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Broccoli Processing Wastes as a Source of Peroxidase

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A peroxidase isozyme (BP) was purified to homogeneity from broccoli stems (*Brassica oleraceae* var. maraton) discarded from industrial processing wastes. BP specific activity was 1216 ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] units/mg, representing 466-fold that of crude extract. BP is a monomeric glycoprotein containing 16% carbohydrates, with a molecular mass of 49 kDa and an isoelectric point close to 4.2. From kinetic data it showed a two-substrate ping-pong mechanism, and the catalytic efficiency measured as the rate-limiting step of free BP regeneration was 3.4×10^6 M^{-1} s^{-1}. The ABTS K_m value was 0.2 mM, which was about 20 times lower than that reported for acidic commercial horseradish peroxidase (HRP). Assessment of BP secondary structure showed 30% helical character, similar to HRP and cytochrome *c* peroxidase. BP lost only 25% activity after 10 min of heating at 55 °C and pH 6; it was stable in the pH range from 4 to 9 and showed an optimum pH of 4.6 using ABTS as substrate. BP was active on substrates normally involved in lignin biosynthesis, such as caffeic and ferulic acids, and also displayed good catechol oxidation activity in the presence of hydrogen peroxide. Reverse micellar extraction was successfully used as potential large-scale prepurification of broccoli peroxidase, achieving a purification factor of 7, with 60% activity yield. Stems from the broccoli processing industry have a high potential as an alternative for peroxidase purification.

KEYWORDS: Peroxidase; broccoli processing wastes; purification; reverse micelles

INTRODUCTION

Peroxidases (EC 1.11.1.7) are widely distributed in nature; most are heme proteins and contain iron(III) protoporphyrin IX as the prosthetic group (*1*). They catalyze the oxidation of a wide variety of substrates using H_2O_2 or other peroxides. Reduction of peroxide at the expense of electron-donating substrates makes peroxidases useful in a number of industrial and analytical applications (*2*). Coupled to other enzymes in polyenzymatic systems producing hydrogen peroxide, it is used in the determination of many compounds, such as uric acid and cholesterol in blood (*3*, *4*). Peroxidase has been used in enzyme immunoassays for quantification of creatine kinase B in serum from ovarian cancer patients, as a marker for early diagnosis (*5*), and combined with indole-3-acetic acid may be used for antibody- or gen-directed enzyme:prodrug therapies (*6*). Peroxidase has been used in analysis and diagnostic kits, electrochemical biosensors, enzyme immunoassays, organic and polymer synthesis, and bioremediation of phenolic compounds, and it can replace current chemical oxidations such as decolorization of synthetic dyes (*7*). The major source of commercial peroxidase is horseradish roots. However, we have tested alternative peroxidase sources locally available and studied their properties involving innovative purification schemes such as immunoaffinity chromatography and reverse micellar extraction as possible substitutes of horseradish (*8*–*10*). Our group is working on the application of purified native and chemically modified broccoli peroxidase for phenolic compound removal from industrial effluents.

Broccoli is a very good source of dietary fiber, vitamins A, C, K, and B_6, folate, and manganese. Additionally, many studies indicate that a regular intake of broccoli has a strong correlation

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with cancer prevention and inhibition (*11, 12*). Mexico produced 247820 tons of broccoli in 2006 (*13*) and is the largest broccoli exporter to the United States, mostly as frozen product. Industrial processing of broccoli produces large amounts of byproduct, such as leaves, stems, and florets that do not meet the required quality properties. A small amount of this material is used as forage, whereas the rest is discarded as solid waste, which upon degradation represents a pollution problem. Here we propose to use this highly available broccoli processing waste as a potential source of value-added products such as enzymes.

We have found that the peroxidase activity in broccoli stems is similar to that of florets (*14*). We hypothesize that broccoli wastes can be an alternative source of a peroxidase isoenzyme, which may be prepurified using a scalable liquid–liquid extraction technique, having properties that could make it a high value added product. Thus, the objective of our research was to purify and partially characterize peroxidase from broccoli processing wastes (mainly stems separated from the more valuable broccoli heads), which may be useful in many industrial and clinical applications.

