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Purification and Characterization of a Peroxidase Isozyme from Indian Turnip Roots

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A peroxidase isozyme (TP I) from Indian turnip roots (*Brassica rapa*) was purified. TP I had a minimum molecular mass of 45 000 Da as determined from sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). A far-UV circular dichroism (CD) spectroscopy study of TP I revealed the presence of 44% α-helix, 16% β-sheet, and 40% random structure. The N-terminal sequence of TP I was found to be Gln-Phe-Val-Ile-Pro-Thr-Tyr-Ala-Trp-Gln. Pyromellitic dianhydride (PMDA)-modified TP I showed enhanced thermal stability and *p*-chlorophenol removal efficiency. In the absence of polyethylene glycol (PEG), PMDA-modified TP I (dose of 50 units mL^{-1}) converted 100% *p*-chlorophenol, while at the same time, native TP I could convert only 85%. In the presence of PEG, PMDA-modified TP I (dose of 0.05 units mL^{-1}) converted *p*-chlorophenol completely in 45 min, while native TP I required 60 min for complete conversion. The K_M value toward the substrates *p*-chlorophenol and *o*-cresol decreased after PMDA modification of TP I, which indicated increased affinity for these substrates.

KEYWORDS: Turnip peroxidase; enzyme purification; Con A sepharose; PMDA modification; wastewater treatment; phenolics removal

INTRODUCTION

Peroxidases (EC 1.11.1.7) are hemeproteins that catalyze the oxidation of a variety of molecules at the expense of hydroperoxides, most often H_2O_2. Peroxidases are found ubiquitously in animals, plants, and microorganisms and efficiently catalyze the oxidation of a variety of substrates (*1*). The best studied peroxidases are plant peroxidases, which are monomeric proteins with molecular weights of 30−40 kDa and contain a single, noncovalently bound heme group (*2*). Peroxidases find widespread applications in bioremediation, biocatalysis, diagnostics, biosensors, and therapeutics (*3*). Because of their broad substrate specificity, peroxidases have been exploited for the detoxification of various aromatic pollutants, such as phenols, aromatic amines, and dyes, present in wastewater/industrial effluents (*4*, *5*).

Over the years, peroxidase from horseradish (*Armoracia rustanica*) has been widely studied and has been the only commercial source for peroxidase. Obviously, a need has been felt to evaluate peroxidases from other plant sources in terms of substrate specificity, pH, and temperature stability and to use these peroxidases in bioremediation and immunoassays. Peroxidase obtained from soybeans (*Glycin max*) has high pH stability (pH 2−10), as compared to horseradish peroxidase (HRP) (pH 4−8) (*3*). Turnip root (*Brassica rapa*) is another source of peroxidase, which had been identified in 1968 by Mazza and coworkers (*6*). Recently, one group from Brazil (*7*−*9*) and another group from Iran (*10*, *11*) have isolated peroxidase from turnips grown in their respective regions. In India, Matto and Husain (*12*, *13*) have reported application of partially purified turnip peroxidase for the decolorization of textile dyes. The same group had earlier reported bioaffinity immobilization of turnip peroxidase (*14*, *15*).

The present work describes the purification of a peroxidase isozyme from the roots of Indian turnip. One important goal was to use it for bioremediation by taking *p*-chlrophenol as a model system.

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MATERIALS AND METHODS

Materials. Fresh turnip roots were purchased from a local market. Concanavalin A−sepharose 4B (Con A−sepharose 4B) was purchased from GE Healthcare, Piscataway, NJ. (diethylamino)ethyl (DEAE)-cellulose, methyl-α-D-mannopyranoside, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co., St. Louis, MO. Pyromellitic dianhydride (PMDA) was purchased from Eastman Kodak Company, Rochester, NY. All other reagents and chemicals were of analytical grade.

Peroxidase Activity Determination. Peroxidase activity was measured using the well-established and frequently used spectrophotometric assay with TMB as a substrate (*16*). The substrate solution was prepared as follows: 0.002 mL of 30% (v/v) H_2O_2 and 1 mL of dimethyl sulphoxide (DMSO) solution containing 1.5 mg of TMB were added to 15 mL of 0.03 M citrate−phosphate buffer at pH 5.5. The reaction mixture contained 0.5 mL of appropriately diluted enzyme and 1.5 mL of substrate solution. Incubation was carried out for 10 min at 25 °C. The reaction was stopped with 0.5 mL of 2 M H_2SO_4. Absorbance was read at 450 nm. A total of 1 unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of TMB/min at 25 °C into a colored product ($\varepsilon = 5.9 \times 10^4\ M^{-1}\ cm^{-1}$ at 450 nm).

Protein Estimation. The protein concentration was determined according to the procedure described by Bradford (*17*), using bovine serum albumin (BSA) as a standard protein.

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Preparation of Crude Turnip Extract (*8*). Turnip roots (500 g) were washed and cut into pieces and homogenized in 500 mL of 0.1 M Tris-HCl at pH 7.0. The extract was centrifuged at 12000*g* for 15 min at 4 °C, and the supernatant was used for further purification.

Affinity Chromatography on Con A−Sepharose 4B. The crude extract (150 mL) was precipitated by adding solid ammonium sulfate up to 85% saturation. After the precipitates stood overnight at 5 °C, they were collected by centrifugation at 12000*g* for 15 min. The precipitates were redissolved in a minimum volume of 0.1 M Tris-HCl buffer and dialyzed extensively against 0.1 M Tris-HCl at pH 7.0 containing 0.5 M NaCl and 0.001 M $CaCl_2$, $MgCl_2$, and $MnCl_2$ for 24 h.

