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Chemical Modification of Turnip Peroxidase with Methoxypolyethylene Glycol Enhances Activity and Stability for Phenol Removal Using the Immobilized Enzyme

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Peroxidase from turnip roots (TP) was isolated followed by modification with methoxypolyethylene glycol (MPEG). The catalytic activity of the modified TP (MTP) on ABTS increased 2.5 times after 80 min of reaction. MTP showed a K_M similar value to that of TP, but a significantly greater k_{cat} for ABTS oxidation, in aqueous buffer. Chemical modification produced an enhanced stability in organic solvents and increased thermal stability of about 4 times that of TP, in aqueous buffer at 70 °C. Circular dichroism showed that MPEG modification decreased TP α-helical structure from 26 to 16% and increased β-turns from 26 to 34%, resulting in an enhanced conformational stability. The temperature at the midpoint of thermal denaturation (melting temperature) increased from 57 to 63 °C after modification. MTP was immobilized in alginate beads (IMTP) and tested for oxidative polymerization of concentrated phenolic synthetic solutions, achieving 17 effective contact cycles removing >65% phenols. IMTP may be useful for the development of an enzymatic process for wastewater effluent treatment.

KEYWORDS: Turnip peroxidase; chemical modification; MPEG; thermostability; organic solvents stability

INTRODUCTION

Peroxidase (EC 1.11.1.7) is an oxidoreductase that catalyzes the oxidation of various electron-donor compounds such as phenols and aromatic amines with H_2O_2 as final electron acceptor. The peroxidase reaction is an efficient way to remove pollutant compounds from wastewater by oxidative polymerization and may also expand the industrial applications of plant peroxidases to the synthesis of fine chemicals and polymers, in addition to its use in biosensors and medical diagnostic kits, among others (*1*).

The commercially available peroxidase from horseradish roots (HRP) is the most studied of peroxidases. However, other plant species may provide peroxidases with similar or even improved properties. Turnip roots (*Brassica napus*) are a good source of peroxidase (*2*). Water is a key component in agriculture and food industry, and thus a safe water supply is a requirement (*3*). Phenol, 2-chlorophenol, and 2,4-dichlorophenol are ranked within the 250 most hazardous pollutants (*4*) and can accumulate in the food chain. In addition, chlorophenols are commonly found in chlorinated water, because phenol can react with chloride (*5*). In a previous study we isolated, characterized, and used some peroxidase isozymes from turnip roots for phenolic compound (phenol, 2-chlorophenol, 3-chlorophenol, and 2,4-dichlorophenol, 0.25 mM each) removal from a synthetic wastewater and a real effluent from a local paint factory (*6*).

Despite its relatively high stability, the application of turnip peroxidase as reaction catalyst is limited due to its inherent instability under stress conditions, such as the presence of organic solvents and elevated temperatures. Enhancement of operational stability will clearly broaden the range of peroxidase applications.

Chemical modification of proteins is widely used as a tool to maintain a native conformation, improving stability. HRP has served as a model for exploring the use of chemical modification to improve the stability of plant peroxidases in aqueous mixtures and in a variety of organic solvents (*7*−*10*). Chemically modified HRP may be used for treatment of wastewaters containing 1 mM phenol, at high temperature (70 °C) (*11*). However, low removal efficiencies have been reported

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for phenol concentrations ≥4 mM (*12*). Chemical modification of unglycosylated recombinant HRP with ethylene glycol bis(succinic acid *N*-hydroxysuccinimide ester) increased heat stability and tolerance to an organic solvent (*13*). In addition, the pegylation of proteins enhances significantly their thermal stability (*14*). Most of the stabilized chemical derivatives of HRP reported to date have involved lysine modifications (*15*), but no reports were found of such modifications on turnip peroxidase.

Due to the importance of peroxidase stabilization and its potential uses described above, the objective of this work was to investigate the effect of covalent modification of turnip peroxidase with methoxypolyethylene glycol (MPEG) on protein structure, activity, and stability. We hypothesized that chemically modified and immobilized turnip peroxidase can efficiently remove phenolic compounds from solutions as high as 10 mM, in a recycling process, which is not achievable by using the native enzyme.

MATERIALS AND METHODS

Materials. Methoxypolyethylene glycol (MPEG, average molecular mass = 5000 Da) activated with cyanuric chloride and phenol, 2-chlorophenol, 3-chlorophenol, and 2,4-dichlorophenol (all with purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol (PEG; average molecular mass = 3350 Da), 4-aminoantipyrine, $K_3Fe(CN)_6$, ABTS [2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)], 2,4,6-trinitrobenzenesulfonic acid (TNBS), and sodium alginate were also supplied by Sigma-Aldrich. Hydrogen peroxide (30% w/v) was purchased from J. T. Baker (Phillipsburgh, NJ). Catalase (EC 1.11.1. 6.65 units μg^{-1}) was supplied by Boehringer Mannheim (Germany) and Affi-gel 10 from Bio-Rad (Hercules, CA). All solutions were prepared with type I deionized water. Other chemicals used were of analytical grade or better.