MATERIALS AND METHODS

Samples and Crude Extract. Broccoli (*Brassica oleracea* L. var. maraton) stems were obtained from Exportadora de Hortalizas (Expo-Hort), Queretaro, Mexico, and transported at 4 °C to the laboratory, where they were stored at −70 °C until used. Broccoli stems (250 g) were washed with distilled water and homogenized at 4 °C in a blender using 50 mM potassium phosphate buffer, pH 6.0. The extract was passed through a cheesecloth and centrifuged at 12000*g* for 15 min, and the supernatant was labeled as crude extract.

Protein Determination and Peroxidase Activity. Total protein content was estimated using the Bradford method (*15*) with bovine serum albumin as standard. Peroxidase activity was determined spectrophotemetrically using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as hydrogen donor. The 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. Peroxidase activity was determined by the change in absorbance at 414 nm due to ABTS oxidation in the presence of H_2O_2 considering an $\varepsilon_{414} = 36\ mM^{-1}\ cm^{-1}$. The assay was performed at 25 °C using a Lambda 2S Perkin-Elmer (*16*). One unit of enzyme is defined as the amount (micrograms) of substrate (ABTS) consumed in 1 min.

Peroxidase Purification. The protein of the crude extract was precipitated with cold acetone (−20 °C), in a 2:1 ratio (acetone/extract). The precipitate was centrifuged (12000*g*, 10 min, 4 °C), redissolved in 20 mM diethylethanolamine buffer, pH 9.0, and dialyzed at 4 °C against the same buffer. This sample was applied to a DEAE-cellulose (Sigma) column (1.5 × 20 cm, Bio-Rad) fitted to a Gradifrac (Pharmacia) system, which was equilibrated with the same buffer at a 40 mL/h flow rate. Two milliliters of the enzyme solution was injected, and 4 mL fractions were collected. The retained protein was eluted with a linear NaCl gradient in the above buffer from 0 to 0.4 M (fractions 8–23) and from 0.5 to 1.0 M for fractions 24–34. Fractions showing peroxidase activity were pooled, dialyzed against deionized water, and freeze-dried. A Sephadex G-100 column (1.5 × 50 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.0, containing 0.3 M NaCl, was injected with 5 mL of the resuspended and filtered sample (0.22 μm pore size). Fractions of 3 mL were collected at a flow rate of 9 mL/h. Active fractions were used for further purification, dissolved in 20 mM diethylethanolamine buffer, pH 9.0, and 500 μL was injected onto a Resource Q column (Pharmacia) coupled to a HPLC (Waters), equilibrated with the same buffer, at a 20 mL/h flow rate. The retained enzyme was eluted using a linear gradient of 0.0−0.3 M NaCl in the above buffer. The fractions showing peroxidase activity were collected, pooled, and dialyzed against deionized water and were used for further characterization studies.

Broccoli Peroxidase Characterization. The carbohydrate content of the purified broccoli wastes peroxides (BP) was evaluated by using the phenol−sulfuric acid method with mannose as standard (*17*). UV–visible spectra of BP solutions were obtained in the range of 240–700 nm.

Gel Electrophoresis. SDS-PAGE of BP was performed in 12% T [acrylamide plus bis(acrylamide)] (*18*), in a Mighty Small SE 250 (Hoeffer) electrophoresis cell. Running conditions and protein band detection using silver staining were conducted as previously described (*19*). The gels were also stained for glycoproteins using a glycoprotein detection kit (Sigma). Non-denaturing gel electrophoresis was carried out using the same buffer system without 2-mercaptoethanol in the sample buffer, and gels were stained for peroxidase activity (*20*). Isoelectric focusing of BP was conducted according to the method given in ref *9*.

Secondary Structure. The far-UV CD spectrum of BP (0.1 mg/mL), pH 6, was recorded using a JASCO J-715 spectrometer in a 0.1 cm path length quartz cell, fitted with a thermoelectric temperature control under constant nitrogen flush. The proportions of each type of secondary structure (α-helix, β-sheet, and random) were calculated using the K2D program (*21*).

Optimum pH for Activity. The optimum pH of BP was determined according to the procedure of ref *8*. The maximum BP conversion rate was analytically found by using Michaelis kinetics as a function of pH and assuming no interaction among the prototropic groups (*1*)

$$\frac{V^{H+}{}_{max}}{V_{max}} = \frac{1}{\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right)} \tag{1}$$

where $V^{H^+}{}_{max}$ is the apparent V_{max} at the different pH values tested, whereas V_{max} is the pH-independent maximum velocity. K_1 and K_2 are BP true ionization constants. The parameters of this equation were obtained by using the Marquardt–Levenberg algorithm from the curve fitting option of the Sigma-Plot v. 10.0 software (*22*). The optimum pH may be considered as the average of the two p*K* values (*1*).