The dialyzed fraction (75 mL) was applied on a Con A−sepharose 4B column (1 × 20 cm, 10 mL bed volume) previously equilibrated with 0.1 M Tris-HCl at pH 7.0 containing 0.5 M NaCl and 0.001 M $CaCl_2$, $MgCl_2$, and $MnCl_2$. The column was washed with the same buffer to remove weakly bound proteins, and then the adsorbed protein was eluted with a linear gradient of 0−0.1 M methyl-α-D-mannopyranoside. Fractions of 2 mL were collected; absorbance was read at 280 nm; and the peroxidase activity and protein content were checked in each fraction.

Anion-Exchange Chromatography on DEAE−Cellulose. The fractions from a Con A−sepharose 4B column containing peroxidase activity were pooled and dialyzed against 0.05 M Tris-HCl at pH 8.6. The dialyzed sample (10 mL) was applied on a DEAE−cellulose column (1 × 20 cm, 10 mL bed volume) previously equilibrated with the above buffer. The column was washed with the same buffer to remove weakly bound proteins. The adsorbed protein was eluted with a linear gradient of 0−0.5 M NaCl in the same buffer. Fractions of 2 mL were collected; absorbance was read at 280 nm; and the peroxidase activity and protein content were checked in each fraction.

Polyacrylamide Gel Electrophoresis (PAGE). The purity of the sample obtained at various stages of purification was checked by sodium dodecyl sulfate (SDS)−PAGE. It was performed using 12% gel according to the procedure described earlier (*18*) on a Genei gel electrophoresis unit (Bangalore Genei Pvt. Ltd., Banglore, India) and using standard molecular weight markers (Bangalore Genei Pvt. Ltd., Bangalore, India).

N-Terminal Sequencing of Turnip Peroxidase Isozyme I (TP I). The N-terminal sequencing was performed at the All India Institute of Medical Sciences, New Delhi, India. The pure protein sample was subjected to PAGE (12%) and electroblotted on a polyvinylidene fluoride (PVDF) membrane. The first 10 amino acids from the N-terminal end of TP I were sequenced by the Edman degradation method on an automated sequencer (PPSQ 21A Shimadzu protein sequencer).

Chemical Modification of TP I. The acetylation of TP I with PMDA was performed as described earlier (*19*). PMDA solution prepared in dimethylsulfoxide (0.002 M, 1 mL) was added dropwise at 4 °C to 0.9 × 10^{-3} μg L^{-1} TP I solution (10 mL; prepared in 0.1 M KH_2PO_4 buffer at pH 8.0) with stirring. The reaction mixture was further stirred for 2 h followed by dialysis against the same buffer.

Determination of the Degree of Modification. The determination of amino groups modified was carried out using trinitrobenzenesulphonic acid (TNBS) according to the procedure described earlier (*20*). TNBS (0.03 M, 0.025 mL) was added to 1 mL of sample solution in 0.1 M borate buffer at pH 9.3 and incubated at 25 °C for 1 h. Absorbance was read at 420 nm. Pure glycine was used as a standard.

Substrate Specificity of TP I. Substrate specificity of TP I toward various substrates, viz. TMB, ABTS, guaiacol, phenol, *p*-chlorophenol, and *o*-cresol, was studied by assaying the enzyme using varying concentrations of each substrate at a fixed concentration of H_2O_2. The data were fitted in the Hanes−Woolf equation using Leonora software to calculate the kinetic parameters (*21*).

Circular Dichroism (CD) Spectroscopy. CD measurements were carried out on a JASCO J-815 spectropolarimeter (Jasco Corporation, Tokyo, Japan) equipped with a Peltier-type temperature controller and a thermostatted cell holder, interfaced with a thermostatic bath. All solutions were filtered with a 0.22 μm pore nylon filter before performing the experiments. Far-UV CD spectra (200−260 nm) were recorded on a 1 cm path length quartz cell at a protein concentration of 0.5 mg mL^{-1} in a 0.01 M sodium phosphate buffer at pH 7.0. Mean residual ellipticity [θ] in deg cm^2 $dmol^{-1}$ was calculated using the formula: $[\theta] = 0.1[\theta_o]M_o/cl$, where $[\theta_o]$ is the observed ellipticity in millidegrees, M_o is the mean residue weight of the protein (M_o = 110), *c* is the protein concentration (mg cm^{-3}), and *l* is the path length (cm). The percentage of different secondary structures was estimated using the K2D program (*22*).

Table 1. Purification of TP I

sample	total protein (mg)	total enzyme activity (U)	specific activity (U mg^{-1})	fold purification	yield (%)
crude extract	150	18770	125		100
85% $(NH_4)_2SO_4$ precipitation	98	18395	188	1.5	98
Con A−sepharose	6	17475	2912	23	93
DEAE−cellulose	2.8	9635	3441	28	51

Thermal Stability. Purified TP I (native and PMDA-modified; 0.5 mg mL^{-1}) was incubated at different temperatures (30−80 °C) for 15 min. The samples were then cooled to room temperature and assayed for peroxidase activity using TMB as a substrate at 25 °C. The starting activity at 0 min was taken as 100%.

***p*-Chlorophenol Removal Using Native and PMDA-Modified TP I.** In a glass vial (30 mL), reagents were added in the following order: (1) 0.01 M phosphate buffer at pH 7.5, (2) TP I (native and PMDA-modified), (3) PEG (0.1 mg mL^{-1}), and (4) *p*-chlorophenol solution (0.001 M). The final reaction volume was 5 mL. The reaction was initiated by adding H_2O_2 (0.001 M). For the time-course study, aliquots were withdrawn at particular time intervals and an excess of catalase was added to terminate the reaction. The samples were centrifuged at 10000*g* for 20 min at 25 °C, and *p*-chlorophenol was estimated in the supernatant.

