Purification and Properties of a Neutral Peroxidase Isozyme from Turnip (*Brassica napus* L. Var. Purple Top White Globe) Roots

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A neutral peroxidase isozyme (p*I* 7.2) from turnip roots (TNP) was purified to homogeneity and partially characterized. TNP is a monomeric glycoprotein with 9.1% carbohydrate content and a molecular weight of 36 kDa. Optimum pH values for activity using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and guaiacol as H donors were 4.5 and 5.5, whereas the K_m values were 0.7 and 3.7 mM, respectively. The ABTS K_m was ∼7 times higher than that reported for basic commercial horseradish peroxidase (HRP-C). TNP retained ∼70% activity after 11 min of heating at 65 °C, whereas the activation energy for inactivation (132 kJ/mol) was higher than or comparable to that of other peroxidases. The low ABTS K_m and high specific activity (1930 units/mg) gave a high catalytic efficiency (500 M^{-1} s^{-1}). These properties make TNP an enzyme with a high potential as an alternative to HRP in various applications.

Keywords: *Neutral peroxidase; turnip roots; protein purification*

INTRODUCTION

Peroxidases (donor:hydrogen peroxide oxidoreductase; EC 1.11.1.7) are heme enzymes found in many fruits and vegetables. They may contribute to deteriorative changes in flavor, texture, color, and nutrition, both in raw foods such as fruits and vegetables and in processed products (*1*, *2*). In plants, peroxidases play important physiological roles: as protective mechanisms in physically damaged or infected tissues (*3*); in indoleacetic acid degradation during maturity and senescence of fruits and vegetables (*4*); and in the late stage of the lignin-forming process (*5*).

In fruits and vegetables there is a wide range of peroxidase isozymes having p*I* values from about 3.5 to 10.0 (*6*). In turnip roots anionic (p*I* 4.5−5.0; *7*) and cationic (p*I* 8.3; *8*) peroxidases have been identified, but there are no reports about neutral isozymes.

Peroxidases are generally glycosylated; the carbohydrate moieties can contribute up to 26% of the enzyme molecular weight (*9*).

In the food industry peroxidase has been considered one of the most heat stable enzymes in plants, and under limited heat treatment it can regain activity during storage, resulting in loss of flavor (*10*) or development of off-flavor, especially in vegetables (*11*).

Commercially, peroxidase is important because it is well-suited for the preparation of enzyme-conjugated antibodies (ELISA), due to its ability to yield chromogenic products at low concentrations and its relatively good stability (*12*).

About 90% of the world production of peroxidase is obtained from horseradish (*Armoracia rusticana* L.; *12*), but this plant does not grow well in Mexico. We have been testing alternative sources locally available and studying their properties as possible substitutes for horseradish peroxidase (*8*, *13*). This work deals with the physicochemical properties of a new turnip neutral isoperoxidase.

MATERIALS AND METHODS

Fresh turnip roots were obtained from the local market. Soil was removed from the roots, and they were sorted, rinsed with distilled water, drained, frozen, and stored at −20 °C until used.

Crude Extract. Turnip roots (1 kg) were removed from frozen storage and homogenized at 4 °C using 1 L of 10 mM potassium acetate buffer, pH 6. The extract was centrifuged at 12000*g*, and the supernatant was used for further purification.

Protein Determination. Protein was determined using the dye-binding method of Bradford (*14*) with bovine serum albumin (BSA) as standard.

Peroxidase Activity. Peroxidase activity was determined spectrophotometrically using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and guaiacol as hydrogen donors. The final reaction mixture (1.5 mL) contained 1 mM ABTS, 50 μL of enzyme, 5 mM H_2O_2, and 10 mM potassium phosphate buffer, pH 6.0, or 20 mM guaiacol, 8 mM H_2O_2, 50 μL of enzyme, and 10 mM potassium citrate buffer, pH 5.5. The change in absorbance of the ABTS radical cation was followed at 414 nm, considering an $\epsilon_{414} = 36$ mM^{-1} cm^{-1} (*15*). The guaiacol oxidation product (cyclic tetraguaiacol) was followed at 436 nm, considering an $\epsilon_{436} = 25.6$ mM^{-1} cm^{-1} (*16*). The assay was performed at 25 °C using a Lambda 2S Perkin-Elmer spectrophotometer. One unit of enzyme is defined as the amount of substrate (micromoles) consumed in 1 min.

