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ScienceDirect
Food Chemistry 105 (2007) 1312–1320
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Purification and characterization of an intracellular peroxidase from genetically transformed roots of red beet (*Beta vulgaris* L.)

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Received 6 March 2007; received in revised form 16 April 2007; accepted 16 April 2007

Abstract

An intracellular peroxidase (POD) produced by genetically transformed root cultures of red beet (*Beta vulgaris* L.) was purified using a combination of $(NH_4)_2SO_4$ fractionation and ion exchange chromatography resulting in 15-fold enhancement of activity. This enzyme exhibited highest activity (10,500 U mg^{-1} protein) and stability at pH 5.0 and retained over 70% of the activity for 20 min at 70 °C where horseradish peroxidase (HRP) – a vastly used commercial source, had lost its activity after 11 min. The purified enzyme showed highest preference for H_2O_2 as the substrate (K_m value of 0.1). Among the ·H donors, the enzyme appeared to have affinity in the order of orthodianisidine > 2,2′-azino-bis(3-ethylbenz-thiazoline)-6-sulfonic acid > guaiacol. The purified POD was completely and competitively inhibited by periodate (HIO_6^-, $K_i = 0.2$ mM) whereas sodium azide (NaN_3) was a non-competitive inhibitor. The purified POD had a molecular mass of 45 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and activity staining. This is the first report describing purification and characterization of POD from red beet hairy roots showing its better efficacy than commercial HRP.

Keywords: *Agrobacterium rhizogenes*; Hairy root; T-DNA; Transformation; Peroxidase; HRP; Purification; Characterization; Isozymes

1. Introduction

Peroxidase (E. C. 1. 11. 1.7) (POD) involves a group of enzymes known to play a very crucial role in scavenging free radicals (peroxides) within the plant system (Regalodo, Garcia-Almandarez, & Duarte-Vazquez, 2004) in addition to their involvement in various developmental and metabolic processes. The enzyme has also been exploited for several commercial applications, the major one being its use as an important component in chemical diagnostics and laboratory experiments (Regalodo et al., 2004; Torres, Tinoco, & Vazquez-Duhalt, 1997). Because of its broader catalytic activity, a wide range of chemicals can be modified using POD and hence can be used for the applications such as synthesis of various aromatic compounds, removal of phenolics from waste waters and the removal of peroxides from foodstuffs, beverages and industrial wastes (Torres et al., 1997).

Over the years horseradish has been the only commercial source of POD (Veitch, 2004). However, other sources can also provide peroxidases with similar or better substrate specificities, yield and economic feasibility. Higher cost of production and purification from crude sources limits the use of this versatile enzyme in many industrial applications. Therefore, the constant effort for finding alternatives for the enzyme has resulted in the exploration of different sources. Among the various sources, *in vitro* hairy root culture systems appear promising because of

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doi:10.1016/j.foodchem.2007.04.050

their amenability for automation to produce in large bioreactor systems (Thimmaraju et al., 2005).

Hairy roots, obtained after genetic transformation with *Agrobacterium rhizogenes*, have been reported for a number of crops, including red beet (Thimmaraju et al., 2005; Thimmaraju, Bhagyalakshmi & Ravishankar, 2004; Thimmaraju, Bhagyalakshmi, Venkatachalam, Sreedhar, & Ravishankar, 2006). About 19.2 U g^{-1} FW of POD activity has been reported in carrot hairy root cultures which is higher compared to its suspension cultures (Uozumi, Kato, Nakashimada, & Kabayashi, 1992). Similarly, horseradish hairy root cultures are reported to produce considerably high level of PODs (Flocco, Alvarez, & Guilietti, 1998; Uozumi et al., 1992). Use of hairy root culture is expected to improve the production through strategies such as selection of clone, changing growth conditions, particularly with reference to hormones and nutrients. Another interesting strategy is the exposure of cultures to changed environmental conditions such as treatment with elicitors. For example, treatment with different abiotic elicitors such as $AgNO_3$ and $CuSO_4$ and fungal extracts of *Verticillium* sp., *Monodyctis cataneae* and *Aspergillus niger* caused about 100% increase in POD activity in transformed root cultures of horseradish. Further, the metal ions caused secretion leading to an overall productivity of about 12-fold higher POD enzyme (Uozumi et al., 1992). The heterotrophic hairy roots of *Ipomoea aquatica* were also found to produce POD at levels 250 U g^{-1} FW. When such roots were made autotrophic, the POD activity within the tissue nearly doubled, which the authors related to the requirement of POD for scavenging the high release of toxic oxidants, the peroxides (Kino-Oka, Hongo, Taya, & Tone, 1992).