Turnip Peroxidase Extraction. Turnip peroxidase (TP) purification including anion-exchange (DEAE-cellulose) chromatography was performed as described by Duarte-Vázquez et al. (*16*). Fresh turnip (*B. napus* L. var. Esculenta DC) roots were homogenized at 4 °C in a blender using 15 mM potassium phosphate buffer, pH 6. The extract was centrifuged at 12000*g* for 15 min, and the supernatant was 10 times concentrated by ultrafiltration using a Pellicon unit (Millipore, Bedford, MA), with a 10 kDa molecular weight cutoff membrane (Millipore). This extract was precipitated using cold acetone (−20 °C; 2:1 acetone/extract), and the precipitate was collected by centrifugation at 12000*g* for 10 min, redissolved in 0.05 M Tris-HCl buffer, pH 8.6, and dialyzed for 48 h against the same buffer. The active fractions were subjected to gel filtration chromatography as previously reported (*6*). In brief, 2 mL of the sample was dissolved in 15 mM phosphate buffer, pH 6.0, and injected into a column containing Sephacryl S-100 (Amersham Biosciences, Uppsala, Sweden), at a flow rate of 20 $cm^3\ h^{-1}$. Fractions showing peroxidase activity, and a Reinheitszahl value ($Rz = A_{403}/A_{280}$) > 3, were collected, dialyzed against deionized water, and lyophilized. Protein content was determined using the dye binding method of Bradford (*17*), with bovine serum albumin as standard.

Assay for Peroxidase Activity. Peroxidase activity was determined using ABTS as hydrogen donor, by the change in absorbance at 414 nm using an extinction coefficient of 36 $mM^{-1}\ cm^{-1}$ (*18*), at 25 °C using a Lambda 40 spectrophotometer (Perkin-Elmer). The final reaction mixture (1.5 mL) contained 1 mM ABTS, 0.5 mM H_2O_2, and 0.4 mg L^{-1} of enzyme in a 15 mM potassium phosphate buffer, pH 6.0. One unit of activity is defined as the micromoles of ABTS consumed per minute at pH 6.0 and 25 °C.

Gel Electrophoresis. SDS-PAGE of purified TP was conducted in 10% T [acrylamide plus bis(acrylamide)], in a Mighty Small II (Amersham Biosciences) electrophoresis cell. Running conditions and protein band detection using Coomassie blue dye were conducted as previously described (*19*).

Chemical Modification. Purified TP was modified using MPEG activated with cyanuric chloride, as described by Tinoco and Vazquez-Duhalt (*20*), and the modified TP was named MTP. Before the modification, TP stability was evaluated by incubating a 0.5 mg mL^{-1} solution, at pH values of 8, 9, and 10 for 1 h, at room temperature (25 ± 2 °C), and the inactivation was determined by measuring the residual enzyme activity. From a TP sequence, six ε-amino groups are available for modification (*21*), and MPEG was added in a 5:1 molar ratio.

A mixture of 5 mg of TP and 24 mg of PEG in 10 mL of 40 mM borate buffer, pH 9.0, was gently stirred using a rotary shaker (Labquake, Barnstead, IA; series 1104, 20 rpm) at room temperature (25 ± 2 °C) and pH 8.5. Samples were withdrawn at time intervals (0−120 min), and the extent of modification was determined by titration of the residual free amino groups using the TNBS assay (*22*). Absorbance was measured at 334 nm using a molar extinction coefficient $\varepsilon_{334} = 1.09 \times 10^{-4}\ M^{-1}\ cm^{-1}$. The effect of chemical modification on enzyme activity was evaluated, as well as the absorbance in the Soret band (403 nm, associated with the heme group). The modified TP (MTP) was ultrafiltered and diafiltered (Amicon stirred cell model 8050), using a 15 mM phosphate buffer, pH 6, followed by residual activity determination.

Kinetic Parameters. Kinetic constants, V_{max} and K_M, of native TP and MTP were determined using ABTS as substrate. Hyperbolic saturation curves were obtained by varying the ABTS concentration from 0.1 to 5 mM with an enzyme-saturating H_2O_2 concentration (2 mM), found from preliminary experiments. Kinetic constants were calculated from a Hanes−Woolf plot of [S] (initial velocity, v)$^{-1}$ versus [S] (*23*). The value of k_{cat} was obtained by dividing V_{max} by the total enzyme (peroxidase) concentration.

Thermal and pH Stability. Thermal inactivation curves for native TP and MTP were obtained by incubating the enzyme in 4 mm (i.d.) × 50 mm long Pyrex test tubes at temperatures from 50 to 90 °C, in 15 mM phosphate buffer, pH 6.0, using a water bath (Shell Laboratory), and the enzyme was added to the preheated solution. The tubes were slightly agitated for a designated temperature, and aliquots were withdrawn onto ice−water at defined time intervals. Samples were assayed for peroxidase activity and residual activities calculated as percentage of that measured at time zero (1.2 units mL^{-1}). The transition state free energy (ΔG) and enthalpy (ΔH) for enzyme denaturation were calculated for TP and MTP at 70 °C, according to the theory of absolute reaction rates (*24*). pH optimum and pH stability were evaluated using 5 mL aliquots of TP, MTP, and immobilized MTP (IMTP) samples added to different test tubes and adjusted between pH 3 and 10 using the following 10 mM buffers: citrate, pH 3−5.5; phosphate, pH 6−7, Tris-HCl, pH 8; borate, pH 9; carbonate, pH 10. For pH stability, samples were kept for 4 h at 25 °C, and the residual activity was evaluated at optimum pH.

