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Purification and Kinetic Properties of Guaiacol Peroxidase in Turnip (*Brassica napus* var. okapi) Root During Different Growth Stages

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ABSTRACT

In this study, we present changes of peroxidase (E. C. 1.11.1. 7) activities in the root of *Brassica napus* var. Okapi during rosette stage until ripening of the fruits. Peroxidase activity was determined seasonally over an eight month period. Results showed that total peroxidase activity in the root was highest in mid June and the lowest activities were observed from time of winter dormancy until the beginning of April at which time morphogenic competence of tissues started to increase. Pattern of isoperoxidase bands on poly acrylamide gels (PAGE) showed that there was six inducible isoforms, named as TPA1, TPB1, TPB2, TPC1, TPC2 and TPC3. The isoform TPC3 was purified and partially characterized. Purification of peroxidase from turnip root was achieved by two ammonium sulphate precipitation steps followed by DEAE-sephadex chromatography. The effects of pH and temperature on enzyme activity were determined with guaiacol as electron donor. Highest activity was obtained at pH 6.0-6.5 and at a temperature of 50 °C. The enzyme was active at pH values below 7.0 even after 24 h and remained active after heat treatment at 70 °C for 30 min. It was inhibited by sodium cyanide rather than sodium azide. Loss of TPC3 peroxidase activity in the extracts of non-senescent root tissues suggests that it might play a role in the senescing process.

Keywords: *Brassica napus*, DEAE-sephadex chromatography, Guaiacol peroxidase, purification, seasonal changes

Abbreviations: ANOVA —one way variance analysis AOS—activated oxygen species; DEAE—diethylaminoethyl; GP—guaiacol peroxidase; PAGE—polyacrylamide gel electrophoresis; PBS—phosphate-buffered saline; SDS—sodium dodecyl sulphate.

Introduction

Peroxidases (EC 1.11.1.7.) belong to a large family of enzymes that are ubiquitous in fungi, plants, and vertebrates. The enzyme usually contains a ferriprotoporphyrin IX prosthetic group and oxidizes several substrates in the presence of hydrogen peroxide (Vianello *et al.,* 1997). Higher plants are known to possess a large set of peroxidases. They have multiple molecular weights and a broad subcellular distribution. During plant development, patterns of the peroxidase isoforms appear to differ depending on source, organ or growth stage of sample being analyzed. Thus, it seems that the expression pattern of peroxidases is tissue specific and developmentally regulated (Klots *et al.,* 1998). This enzyme has been widely used as a reagent for

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organic synthesis and biotransformation as well as in coupled enzyme assays, chemiluminescent assays, immunoassays and treatment of waste waters (Veitch, 2004). Horseradish (*Armoracia sp*.) roots represent the traditional source for commercial production of peroxidases (Krell, 1991), but it is possible that other plant could provide some isoperoxidases that exhibit similar or improved characters such as better substrate specificity, high thermostability, high yield and economic feasibility (Macek *et al.* 1993). Turnip (*Brassica napus*) is an annual herb cultivated in many regions of the world mainly for its valuable seed oil and also as a protein-rich feedstuff (Mestek *et al.,* 2007). There are few reports about turnip peroxidase; Mazza *et al.* (1968) have detected five peroxidase isoenzymes (three anionic and two cationic) in turnip roots. Agostini *et al.* (2002) used peroxidase isoenzyme secreted by turnip hairy-root cultures for phytoremediation of 2, 4-dichlorophenol and diagnostic kits.

Growth stages of the turnip plant are consist of germination, plantlet, rosette, winter dormancy, shooting, flowering, and ripening stages. The time of each stage is depends on nutrition, light intensity and environmental conditions. In order to be able to produce and purify peroxidases locally, we sought an appropriate source for the enzyme. The aims of this study were to determine changes in quantitative and qualitative expression of the peroxidase isozymes in the turnip root during rosette to ripening stages, introduce a purification protocol for isolation of the one of the isozymes and determine the kinetic properties of the partially purified enzyme.