In our previous study we reported a hairy root clone of red beet (*Beta vulgaris* L.) capable of producing POD as high as 9000 U g^{-1} FW and 1.18×10^6 U l^{-1} (specific activity of 600 U mg^{-1} protein) on hormone-free MS liquid medium, both in shake-flask and bioreactor (Thimmaraju et al., 2005). An extensive screen of biotic and abiotic elicitors was made and an elicitation of POD activity by about 3.52-fold was obtained when the root cultures were treated with dried cell powder (DCP) of Candida versatalis and about 3.44-fold higher when treated with glutathione (GSH). These observations resulted in evolving a best strategy for elicitation of POD, which involved combined elicitation using GSH (1 mM) and DCP of Rhizophus oligosporus resulting in a 4-fold enhancement, accounting for 10.9×10^6 U l^{-1} (Thimmaraju et al., 2006). Though such highly productive root cultures are available, the purification of the enzyme is a major hurdle for the realization of its commercial potential. Different strategies such as ion exchange chromatography, FPLC (fast protein liquid chromatography) have been employed for POD purification in different systems (Aruna & Lali, 2001; Brownleader, Ahmed, Trevan, Chaplin, & Dey, 1995; Christensen, Bauw, Welinder, Montagu, & Bioerjan, 1998; Nair & Showalter, 1996; Kristensen, Bloch, & Rasmussen, 1999; Wititsuwannakul, Wititsuwannakul, Sattaysevana, & Pasitkul, 1997). Srinivas, Rashmi, and Raghavarao (1999) used a different strategy which involved an aqueous two phase extraction coupled with gel filtration for purification of a POD extracted from the leaves of *Ipomea palmata*. However, to the best of our knowledge, there is no report on the purification and characterization of POD from red beet hairy roots cultures. In the present study we report for the first time a simple two step process of purification and also partial characterization of an intracellular POD from a high yielding red beet hairy root clone.

2. Materials and methods

2.1. Establishment and maintenance of hairy root cultures

Transformation and initiation of hairy root clone LMG-150 used in the present study has been reported earlier (Thimmaraju et al., 2005). The culture was maintained by sub culturing the root tips every three weeks into fresh liquid culture medium. The cultures were incubated on a gyratory shaker at 90 rpm in dark at 25 ± 2 °C, unless otherwise mentioned.

2.2. Extraction and assay of POD and estimation of total protein

POD was extracted by homogenizing a known weight of fresh tissue of hairy roots, in 0.2 M sodium phosphate buffer (pH 6). After homogenization the homogenate was centrifuged at 5000*g* twice and the supernatant was used for further assay and purification. The POD assay was done following the method described by Wititsuwannakul et al. (1997). Briefly, 1 ml assay mixture was prepared which consisted of 100 µl of 0.08 M H_2O_2, 100 µl of 0.008 M orthodianisidine hydrochloride (Sigma, St. Louis, Mo, USA) obtained from a stock solution of 0.25% (w/v) (7.88 mM ~ 0.08 M), 10 µl of enzyme extract and 790 µl of respective buffer. The change in OD at 460 nm per minute (dA min^{-1}) at 27 °C was recorded using kinetic program in UV–visible spectrophotometer (Shimadzu UV-160A). The results were expressed as units per volume of fresh and the purified fractions. Activity was quantified on the basis of standard curve of horseradish POD enzyme (ICN, California). One unit of enzyme activity refers to the rate of change of 1 OD per minute. The specific activity was expressed as enzyme units per milligram protein. Initial estimation of total protein content in the crude extracts was done by adding reducing agents such as NaCl and cysteine to the extract. This was followed by precipitation of the total protein using trichloroacetic acid, followed by re-dissolving in 2 N NaOH to determine total protein concentration, following the method of Lowry, Rosenberg, Farr, and Randall (1951) with bovine serum albumin as a standard. However, the step of reducing agent treatment was omitted for estimating total protein content in the purified fractions.

2.3. Screening for isozymes

Since POD has several isozymes, we screened for the abundance of different isozymes for further purification. The POD zymogram was prepared by polyacrylamide gel electrophoresis (7.2%) (PAGE) carried out at 120 V for 4 h using 12 × 14 × 0.3 cm gel without SDS using standard protocol. The gel was stained for POD activity with a 100 ml solution of sodium phosphate buffer containing 10 ml of 0.25% orthodianisidine hydrochloride and 10 ml of 1% hydrogen peroxide (Wititsuwannakul et al., 1997) and immediately photographed. Due to the abundance of intracellular isozymes than those leached into the medium (Fig. 1a), we chose to characterize the former.

2.4. Purification of enzyme

Starting from the enzyme extraction from the crude biomass, all the purification steps were performed at 4–6 °C.

2.5. Ammonium sulfate fractionation

Ammonium sulfate fractionation was done by using the finely ground ammonium sulfate. The powder was weighed and added slowly by constant stirring to ensure complete solubility and the solution was kept at 4 °C overnight for complete precipitation. Different degrees of saturation were achieved by progressively adding the specified quantity of ammonium sulfate as per the relevant saturation chart. After each saturation step the precipitate was collected by centrifuging the enzyme extract at 8000*g* for 20 min at 4 °C. The collected fractions (0–20%, 20–40%, 40–60%, 60–80% to 80–90%) were analyzed for enzyme activity and total protein content, the specific activity was calculated and the values were expressed in terms of fold purification. The fraction with maximum specific activity was selected and dialyzed in a dialysis tube of 12000 kDa cut-off range for further use.