Catalytic Stability of TP and MTP in Organic Solvents. The stability of TP and MTP in organic solvents was measured using dimethylformamide (DMF), methanol (MET), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and 1,4-dioxane (DO). TP and MTP samples (10 $\mu g\ L^{-1}$ final concentrations) were incubated in 10 mM phosphate buffer, pH 7.0, at 25 °C, and from 0 to 90% (v/v) solvent for 60 min. Samples were withdrawn from each reaction mixture and assayed for peroxidase activity under the standard conditions described above.

Circular Dichroism (CD) Measurements. The effect of chemical modification on protein structure and thermal stability was investigated by using CD. CD spectra were recorded on a JASCO J-715 spectropolarimeter fitted with a thermoelectric temperature control.

CD in the UV region (200−250 nm) was monitored using a protein concentration of 0.1 mg mL^{-1} in a 0.1 cm path length quartz cell under constant nitrogen flush. This wavelength range was used to provide quantitative estimates of the secondary structure, and CD data were expressed as molar ellipticity [θ] (millidegrees $cm^2\ mmol^{-1}$), based on an assumed mean amino acid residue weight (MRW) of 110 (*25*). The molar ellipticity was determined as $[\theta] = (\theta \times 100\ MRW)\ (c\ l)^{-1}$, where c is the protein concentration in mg mL^{-1}, l is the light path length in cm, and θ is the measured ellipticity at wavelength λ. CD spectra were reported as the average of three scans recorded at a speed of 100 nm min^{-1} and resolution of 1 nm, corrected by subtracting the adequate blank runs on TP-free solutions. The proportions of the secondary structure (α-helix, β-sheet, and random) were calculated using the K2D program (*26*).

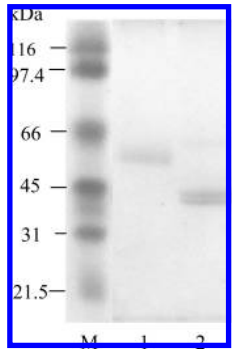

Figure 1. SDS-PAGE under reducing conditions of turnip root peroxidase. Lanes: M, low range molecular weight markers (Bio-Rad); 1, modified turnip peroxidase (MTP, 5 μg); 2, native TP (50 ng). Protein bands were stained with Coomassie blue dye.

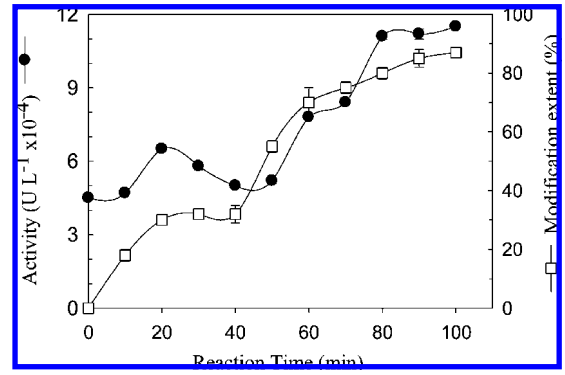

Figure 2. Modification extent and catalytic activity in relation to modification reaction time (means of five replicates ± standard deviation). The reaction mixture contained a ratio of 5:1 PEG/free amino group on a molar basis.

Thermal Denaturation. Thermal denaturation curves for TP and MTP were evaluated using the CD technique by heating the samples contained in 3 mL quartz cuvettes at a constant rate of 1 °C/min from 30 to 90 °C while the ellipticity (θ) was monitored at 222 nm. Melting temperature was defined as the midpoint of the transition from helical to random structure (*27*)

Immobilization of Modified TP. MTP (Rz = 3.0) was immobilized in a calcium alginate matrix with PEG. Immobilization was achieved by mixing 1.77 mg of protein of MTP in 20 mL of 15 mM sodium phosphate buffer, pH 7.0, with 0.44 g of sodium alginate solution (dry wt) and 100 mg L^{-1} of PEG, and the solution was mixed overnight at 4 °C. This mixture was added dropwise into 50 mL of 4% (w/v) calcium chloride solution, under constant stirring, to form calcium alginate beads, which showed an equilibrium total volume of 6.6 ± 0.3 mL, about 1 h after the last drop was added. The total number of spheres from one batch (210) was recovered by using a strainer, and after shaking, each bead showed an average weight of 25 ± 0.5 mg, with an average diameter of 0.39 ± 0.03 cm, which was used to calculate its corresponding volume.

Immobilized Activity Determination. The specific activity of IMTP was calculated on the basis of the preparation dry weight. The IMTP activity was calculated on the basis of the volume of the phenolic mixture inside the batch reactor. The number of alginate beads formed per milliliter of alginate solution was constant. Each of the 210 beads resulting from an immobilization batch was evaluated, resulting in a homogeneous peroxidase activity with a variability within 5%. One alginate bead was used to measure both initial and remaining IMTP activity (assuming a uniform activity loss for each bead), which was evaluated by adjusting the reaction volume (1.5 mL) with phosphate buffer. The total activity was calculated by knowing the total number of spheres, and the activity was expressed as units per gram of alginate beads, considering the average weight of each one. Peroxidase activity in the reactor may be expressed as units per milliliter of phenolic solution (considering bead volume) (*6, 28, 29*).