Estimation of the Concentration of *p*-Chlorophenol. The concentration of *p*-chlorophenol was determined according to the assay procedure reported earlier (*23*). The samples obtained were subjected to *p*-chlorophenol determination as follows: 0.15 mL of ammonium buffer [0.7 g of ammonium chloride and 6.5 mL of 25% (v/v) liquor ammonia were dissolved in 10 mL of distilled water] was mixed with 2.55 mL of sample (appropriately diluted in distilled water). A total of 0.15 mL of 8 g L^{-1} 4-aminoantipyrine aqueous solution was added to the mixture followed by the addition of 0.15 mL of 36 g L^{-1} potassium ferricyanide aqueous solution. The *p*-chlorophenol concentration was determined by measuring the absorbance at 510 nm. The total *p*-chlorophenol concentration was determined from a calibration graph based on pure *p*-chlorophenol.

RESULTS AND DISCUSSION

Purification of Turnip Peroxidase. *Affinity Chromatography on Con A−Sepharose 4B.* Con A is known to bind to several important glycoproteins (*24*). Con A recognizes α-D-glucose and α-D-mannose with free hydroxyl at the 3, 4, and 6 positions (*25*). Purification of HRP using Con A−sepharose has been reported earlier (*26*, *27*). Results of purification of turnip peroxidase using Con A−sepharose 4B column chromatography are shown in **Table 1**. All of the peroxidase activity (100%) bound to the affinity matrix. At the same time, 78% of loaded protein came in the unbound fraction (**Figure 1a**). When bound peroxidase activity was eluted with a linear gradient of 0−0.1 M methyl-α-D-mannopyranoside, 93% of total peroxidase activity could be recovered (**Table 1**), with an overall fold purification of 23 and RZ value of 1.0 [The ratio of absorbance of the Soret band (403 nm) to that at 280 nm is defined as the RZ value. It is used as a measure to ascertain the degree of purity of peroxidase preparation (*28*)]. RZ = 1 reflects impure peroxidase preparation. Highly pure HRP has a RZ value of 3.04 (*26*). However, RZ values for pure peroxidases are different among peroxidases from different sources because of different aromatic amino acid compositions (*29*).

Anion-Exchange Chromatography on DEAE−Cellulose. Several isozymes of turnip peroxidase have been reported in the literature (*6*−*9*). Mazza and co-workers (*6*) have reported seven isozymes of turnip peroxidase. They purified five of the seven isozymes, which were designated as P_1, P_2, P_3 (acidic proteins), P_6, and P_7 (basic proteins) using a combination of anion- and cation-exchange chromatography. P_1, P_2, and P_3 having molecular