Materials and Methods

Plant material

During December to June, turnip (B*rassica napus* var. Okapi) roots were collected from a field in Hamedan (Hamedan Province, Iran) at four growth stages (rosette, winter dormancy, shooting and ripening stages). Roots were quickly transferred to the laboratory, where they were cleared from dirt particles and maintained in -20˚C until use.

Protein and peroxidase assay

Turnip roots were grinded using a blender placed in a cold room (4 °C), homogenized in PBS (20 mM, pH 6.8) containing 2% polyvinyl polypyrrolidone (PVPP) and then centrifuged (30000 g, 45 min, 4 °C).

Protein concentration was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as standard protein. Also, peroxidase activity of the crude extracts was measured at four growth stages. Guaiacol peroxidase (GP) activities were estimated as oxid ation of guaiacol to the formation of tetraguaiacol (ε =26.6 mM^{-1} cm^{-1}) in 3 ml reaction mixtures containing 0.95 ml of citrate buffer (100 mM, pH 4.6), 0.95 ml of H_2O, 1 ml of guaiacol 15 mM, 50 µl of H_2O_2 32 mM, 50 µl enzyme extract (Liu *et al.,* 1999). The reaction was started by addition of guaiacol; assays were carried out at 25 °C and initial rates were calculated from time-dependant absorbance changes (increase of absorption at 470 nm). One unit of peroxidase activity represents the amount of enzyme that catalyzes the oxidation of one micromole of guaiacol in one minute. All experiments were carried out with three replications per each treatment.

Peroxidase purification

Roots in ripening stage (mid June) was selected for purification of the enzyme because of observation of highest activity at that stage. Three hundred grams of roots were homogenized (1:5 w/v) in a warring blender. The crude extract was brought to

30% saturation with solid ammonium sulfate, allowed to stand 8 hours at 4 °C and then centrifuged (15000 g, 20 min, 4 °C). The supernatant was brought to 80% saturation with solid ammonium sulfate and stirred over night in a cold room (4 °C). Peroxidase was concentrated in a precipitate obtained by centrifugation (26000 g, 20 min, 4 °C), dispersed in the extraction buffer, dialyzed against the same buffer, and applied to a DEAE-Sephdex A-50 column (4x14 cm) previously equilibrated with the extraction buffer at 4 °C. Peroxidase was eluted from column in two steps; at first, elution was performed with 100 ml of PBS (0.02 M, pH=6.8), then with 0-1.5 M NaCl gradient in the same buffer. Elutes were monitored at 280 nm for protein and at 403 nm for Soret bands and peroxidase activity. Protein fractions which showed both 403 nm absorbance and peroxidase activity were pooled and considered the peroxidase extract.

Electrophoresis

Seasonal changes in pattern of peroxidase activity in the root tissues were investigated by gel electrophoresis according to Hames and Rickwood (1990). The protein extracts were run on 7.5% polyacrylamide gel (PAGE) in a cold room (40 mA, 6 h). Peroxidase bands were detected on the gel by submerging the gel in a staining solution containing 80 ml of acetate buffer (0.1 M, pH 5), 8 ml of H_2O_2 3% and 4 ml of benzidine 0.04 M in methanol 50% (Van Loon, 1971). Purity and molecular weight of TPC3 was examined on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Hames and Rickwood, 1990). Proteins were visualized by staining with Coomassie brilliant blue R_{250}. Molecular weight markers were obtained from Fermentas (Lithuania; Mw = 11-170 kDa).

Kinetic properties of TPC3

The apparent Michaelis-Menton constant for H_2O_2 as oxidant substrate was calculated. The reaction mixture contained 100 mM citrate buffer pH 4.6, fixed saturate concentration of guaiacol and H_2O_2, during one minute. H_2O_2 was used at concentrations in the range of 0-3 mM. Initial estimates of Km were obtained from Lineweaver-Burk plots, a linear transformation of the Michaelis equation. Km H_2O_2 and Vmax were calculated from primary and secondary plots of $1/v_0$ versus $1/[S_0]$.

The pH optima were determined at 25 °Cusing two buffer systems, 100 mM phosphate-citrate ranging from pH 2.5 to pH 8 and 100 mM glycine-NaOH buffer ranging from pH 8.5 to pH 9.5. Determination of pH stability for peroxidase was performed by transferring 50 µl of purified enzyme into 950 µl phosphate-citrate buffer (pH 2.5- 8) for 1 and 24 hour at 25˚C, and the samples were assayed for remaining peroxidase activity.