Phenolic Solution. A synthetic phenolic solution was prepared from a mixture containing 2.5 mM each of phenol, 2-chlorophenol, 3-chlorophenol, and 2,4-dichlorophenol. The solution was adjusted to pH 6.0 with 0.1 N H_2SO_4.

Phenol Assay. The concentration of total phenols was determined by a colorimetric method using 4-aminoantipyrine (20 mM in 250 mM $NaHCO_3$) and ferricyanide [83.4 mM of $K_3Fe(CN)_6$ in 250 mM $NaHCO_3$] (*28*). The reaction products were monitored at 510 nm, and the phenol concentration was estimated by a standard curve. The assay mixture contained 0.10 mL of ferricyanide solution, 0.10 mL of 4-aminoantipyrine solution, and 0.80 mL of phenolic sample. All results are reported as the quantity of phenol equivalents removed.

Enzymatic Phenol Removal. Phenol polymerization with IMTP was conducted in a 30 mL batch reactor containing an aqueous phenolic mixture, and the reaction was initiated by adding a known amount of H_2O_2. The reaction mixture was continuously stirred using an orbital shaker (Lab-line, 150 rpm) at 25 °C, for 10 min (*6*). Reactions were stopped by adding catalase to the reaction mixture to a final concentration of 125 units mL^{-1}. IMTP and free TP (used as control) activities were 4 units (g of alginate bead)$^{-1}$. Samples were then treated with alum [$Al_2(SO_4)_3 \cdot 14H_2O$] to a final concentration of 40 mg L^{-1} to enhance colloidal particle coagulation, the pH was adjusted to 6.3, and then the samples were centrifuged for 10 min at 12000*g* (Eppendorf 580R) (*29*). The supernatant was analyzed for residual phenol concentration as described above. Experiments were conducted in triplicate, and the standard deviation was determined.

Reusability of Immobilized Enzyme in Phenol Removal. The reusability of IMTP for removal of aqueous phenols was monitored using 10 minute reaction cycles under the conditions described. After each reaction cycle, the IMTP was separated by using a strainer, washed with distilled water, stored for 10 min in 4% $CaCl_2$, and immediately used for the following phenol removal cycle. Peroxidase activity and phenol removal efficiency were calculated for each cycle (*6*).

RESULTS AND DISCUSSION

TP Purification. After gel filtration chromatography, the specific activity of TP reached 1323 units mg^{-1}. The Rz value of purified TP was 3.0. The yield of pure TP was relatively low [885 μg of TP (kg of fresh roots)$^{-1}$]. This purified fraction was used for entrapment on alginate beads. The estimated molecular mass of TP was 40 kDa (**Figure 1**).

Conditions for Chemical Modification of TP. The effect of high pH on TP stability was determined. The activity decreased 3.5, 8.0, and 36% when TP was incubated for 1 h at pH values of 8.0, 9.0, and 10, respectively. The modification reaction of TP with MPEG implicated the ε-amino group of lysine; thus, we decided to carry out this modification at pH 9.0, due to the presence of a sufficient number of unprotonated species, combined with a small loss in enzyme activity. Garcia et al. (*30*) modified HRP with MPEG at the same pH with a low enzyme activity loss.

In an attempt to improve TP modification some other modifying conditions were assayed such as reaction time and MPEG/TP ratio. The effect of chemical modification on enzyme activity was evaluated, as well as the absorbance at the Soret band (403 nm, associated with the heme group). Native TP showed a Soret peak at 405 nm, whereas MTP using a 5:1 MPEG/TP ratio caused a minor absorbance increase and a shift of the Soret band to 408 nm (data not shown). This shift in the absorbance peak suggests changes of the conjugated double bonds in the porphyrin ring of the TP heme group, reflecting the bonding of the MPEG to the protein moiety in the vicinity of the heme (*31*). Thus, the 5:1 MPEG/protein ratio was chosen for further studies.

Chemical modification with MPEG enhanced the TP activity, and there is a correlation between the peroxidase activity and the modification extent (**Figure 2**). After 100 min of reaction

Table 1. Kinetic and Thermodynamic Parameters for Native and Modified TP

enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	E_a[a] (kJ/mol)	ΔG[a] (kJ/mol)	ΔH[a] (kJ/mol)
native	0.56 ± 0.04a	33000 ± 70a	59000 ± 1700a	113.9 ± 5a	103.2 ± 4a	111.0 ± 5a
modified	0.60 ± 0.03a	38000 ± 55b	63000 ± 1800a	168.2 ± 7b	106.5 ± 5a	168.3 ± 6b

[a] Evaluated for thermal denaturation of native and modified TP at 70 °C. Values within columns without the same letter are significantly different ($p < 0.05$) using the Tukey test.

time, the modification of TP reached 87 ± 1% of free amino groups available, according to the TNBS assay, whereas its peroxidase activity was 2.5 times higher than that of the unmodified TP. Both activity and modification extent showed a double kinetics with an intermediate plateau at 40 min, probably due to the exhaustion of more reactive amino groups. On the other hand, MTP activity loss was expected due to protein denaturation by effect of the pH (*2*). HRP modified with MPEG activated with *p*-methyl carbonate showed a modification extent of 28% using the same pH and temperature conditions but longer modification time (*30*). This was probably due to changes associated with the protein in the vicinity of the heme group, as shown by the absorbance change in the Soret band. This effect may have been responsible for a better accessibility of the substrate to the active site of MTP. In addition, the increased activity of MTP may be due to a stabilization effect associated with a decrease in the positive net charge of the protein, leading to a reduced electrostatic repulsion (*30*).