Kinetic Constants for ABTS and H_2O_2. Kinetic constants were calculated as previously described (*8*), using 10 μg of protein in a 1.5 mL reaction volume, at the optimum pH. K_m values were determined using the Lineweaver–Burk plot for the two-substrate mechanism followed by peroxidase (*1*). Equation 2 was used to obtain the K_m values for ABTS and H_2O_2 and also to distinguish among ordered, random, and ping-pong mechanism (*1*)

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_a}{A_0 V_{max}} + \frac{K_b}{B_0 V_{max}} \tag{2}$$

where V_{max} is the maximum velocity, K_a and K_b are K_m values for substrates A (H_2O_2) and B (ABTS), respectively, and A_0 and B_0 are substrate A and B concentrations.

The initial velocities (v_0) were determined as a function of both substrate concentrations. When A_0 is constant, eq 2 yields a slope and intercept given by

$$\text{slope} = \frac{K_b}{V_{max}} \tag{3}$$

$$\text{intercept} = \frac{1}{V_{max}} + \frac{K_a}{A_0 V_{max}} \tag{4}$$

A replot of *y*-intercepts obtained by using eq 4 versus $1/(A_0)$ will produce a straight line with a slope and intercept given by

$$\text{intercept} = \frac{1}{V_{max}} \tag{5}$$

$$\text{slope} = \frac{K_a}{V_{max}} \tag{6}$$

Therefore, constants K_a, K_b, and V_{max} can be determined from eqs 3, 5, and 6.

Table 1. Conditions and Activity Determination of BP over Different Substrates (± Standard Deviation)

substrate	λ (nm)	E ($M^{-1}cm^{-1}$)	[H_2O_2] (mM)	[substrate] (mM)	ref	specific activity (μM min^{-1} mg of $protein^{-1}$)
ferulic acid	318	31100	0.3	0.02	*23*	1.74 ± 0.09
ascorbic acid	290	2.8	0.5	0.5	*24*	35.7 ± 3.5
coumaric acid	240	17900	0.5	0.1	*25*	0.034 ± 0.002
catechol	295	1700	2	20	*23*	76.5 ± 3.5
caffeic acid	285	34000	0.5	0.05	*26*	9.65 ± 0.4

Considering that the rate-limiting step in peroxidase catalysis is the regeneration of free enzyme (*23*), the apparent second-order rate constant (k_{app}) may be used to more accurately describe the catalytic efficieny, which can be written as

$$\text{rate}(V_{max}) = k_{app}[\text{E(II)}][\text{AH}_2] \quad (7)$$

where [E(II)] is the initial BP concentration and [AH_2] is the ABTS concentration for optimal catalysis.

Substrate Specificity. The enzymatic activity of the peroxidase toward different substrates was performed at 25 °C in a final volume of 1 mL containing 50 mM potassium phosphate buffer, pH 5.0, under conditions shown in **Table 1**. Three nanomolar enzyme concentration was used in all determinations, and reaction was started by the addition of H_2O_2. All data were corrected for nonenzymatic substrate oxidation by H_2O_2. The enzymatic activities were calculated by dividing the change in absorbance per minute by the extinction coefficient of the substrate. One activity unit was defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute.

Thermal and pH Stability. ABTS was the substrate used to perform these tests. The thermal stability of BP was assayed using a water bath (Shell Laboratory) at temperatures ranging from 35 to 75 °C, during 10 min (*27*). 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 for a designated temperature. After heating, the solutions were rapidly cooled in ice–water, and residual peroxidase activity was immediately determined. pH stability was evaluated using 5 mL aliquots of BP solution added to different test tubes and adjusted between pH 3 and 11 using the following buffers: 10 mM citrate, pH 3–6; 10 mM phosphate, pH 7.0; 10 mM Tris-HCl, pH 8.0; 10 mM borate, pH 9; 10 mM carbonate, pH 10–11. Each sample was kept for 4 h at 30 °C, and the residual activity was evaluated at optimum pH, as described before.