Effect of temperature on enzyme activity was determined at various temperatures. For determination of enzyme thermostability during incubation, 50 µl of solubilized enzyme was preincubated in 0.1 M citrate buffer, pH 4.5 for 10, 20 and 30 min at different temperatures (30-80 °C). Then remaining activity was determined and expressed as percentage of initial enzyme activity.

Effect of inhibitors including sodium azide and sodium cyanide on TPC3 activity was investigated after a 3 min preincubation of purified extract with selected inhibitor concentrations in 100 mM citrate buffer. The concentration ranges used were 0-2 mM NaN_3 and NaCN, and these ranges were and chosen as a function of the sensitivity of the extracts towards the inhibitors.

Statistical analysis: Data were subjected to ANOVA (one-way variance analysis) using

statistical software SPSS 10 (SPSS Inc, Chicago, USA). General Linear Model (GLM) procedure was performed to examine the effects of temperature, pH and interaction of each factor with time on the stability of enzyme. The means were presented for averages of experiments that were repeated at least three times. Means values were compared by post hoc Tukey test. The term significant indicates differences for which $p<0.05$.

Results And Discussion

Seasonal changes of G P activity

Specific activity of Peroxidase increased in the root tissue throughout the growing period as turnip ripened (Fig. 1). The lowest total activity was observed in the turnip root during late December (rosette stage) to beginning of April when morphogenic competence of organs started to increase so as to longitudinal growth of the shoot. The highest activity was detected at mid June when turnip seeds developed to maturity and roots were very stiff and lignified.

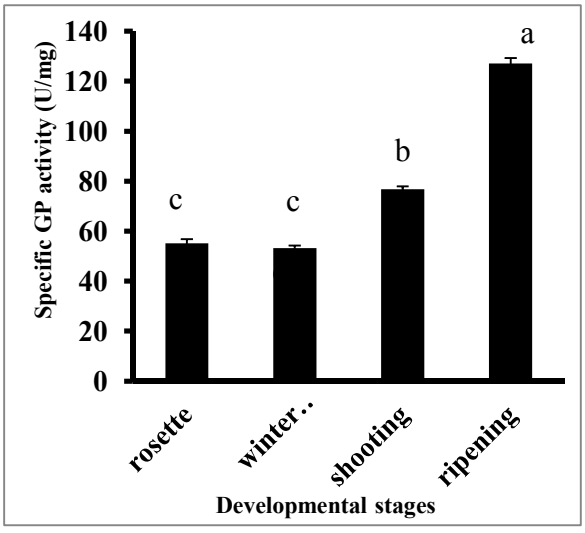

Figure 1. Seasonal variation of total peroxidase activity in crude extracts of turnip roots collected from late December to mid June. Data are expressed as means of three replicates and standard errors are shown by vertical bars. Specific activity is expressed as units (U) per mg of protein.

Seasonal changes of peroxidase isozymes clearly revealed six isoforms depending on root age. The six isoforms were named TPA1, TPB1, TPB2, TPC1, TPC2 and TPC3 based on their mobility in the PAGE analysis (Fig. 2). TPA1 with the fastest mobility was common to all profiles of root extracts but it was strongest in the April profile. In addition, TPC1 with the slowest mobility (Rm=0) was also observed in all growth stages too but its expression was poor after end of the dormant period. It seems that TPC2 and TPC3 were expressed from two different peroxidase genes; each isoenzyme appeared as a strong band accompanying weak bands in polyacrylamide gels which had been stained for peroxidase (Fig. 2). The peroxidases with intermediate mobility as compared with other isozymes were TPB1 and TPB2. The first isozyme was expressed by a heterozygous gene which appeared as two monomeric bands with same intensity and it was active in the turnip root with the exception of shooting stage. The second, TPB2, had overexpresion pattern only in the shooting stage and most likely had homozygous form.