Catalytic Properties of MTP. After 80 min of reaction time, the chemical modification of TP resulted in a protein with 80% of its amino groups linked to MPEG moieties. This preparation showed less electrophoretic mobility than the unmodified TP (**Figure 1**), doubtless due to a large amount of MPEG molecules covering the protein.

Chemical modification also produced changes in catalytic properties of TP. Both TP and MTP showed apparent Michaelis−Menten kinetics with ABTS as substrate, and the apparent kinetic constants are shown in **Table 1**. Compared with native TP, MTP showed a similar apparent Michaelis constant (K_M) value and a significantly higher k_{cat} value, whereas the efficiency parameter k_{cat}/K_M slightly increased upon this modification. A variety of results have been reported about the effect of pegylation on the enzyme activity: A laccase modified by PEG showed 1300 times increase in k_{cat} compared to the unmodified enzyme, for syringaldazine oxidation, which was attributed to a higher hydrophobicity of the modified enzyme (*32*). An alternative to pegylation is the use of nonionic amphiphilic polyoxyethylene lauryl ether (Brij 35) covalent modifier, which has been applied to catalase (*33*). The Brij 35 catalase exhibited 15−20-fold greater activity than the native enzyme in aqueous solution. This study also showed that Brij 35 modification of catalase gave higher overall activity than PEG modification in aqueous solution (*33*). On the other hand, the use of cyanuric chloride to activate MPEG sometimes leads to a substantial loss of enzymatic activity due to the low selectivity of this reagent as protein modifier (*34, 35*). Depending on the substrate, the effect of PEG modification on catalytic efficiency (k_{cat}/K_M) of a laccase from *Myceliophthora thermophila* showed a decrease when ABTS was employed (*36*). Finally, close to our results the modified and native manganese peroxidase showed similar catalytic properties in the oxidation of Mn(II) and other substrates such as 2,6-dimethoxylphenol, veratryl alcohol, guaiacol, and ABTS (*37*).

The specificity of native TP and MTP for oxidative polymerization of phenol only was evaluated, giving activities of 2120 ± 35 and 1740 ± 25 μmol of phenol consumed [(min) (mg of enzyme)]$^{-1}$ at 25 °C, respectively. From data of a previous work we calculated a value of 1584 ± 25 μmol of phenol consumed [(min) (mg of enzyme)]$^{-1}$ at 25 °C, using peroxidase from *B. napus* var. Purple Top White Globe (*29*). Similar values were obtained for the phenolic compounds mixture used in this work, indicating high specificity.

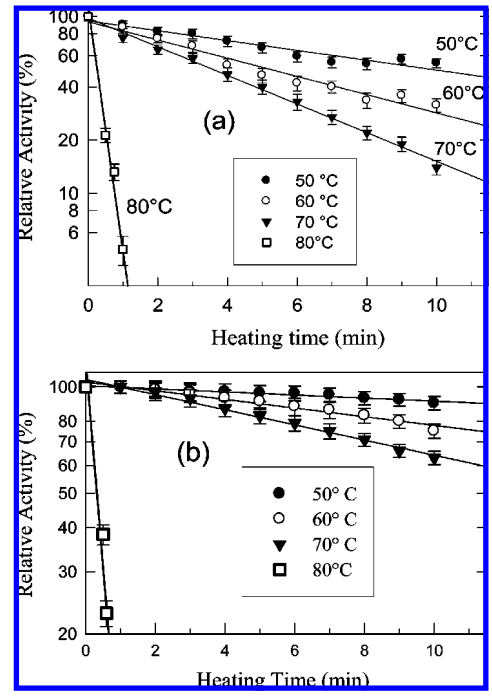

Figure 3. Thermostability of native (TP) (**a**) and modified turnip peroxidase (MTP) (**b**) in aqueous buffer (15 mM phosphate, pH 6.0). The ordinate represents relative activity, that is, the ratio of the activity to the initial activity before heating, expressed as percentage.