Peroxidase Purification. The crude extract (1 L) was 10 times concentrated by ultrafiltration using a Minitan II (Millipore) tangential ultrafiltration unit with 10 kDa molec-

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ular weight cutoff (MWCO) membranes (Millipore). The retentate (0.1 L) was precipitated using cold acetone (−20 °C; 2:1 ratio acetone/extract). Acetone precipitation, besides improving purification when compared to the use of ammonium sulfate, removed most of the extract pigments, which otherwise caused blocking of the ion-exchange column. The precipitate was collected by centrifugation (12000*g*, 10 min), redissolved in 10 mL of 0.05 M Tris-HCl buffer, pH 8.6, and dialyzed for 36 h against the same buffer (4 L).

The dialyzed sample was centrifuged at 12000*g* for 5 min to remove precipitate. The supernatant was applied to a 1.5 × 20 cm Bio-Rad column packed to a 15 cm bed height with DEAE-cellulose (Sigma, exchange capacity = 0.89 mequiv/g) fitted to a Gradifrac (Pharmacia) system. The column was previously equilibrated with 0.05 M Tris-HCl buffer, pH 8.6, using a 20 mL/h flow rate. Two milliliters of the enzyme solution was injected into the column, and 4 mL fractions were collected. The retained protein was eluted at the same flow rate using a linear gradient of 0.0−1.0 M NaCl in the above buffer, from fractions 14 to 30. The 280 nm absorbance and peroxidase activity of the eluted fractions were recorded.

A nonretained peroxidase peak having most of the activity was pooled, dialyzed against deionized water, and freeze-dried (Virtis 5L equipment). The dry sample was redissolved in 2 mL of 0.05 M potassium phosphate buffer, pH 7, containing 1.0 M ammonium sulfate (buffer A). The sample was injected onto a column previously equilibrated with buffer A, packed with methyl-HIC support (Bio-Rad), and fitted to a Gradifrac System (Pharmacia). The column was washed with 40 mL of buffer A at a flow rate of 30 mL/h, and 4 mL fractions were collected. The retained protein was eluted by decreasing the ammonium sulfate concentration in a linear gradient from 1 M (fraction 10) to 0 M (fraction 28), replacing buffer A with 0.05 M potassium phosphate buffer, pH 7.0 (buffer B).

One peak with peroxidase activity was pooled, dialyzed against distilled water, freeze-dried, and assessed for physicochemical properties. All chromatographic steps were performed at room temperature.

Enzyme Characterization. *SDS-PAGE*. The purity and molecular weight of peroxidase fractions were analyzed using denaturing polyacrylamide gel electrophoresis (SDS-PAGE; *17*), under reducing and nonreducing conditions, to check the presence of subunits. A vertical chamber (Mighty Small SE 250, Hoeffer Instruments) was used. The stacking gel had 4*T* [percent of the mixture acrylamide plus *N*,*N*′-methylenebis-(acrylamide) in the gelling solution, w/v] and 2.6*C* [percent *N*,*N*′-methylenebis(acrylamide) in the mixture acrylamide plus bis(acrylamide), w/w], whereas the separating gel had 10*T* and 2.6*C*. Runs were performed at constant current (10 mA/plate in the stacking gel and 15 mA/plate when samples moved to the separating gel). Low molecular weight markers (6.5−66 kDa; Sigma) were used. Protein band positions were obtained using the rapid silver staining method (*18*). Turnip peroxidase molecular weight was interpolated from a plot of log (protein standard molecular weights) versus R_F.

Isoelectric Focusing (IEF). A horizontal cell (Mini IEF, model 111, Bio-Rad) with a homogeneous polyacrylamide gel and Pharmalyte (Pharmacia) carrier ampholytes with p*I* range 3−9, were used. Two microliters of the purified peroxidase was loaded at the middle of the gel. The run was conducted under constant voltage, 100 V for 15 min, then 200 V for another 15 min, and finally 450 V for 1 h. The gel was tested for peroxidase activity using a fresh solution containing 0.2 M phosphate buffer, pH 6.0, 0.5% (w/v) ABTS, and 0.03% (w/v) H_2O_2 (*8*). After the gel had been rinsed twice with deionized water, it was silver stained (silver stain kit, Bio-Rad). Calibration proteins for p*I* measurements were carbonic anhydrase II from bovine erythrocytes (p*I* 5.9), carbonic anhydrase I from human erythrocytes (p*I* 6.6), and myoglobin from horse heart (pI 6.8 and 7.2).