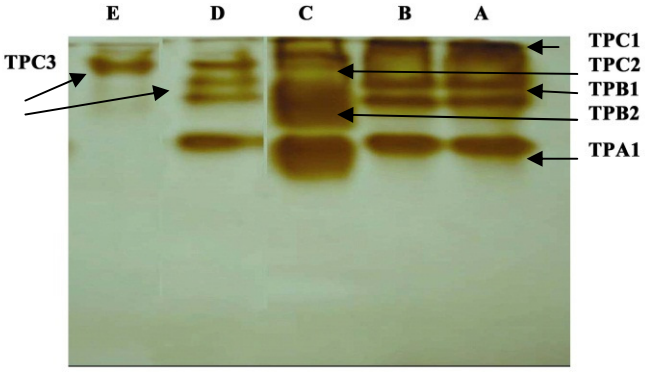

Figure 2. PAGE analysis of the turnip root peroxidases in different growth stages including, A: late December (rosette stage), B: February (winter dormancy stage), C: April (shooting stage), D: mid June (ripening stage).

E represents the purified isoenzyme of ripening stage obtained by DEAE-Sephadex chromatography.

A similar pattern was observed in samples collected during late December until mid February; the pattern consisted of TPA1, TPB1 and TPC1 (profile 1 and 2 in Fig. 2). Broadening of the TPA1 and TPB2 bands on the PAGE gel may be attributable to the high glycosylation of peroxidases because most reported plant peroxidases have oligosaccharide chains linked to asparagines (O'Donnell *et al.,* 1992). TPB2 and TPC2 were expressed in shooting stage and TPC3 in the ripening stage. TPC2 and TPC3 are time-specific enzymes because they were activated only at the shooting and ripening stages, respectively.

Table 1. Partial purification of a peroxidase isoform purified from turnip root at ripening stage.

Purification procedure	Total protein (mg ml^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)	RZ
Crude extract	230. 32	1493. 96	6. 48	1	100	0. 077
Ammonium sulfate precipitate	92. 30	775. 48	8. 40	1. 29	51. 9	nd
TPC3	3. 4	231. 68	67. 94	10. 47	15. 5	0. 159

Units (U) are expressed as µmol of guaiacol oxidized per min and specific activity is expressed as units (U) per mg of protein. Data presented are average of three replicates, nd: not determined.

In higher plants, the number of isoenzymes may be extremely high; up to 40 genes corresponding to isoperoxidases for each plant and several other isoforms can be generated by posttranscriptional and posttranslational modifications (Johri *et al.,* 2005). Maximum number of the GP bands was observed after winter dormancy stage, when biological activities restart. Several physiological functions have been suggested for peroxidase, for example cell wall lignifications (Vitali, 1998), cell wall stiffening (Fry, 1986), auxin metabolism (Lagrimini *et al.,* 1997) and root elongation (Cordoba-Pedregosa *et al.,* 1996). Also a significant increase in antioxidant enzymes, especially guaiacol and ascorbat peroxidase activity, helps to detoxificate activated oxygen species (AOS) under stress (Dabrowska *et al.,* 2007; Soda *et al.,* 1991). The GP isoenzymes detected in turnip root may be involved in some of the functions mentioned above.

Extraction and purification of TPC3

Root peroxidase isozymes existing at the ripening stage were partially purified; the procedure is summarized in Table 1. Protein yield and peroxidase activity of the TPC3 at each purification stage were estimated. Crude extract (6.48 U mg^{-1}) after concentration and dialysis was fractionated on DEAE-Sephadex A-50 column chromatography into an unadsorbed peak with high peroxidase activity (67.94 U mg^{-1}) and an adsorbed peak with low peroxidase activity (1.14 U mg^{-1}). The elution profile in Figure 3 shows two sharp

peaks of peroxidase activity. Peak No. 1 consisting of fraction numbers 8-40 was TPC3 and peak No. 2 which consisting of fraction numbers 50-68 was TPA1. TPC3 was not adsorbed to the column and was eluted with first elution, but TPA1 was adsorbed to the column at first elution and subsequently was eluted in increased concentrations of NaCl. Kinetic properties of the TPC3 from two isolated fractions were studied. An overall recovery of 15.5% (3.4 mg) and a 10.47-fold purification of the TPC3 were achieved (Table 1).