Thermal Stability. The thermal stability of TP and MTP was monitored after incubation at 50−90 °C at various time intervals. MPEG modification of TP produced a significant enhancement of its thermal stability in aqueous buffer between 50 and 70 °C (**Figure 3**). TP retained ∼40% of the original activity after 5 min of incubation at 70 °C, whereas MTP exhibited about 85% of its original activity under the same conditions. After exposure for 10 min at 50 and 60 °C, TP retained only 55 and 35% activity, respectively, whereas MTP retained 90 and 75% activity, respectively, at the same temperatures. The half-lives of TP at 70 and 80 °C were 3.8 ± 0.5 and 0.27 ± 0.05 min, whereas those for MTP were 15.8 ± 1.5 and 0.31 ± 0.07 min, respectively. Neither TP nor MTP showed any remaining activity after 20 s of heating at 90 °C. MTP increased about 4 times its stability at 70 °C compared to native TP, whereas HRP modified with bifunctional agents, PEG−bis(*N*-hydroxysuccinimidyl succinate) and phthalic andhydride, also showed an increased thermostability of 4 and 5 times, respectively, at 65 °C (*8, 13*). It is well-known that polyethylene glycol modification of enzyme surface amino groups is a practical method to enhance biocatalyst stability (*38*).

From Arrhenius plots, the activation energy for TP heat inactivation, and from the absolute reaction rates theory ΔG

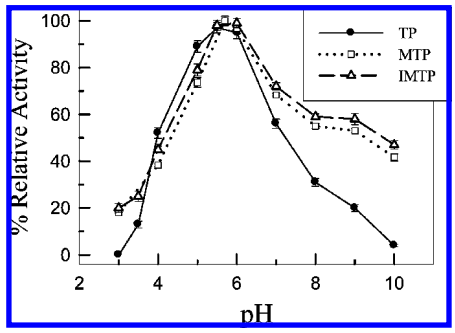

Figure 4. pH stability of native (TP), modified (MTP), and immobilized modified (IMTP) turnip root peroxidase. The ordinate represents relative activity, that is, the ratio of the activity to the maximum activity, expressed as a percentage. Samples were kept for 4 h at the specified pH, followed by residual activity determination at pH optimum. The 10 mM buffers used were as follows: citrate, pH 3−5.5; phosphate, pH 6−7, Tris-HCl, pH 8; borate, pH 9; carbonate, pH 10.

and ΔH at 70 °C were calculated (**Table 1**). Native TP showed lower values of the thermodynamic parameters evaluated (E_a, ΔG, and ΔH), when compared to those obtained for MTP, corroborating a higher thermal stability in accordance with the above-mentioned thermal stability studies (**Figure 3**). The thermostability of MTP makes it a good alternative for many biotechnological processes in which a thermally stable enzyme is required. This feature indicates that MTP may have a performance similar to or better than native TP in environmental applications while tolerating more extreme conditions and displaying longer operational lifetime, as discussed later.

MPEG may stabilize TP in different ways. The modification of the amino groups of the enzyme, mainly lysine residues, alters the protein positive charge considering that MPEG was linked to about 80% of the total amino groups of TP. Thus, the greater stability of MTP may arise from the neutralization of positive charges leading to decreased charge repulsion within the polypeptide, as suggested by Liu and Wang (*39*). Another possibility is that a primary shield of rigid water molecules solvates the hydrophilic region of bound PEG, helping to maintain the structure of the protein, whereas the hydrophobic regions in PEG interact with the hydrophobic clusters on the protein surface. Those sets of interactions could produce a shell-like structure, in which PEG is coiled on the protein surface, avoiding direct contact with water molecules in the solvent and producing a more conserved structure around heme. The global effect of those interactions is a decrease in unfolding rate of the active site. Finally, our results suggest that the thermostability might be the result of two factors: a more conserved structure of the heme pocket and a decreased unfolding rate of the active site (*14*).

pH Stability and Optimum pH. The optimum pH values for TP, MTP, and IMTP were 5.5, 5.8, and 6.0, respectively (data not shown). The pH of maximum stability of both MTP and IMTP was between 5.8 and 6.0, whereas that of TP was 5.5 (**Figure 4**). **Figure 4** shows that modified TP had a tendency for increased pH stability at alkaline pH values, as compared to native TP.

Catalytic Stability of MTP in Organic Solvents. Polymerization of phenols or its derivatives constitutes a prominent application for plant peroxidases. Organic solvents are sometimes needed to increase solubility of monomeric substrates to reduce the mass transfer limitations. MTP showed a higher tolerance to the solvents tested than native TP (**Figure 5**). Both enzymatic preparations showed an activity decrease as the organic solvent concentration increased. However, the MTP