Glycoprotein Identification. The glycosylated nature of the peroxidase isozyme was evaluated by staining, after SDS-PAGE, using a glycoprotein detection kit (Sigma). This test is based on the oxidation of the oligosaccharides by periodic acid and staining with Schiff reagent. The aldehyde groups (from, e.g., mannose and galactose) produce abstraction of SO_2 from the colorless complex of leuco-magenta {4-[(4-aminophenyl)-(4-imino-2,5-cyclohexadien-1-ylidene)methyl]-2-methylbenzenamine monohydrochloride}, resulting in the release of the pink dye (*19*).

Carbohydrate Content. The carbohydrate content of the purified isozyme was evaluated by using the phenol−sulfuric acid method with mannose as standard (*20*).

Optimum pH for Activity. The following buffers were used for ABTS and guaiacol as substrates: 10 mM citrate buffer, pH 3−6; 10 mM phosphate buffer, pH 7; 10 mM Tris-HCl buffer, pH 8.0; and 10 mM borate buffer, pH 9.0. The ionic strength was kept constant at 430 mM by adjustment with KCl. We obtained the same activity results for each substrate using Tris-HCl or phosphate buffers at the pH range 7−8. However, the change of buffers outside this pH range may lead to some inaccuracy in true peroxidase activity behavior. When universal buffer (*21*) was used, erratic peroxidase activity was observed in the pH range 3−6.

K_m Determination and Kinetic Mechanism. For guaiacol as substrate, individual experiments for each H_2O_2 concentration were performed at guaiacol concentrations of 1, 5, 10, 15, and 20 mM. The following H_2O_2 concentrations were used: 0.06, 0.2, 0.5, 0.75, 1, and 1.5 mM. The reaction volume was 1.5 mL.

ABTS K_m values were determined at ABTS concentrations of 0.1, 0.2, 0.5, 1, and 1.5 mM, while H_2O_2 concentration was kept constant (0.01, 0.25, 0.5, 1.0, and 1.5 mM) in individual experiments for each ABTS concentration. pH, ionic strength, and temperature were kept constant in these series of experiments, where the reaction volume was 1.5 mL. K_m values were determined using the Lineweaver−Burk reciprocal plot graphic method for the two-substrate ping-pong mechanism followed by peroxidase. Assuming initial rates (v_0), a general equation for this mechanism in the forward direction was given by Whitaker (*22*)

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_a}{A_0 V_{max}} + \frac{K_b}{B_0 V_{max}} \quad (1)$$

where V_{max} = maximum velocity, $K_a = K_m$ for substrate A (hydrogen peroxide), A_0 = substrate A concentration, $K_b = K_m$ for substrate B (H donor), and B_0 = substrate B concentration. From a plot of eq 1, enzyme systems following a sequential mechanism (random or ordered) give lines that intersect to the left of the *y*-axis, whereas systems that follow a ping-pong mechanism give parallel lines (*22*). When A_0 is constant, eq 1 will yield a slope and intercept given by

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

$$\text{intercept} = 1/V_{max} + K_a/(A_0)V_{max} \quad (3)$$

According to the intercept terms of eq 3, a replot of *y*-intercepts versus $1/(A_0)$ will produce a straight line with a slope and intercept given by

$$\text{intercept} = 1/V_{max} \quad (4)$$

$$\text{slope} = K_a/V_{max} \quad (5)$$

Therefore, the constants K_a, K_b, and V_{max} can be determined from eqs 2, 4, and 5.