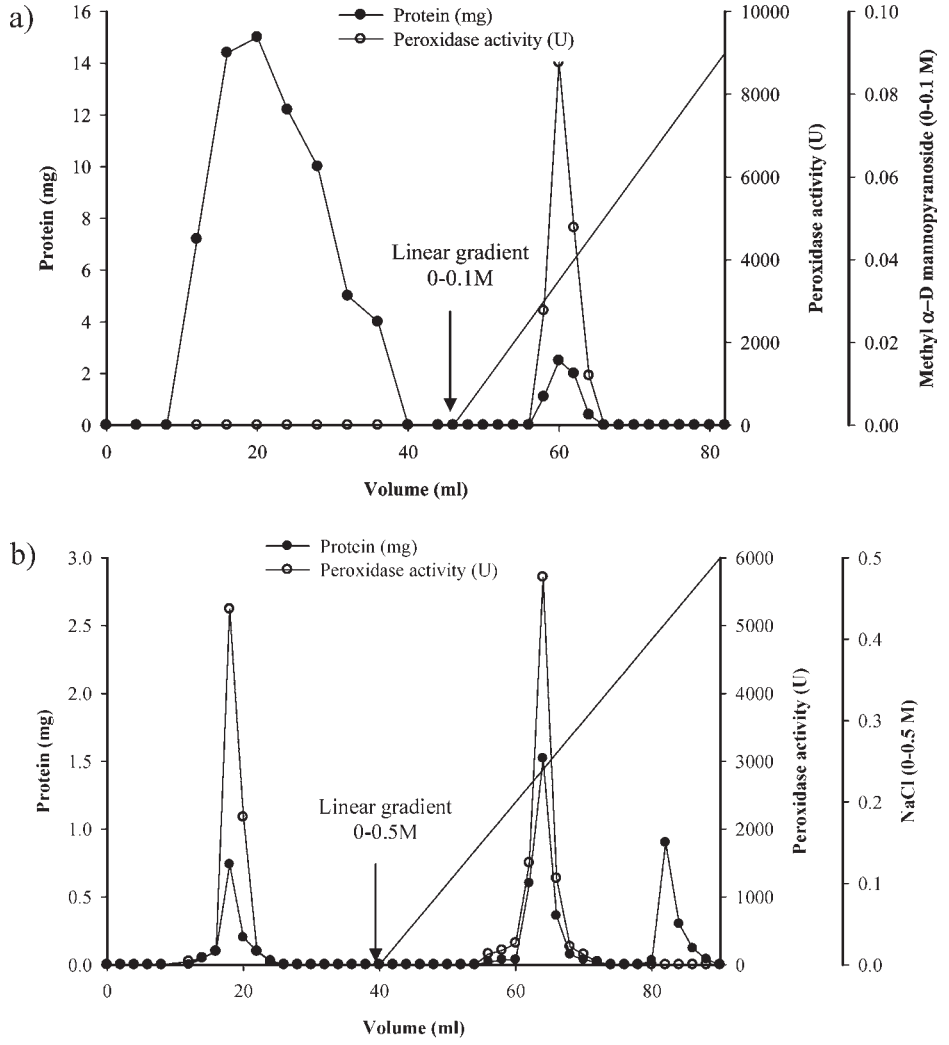

Figure 1. (a) Column chromatography of turnip peroxidase using Con A−sepharose 4B. The matrix (10 mL) was packed in a Supelco glass column (1 × 20 cm) and equilibrated with 0.1 M Tris-HCl buffer containing 0.5 M NaCl and 0.001 M $CaCl_2$, $MnCl_2$, and $MgCl_2$ at pH 7.0. The dialyzed fraction (75 mL) obtained after ammonium sulfate precipitation was applied to a column at a flow rate of 24 mL h^{-1}. The unbound protein was removed by washing the column with the same buffer. The bound protein was eluted by a linear gradient of 0−0.1 M methyl-α-D-mannopyranoside. (b) Purification of turnip peroxidase isozymes using DEAE−cellulose. Matrix (10 mL) was packed in a Supelco glass column (1 × 20 cm) and equilibrated with 0.05 M Tris-HCl buffer at pH 8.6. The dialyzed fraction (10 mL) obtained after a Con A−sepharose 4B column was applied to a column at a flow rate of 24 mL h^{-1}. The unbound protein was removed by washing the column with the same buffer. The bound proteins were eluted by a linear gradient of 0−0.5 M NaCl.

weights of 55 000, 56 000, and 45 000 Da, respectively, constituting 12.5, 9, and 23.7% of total peroxidase activity. P_1, P_2, and P_3 had pI (isoelectric point) values of 3.3, 3.5, and 3.7, respectively. P_6 and P_7 isozymes were basic proteins and had molecular weights of 44 000 and 34 000 Da, with 15.5 and 39% of total peroxidase activity, respectively. P_7 was described as a major isozyme, having a pI value of 11.6. Duarte-Vazquez and co-workers (*8*) have reported two neutral isozymes of turnip peroxidase, which were obtained from unbound fractions of DEAE−cellulose chromatography followed by methyl hydrophobic interaction chromatography (HIC). These isozymes named F1 and F2 constituted 7.77% (36 000 Da) and 13.5% (39 400 Da) of the overall peroxidase yield, respectively. The same group (*9*) has reported an acidic isozyme of turnip peroxidase obtained after the elution from a Resource-Q anion-exchange column having a molecular weight of 49 000 Da, which constituted 20.3% of total peroxidase activity. Hamed et al. (*10*) have reported two isozymes, i.e., main soluble (SP_2) and ionically bound (IBP_4), from an Iranian variety of turnip. These isozymes were purified using a combination of ammonium sulfate precipitation, cellulose phosphate, and phenyl-sepharose chromatography. SP_2 constituted 38% while IBP_4 constituted 13% of total peroxidase activity.

The peroxidase activity containing fractions obtained from the Con A−sepharose 4B column were loaded to DEAE−cellulose. The peroxidase activity was distributed into two peaks (**Figure 1b**). The unbound fraction constituted 43% of the starting activity and had a RZ value of 1.7, while the eluted fraction constituted 51% of the starting peroxidase activity and had a RZ value of 2.5 (**Table 1**).

SDS−PAGE Analysis and Molecular-Weight Determination. The SDS−PAGE pattern of the fractions obtained after ammonium sulfate precipitation, Con A−sepharose 4B, and DEAE−cellulose is shown in **Figure 2**. The peroxidase fraction eluted from DEAE−cellulose showed a single band having a minimum molecular mass of 45 000 Da, as determined by SDS−PAGE. The turnip peroxidase isozymes are monomeric proteins, and their molecular weights are in the range of 34 000−51 500 Da, as reported by earlier workers (*6*−*9*).

Because TP I eluted from DEAE−cellulose constituted half of the initial peroxidase activity, it was selected for further characterization.

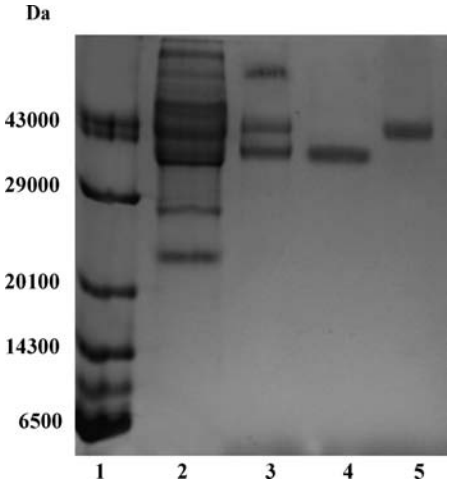

Figure 2. SDS−PAGE analysis of the turnip peroxidase. The polyacrylamide gel (12%) was stained with Coomassie Brilliant Blue. Lane 1, molecular-weight marker; lane 2, ammonium sulfate precipitation; lane 3, Con A−sepharose fraction; lane 4, DEAE−cellulose unbound fraction; and lane 5, DEAE−cellulose eluted fraction.

