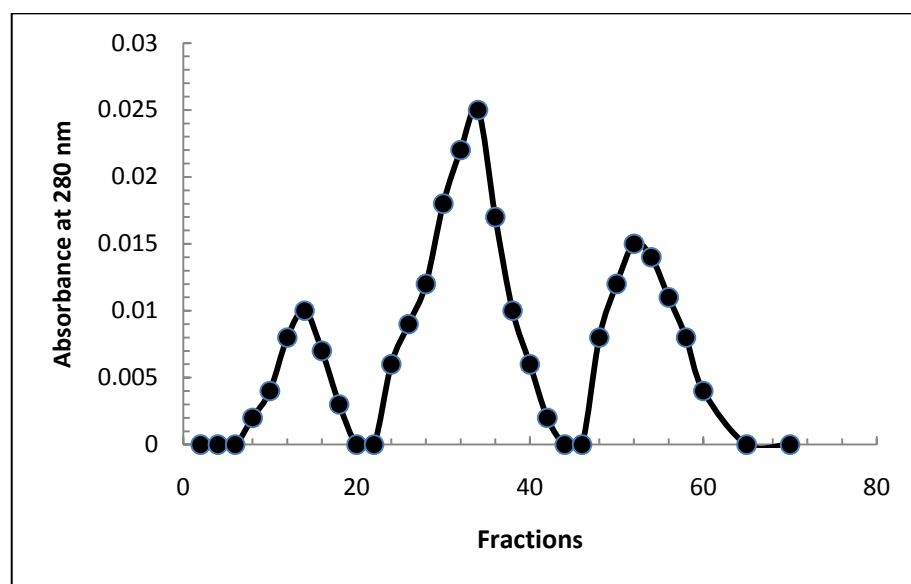


Fig. 1. Absorbance of fractions of DEAE-cellulose after chromatography at 280 nm.

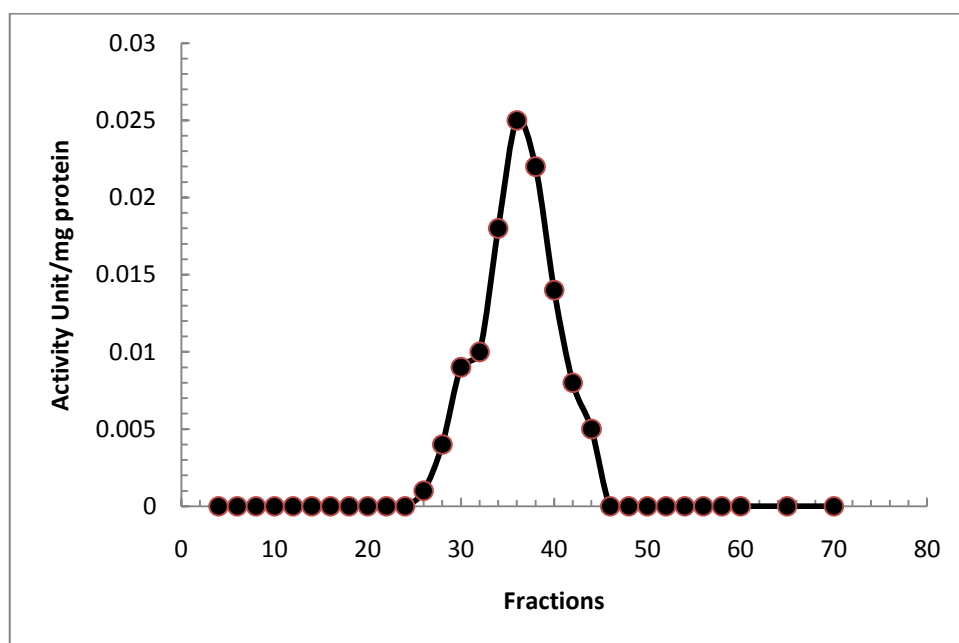


Fig. 2. Peroxidase activity of fractions in presence of guaiacol (150mM) and H₂O₂ (10mM).