Purification Using Reverse Micelles. Once we found the importance of having large amounts of BP, we tried this liquid–liquid extraction technique for possible purification scale-up. Cetyltrimethylammonium bromide (>99%, Fluka) (CTAB) was used for reverse micellar extraction of broccoli peroxidase crude extract. CTAB (0.2 M) was dissolved in a mixture of isooctane (spectrophotometric grade, 99%, Sigma) with either *n*-pentanol, butanol, hexanol, or isooctanol (Sigma), in a 9:1 ratio. pH varied from 7 to 11, using 30 mM phosphate buffer. The ionic strength (IS) was adjusted from 0.1 to 0.6 M with either KCl, KBr, or KF (Sigma). Phase transfer was accomplished in well-stirred closed vessels, at room temperature (25 ± 2 °C). Forward and backward transfers (5 mL of each phase) were conducted until equilibrium was reached (8 and 15 min, respectively). A centrifuge (Eppendorf) was used to achieve phase separation (5000*g* for 2 min at room temperature). Back extraction was performed using an equal volume of fresh aqueous buffer (80 mM citrate, pH 4.0) containing 2 M KCl (*10*).

RESULTS AND DISCUSSION

BP Purification. Peroxidase activity in the crude extract of broccoli stems was 2.6 ABTS units/mg of protein. This value was higher than those reported for Brussels sprouts (0.4 unit/mg) and broccoli var. italica (0.5 unit/mg) extracts (*9*), but lower than the 6.5 units/mg found for turnip extract (*20*). Peroxidase activity per gram of fresh material was about 3.5 units; thus, broccoli wastes appears to be a good source for peroxidase purification.

Table 2. Summary of the Purification Steps of BP (250 g) (Mean of Five Replicates with Standard Deviation within 5% of the Mean)

sample	protein (mg)	activity (units)	specific activity (units/mg)	fold[a]	yield
crude extract	2166	5651.92	2.61	1.00	100.00
acetone precipitation	384.1	3968	4.19	1.61	70.2
AEC[b]	23.28	1855	79.70	30.5	32.8
gel filtration	2.65	600	226.4	86.8	10.6
HPLC-AEC[c]	0.13	151.7	1217	466	2.68

[a] Ratio of actual to initial specific activity. [b] Anion exchange chromatography (DEAE-cellulose), retained fraction. [c] Resource Q column.

The purification procedure for BP is summarized in **Table 2**. The crude extract was precipitated with cold acetone, which improved peroxidase purification and also helped to concentrate the extract for further purification. The activity yield and purification factor after this step were 70% and 1.6, respectively. In this novel purification scheme acetone was used instead of ammonium sulfate, which removed color material that tended to clog the ion exchange chromatography column, whereas acetone was commonly recycled. Activity recovery after this purification step was higher than other report values using a two-step differential precipitation with ammonium sulfate, which produced only a 34% activity recovery (*28*).

Conventional chromatographic methods for peroxidase purification included anion exchange (AEC) (**Figure 1A**), gel filtration (**Figure 1B**), and HPLC anion exchange (**Figure 1C**). Two peaks with peroxidase activity were obtained from DEAE-cellulose; one eluted in the void volume, whereas the other eluted at about 0.18 M NaCl gradient (**Figure 1A**). The specific activity of the retained pooled fractions was 79.7 units/mg, with a RZ value ($\mathbf{A}_{403}/\mathbf{A}_{280}$ nm) of 1.0, which was greater than those of the nonretained pooled fractions (22.4 units/mg and RZ = 0.3, respectively), indicating a higher purity. Therefore, the retained pooled fractions were chosen for further purification. The relatively low RZ value of the retained fractions (1.0) suggested a poor degree of purification; however, this value was higher than that reported by Duarte-Vázquez (*20*) (0.62) for turnip peroxidase after ammonium sulfate precipitation and AEC. The elution profile after gel filtration chromatography showed two peaks containing peroxidase activity (I and II, **Figure 1B**). Peak I came out close to the void volume, which indicated a high molecular weight, probably due to protein aggregation, but it contained >90% of the total peroxidase activity with an RZ value of 1.6. A large amount of the 280 nm absorbing material of the retained fraction from AEC was removed using this purification step, although, this peak was still relatively impure, because highly purified horseradish peroxidase has RZ = 3.0.