				% Identity
TP I (*B. rapa*)	1	QFVIPTYAWQ	10	-
C. Sativus	84	QFPIPSYADF	93	60
S. aureus	96	DFLIPTYSYV	105	50
Cyanothece sp. PCC 8802	301	KSDIPTYAVT	310	50
L. xyli CTCB07	307	FPVIPTYAHI	316	60
T. vibrio K90	643	NLTDMAYAWQ	652	40
M. domestica	496	QFTVIPTDQT	505	60

Figure 3. N-Terminal sequence of TP I. The pure protein sample was subjected to PAGE (12%) and electroblotted onto a PVDF membrane. The first 10 amino acids from the N-terminal end of TP I were sequenced by the Edman degradation method on an automated sequencer (PPSQ 21A Shimadzu protein sequencer).

N-Terminal Sequencing of TP I. The first 10 residues from the amino terminal of TP I were sequenced. The sequence was Gln-Phe-Val-Ile-Pro-Thr-Tyr-Ala-Trp-Gln. The BLAST search against the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) database indicated that TP I is structurally related to the peroxidase family (**Figure 3**).

CD Spectroscopy. The far-UV CD spectrum of TP I showed characteristic α-helix structure, such as negative bands at 208 and 222 nm (**Figure 4a**), in agreement with other reports on turnip peroxidase and HRP (*30*−*32*). Estimation of secondary structure components using the K2D program revealed 44% α-helix, 16% *β*-sheet, and 40% random structure. HRP is found to have 35% α-helix, 14% *β*-sheet, and 51% random structure (*30*). The process of thermal denaturation of TP I was monitored directly by following ellipticity changes at 222 nm (**Figure 4b**). The melting temperature (T_m) calculated from the first-order derivatives of the ellipticity−temperature plot was 50 °C for TP I. Guerrero and co-workers (*32*) have reported T_m of 57 °C for turnip peroxidase isozyme.

Substrate Specificity. The substrate specificity of TP I was examined (**Table 2**). Chemical modification of TP I produced changes in catalytic properties of an enzyme. TNBS analysis showed 0.75° of modification of *ε*-amino groups in the PMDA-modified TP I. **Table 2** also shows the values of apparent kinetic parameters of PMDA-modified TP I studied with various substrates. The values of kinetic parameters (K_M and V_{max}) for TP I

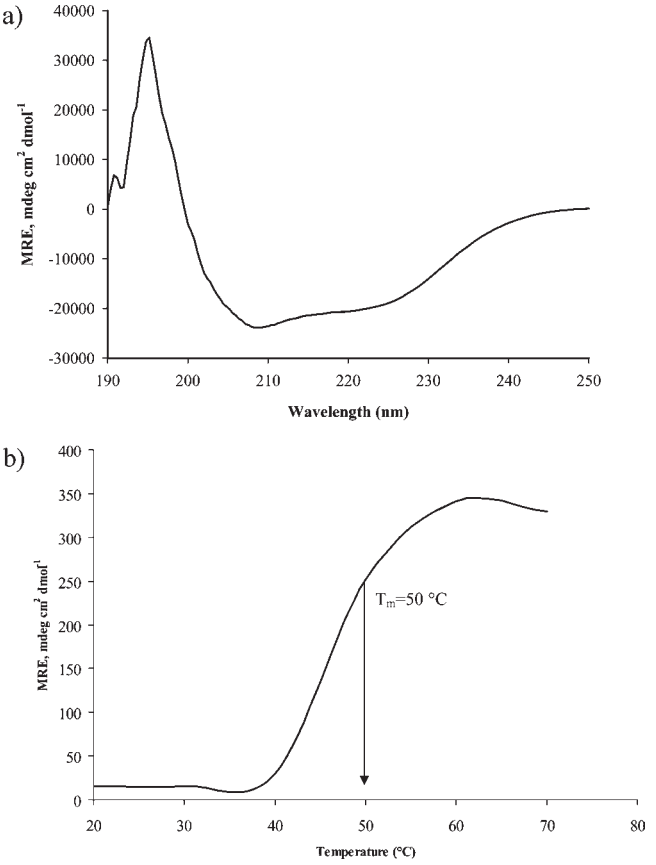

Figure 4. (a) Far-UV CD spectrum of purified TP I for estimation of secondary structure components. (b) CD melting curve by measuring the change in ellipticity at 222 nm during thermal denaturation of TP I (first-derivative curve).

Table 2. Determination of Kinetic Parameters for Native and PMDA-Modified TP I[a]

substrate	kinetic parameter	native TP I	PMDA-modified TP I
TMB	K_M (M)	0.045×10^{-3}	0.045×10^{-3}
	V_{max} (M min^{-1})	4.33×10^{-6}	4.85×10^{-6}
	V_{max}/K_M	96.2	105.4
ABTS	K_M (M)	0.19×10^{-3}	0.18×10^{-3}
	V_{max} (M min^{-1})	0.12×10^{-6}	0.13×10^{-6}
	V_{max}/K_M	0.63	0.72
guaiacol	K_M (M)	0.14×10^{-3}	0.14×10^{-3}
	V_{max} (M min^{-1})	0.99×10^{-6}	1.1×10^{-6}
	V_{max}/K_M	7.07	7.9
phenol	K_M (M)	1.17×10^{-3}	1.12×10^{-3}
	V_{max} (M min^{-1})	50×10^{-3}	55.8×10^{-3}
	V_{max}/K_M	42.7	50
p-chlorophenol	K_M (M)	1.5×10^{-3}	1.1×10^{-3}
	V_{max} (M min^{-1})	52.3×10^{-3}	55.2×10^{-3}
	V_{max}/K_M	35	50
o-cresol	K_M (M)	16.7×10^{-3}	9.1×10^{-3}
	V_{max} (M min^{-1})	44.6×10^{-3}	44.1×10^{-3}
	V_{max}/K_M	2.6	4.8

[a] Kinetic parameters were calculated at 25 °C at various concentrations of each substrate. Experiments were carried out in triplicate and percentage error was within 5% in each set of readings.