2.6. Anion exchange chromatography (AEC)

About 10 g of ion exchange resin DEAE cellulose (Sigma, St. Louis, Mo, USA) soaked in double distilled water for 48 hours were activated by following standard protocol. Briefly, the matrix was washed with 0.1 M HCl for 10 min and the pH of the matrix was brought back to neutral by repeated washing with excess of double distilled water. Again the matrix was treated with 0.1 M NaOH for 10 min and washed several times to bring the pH to neutral. The activated matrix was equilibrated with pH 6.0 sodium phosphate buffer (0.2 M) and packed onto a column of bed volume 25 ml.

The packed resin was equilibrated with sodium phosphate buffer (0.2 M pH 6.0) by washing overnight at a flow rate of about 12 ml h^{-1}. The column was loaded with 80% dialyzed ammonium sulfate fraction of BHR-POD. The unbound enzyme was collected 2 ml fractions by washing the loaded column with approximately 3 bed volumes of buffer. The bound fractions were eluted by a linear gradient of 0–0.5 M NaCl prepared in 0.2 M sodium phosphate buffer (pH 6.0), collecting fractions of 2 ml each. The OD at 280 nm and activity of POD in each fraction was monitored and plotted. The fractions corresponding to the peak with maximum activity were pooled and concentrated by lyophilization and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

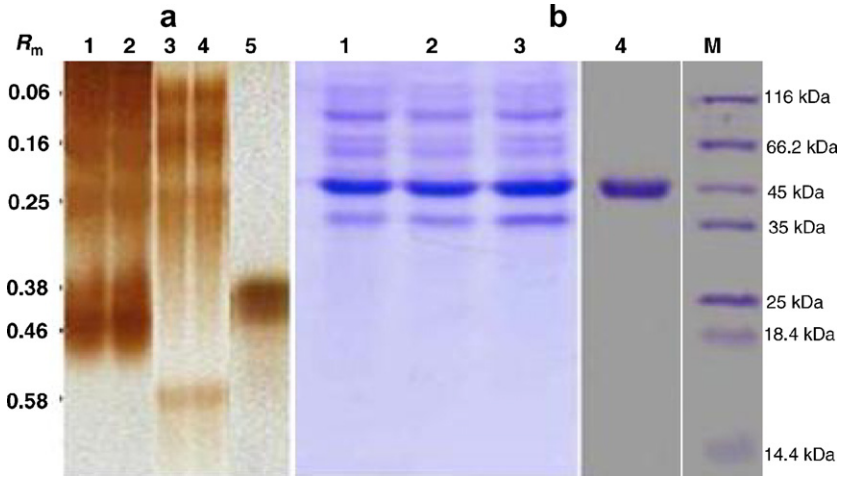

Fig. 1. (a) Zymograms of peroxidase isozymes from roots (lanes 1 and 2) and from spent medium (lanes 3 and 4) developed by activity staining of the crude enzyme separated on 7.2% native polyacrylamide gel and partially purified fraction (lane 5). R_m represents the relative mobility of respective isoform. (b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis of crude (lanes 1–3) and purified fraction of (lane 4) of BHR-POD. The electrophoresis was carried out by loading about 20 μl each of crude enzyme prepared from both biomass and spent medium and purified fraction (lane 4) in a 7.2% native PAGE (12 × 14 × 0.3 cm) and 10% SDS–PAGE were carried out at 120 V for 4 h. Lane M represents protein molecular weight markers.

2.7. Electrophoretic analysis

The POD zymogram was prepared by 7.2% polyacrylamide gel electrophoresis and carried out at 120 V for 4 h using a $12 \times 14 \times 0.3$ cm gel without SDS according to the standard protocol. After electrophoresis at 120 V for 4 h, the gels were washed thrice with 2.5% TritonX-100 solution to remove SDS. The gel was stained for POD activity with a 100 ml solution of sodium phosphate buffer containing 10 ml of 0.25% orthodianisidine hydrochloride and 10 ml of 1% H_2O_2 and immediately photographed. SDS–PAGE of purified enzyme was carried under similar conditions as described by Laemmli (1970) and stained with Coomassie blue (Blum, Beier, & Gross, 1987). The molecular weight of the purified enzyme was determined by SDS–PAGE on a 7.2% polyacrylamide gel. SDS–PAGE molecular weight standards were used as the marker proteins.

2.8. pH optima for the activity and stability of crude and purified enzyme

The optimum pH value for the activity of the HR-POD was found by assaying enzyme activity at different pH levels. The assay was carried out by taking buffers of different pH such as (pH 3–5, 0.1 M sodium citrate; pH 6–8, 0.2 M Sodium phosphate; pH 9, 0.2 M Tris–HCl) pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 separately in an assay mixture. The total 1 ml assay mixture consisted of 10 μl of the 60–80% dialyzed fraction of the enzyme, 100 μl 0.25% orthodianisidine hydrochloride, 100 μl of 1% H_2O_2 and 790 μl of buffers of different pH. The experiment was repeated using standard HRP. For the assay measuring optimum pH for stability, 100 μl of the 60–80% dialyzed fraction (crude) and from AEC-unbound fraction purified were taken and mixed with 100 μl each of different buffers such as pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 separately. The mixture stayed at room temperature (24 ± 1 °C) overnight (ca. 12 h). The activity was measured as explained earlier at pH 6.0. The experiment was repeated using standard HRP.