Peroxidase Inhibitors. Potassium cyanide inhibition of the purified turnip peroxidase (TNP) was determined using a constant ABTS concentration, in individual experiments in which the KCN concentration ranged from 0 to 50 μM. H_2O_2 was kept constant at each of the following concentrations: 0.50, 0.60, and 0.90 mM. Fifty microliters of KCN was added to 1.45 mL of a mixture containing 1.5 mM ABTS and 4 μg/mL of TNP in 0.1 M citrate buffer, pH 4.5. The mixture was incubated for 5 min at 25 °C followed by addition of the appropriate amount of hydrogen peroxide. The blank consisted

Table 1. Summary of the Purification Stages of TNP (Mean of Five Replicates with Standard Error within 5% of the Mean)

sample	protein (mg)	activity (units)	specif act. (units/mg)	fold	yield (%)
1. crude extract	5040	48500	9.60	1.00	100
2. ultrafiltration	2560	38000	14.8	1.54	78.4
3. acetone precipitation	1260	23700	18.8	1.96	48.9
4. AEC[a]	33.8	11100	328	34.2	22.9
5. HIC[b] fraction 1	3.35	3770	1130	118	7.77
fraction 2 (TNP)	3.40	6560	1930	201	13.5

[a] Anion-exchange chromatography (DEAE cellulose), nonretained fractions. [b] Hydrophobic interaction chromatography (methyl-HIC).

of all reagents except KCN and H_2O_2. K_i was determined from the intercept of a plot of $1/v_0$ versus KCN concentrations (*23*).

For sodium azide inhibition of TNP, H_2O_2 was kept constant at the following concentrations: 0.50, 0.60, and 0.90 mM. The concentration of ABTS was 1.5 mM, and the sodium azide concentrations ranged from 0.1 to 8 mM for each H_2O_2 concentration. The sequence for the inhibition studies and ABTS concentrations were the same as indicated for KCN.

Peroxidase Thermal Inactivation. Heat inactivations of turnip peroxidase solutions were conducted using a water bath (Shell-Lab) at 65−80 °C, during 0−12 min. A fixed enzyme aliquot was added to 0.5 mL of 50 mM phosphate buffer, pH 6.0, in 12 × 75 mm test tubes. Tubes were slightly agitated while heated for a designated time at a fixed temperature.

After heating, the solutions were rapidly cooled in ice water, and residual peroxidase activity was immediately determined. Rate constants were calculated from the slope of semilog plots of relative activity against heating time at constant temperature. These rates were plotted against the reciprocal of the absolute temperature, and assuming an Arrhenius-type behavior, the activation energy for inactivation of peroxidase (E_a) was calculated.

RESULTS AND DISCUSSION

The crude extract was concentrated by ultrafiltration. Because the membrane MWCO was 10 kDa, salts, carbohydrates, and some low molecular weight peptides were probably removed.

Acetone precipitation was conducted as a further step and not only improved peroxidase purification but also helped to concentrate the crude extract. The activity yield and purification factors using these two steps are shown in Table 1.

The next stage in the purification was anion-exchange chromatography (DEAE-cellulose). Peroxidase distributed into two peaks; one of them was eluted in the void volume while the other eluted with the salt gradient (Figure 1A). The nonretained pooled fractions' (NPF) specific activity (328 units/mg) and RZ value (A_{403}/A_{280} = 0.8) were greater than those of the retained fractions (105 units/mg and RZ = 0.4, respectively). Therefore, the NPF were chosen for further purification. Sometimes proteins are not adsorbed by anion exchangers when pH >p*I*, as in this case. This result may be attributed partly to the uneven distribution of charges over the protein surfaces (*24, 25*) and partly to nonelectrostatic interactions (*26*). The relatively low RZ value of the NPF indicated a poor degree of purification; nevertheless, we were able to remove a large amount of other proteins.

The NPF fraction was injected onto a methyl-HIC column equilibrated with 0.05 M potassium phosphate buffer containing 1.0 M ammonium sulfate. Turnip peroxidase was bound to the HIC column and required a decreased ionic strength (0.05 M phosphate buffer) to