RZ value and molecular mass determination

The purity of peroxidase preparations was determined by measuring the ratio of the heme absorbance (at 403 nm) to the protein absorbance (at 280 nm). This ratio is denoted the RZ (Reinheistszahl) value. PurifiedTPC3 gave a preparation with a low RZ value (0.159) in comparison with that of the highest grade of commercial HRP, suggesting the presence of non-peroxidase proteins with similar chromatographic behavior to that of the enzyme. Purified fraction of TPC3 appeared as two major bands with molecular masses of 34 and 38 kDa in SDS-PAGE (Fig. 4). On the other hand, it was illustrated two bands on the PAGE gel as one very weak and another stronger band. It is possible that the two major polypeptides are two subunits which are belong to a dimeric structure of TPC3. Thus, average molecular masses of the native purified peroxidase may be was between ~68 to ~76 kDa. Also, the existence of weak bands together with TPC2 and TPC3 may reflect artifacts resulting from proteolytic cleavage, altered glycosylation or existence of different forms of the dimeric structures. Future findings are expected to clarify this issue. A wide range of molecular weights has been reported for isoperoxidases from different sources. In the previous reports, the molecular weight of turnip peroxidase that purified as a single polypeptide was found to be 37-39 kD with matrix assisted laser desorption ionization mass spectrometer (Singh *et al.,* 2002).

Characterization of the TPC3 peroxidase

The apparent K_m and V_{max} values of TPC3 for H_2O_2 were calculated. K_m was 55 µM and V_{max} was 5 nmol S^{-1} (Fig. 5). Purified TPC3 isoperoxidase from turnip root had low Km, but substrate inhibition was not observed even by 9 mM H_2O_2. Agostini *et al.* (2002) reported a peroxidase isoform (secreted by hairy roots of *Brassica napus*) whose apparent K_m value was 79 µM and which remained active at higher concentrations of H_2O_2 (up to75 mM) (Agostini *et al.,* 2002). Some studies report inhibition of peroxidases at higher concentrations of H_2O_2 (Hiner *et al.,* 2001; Moosavi Movahedi *et al.,* 1999). Peroxidases exhibit catalase activity, which is the reduction of hydrogen peroxide to water outside of the normal enzymatic cycle. It seems that catalase activity of peroxidases is dependent on the concentrations of the enzyme and H_2O_2 present in the solution.

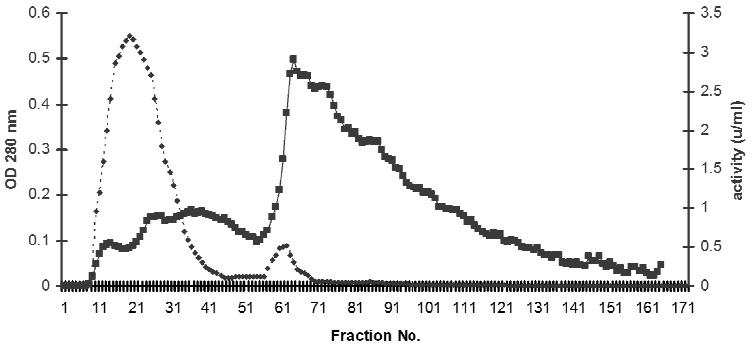

Figure 3. Purification of turnip root peroxidase by successive chromatography. Gradient elution profile from DEAE-Sephadex A-50 column. Absorbance at 280 nm (■); guaiacol peroxidase activity (♦).

As shown in Figure 6a, the highest activity of TPC3 was observed between pH 6.0 and 6.5 with guaiacol as a substrate. There were

significant differences between treatment levels ($P<0.05$). The pH stability was studied between 2.5 and 8 values. After 24 h incubation at 25 °C, the enzyme was stable between pH 4.5 and pH 7.5 and its activity was kept higher than 50% of the control (Fig. 6b). However, activity decreased remarkably between pH 3.5 and pH 4, and even TPC3 was totally inactivated after 24 h incubation in pH 2.5-3.0 (Fig. 6b). Effect of pH, incubation time and interaction of these factors on TPC3 stability showed differences at $P<0.01$ level.