2.9. Thermal inactivation study

Temperature effects on the crude and AEC-derived enzymes were carried out by measuring the residual activity after incubating 100 μl of the enzyme for 45 min at different temperatures in a water bath.a Aliquots (10 μl) taken at different time intervals were assayed immediately and remaining activity was expressed as per cent decrease from the original activity.

2.10. Determination of K_m

The initial experiment was carried out to determine the K_m and V_{max} values for H_2O_2 by assaying the activity at different concentrations of H_2O_2 ranging from 0.01 to 0.2 mM at a constant orthodianisidine hydrochloride concentration (2 mM). Based on this experiment a constant concentration of 0.5 mM H_2O_2 was selected for determining the K_m and V_{max} values for other substrates such as ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline)-6-sulfonic acid) (1–10 mM), orthodianisidine hydrochloride (0.1–2 mM) and guaiacol (30–50 mM). The K_m and V_{max} values were determined by using the Lineweaver–Burk reciprocal plot graphic method (Segel, 1993) for the two substrate ping-pong mechanism followed by POD. Assuming initial rates (v_0), a general equation for this mechanism in the forward direction was given by Whitaker (1994) is as below

$$1/v_0 = 1/V_{max} + K_a/A_0V_{max} + K_b/B_0V_{max} \quad (1)$$

where V_{max} = maximum velocity, $K_a = K_m$ for substrate A (H_2O_2), A_0 = concentration of substrate A, $K_b = K_m$ for substrate B ('H donor), and B_0 = concentration of substrate B, from a plot of Eq. (1), enzyme systems following sequential mechanism (random or ordered) give lines that intersect to the left of y-axis, whereas, the systems that follow a ping-pong mechanism give parallel lines (Whitaker, 1994). When A_0 is constant, Eq. (1) will yield a slope intercept given by

$$\text{Slope} = K_b/V_{max} \quad (2)$$

Therefore, the constants K_m and V_{max} were finally determined from Eq. (2).

2.11. Enzyme activity inhibition

The potassium periodate, and sodium azide inhibition of the AEC partial purified POD was determined by using a constant orthodianisidine hydrochloride concentration (0.5 mM). The individual experiments in which the concentrations of potassium periodate and sodium azide ranged from 0.1 to 0.5 mM at three different concentrations of H_2O_2 (0.5, 0.6 and 0.9 mM) were carried out. The required aliquots of potassium periodate and sodium azide were separately added to a 1 ml reaction mixture containing 0.5 mM orthodianisidine hydrochloride and individual concentrations of H_2O_2. The blank consisted of all the reagents except potassium periodate (or sodium azide) and H_2O_2. The K_i was determined from the intercepts of a plot of $1/v$ (reciprocal of initial velocity) versus potassium periodate (or sodium azide) concentrations.

3. Results and discussion

3.1. Intracellular and extra-cellular POD

Fig. 1a shows the active staining of PODs of intra- and extra-cellular ones separated on 7.2% polyacrylamide gel. POD from the hairy roots of clone LMG 150 showed five distinct bands each corresponding to relative mobility (R_m) of 0.06, 0.16, 0.25, 0.38 and 0.46. Of these 0.06 0.16 and 0.25 were found in the spent medium. The bands 0.38 and 0.46 were not present in the medium, whereas the band with R_m value of 0.58 was found only in the medium, which

was absent in the biomass and this may be the isozyme that was completely secreted in to the medium. Therefore, red beet hairy roots totally produced about 6 POD isoforms of which three were secreted partially into the medium and only one was completely secreted into the medium. The protein molecular markers on the extreme right lane of Fig. 1a gives a rough idea about the nature of isozymes though a native gel should not be compared with such markers.

PAGE with active staining (Fig. 1a) showed four distinct bands of R_m 0.06, 0.16, 0.25, 0.38 and 0.46 in the biomass and bands at R_m 0.06, 0.16, 0.25 and one extra band of R_m 0.575 in the spent medium where isozymes of R_m 0.38 and 0.46 were totally absent. This pattern indicates that four of the six isozymes were of secretary-type-proteins but the degree of secretion varied allowing a good scope for improving the secretion. The involvement of specific signals for leaching a specific type of POD isozyme forms an interesting study for the future. Further, there was a clear distinction between the different isoforms differing by R_m value of at least 0.15, which implies that the isoforms are amenable for easy separation and further purification (Fig. 1).

Table 1
Levels of purification of red beet hairy root peroxidase obtained after the application of different purification steps leading to the improvement in the activity of enzyme

Purification step	Protein (mg)	Activity (units)	Specific activity (U mg^{-1} protein)	Fold (s) of activity
Crude extract	168.0	1.17×10^6	700.0	1.0
Ammonium sulfate precipitation	123.5	2.16×10^6	1750.0	2.5
AEC[a] (DEAE Cellulose[b])	9.5	0.99×10^6	10500.0	15.0

[a] Anion exchange coloum.
[b] Diethyl amino ethyl cellulose.