The active fractions comprising peak I from gel filtration were dialyzed against distilled water, freeze-dried, and redissolved in the equilibration buffer of the anion exchange column fitted to a HPLC system. The elution profile (**Figure 1C**) showed two peaks having peroxidase activity (peak III and purified broccoli wastes peroxidase BP with RZ = 2.8). BP was collected

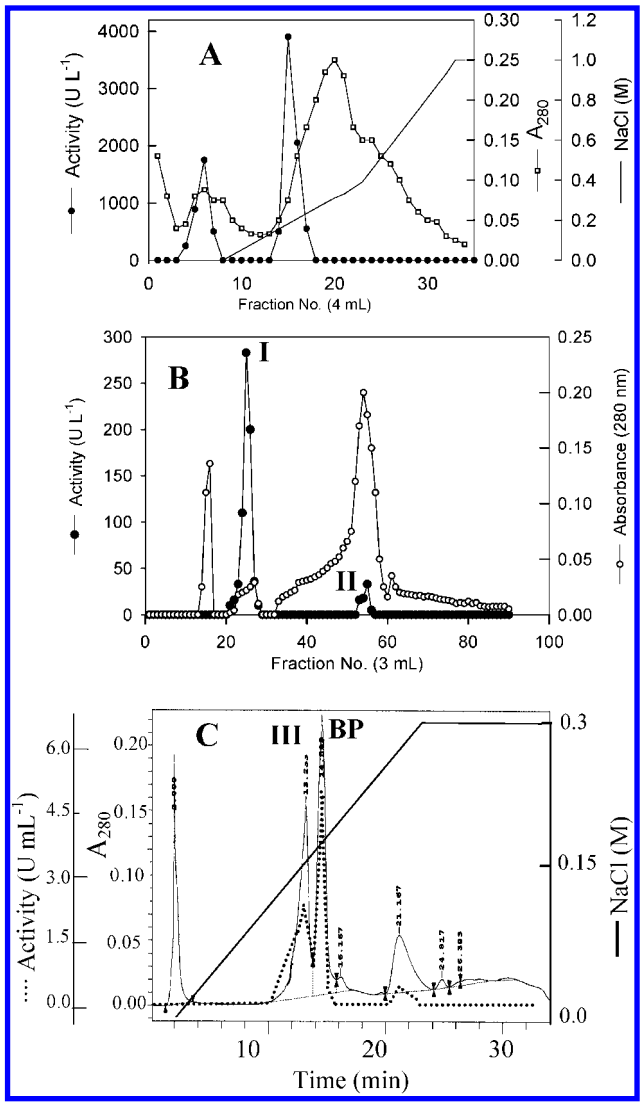

Figure 1. Chromatographic purification of broccoli waste peroxidase: (**A**) anion exchange, using DEAE-cellulose, 15 mg injected protein (buffer A, 20 mM diethylethanolamine, pH 9.0; buffer B, A + 1 M NaCl; flow rate, 40 mL/h); (**B**) gel filtration using Sephadex G-100, 30 mg injected protein (buffer, 20 mM Tris-HCl, pH 7.0, + 0.3 M NaCl; flow rate, 8 mL/h; I, II, peaks showing >90%, and <10% peroxidase activity, respectively); (**C**) HPLC anion exchange (1 mL Resource Q column), 0.5 mg injected protein [buffer A, same as in (**A**); buffer B, that in (**A**) + 0.3 M NaCl; flow rate, 0.5 mL/min; BP, purified broccoli waste peroxidase].