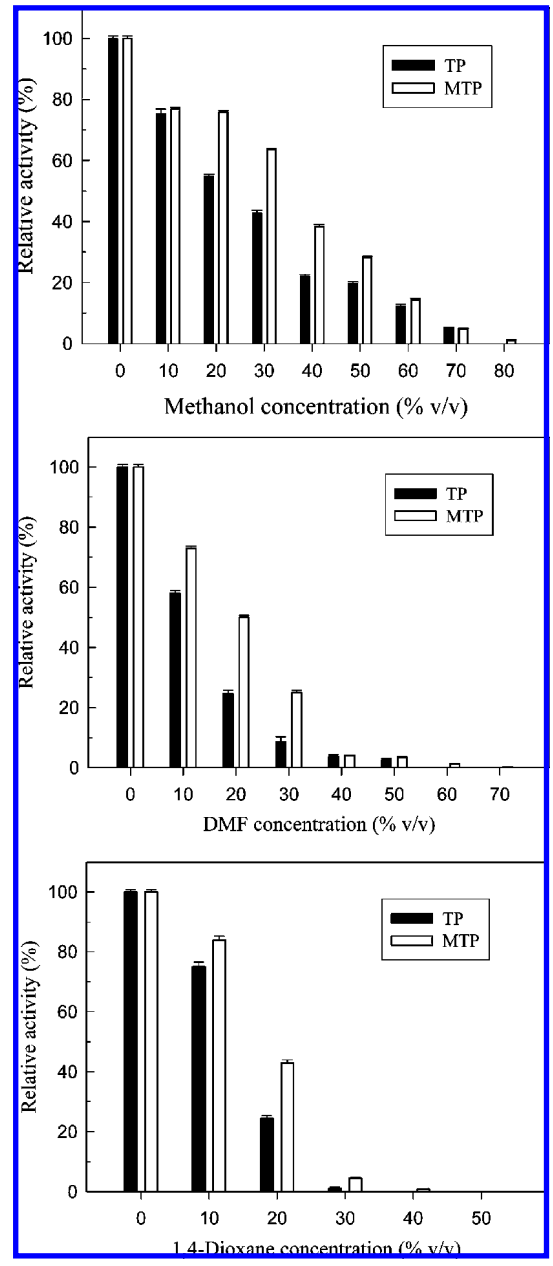

Figure 5. Effect of solvents on native (TP) and modified (MTP) turnip root peroxidase at room temperature for 1 h. The ordinate represents relative activity, that is, the ratio of the activity to the initial activity before exposure to aqueous−solvent mixtures, expressed as percentage.

showed, in all cases, higher solvent tolerance than the unmodified preparation. For example, in a reaction mixture containing 30% (v/v) of methanol, the MTP activity represented 65% of the original activity, whereas the unmodified TP showed only 40%. Also, in the case of DMF at 30% (v/v), the MTP retained 25% of the original activity, whereas TP showed only 5% of the activity in aqueous medium. It is well-known that pegylation stabilizes and enhances enzyme activity in organic solvents (*38*). However, as solvent concentration was increased to 80% for methanol, 60% for DMF, and 30% for 1,4-dioxane, a negligible activity was observed for either TP or MTP. Activity loss could be a result of the effect of organic solvent on the tertiary or secondary structure of TP. No activity was detected at any THF concentration for either TP or MTP, in contrast to the results reported for HRP modified with maleic and citraconic anhydrides, which showed better tolerance to DMF, DMSO, and THF than the native form (*10*). An increase of solvent tolerance has

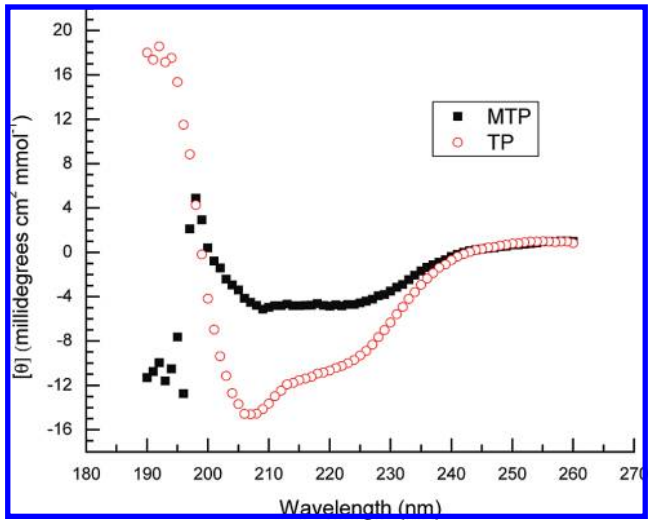

Figure 6. CD spectra of native (TP) and modified (MTP) turnip root peroxidase in 15 mM phosphate buffer, pH 6.0. Protein concentration was 15 μM.

Table 2. Percentage of Secondary Structure Elements of Native and Modified TP, Estimated from CD Spectra Using K2D Software (Mean of Three Determinations)

enzyme	α-helix	β-structure	random coil
native TP	26	26	48
modified TP	16	34	50

been reported (*36*) with a modified laccase from *M. thermophila*. An increase in catalytic efficiency (up to 50%) in 15% DMF was also reported for chemically modified chloroperoxidase with citraconic, maleic, or phthalic anhydrides (*39*).

CD Studies. The effect of PEG modification on the secondary structure of TP was investigated by far-UV CD. The far-UV CD spectrum of native TP showed characteristic α-helix structure, such as negative bands at 208 and 222 nm (**Figure 6**), in agreement with other reports for HRP (*10, 15*) and for chloroperoxidase (*39*). As a result of modification, the overall shape of the spectrum showed significant changes with lower negative ellipticities. The percentage of secondary structure elements calculated using the K2D program is summarized in **Table 2**. Data on percentage of secondary structure suggest a marked decrease in helical structure (from 26 to 16%) combined with an increase in β-turns (from 26% in TP to 34% in MTP), whereas no significant changes were observed in random structure. These spectra suggest a relatively more compact structure upon modification.