Effect of temperature on the GP activity and its stability are presented in Figure 7. At low temperatures, the activity of TPC3 was about 2-fold lower compared with 50 °C. The activity noticeably increased when temperature was raised to 50 °C. TPC3 showed a maximum activity at 50 °C and it decreased dramatically afterward. TPC3 was most active between 40 °C and 60 °C (Fig. 7a). There are significantly differences between applied temperature levels ($P<0.05$).

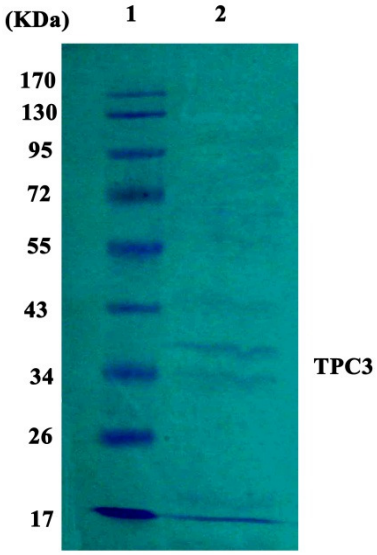

Figure 4. Electrophoresis of purified TPC3 on ten percent SDS-PAGE. Lane 1, Protein molecular weight marker (11-170 kDa, Fermentas, Lithuania) and lane 2, purified TPC3 fraction.

This enzyme exhibited high thermal stability; it could tolerate heating for 30 min at temperature up to 70 °C without being inactivated (Fig. 7b). The enzyme lost about 45% of its activity within 10 min at 60 °C and then GP activity has been stabled. While, peroxidase activity of TPC3 had not changed during incubation at 30 °C for 30 min, it was completely inactivated at 80 °C and after boiling for 10 min. Statistical analysis (GLM) revealed that effects of temperature, incubation time and interaction of those factors on TPC3 stability were significantly different at $P<0.01$, $P<0.05$ and $P<0.05$ levels, respectively.

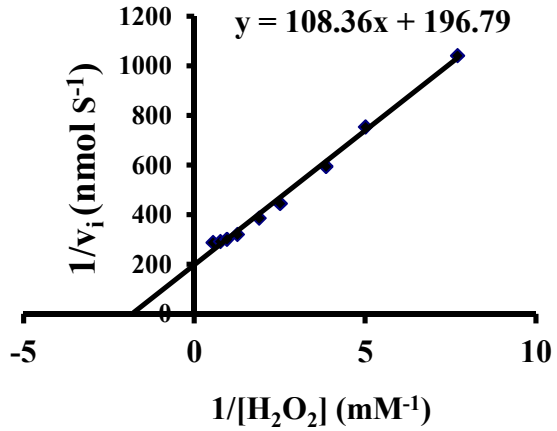

Figure 5. Lineweaver-Burk plot of purified TPC3, guaiacol peroxidase was assayed in the presence of increasing H2O2 concentrations. Data presented are average of three separate experiments.

Apoplastic, cytosolic, and soluble peroxidases of several plant tissues showed temperature optima between 30 °C and 60 °C, the most between 50 °C and 60 °C (Bernards *et al.*, 1999; Loukili *et al.,* 1999; Nair and Showalter, 1996; Soda *et al.,* 1991). To confirm that thermal stability of TPC3, a similar non-linear inactivation curve has been reported for other plant peroxidases (Deepa and Arumughan, 2002). Thermal stability of peroxidases has been attributed to the presence of large number of

cystein residues in the polypeptide chain (O'Donnell *et al.,* 1992). However, this is an overlay of two quite different processes; as increase in molecular motion, the rate of chemical reactions increases with temperature by a factor of 2-3 every 10 °C according to an empirical rule. This also holds for enzyme reactions and the increase may continue to extremely high temperatures. But protein nature of the enzyme is very sensitive to higher temperatures and denaturizing predominates and the enzyme becomes inactive (Bisswanger, 2004).