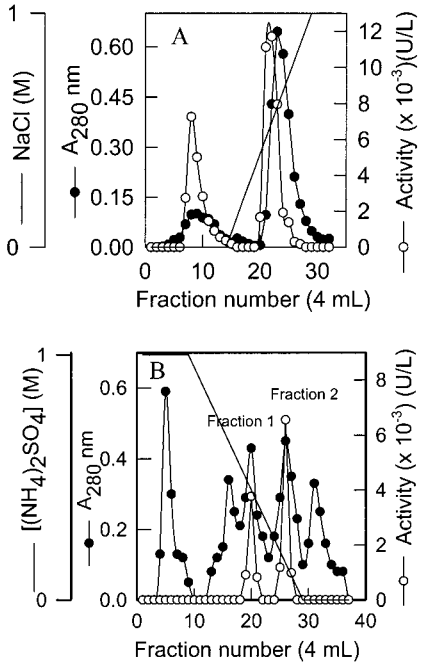

Figure 1. Chromatographic separation used to obtain purified TNP: (A) anion-exchange chromatography (DEAE-cellulose) of turnip root peroxidase (Tris-HCl 0.05 M buffer, pH 8.6; the same buffer with added 1.0 M NaCl was used for gradient elution); (B) HIC using methyl-HIC support of NPF (buffer was 20 mM phosphate, pH 6.0, with 1.0 M ammonium sulfate; the same buffer without added salt was used for gradient elution).

elute. The elution profile (Figure 1B) showed two peaks having peroxidase activity (fractions 1 and 2). Fraction 2 had high specific activity and purification factors (Table 1), with an RZ value of 2.3. Because highly purified HRP has an RZ = 3.0 (*27*), our extract might have an impurity. However, RZ values are different among peroxidases from different sources due to different aromatic amino acid compositions (*22*). The combined activity of the two peroxidase fractions was ∼20% of the crude extract activity. These fractions were dialyzed, freeze-dried, and stored for further studies.

Much of the difficulty in characterizing peroxidases is due to the presence of multiple isozymes in crude extracts. Therefore, it is essential to isolate first the individual isozyme.

Molecular Weight and Purity. The molecular weight and purity of peroxidase fractions were analyzed by SDS-PAGE (Figure 2A). After silver staining, small impurities were observed in fraction 1, whereas a single band was detected in fraction 2. Therefore, fraction 2 was used for further studies due to its higher degree of purity.

Fraction 2 was subjected to reducing (2-mercaptoethanol) SDS-PAGE, and a single band was also detected, confirming the enzyme purity and that it is composed of a single polypeptide chain (results not shown). The molecular weight was calculated as 36 kDa, similar to those of wheat germ peroxidase (35−36.5 kDa, *28*), soybean anionic peroxidase (37 kDa; *29*), and tea leaves peroxidase (34.5 kDa; *30*), but lower than those of HRP (40−46 kDa; *31*), poplar xylem peroxidase (46−54 kDa; *32*), pepper fruit acidic peroxidase (50 kDa; *33*), and sycamore maple peroxidase (42 kDa; *34*).

According to Srivastava and van Huystee (*35*), the molecular weight of peroxidases from various sources

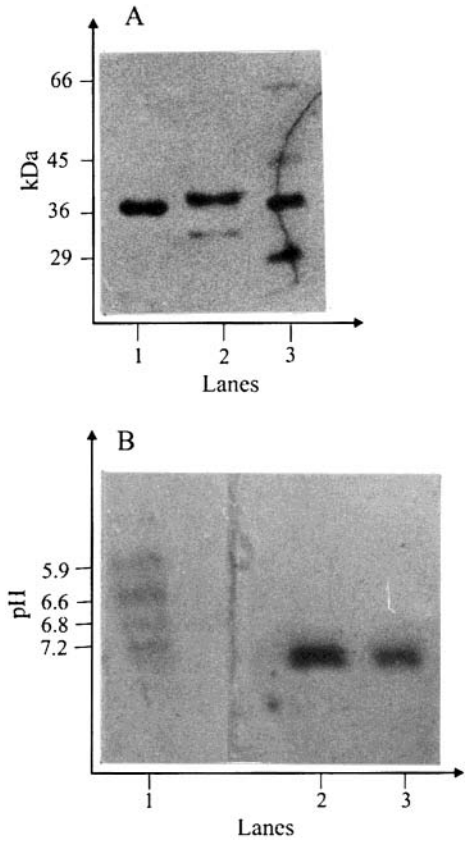

Figure 2. Electrophoretic analysis of fractions having peroxidase activity from hydrophobic interaction HIC column: (A) SDS-PAGE (8 μg per lane) [lane 1, TNP (fraction 2); lane 2, fraction 1; lane 3, molecular weight markers (Sigma)]; (B) IEF [lane 1, p*I* markers (Sigma; 4 μg of each marker); lane 2, TNP (20 μg) activity band (ABTS as substrate); lane 3, TNP (20 μg) protein band (silver stained)].

ranges from 30 to 60 kDa. This variability has been attributed to post-translational modifications of the polypeptide chain including the number and composition of glycan chains present in plant peroxidases (*9*).