3.2. Purification of POD

3.2.1. Fractionation

The crude enzyme extract was concentrated by progressive fractionation by ammonium sulfate precipitation from 0–20, 20–40, 40–60, 60–80 and 80–100%. The fraction obtained with 60–80% showed maximum activity and minimum total protein content (data not shown). Further, the fraction was dialyzed for 48 h using 12,000 kDa cut-off range dialysis membrane against 0.2 M sodium phosphate buffer (pH 6.0). This primary purification resulted step in about 2.5-fold purification of POD from the crude enzyme extract (Table 1). The dialyzed fraction was used for further purification by anion exchange chromatography.

3.2.2. Anion exchange chromatography (AEC)

Partial purification of the enzyme was achieved by ion exchange chromatography by loading the dialyzed 60–80% $(NH_4)_2SO_4$ fraction on to a DEAE (diethyl amino ethyl) cellulose column that had been activated, prewashed and equilibrated with 0.2 M sodium phosphate buffer of pH 6.0. As shown in Fig. 2 most of the activity was recovered in the void when eluted with 0.2 M sodium phosphate buffer (pH 6.0). This resulted in the elution of a total of two peaks, a major and a minor, whereas a negligible level of POD activity was observed in the fractions eluted 0–0.5 M NaCl gradient in 0.2 M sodium phosphate buffer (pH 6.0) (data not shown). Further the major peak recovered from AEC column was dialyzed against double distilled water for about 48 h. The enzyme activity and protein content were estimated and the results expressed in terms of specific activity showed about 15-fold increase in specific activity when compared to crude (Table 1). The fraction was concentrated by lyophilization (here onwards represented as BHR-POD) and used for further characterization.

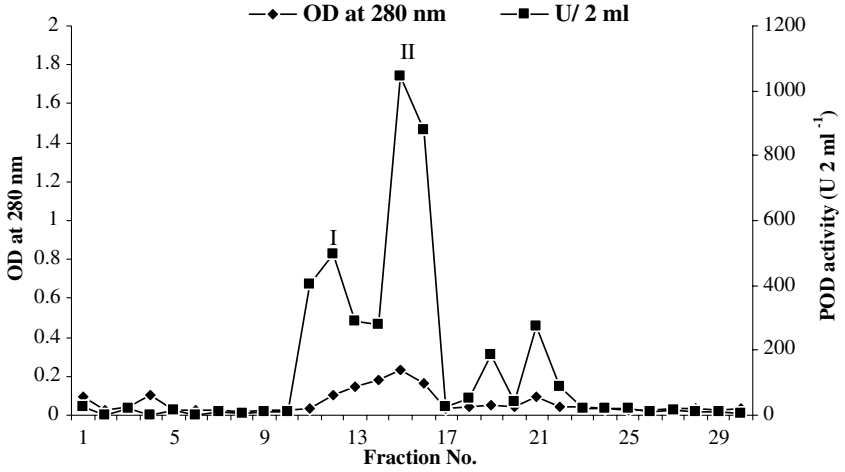

Fig. 2. Anion exchange chromatography of BHR-POD: Elution profile of unbound fraction from DEAE cellulose column obtained in 0.2 M sodium phosphate buffer (pH 6.0) as 2 ml fractions. The absorbance of protein at 280 nm is presented on the left hand *y*-axis whereas the POD activity of individual fraction is presented on the right hand *y*-axis.

3.3. Biochemical characterization of BHR-POD

3.3.1. Molecular weight and purity

The molecular weight and the purity of the enzyme were analyzed by SDS–PAGE and native PAGE analysis (Fig. 1). Coomassie brilliant blue staining of SDS–PAGE gel and the activity staining of the native gel of the peak-I showed some impurities, whereas the peak-II analysis showed a single band (Fig. 1b) and hence this fraction was used for further studies. The authors, however, do not claim this as a totally pure preparation, since there are chances for different types of PODs having similar properties and are difficult to separate by biochemical methods. The specific activity was 10,500 U mg^{-1} protein, which is very high compared to PODs from various other sources (Duarte-Vazquez, Garcia-Almendarez, Regalado, & Whitaker, 2001).

The peak-II when subjected for SDS–PAGE analysis under reducing (ß-mercaptoethanol) conditions showed a single band when stained with Coomassie brilliant blue, confirming the purity of the enzyme and also indicating that it is a single polypeptide chain. The molecular weight was found to be 45 kDa (Fig. 1b), which is similar to that of HRP (40–46 kDa) (Paul & Stigbrand, 1970), and sycamore maple POD (42 kDa) (Dean, Sterjiades, & Eriksson, 1994). Molecular weights of most of the PODs vary from 30 to 60 kDa (Srivastava & van Hyustee, 1977). This variability in the molecular weights of PODs was attributed to the post translational modifications and also the length and number of glycan chains in the polypeptide chain (Van Huystee, Sesto, & O'Donell, 1992).