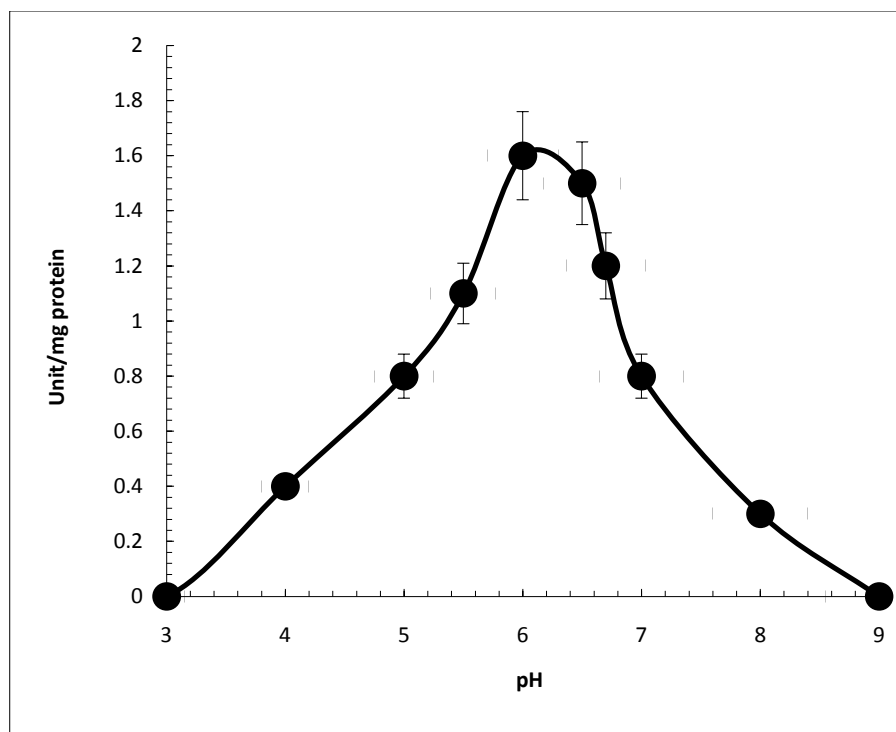


Fig. 3. Effect of pH value on the peroxidase activity partial purified from pyrus communis.

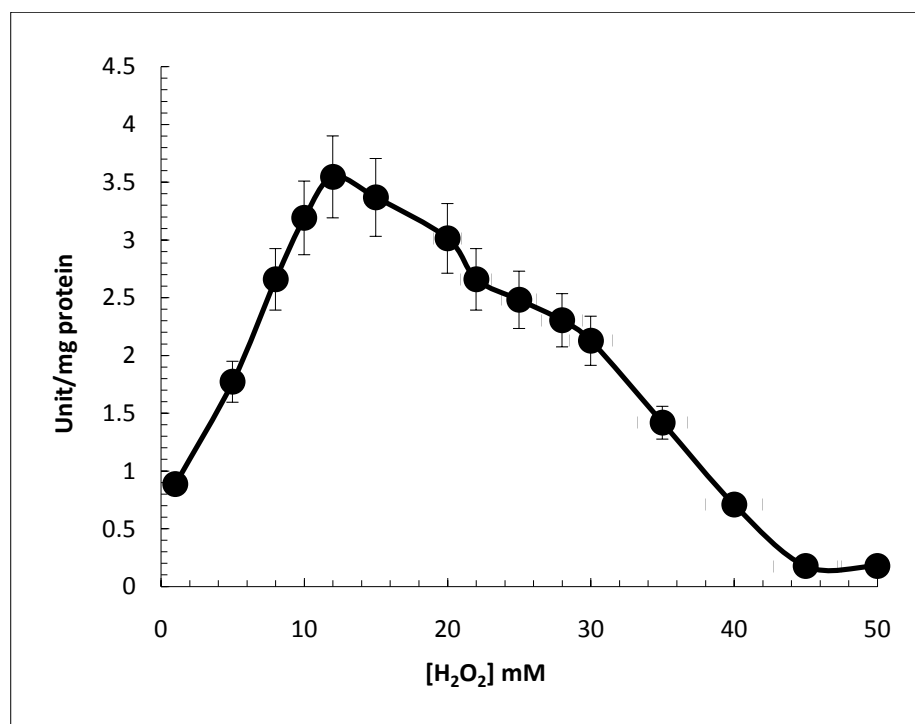


Fig. 4. Activity of wild pear peroxidase in presence of constant concentration of H_2O_2 (10mM) and different concentration of guaiacol.