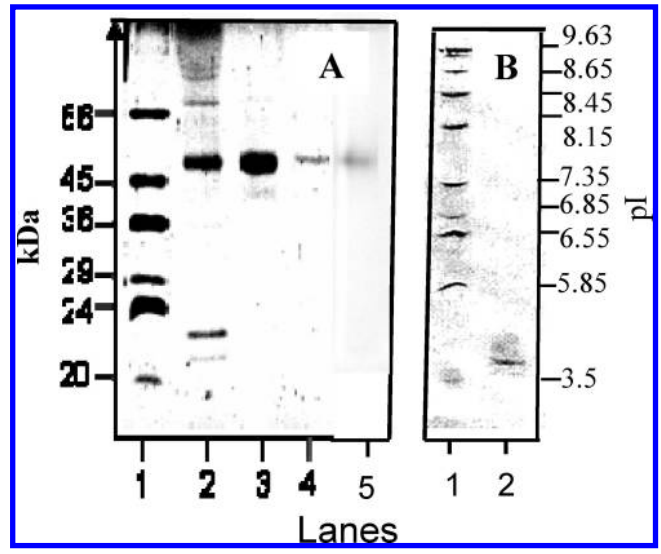

Figure 2. (**A**) SDS-PAGE under reducing conditions of broccoli extracts [lanes 1–4, 3.8 μg of protein/lane; lane 1, low molecular weight markers (Sigma); lane 2, retained fraction after anion exchange chromatography; lane 3, purified fraction from gel filtration chromatography; lane 4, purified BP from HPLC, all previous bands silver stained; lane 5, activity bands of purified fraction from gel filtration, after non-denaturing PAGE]. (**B**) Isoelectric focusing of BP [lanes 1 and 2, 5 μg of protein/lane; lane 1, p*I* markers (Pharmacia); lane 2, BP isoelectric point].

and used for further studies because it showed a higher peroxidase activity and purification factor than peak III. The purification scheme resulted in a 466-fold purification and a specific activity of 1216 units/mg (**Table 2**). Because our purification scheme involved a higher resolution technique (HPLC), the purification factor was about 3 times higher than that reported for another acid broccoli peroxidase (*28, 29*). Our aim was to obtain a highly purified enzyme with the purpose of using it for enzyme immunoassays, biosensors, or immunohistochemistry studies. Despite the low yields (2.7%) of BP, the large amounts of broccoli processing waste make this an attractive source of commercial peroxidase.

Molecular Weight and Purity. The purified BP exhibited a single band when subjected to reducing and nonreducing SDS-PAGE, indicating protein homogeneity and a single polypeptide chain, with a calculated molecular mass of about 49 kDa (**Figure 2A**, lane 4). However, when the semipurified protein was subjected to gel filtration on Sephadex G-100, the protein eluted in a single peak corresponding to an estimated M_r of about 200 kDa. SDS-PAGE showed that this peak corresponded to two protein bands of similar size (**Figure 2A**, lane 3), which were identified as peroxidase isozymes, because the same bands showed activity following non-denaturing PAGE (**Figure 2A**, lane 5). Dynamic light scattering measurements have shown that some plant peroxidases tend to form aggregates (*30*). Thus, this was probably the reason for such a high molecular mass, of possibly an aggregate of four peroxidase molecules. The molecular masses of fruit and vegetable peroxidases range from 30 to 54 kDa, which agree with the results obtained here. BP showed an isoelectric point (p*I*) of about 4.2 (**Figure 2B**, lane 2), which is similar to those of two Brussels sprouts isozymes (pI = 4.3 and 4.7) (*9*), and was classified as an acid isozyme. This isoperoxidase is different from the acid broccoli peroxidase initially reported with a p*I* close to 4 (*28*) and later corrected to pI < 3 (*29*).

Glycoprotein Nature and Carbohydrate Content. Purified BP was examined qualitatively for glycosylation by periodic acid staining after SDS-PAGE, and a pink band characteristic of a glycoprotein was observed (results not shown). The purified BP contained 16% carbohydrate, which is similar to that of Japanese radish (18%) (*31*), hull of soybean (18.7%), (*32*), and an acidic isozyme from turnip (18%) (*33*) and lower than that of HRP C (22%) (*34*).

Lige et al. (*35*), using a site-directed replacement of each of the three glycosylation sites of cationic peanut peroxidase, showed that only the glycan linked to Asn185 was important for the thermal stability. Tams and Welinder (*36*) studied the influence of *N*-glycans on the stability of HRP C. They demonstrated that the thermodynamic stability of HRP C in equilibrium at various temperatures is indistinguishable from that of chemically deglycosylated HRP (dHRP), whereas the unfolding rate constant of dHRP in guanidium chloride, and hence the kinetic stability, decreased 2–3-fold. After N-deglycosylation no differences were found between the thermal

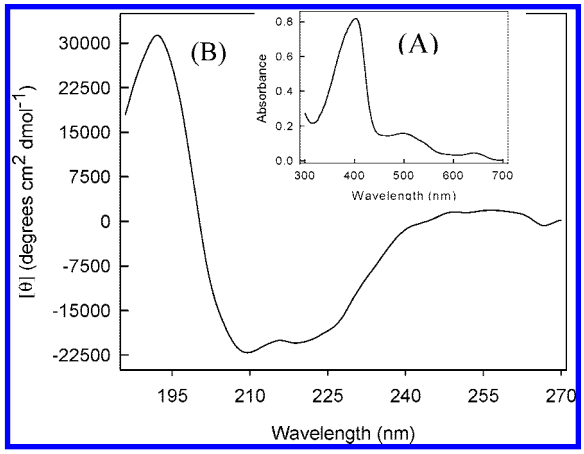

Figure 3. Spectra of purified BP at 25 °C: (**A**) UV–vis absorption (0.2 mg/mL); (**B**) CD far-UV spectra (0.1 mg/mL). Means of three replicates with standard deviation within 5% of the mean are shown.