3.4. Kinetic studies

3.4.1. Substrate specificity

Generally peroxidases are specific for H_2O_2 as a substrate, but can also use a number of ·H donors, one such being the orthodianisidine hydrochloride (Duarte-Vazquez et al., 2001). Hence, the experiments were carried out to test the substrate specificity of BHR-POD. The AEC-purified fraction was assayed at various concentrations of different ·H donors and H_2O_2. The Eqs. (1) and (2) were used to determine the K_m and V_{max} values from the Lineweaver–Burk plots for various POD substrates such as H_2O_2, orthodianisidine hydrochloride, ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline)-6-sulfonic acid) and guaiacol (Fig. 3). The Fig. 3a is the Lineweaver–Burk plot for H_2O_2 at 2 mM othodianisidine, which shows lowest K_m value of 0.1 indicating the specificity of BHR-POD to H_2O_2. Among the ·H donors studied the enzyme appeared to have the affinity in the order orthodianisidine > ABTS > guaiacol, meaning, the enzyme showed

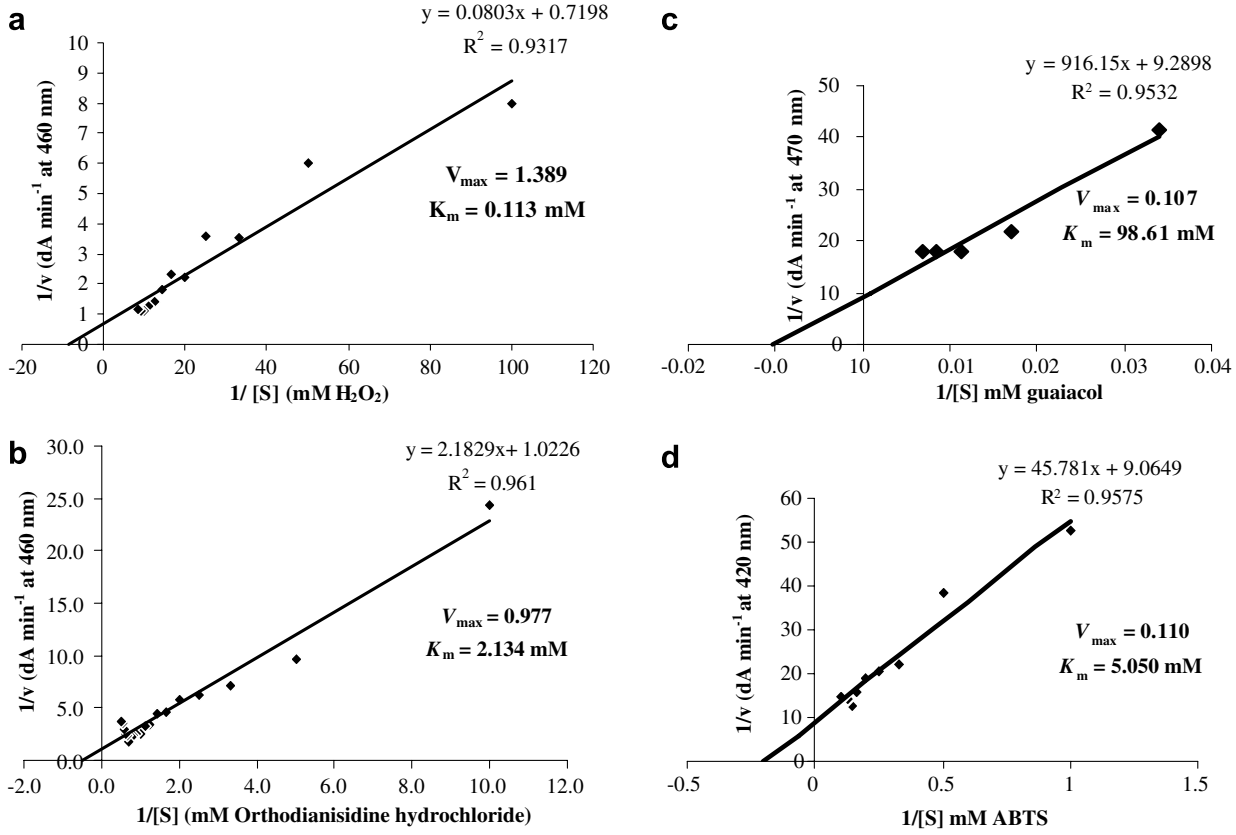

Fig. 3. Lineweaver–Burk double reciprocal plots for POD activity at various concentrations of H_2O_2 at 2 mM orthodianisidine hydrochloride (a); orthodianisidine hydrochloride at 0.5 mM H_2O_2 (b) and Lineweaver–Burk double reciprocal plots for POD activity at various concentrations of ABTS at 0.5 mM H_2O_2 (c) and guaiacol at 0.5 mM H_2O_2 (d). The assay was carried out at 25 °C using 0.2 M sodium phosphate buffer (pH 6.0).

more affinity towards orthodianisidine as indicated by the lowest K_m value among the ·H donors (Fig. 3b–d). The orthodianisidine K_m value was 2.134 mM (Fig. 3b) and this value was lower than those found for guaiacol oxidation by POD from turnip roots (3.7 mM) (Duarte-Vazquez et al., 2001) and Korean radish roots (6.7–13.8 mM) (Lee & Kim, 1994). These results suggest that BHR-POD has higher specificity to orthodianisidine than to other substrates checked in this study.