As shown in Table 2, classical peroxidase inhibitors like sodium cyanide or sodium azide caused a complete loss of the peroxidase activities or decreased the rates by more than 90%. Estimated IC_{50} value (the concentration of inhibitor that causes 50% loss of enzymeactivity) for sodium azide and sodium cyanide effects on GP activity of TPC3 were 300 µM and 1.3 µM, respectively (Fig. 8). Inhibitory effect of sodium cyanide via interaction with heme as prosthetic groups was more than sodium azide. According to the Veitch NC (2004), small molecules such as carbon monoxide, cyanide, fluoride and azide bind to the heme iron atom at distal site giving six-coordinate peroxidase complexes that inactivate the enzyme.

Table 2. Effect of cyanide and azide concentration on GP activity of TPC3 purified from turnip root*.

Concentration (µM)	% Residual GP activity	
	NaCN	NaN_3
0	100 a	100 a
3	22. 89 ± 1. 83 b	95. 71±5. 22 a
33	0. 93 ±0. 40 c	83. 64±1. 3 ab
100	0 c	66. 15 ±0. 59 c
500	0 c	34. 31 ±0. 65 d
1000	0 c	26. 75 ±4. 89 d
2000	0 c	10. 67 ±0. 71 e

*Activities were expressed as percentage of the control. Data are expressed as means of three replicates ± standard errors.
Values with same letters in each column had no significant differences ($p<0.05$).

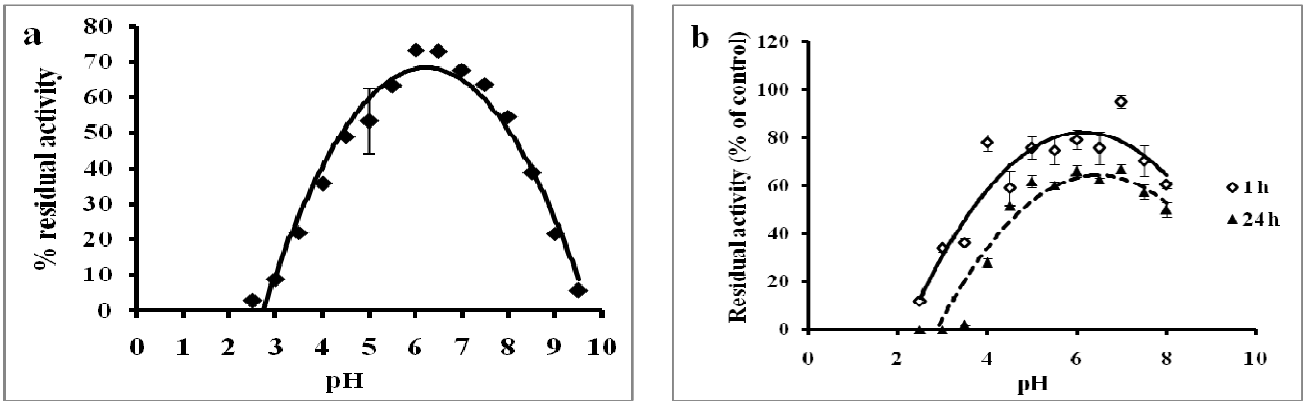

Figure 6. Properties of peroxidase from turnip root. (a) Effect of pH on the enzymatic activities of guiaicol peroxidase from turnip root, and (b) its stability after 1 and 24 h incubation of purified peroxidase within pH range 4 to 8. Data are expressed as means of three replicates ± standard errors bars.