IEF. When fraction 2 peroxidase was subjected to IEF, a single band migrating to pH 7.2 was observed, indicating a neutral peroxidase (TNP; Figure 2B). In this figure lane 2 shows TNP peroxidase activity, and lane 3 shows the silver-stained TNP. A wide range of peroxidase isozymes with p*I* values varying from 3.5 to 10 have been detected in fruits and vegetables (*6*). Acidic peroxidases have been identified in pepper fruit (3.8; *33*) and Brussels sprouts (*13*). Basic peroxidases have been found in barley (9.3; *36*) and strawberry fruits (9.5−10; *37*). In turnip roots acidic peroxidases have been identified (*7*) as well as basic peroxidases (*8*, *38*), but there are no reports of neutral peroxidases such as that purified in this work. The yield of pure TNP was ~0.6 mg/kg of fresh roots.

Glycoprotein Nature and Carbohydrate Content. Because many peroxidases from different higher plants have been characterized as glycoproteins, the glycoprotein nature of TNP was investigated. After staining, the main protein of fraction 1 and the single protein of fraction 2 (TNP) showed a pink color, characteristic of glycoproteins (data not shown). According to the phenol−sulfuric acid reaction, TNP had 9.1% carbohydrate content. Thus, the carbohydrate content of TNP is similar to that of Korean radish (9−14%; *39*) and higher than that of wheat germ (4.1−7.9%; *28*) peroxidases. However, peroxidases having carbohydrate contents higher than that of TNP are those from soybean (15%; *40*), peanut (22%; *41*), and isozyme C of HRP (18%, *42*).

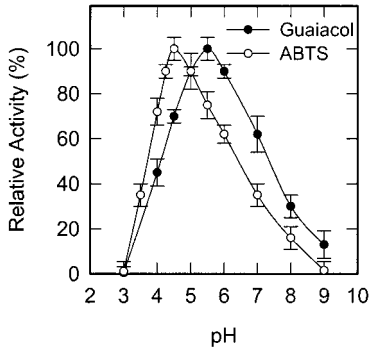

Figure 3. Effect of pH on enzymatic activity of TNP with guaiacol and ABTS as H donors. The ordinate represents relative activity, that is, the ratio of the activity to the maximum activity expressed as percentage. Buffers used were 10 mM citrate, pH 3−6; 10 mM phosphate buffer, pH 7; 10 mM Tris-HCl buffer, pH 8.0; and 10 mM borate buffer, pH 9.0. The ionic strength was kept constant at 430 mM by adjustment with KCl.

TNP Kinetic Studies. *Characterization Using Guaiacol as H Donor.* Peroxidases are specific for H_2O_2 as substrate but can use a number of H donors such as guaiacol. The pH of optimum activity for TNP was 5.5 (Figure 3), very similar to that of strawberry fruit (6.0; *37*), tomato (5.3−5.5; *43*), and soybean (5.5; *44*) peroxidases. These values are comparable due to use of the same activity measurement system. It is known that the optimum pH changes according to the H donor used in the activity assay and even with the buffer solution (*45*).

Eq 1 was used to distinguish among the ordered, random, and ping-pong mechanisms and to obtain the K_m values for both substrates (*22*). The initial velocities (v_0) were determined as a function of both substrate concentrations (*A*, H_2O_2; and *B*, guaiacol or ABTS). Within any one experiment A_0 was kept constant while B_0 was changed, and more experiments were conducted at different fixed concentrations of A_0. When v_0 was plotted versus the substrate concentrations, the effect of both substrates on the initial velocity was very clear (data not shown). Double-reciprocal plots of TNP kinetics, according to eq 1, are shown in Figure 4A, where the parallel lines indicate that TNP follows a ping-pong mechanism. The slopes and *y*-intercepts from Figure 4A were determined. According to eq 3, the calculated intercept values were plotted against $1/A_0$, giving a straight line (Figure 4B). From the intercept of Figure 4B, the V_{max} value [411 μM guaiacol consumed/(mg of protein·min)] was calculated (eq 4), whereas K_a was calculated from the slope (eq 5). Knowing V_{max}, from the constant slopes of Figure 4A, K_b was calculated (eq 2). The turnover number (k_{cat}, substrate molecules consumed per second per molecule of peroxidase) was 0.37 s^{-1}.