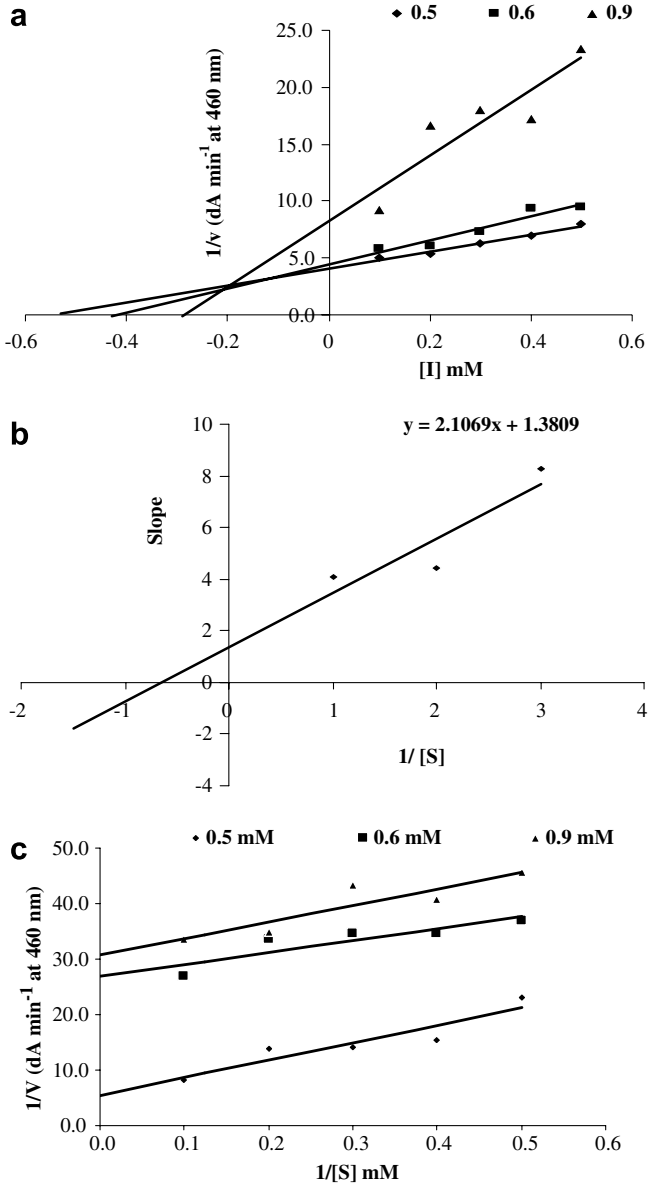

Fig. 4. Dixon plots for beet hairy root POD inhibition, the experiments were conducted by assaying for POD activity at different inhibitor concentrations (0–0.6 mM) of potassium periodate (a), slope re-plot (b) and sodium azide inhibition of hairy root POD (c). From the plot it can be inferred that the periodate acts in a competitive manner in inhibiting the BHR-POD whereas the sodium azide acts in a non-competitive manner in inhibiting the beet hairy root POD. The values are the average of three independent experiments.

3.4.2. Inhibition of HR-POD

Potent inhibitors of hemoprotein catalyzed reactions such as periodate (Fig. 4a and b) and sodium azide (Fig. 4c) (Duarte-Vazquez et al., 2001) were tested for BHR-POD inhibition and the data were analyzed by using Dixon plots. The plots indicate that the periodate acts in a competitive manner (Fig. 4a and b) ($K_i = 0.2$ mM) whereas the sodium azide causes the POD inhibition in non-competitive manner (Fig. 4c). This data indicates a much higher sensitivity of POD to periodate compared to sodium azide. The inhibition by sodium azide is exactly opposite of that reported for turnip POD (Duarte-Vazquez et al., 2001).

3.4.3. pH optima for activity and stability

When checked for pH optima using orthodianisidine hydrochloride as H donor, the crude POD from red beet hairy root showed maximum activity at pH ranging from 5 to 6 whereas the commercial HRP showed highest activity at pH ranging from 4 to 5. The enzyme was stable over a wide range of pH from 4 to 9 exhibiting highest stability at pH 7 and 9 (Fig. 5a). The purified BHR-POD showed highest activity at pH 5 which was stable over a wide range from pH 3 to pH 9 with highest stability between 6 and 8 (Fig. 5b). The pH for optimum activity was very similar to that of strawberry fruit (6.0) (Civello, Martinez, Chaves, & Anon, 1995) tomato (5.3–5.5) (Heidrich, Lorenz, & Schreier, 1983) and soybean (5.4) (Sessa & Anderson, 1981) PODs. The commercial HRP showed highest activity at slightly more acidic pH (4–5) compared to crude and BHR-POD (Fig. 5a).