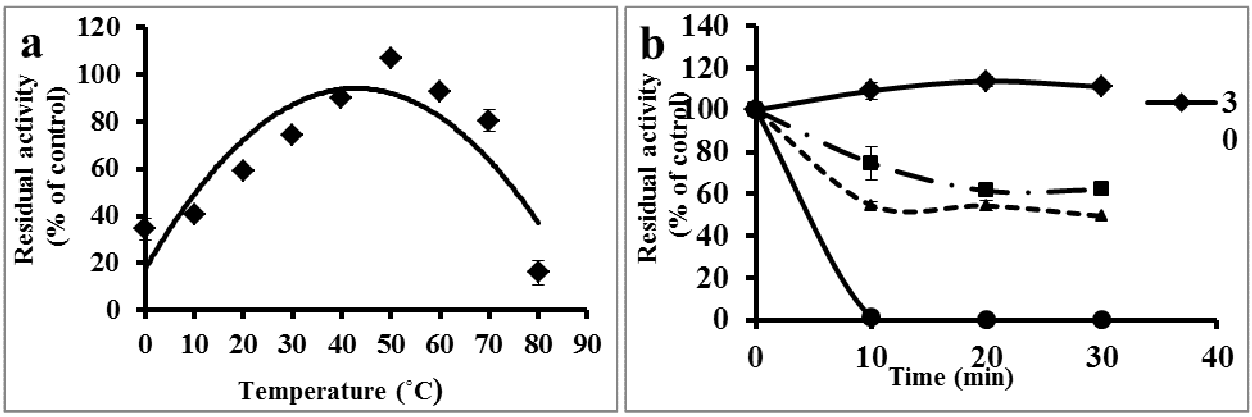

Figure 7. Effect of temperature on peroxidase activity. (a) Dependence of the guaiacol peroxidase activity to temperature in purified TPC3 and (b) Thermal stability of TPC3, soluble enzyme was incubated at 30-80 °C at different time slices. Data presented are average values ± SE of n = 3 experiments. The rates of guaiacol oxidation were determined under the standard assay conditions except for temperature.

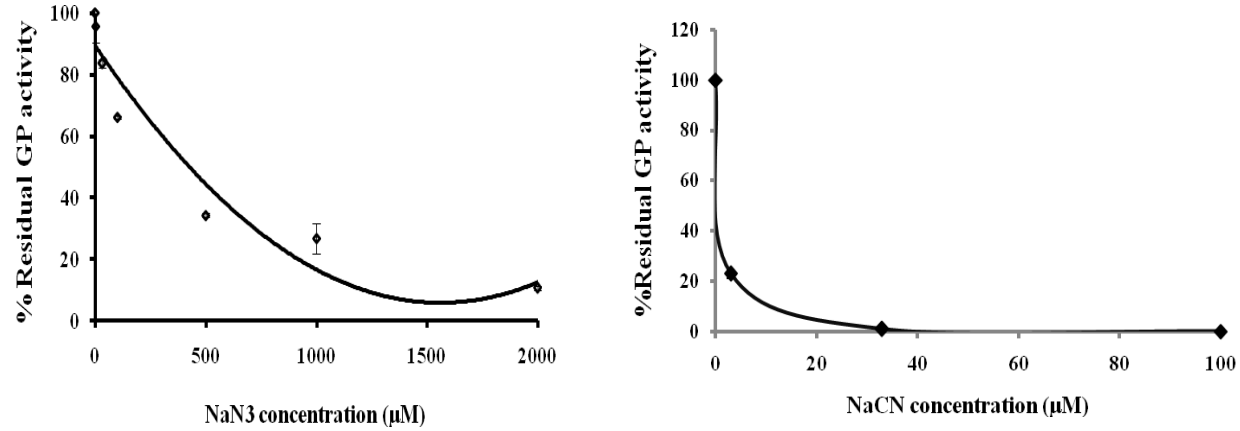

Figure 8. Effect of sodium azide on guaiacol peroxidase activity of TPC3 from turnip root. All assays were performed after 3 min preincubation of extract samples with different concentrations of azide. Activities were expressed as percentage of the control. Data are average of three separate experiments

Conclusion

Results of the present work demonstrated the occurrence of at least six bands with peroxidase activity in the turnip roots. Seasonal changes in the number of isozyme bands could be classified into 3 patterns depending on root age. These patterns were related to developmental process (rosette, shooting and ripening stages). TPC3 purified as a time-specific enzyme belonging to ripening stages appeared as two major bands with an average molecular mass of 34 and 38 kDa in SDS-PAGE. The kinetic study of the enzyme showed that it was thermostable and is an inexpensive and easily available source of peroxidase that retains its activity in alkaline pH. This feature should be beneficial in processes wherein peroxidase treatments are used to obtain processed products in high temperatures and pH. Thus, it can be used as an economic alternative of horseradish peroxidase with high activity. In

future, TPC3 could be efficiently used for the treatment of polluted water/industrial effluents contaminated with aromatic amines. Also, it is a suitable biocatalyst for medicinal applications.

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