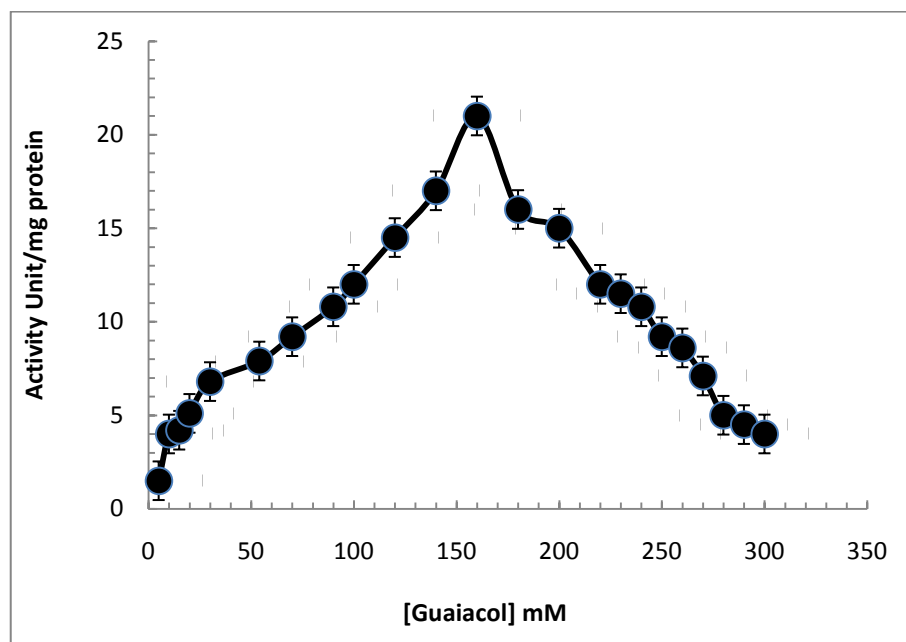


Fig. 5. Activity of wild pear peroxidase in presence of constant concentration of H_2O_2 (10mM) and different concentration of guaiacol.

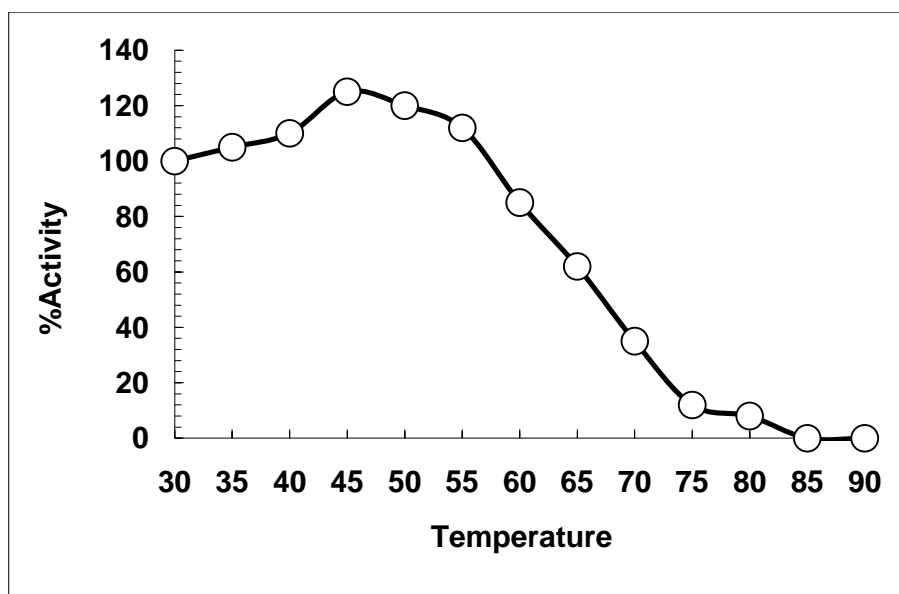


Fig. 6. Effect of different temperature (30- 90)°C on peroxidase activity purified from pyrus communis.

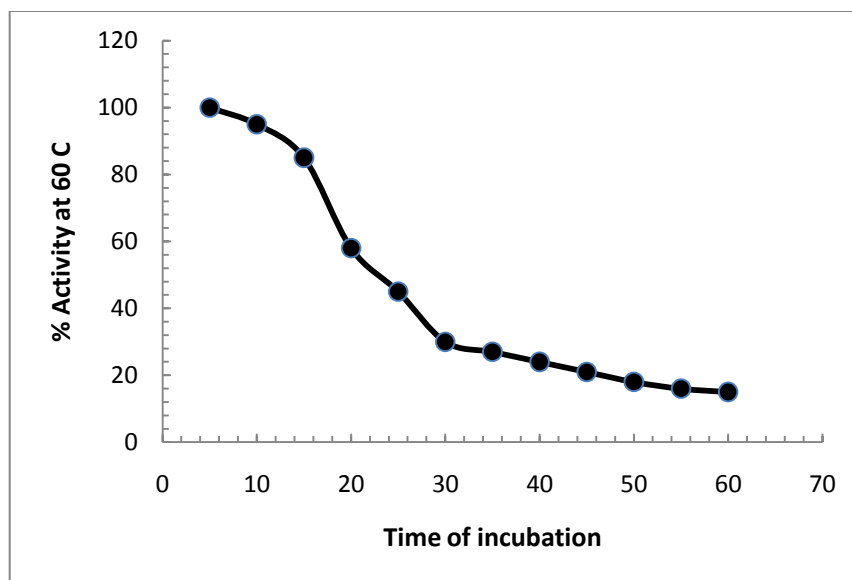


Fig. 7. Effect of time incubation of wild pear peroxidase on activity at 60°C.

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