The guaiacol K_m value was 3.7 mM. This value was lower than those found for guaiacol oxidation by peroxidase from Korean radish roots (6.7−13.8 mM; *39*), green peas (10.2 mM; *45*), and tomato (5−10 mM; *43*) peroxidases. TNP had a catalytic efficiency (k_{cat}/K_m) of 100 M^{-1} s^{-1}.

Characterization Using ABTS as H Donor. Using ABTS as H donor, turnip peroxidase had a maximum

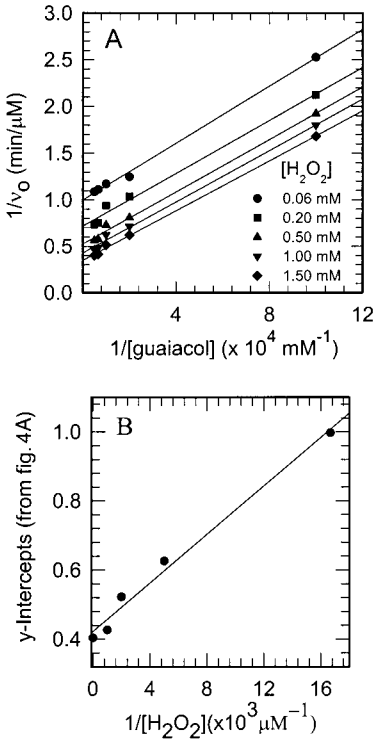

Figure 4. Kinetic behavior of the two-substrate reactions for TNP: (A) plot of the substrate−velocity relationship, according to eq 1; (B) plot of the *y*-intercepts of the lines of Figure 4A versus ($1/H_2O_2$), according to eq 3.

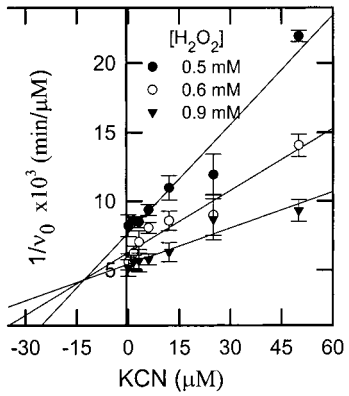

Figure 5. Effect of cyanide on TNP oxidation of ABTS at pH 5.0, at three concentrations of H_2O_2.

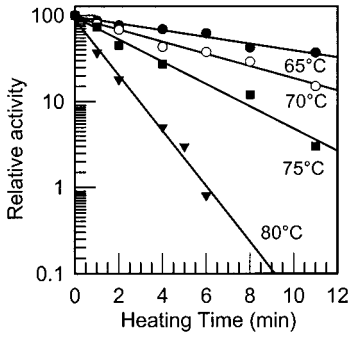

Figure 6. Rates of heat inactivation of TNP. The ordinate represents relative activity, that is, the ratio of the activity to the maximum activity expressed as percentage.

activity at pH 4.5 and showed high activity in the range of pH 4−6 (Figure 3). This optimum pH is similar to those found for two turnip anionic peroxidases (close to 5.0; *8*).

In the same way as for guaiacol characterization, substrate (ABTS) concentration−velocity plots were obtained, and according to eqs 2, 4, and 5, K_m, V_{max} [583 μM ABTS consumed/(mg of protein·min)], and the reaction mechanism were obtained. TNP followed a ping-pong mechanism, as shown by Childs and Bardsley (*15*) for HRP. The ABTS K_m value was 0.7 mM, which is ∼6 times lower than that of acidic HRP but 7 times higher than that of basic HRP, according to Hiner et al. (*46*). This indicates that TNP could be suitable for applications where high sensitivity threshold for ABTS is required, such as enzyme immunoassays or immunohistochemistry. The catalytic efficiency (k_{cat}/K_m) using ABTS was 500 M^{-1} s^{-1}, ∼5 times higher than that obtained for guaiacol. This indicated that TNP had a higher specificity for ABTS than for guaiacol. Additionally, the catalytic efficiency was lower than those from Brussels sprouts acidic peroxidases (1090 and 1120 M^{-1} s^{-1} for isozymes A1 and A2, respectively; *13*).