(native) are matched with those reported earlier by other workers (*7*−*9*, *31*). In all of the cases, except *p*-chlorophenol and *o*-cresol, K_M values were same for both native and PMDA-modified TP I. In the case of *p*-chlorophenol and *o*-cresol, the K_M values decreased after chemical modification, which indicated more affinity for these substrates upon chemical modification. The V_{max} values

improved slightly for all of the substrates upon chemical modification, except *o*-cresol. In the case of *o*-cresol, the V_{max} value decreased negligibly from 44.6×10^{-3} to 44.1×10^{-3} M min^{-1}. The kinetic efficiency parameter V_{max}/K_M for all of the substrates was improved as well after modification of TP I. The improvement in catalytic efficiency after chemical modification of amino groups of peroxidases has been reported earlier (*32*−*34*). Guerrero et al. (*32*) have modified turnip peroxidase isozyme with methoxypolyethylene glycol (MPEG). After modification, turnip peroxidase isozyme showed a K_M value similar to that of native peroxidase isozyme but a significantly greater k_{cat} value (33 000−38 000 s^{-1}) for ABTS oxidation, in aqueous buffer (*32*). Song et al. (*33*) have reported a similar K_M value after modification with phthalic anhydride but a greatly improved k_{cat} value from 1.45×10^6 to 2.88×10^6 min^{-1} for *p*-chlorophenol. Song et al. (*33*, *34*) have reported improved substrate affinity (K_M) and the catalytic efficiency (k_{cat}/K_M) of HRP for various phenolic compounds after phthalic anhydride modification. PMDA converts a single positive charge on the amino group to three negative charges and, hence, is known to have a drastic effect on enzyme properties (*19*).

Thermal Stability. Thermal stability patterns of native and PMDA-modified TP I were compared (**Figure 5**). Thermal stability was found to have increased after PMDA modification.

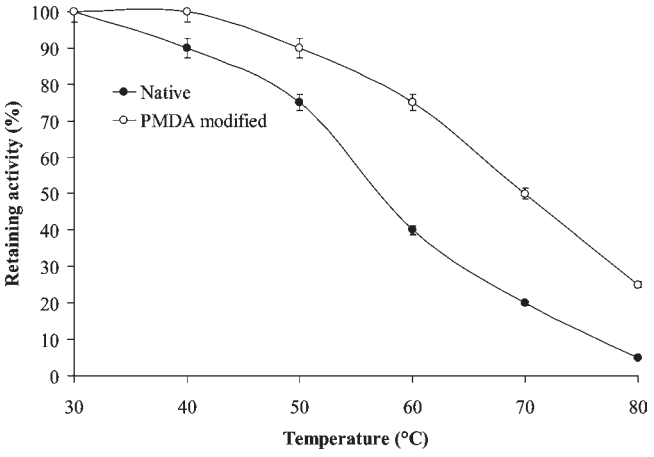

Figure 5. Thermal stability of purified TP I (native and PMDA-modified). Protein samples (0.25 mg mL^{-1}) were incubated at different temperatures (30−80 °C) for 15 min. The samples were then cooled to room temperature and assayed for peroxidase activity using TMB as a substrate at 25 °C. Experiments were carried out in triplicate, and error bars represent the percentage error in each set of readings.

The PMDA-modified enzyme retained 100% activity at 40 °C after 15 min, while the native enzyme lost 15% activity. At 80 °C, the native enzyme lost 95% activity, while at the same time, the PMDA-modified enzyme was able to retain 25% activity. A similar improvement (but to a lesser degree) in thermal stability of chemically modified peroxidases from a variety of sources has been reported earlier (*31*, *35*, *36*). Mogharrab et al. (*31*) have reported an increase in the melting temperature of HRP by 3 °C, and the catalytic efficiency enhanced by 80% after chemical modification of surface-exposed lysine residues. Ryan et al. (*35*) have studied chemical modification of HRP using bifunctional imido esters and succinimides. The $t_{1/2}$ values were improved significantly (3−9 min) after modification of HRP with dimethyl suberimidate (DMS) (*35*). Similarly, when HRP was modified with ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester), $t_{1/2}$ increased from 6 to 147 min (*35*). The improved thermal stability of TP I after chemical modification can make it a potential catalyst for carrying out various synthetic reactions at elevated temperatures (*37*, *38*).

Comparison of Properties of TP1 with Other Known Turnip Root Isozymes. Plants, more than other life forms, produce enzymes with kinetic characteristics depending upon the climate of the region. Quite a few isozymes of peroxidase from turnip roots have already been described in the literature. While some comparisons have already been made during the above discussion, **Table 3** summarizes this comparison in a more extensive way.

One striking observation is that on the basis of percent activity of a particular isozyme, TP1 has 51% of the total peroxidase present in Indian turnip roots. Its specific activity is also highest among isozymes reported by others. It does not seem to be identical to any peroxidase isozyme (from turnip roots) that has been reported in the literature.

p-Chlorophenol Removal Using Crude Turnip Peroxidase. Waster water treatment using *p*-chlorophenol as a model system was studied using a crude extract of turnip peroxidase. Panels **a** and **b** of **Figure 6** show the time-course conversion study of *p*-chlorophenol using crude extract in the presence and absence of PEG, respectively. A very large amount of crude turnip enzyme (200 U mL^{-1}) was required to completely convert *p*-chlorophenol in the absence of PEG (**Figure 6a**). When *p*-chlorophenol treatment was studied using crude turnip peroxidase in the presence of PEG, a minimum of 2 U mL^{-1} crude enzyme was required for complete conversion (**Figure 6b**).