An important feature of the CD spectrum of MTP is the decrease in the positive ellipticity peak at 190 nm, indicating a possible enzyme aggregation after chemical modification. Both the increase in structural compactness and aggregation may have contributed to the higher stability of MTP.

The process of thermal denaturation of TP and MTP was monitored directly by following ellipticity changes at 222 nm, because changes upon heating are significant at this wavelength (**Figure 7**). In both cases, denaturation of the enzyme was accompanied by an increase in ellipticity, without the presence of intermediate stages, which led us to conclude that TP appeared to unfold in a single step.

Melting temperatures (T_m, midpoint of the transition from helical to random structure), calculated from the first-order derivatives of ellipticity−temperature plots, were 63 °C for MTP and 57 °C for TP. From the same data, the onset temperatures

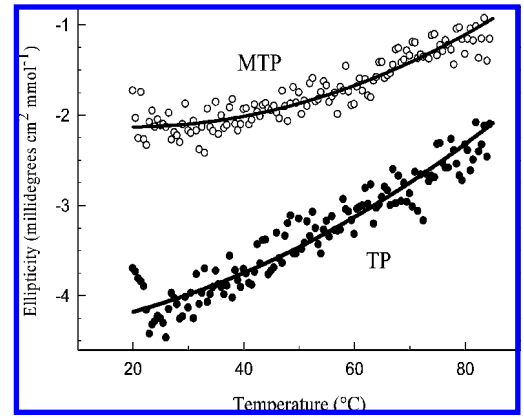

Figure 7. Temperature dependence of molar elipticity at 222 nm for native (TP, solid circles) and modified turnip peroxidase (MTP, open circles).

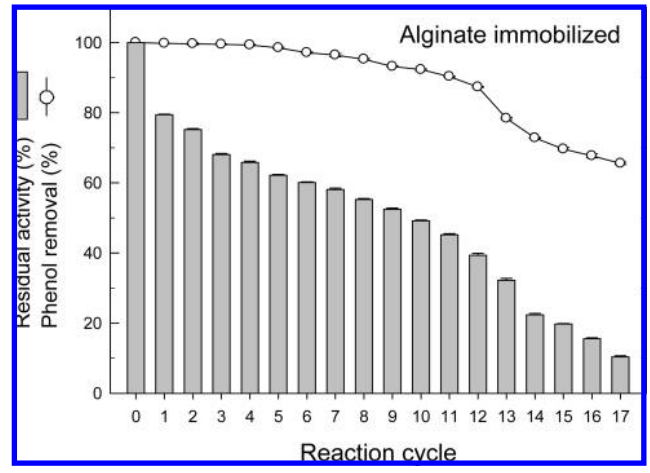

Figure 8. Residual activity and phenol removal efficiency of immobilized modified turnip peroxidase (IMTP). Initial enzyme activity was 1.2 AU mL^{-1}.

of transition (T_o, the temperature at which 5% of the initial ellipticity signal is lost) for both TP and MTP were 35 and 40 °C, respectively. Thus, both T_m and T_o are higher for MTP than for TP, suggesting an enhanced conformational stability of the protein upon modification.

Application of Immobilized MTP on a Synthetic Phenolic Solution. Preliminary work using an immobilized crude extract was successful when low phenol concentrations were tested (<4 mM) only. Under these conditions we obtained four cycles of phenol removal at an efficiency >65%, using a specific activity of 4.9 units (mg of protein)$^{-1}$.

To evaluate the performance of IMTP, a 10 mM synthetic phenolic solution was used. Batch reactions of 10 min were chosen to produce high phenol polymerization while avoiding unnecessary TP inactivation, as previously demonstrated (*6*). This reaction permitted the achievement of a high number of effective removal cycles, defined as >65% phenol removal, with the same immobilized MTP, leading to a more economical process. Seventeen effective removal cycles were obtained using this short reaction time (**Figure 8**), where IMTP showed a phenol removal of >90% after the first 11 reaction cycles. In contrast, using immobilized HRP, Dalal and Gupta (*12*), achieved 10 phenol removal cycles, with a removal efficiency ≥50%, while using a low phenolic solution concentration of 1 mM. The same phenol concentration was used by Quintanilla et al. (*6*) to achieve 15 removal cycles (≥65% removal efficiency) using immobilized TP. Both research groups

reported that higher phenol concentrations (>4 mM) led to enzyme inactivation after first contact.

Therefore, the chemical modification and immobilization of the modified TP showed an enhanced protein conformational and thermal stability, higher solvent tolerance, and a slight increase in catalytic efficiency. This allowed an extended reuse of the IMTP preparation in the transformation of a highly concentrated phenol solution. The thermal stability of MTP may result in a performance similar to or better than that of native TP in environmental applications while tolerating more extreme conditions and displaying longer operational lifetime. This was associated with the chemical modification with MPEG, which probably produced a decrease in the active site unfolding rate, which was not achieved by native TP in a 10 mM phenol solution. Efficient reduction of industrial contaminants such as phenolic compounds may help to avoid possible accumulation in the food chain. Preliminary work suggests a significant toxicity loss after phenol polymerization of the synthetic mixture employed in this study.

A pilot scale enzymatic process for industrial wastewater effluent treatment is under development with IMTP that should be stable enough to produce a robust process.

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