Khan and Robinson (*47*) attributed different peroxidase capacities to oxidize substrates to molecular structure variations and indicated that oxidation rates could be determined by the velocity of free radical release from the enzyme−substrate complex (e.g., tetraguaiacol formation).

Cyanide Inhibition. Cyanide inhibits many iron-containing enzymes. Cyanide is a competitive inhibitor of TNP, as shown by a Dixon plot (Figure 5), when its concentration was >1.0 μM. Competitive cyanide inhibition has been observed for peroxidases from tea leaves (*30*), sweet potato (*48*), and tomato (*43*). The cyanide inhibition constant K_i, at pH 5.0, was 14 μM (Figure 5). Halpin et al. (*45*) reported K_i values of 1.5 and 5 μM for two green pea peroxidases, and for sweet potato peroxidase a K_i of 0.5 μM was obtained (*48*). Thus, it is concluded that TNP is apparently more tolerant than those peroxidases to cyanide inhibition.

Sodium Azide Inhibition. Another potent inhibitor of hemoprotein-catalyzed reactions is sodium azide, which also inhibited TNP in a competitive fashion (data not shown). The K_i was 0.9 mM, indicating a much higher sensitivity to cyanide. This value was similar to that reported for sweet potato peroxidase (1.2 mM; *48*). According to Saunders et al. (*49*), cyanide reversibly inhibits peroxidase at concentrations between 10^{-5} and 10^{-6} M, whereas sodium azide produces the same effect at 10^{-3} M or higher. Cyanide inhibits all peroxidases studied to date and has been used to identify true peroxidase activity (*16*).

TNP Thermal Inactivation. The rate of heat inactivation of TNP increased with temperature and length of heat exposure (Figure 6). When it was heated to 65 °C, ∼70% of the activity was retained after 11 min. Thus, TNP was much more heat resistant than peroxidases from Brussels sprouts (*13*), two anionic and a basic isozyme from turnip (*7*, *8*), and a commercially available HRP (*50*). This plot was used to calculate the activation energy for heat inactivation (E_a). Arrhenius plots of ln(rate constant) versus the reciprocal of absolute temperatures ($1/T$) were prepared, and the slope of the resulting straight lines was used to calculate the activation energy for peroxidase destruction. TNP had an E_a of 132 kJ/mol, similar to the value found by Lu and Whitaker (*10*) for HRP and for unpurified asparagus peroxidase (140 kJ/mol; *51*). The E_a value is similar to or higher than that of other plant peroxidases. However, higher values have been found for peroxidases

from cauliflower (E_a = 194 kJ/mol; *52*) and asparagus (E_a = 174 kJ/mol; *51*). The activity loss and the low E_a value have been attributed to dissociation of the heme group from the protein active site during heat treatment (*10*).

During storage at 2−6 °C TNP retained ~90% activity after 1 month, whereas full activity was retained at −20 °C for 6 months.

The high specific activity (1930 units/mg) and catalytic efficiency for ABTS (500 M^{-1} s^{-1}) shown by TNP make it an alternative source of peroxidase for immunoassays and immunohistochemistry applications. Further studies on structural characterization are presently being conducted. Because lectin affinity purification has produced poor results, we are now working on the production of an antibody against TNP for an easier purification of TNP.

ABBREVIATIONS USED

A_0, substrate A (H_2O_2) concentration; ABTS, 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); B_0, substrate B (H donor) concentration; BSA, bovine serum albumin; *C*, percent *N*,*N*′-methylenebis(acrylamide) in the mixture acrylamide plus *N*,*N*′-methylenebis(acrylamide) (w/w); E_a, activation energy for inactivation, kJ/mol; HIC, hydrophobic interaction chromatography; HRP, horseradish peroxidase; IEF, isoelectric focusing; K_a, K_m for substrate A (hydrogen peroxide); K_b, K_m for substrate B (H donor); k_{cat}, turnover number (number of substrate molecules consumed per second per molecule of enzyme); k_{cat}/K_m , catalytic efficiency (M^{-1} s^{-1}); K_i, inhibition constant; K_m, Michaelis−Menten constant; NPF, nonretained pooled fractions from anion-exchange chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; *T*, percent of the mixture acrylamide plus *N*,*N*′-methylenebis(acrylamide) (w/v); TNP, turnip neutral peroxidase; V_{max}, maximum velocity; v_0, initial velocity.

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