p-Chlorophenol Removal Using Purified Native and PMDA-Modified TP I. PMDA-modified TP I was also examined for wastewater treatment using *p*-chlorophenol as a model system and compared to native TP I. **Figure 7a** represents the time course

Table 3. Comparison of the Properties of Different Purified Turnip Peroxidase Preparations

	turnip peroxidase isozyme					
properties	TP I	SP_2 (*10*)	IBP_4 (*10*)	P_7 (*6*)	F1 (*8*)	TAP (*9*)
purification scheme	ammonium sulfate precipitation, Con A−sepharose, DEAE−cellulose	ammonium sulfate precipitation, cellulose phosphate, phenyl-sepharose	ammonium sulfate precipitation, cellulose phosphate, phenyl-sepharose	DEAE−Sephadex, CM−Sephadex	acetone precipitation, DEAE−cellulose, methyl HIC	acetone precipitation, DEAE−cellulose, Sephadex G-100, Resource-Q
content (%) (on the basis of activity)	51	38	13	39	7.8	20.3
U/mg	3441	2760	896	NM[a]	1130	1810
molecular weight (Da)	45000	NM	NM	34000	36000	49000
RZ value	2.5	NM	NM	2.94	2.3	3.1
optimum pH	5.5	6.5	6	NM	4.5	4
thermal stability	75% at 50 °C for 15 min	NM	NM	NM	70% at 65 °C for 11 min	90% at 60 °C for 25 min

[a] NM = not mentioned.

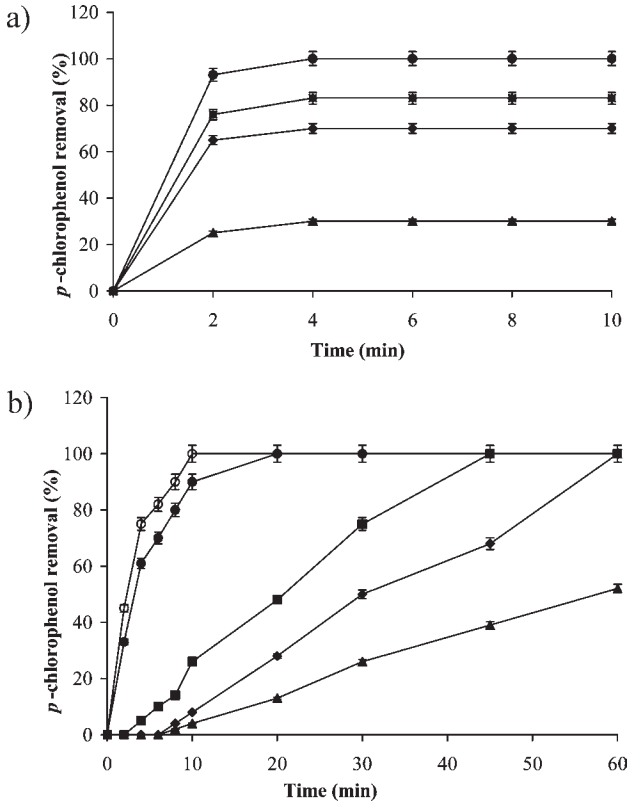

Figure 6. (a) Comparison of the time-course conversion of *p*-chlorophenol by crude turnip peroxidase in the absence of PEG. Reactions were carried at 25 °C with 0.001 M *p*-chlorophenol and 0.001 M H_2O_2. The amount of peroxidase added was varied: (▲) 50 U mL^{-1}, (◆) 100 U mL^{-1}, (■) 150 U mL^{-1}, and (●) 200 U mL^{-1}. Aliquots were withdrawn at various time intervals and analyzed for *p*-chlorophenol concentration. The experiments were performed in triplicate, and error bars represent the percentage error in each set of readings. (b) Comparison of the time-course conversion of *p*-chlorophenol by crude turnip peroxidase in the presence of PEG. Reactions were carried at 25 °C with 0.001 M *p*-chlorophenol, 0.001 M H_2O_2, and 0.1 mg mL^{-1} PEG. The amount of TP I added was varied: (▲) 1 U mL^{-1}, (◆) 2 U mL^{-1}, (■) 3 U mL^{-1}, (●) 4 U mL^{-1}, and (○) 5 U mL^{-1}. Aliquots were withdrawn at various time intervals and analyzed for *p*-chlorophenol concentration. The experiments were performed in triplicate, and error bars represent the percentage error in each set of readings.

for the conversion of *p*-chlorophenol into free radicals and subsequently into polymers as a function of enzyme dose (30, 40, 50, and 60 U mL^{-1}) in the absence of PEG using native and modified TP I. In the absence of PEG, conversion of *p*-chlorophenol was much faster, and while 60 U mL^{-1} native TP I was still required, the 100% conversion of the *p*-chlorophenol was obtained in 4 min. When the native TP I dose was 50 U mL^{-1}, only 80% conversion of *p*-chlorophenol could be obtained in 4 min, and after that, there was no conversion. The conversion was still less with the native TP I dose of 30 and 40 U mL^{-1}. On the other hand, the conversion of *p*-chlorophenol with PMDA-modified TP I was much faster and the enzyme dose required was still less compared to that with native TP I. When the PMDA-modified TP I dose was 60 U mL^{-1}, 100% conversion of *p*-chlorophenol was obtained in 2 min. At the enzyme dose of 50 U mL^{-1}, the PMDA-modified TP I could completely convert *p*-chlorophenol in 4 min.