HRP was stable at a narrow range of pH between 4 and 6 and showing highest stability at pH 9 similar to crude beet hairy root POD (Fig. 5a). Therefore, the versatility of BHR-POD is an added advantage allowing wider applications than HRP.

3.4.4. Thermostability of the purified POD

The thermostability of the crude enzyme has been reported in our earlier communication as the percent increase or decrease of activity as a function of three different temperatures during a period of 45 min. The PODs extracted at acidic and neutral pH showed negligible inactivation up to 50 °C with almost 95% of the activity being retained even after 40 min. However, the POD of basic pH was very sensitive to temperature with 50% loss at 50 °C in 40 min, with the total loss of activity at 60 °C (Thimmaraju et al., 2005). At the latter temperature (60 °C) the acidic and neutral PODs retained more than 70% of the activity up to 40 min with complete inactivation at 70 °C. Nevertheless, the purified enzyme was more stable (Fig. 5c) compared to its crude state as it retained more than 70% activity at 70 °C even after 20 min while the commercial HRP had lost most of its activity within 11 min at 70 °C (Duarte-Vazquez et al., 2001). Therefore, the purified intracellular BHR-POD reported in this communication showed the properties either on par or better than the commercial HRP.

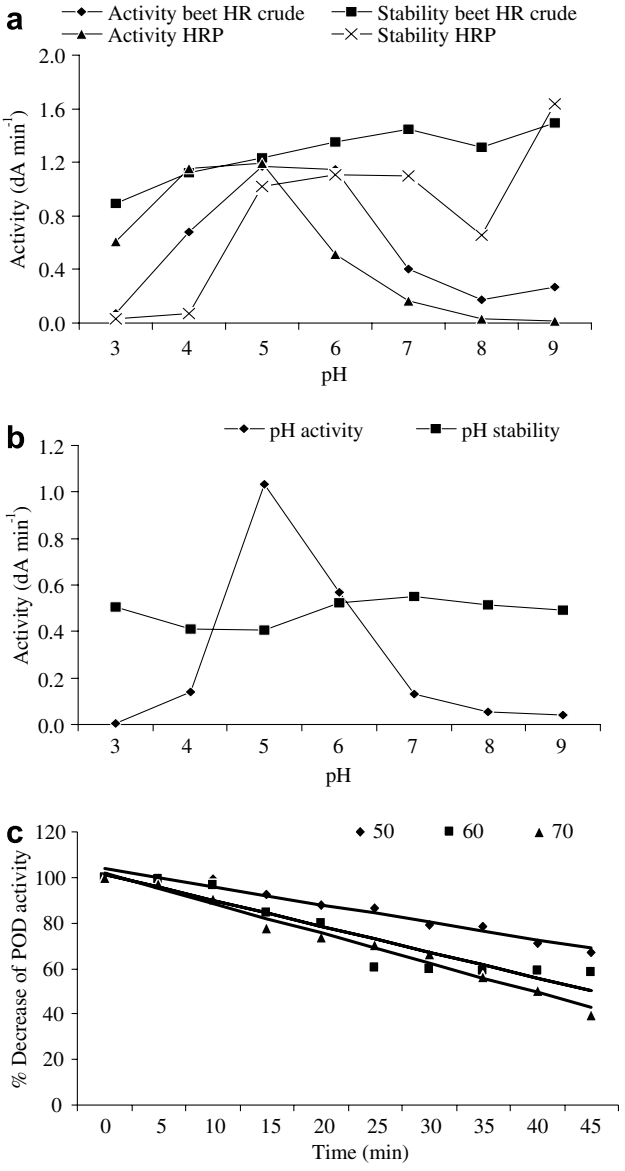

Fig. 5. Effect of pH on the activity and stability (incubated for 12 h) of crude beet hairy root POD, horseradish POD (a), and AEC fraction of BHR-POD (b). The buffers used were pH 3–5 sodium citrate (0.1 M), pH 6–8 sodium phosphate buffer (0.2 M); pH 9 Tris–HCl (0.2 M). Fig. 5c represents thermostability of BHR-POD observed at three different temperatures (50, 60 and 70 °C) over a time scale of 45 min where 500 μl of the purified enzyme in sodium phosphate buffer (pH 6.0) was incubated at three different temperatures and the rate of loss of activity was monitored by taking 10 μl from each of the treatments and measuring the activity i.e., dA min^{-1} at 460 nm. Data presented is an average of 5 replicates of two independent experiments.

4. Conclusion

The present study has shown that red beet hairy roots produce copious levels of POD, which was purified to homogeneity. The purified enzyme showed better thermal stability than the commercial source from horseradish, indicating its wider applications. The present study has also found that the kinetics of purified enzyme of 45 kDa was not much in accordance with the Michaelis rules, making this enzyme an interesting candidate for further studies.

Acknowledgments

TR and LV are grateful to CSIR, India for financial support in terms of Senior Research Fellowships. Valuable suggestions by Dr. Sridevi Annapoorna Singh of Protein Chemistry Department of CFTRI is gratefully acknowledged. The authors thankfully acknowledge the Director, CFTRI and the Head, Plant Cell Biotechnology Department for their constant support and encouragement during the research work.

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