It has been known that PEG can improve the efficiency of phenol removal by forming a protection layer in the vicinity of the active center of peroxidase to restrict the attack of free phenoxy radicals formed in the catalytic cycle and ultimately reduces the

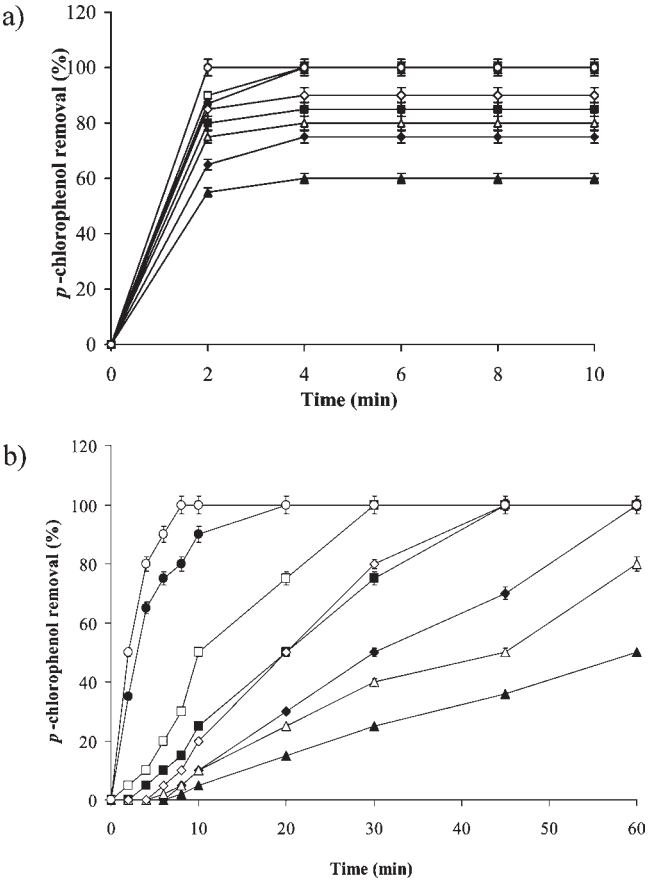

Figure 7. (a) Comparison of the time-course conversion of *p*-chlorophenol by native and PMDA-modified TP I in the absence of PEG. Reactions were carried at 25 °C with 0.001 M *p*-chlorophenol and 0.001 M H_2O_2. Aliquots were withdrawn at various time intervals and analyzed for *p*-chlorophenol concentration. The amount of TP I added was varied. Native TP I: (▲) 30 U mL^{-1}, (◆) 40 U mL^{-1}, (■) 50 U mL^{-1}, and (●) 60 U mL^{-1}. PMDA-modified TP I: (△) 30 U mL^{-1}, (◇) 40 U mL^{-1}, (□) 50 U mL^{-1}, and (○) 60 U mL^{-1}. The experiments were performed in triplicate, and error bars represent the percentage error in each set of readings. (b) Comparison of time-course conversion of *p*-chlorophenol by native and PMDA-modified TP I in the presence of PEG. Reactions were carried at 25 °C with 0.001 M *p*-chlorophenol, 0.001 M H_2O_2, and 0.1 mg mL^{-1} PEG. Amount of TP I added was varied. Native TP I: (▲) 0.025 U mL^{-1}, (◆) 0.05 U mL^{-1}, (■) 0.1 U mL^{-1}, and (●) 0.5 U mL^{-1}. PMDA-modified TP I: (△) 0.025 U mL^{-1}, (◇) 0.05 U mL^{-1}, (□) 0.1 U mL^{-1}, and (○) 0.5 U mL^{-1}. Aliquots were withdrawn at various time intervals and analyzed for *p*-chlorophenol concentration. The experiments were performed in triplicate, and error bars represent the percentage error in each set of readings.

dose of peroxidase required to convert phenol (*39*−*41*). **Figure 7b** represents the time conversion of *p*-chlorophenol using native and PMDA-modified TP I in the presence of PEG. It shows that, in the presence of PEG, only 0.05 U mL^{-1} native TP I was required to completely convert the *p*-chlorophenol but the time required was 60 min. Use of 0.1 U mL^{-1} native TP I completed the conversion (100%) in 45 min. A further increase in enzyme dose made the conversion faster, and with 0.5 U mL^{-1} native TP I, complete conversion was possible in 20 min itself. In the presence of PEG also, the effect of PMDA modification on *p*-chlorophenol was again very significant. When PMDA-modified TP I was 0.025 U mL^{-1}, 80% conversion was obtained after 60 min. *p*-Chlorophenol conversion using 0.025 U mL^{-1} native TP I produced only 50% conversion after 60 min, and after that, there was no conversion. At higher doses of PMDA-modified TP

I (i.e., 0.05, 0.1, and 0.5 U mL^{-1}), the conversion was faster compared to that of native TP I. A 0.5 U mL^{-1} PMDA-modified TP I converted *p*-chlorophenol completely in 8 min itself.

Finally, to conclude, the present work describes purification and characterization of TP I from turnip roots (*B. rapa*). Chemical modification of TP I by PMDA showed enhanced thermal stability and enhanced *p*-chlorophenol removal efficiency, as compared to native TP I. Crude turnip peroxidase (1 U mL^{-1}) brought complete conversion in 60 min in the presence of PEG. Considering that the purified isozyme would be a costly choice for bioremediation of wastewater, it is satisfying to note that crude extracts of turnip roots also led to complete removal of the phenol. The high specific activity (3441 U mg^{-1} for TP I) can make it a good candidate for peroxidase for immunoassays and immunohistochemistry applications. Some properties of TP I have been compared to turnip peroxidase isozymes from different regions reported by earlier workers.

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