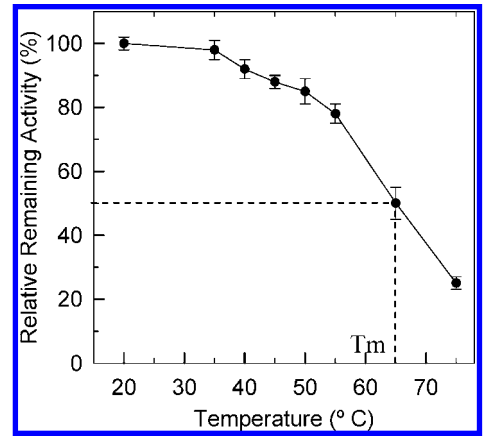

Figure 4. Thermal stability of purified BP (0.5 mg/mL). The ordinate represents relative activity, that is, the ratio of the activity to the initial activity before heating for 10 min, expressed as percentage. Means of triplicates with error bars indicating standard deviation are shown.

stabilities of HRP and d-HRP, with a transition midpoint of 57 °C. Physiological significance of the carbohydrates linked to plant peroxidases is not clear. It has been suggested that the carbohydrate moieties of peroxidase contribute to the characteristic high thermostability of this enzyme (*37*) and resistance to proteolytic attack (*38*, *39*).

UV–Visible Spectra and Secondary Structure of BP. Purified BP showed a Soret band at 403 nm and α- and *β*-bands at 498 and 640 nm (**Figure 3A**). These values are similar to those of class III peroxidases such as those from horseradish (402.5, 498, and 641 nm, respectively) and from soybean (402, 490, and 633 nm, respectively). The CD spectrum of BP in the far-UV region showed bands with minima at 208 and 222 nm typical of α-helix (**Figure 3B**). The CD curve of BP also exhibited a band with a minimum at 216 nm, which represents the *β*-structure of the protein (*40*).

Computer simulation of the CD spectra of broccoli peroxidase using the program K2D (*21*) showed 30% helical character, 15% *β*-strand, and 55% random. Heme plant peroxidases α-helix accounts for 30–40% of the total secondary structure (*41*), but α-helix content may vary, because palm tree has only 14.9% (*42*). A report on a different acidic broccoli peroxidase showed 40% helical character (*29*). The same authors calculated 30% helix structure for a basic (p*I* > 10) HRP, whereas 42% α-helix was reported for HRP C (*43*) and 30% for HRP A1 (*41*). Discrepancies may be expected because algorithms predicting secondary structure use linear combinations of reference spectra of proteins, with 100% α, *β*, or random structure measured from model polypeptides. Other models use linear combinations of protein spectra for which the secondary structure is known from X-ray diffraction pattern analysis (*44*).

Thermal Stability of BP. Peroxidase is reported to be one of the most heat stable enzymes in plants. However, its heat resistance depends on the enzyme source as well as the assay conditions. BP exhibited good thermal stability, because 75% of the initial activity was retained after heating at 55 °C for 10 min, pH 6.0 (**Figure 4**). The BP midpoint inactivation temperature (T_m), defined as the temperature at which 50% of enzyme activity is lost, was 65 °C (**Figure 4**). This is similar to the T_m value of 68 °C reported for chickpea peroxidase (*45*). BP was more thermostable than a heat labile HRP isozyme (A1) (*46*) and commercial HRP (type XII), which lost 50% activity after 8 min of heating at 60 °C and pH 6 (*47*), as well as two peroxidases from turnip roots, which lost >80% of their initial activity after heating at 50 °C for 15 min (*27*). BP was slightly less thermostable than an acidic broccoli peroxidase (*48*), less thermostable than a commercial impure HRP (RZ = 0.59), which showed 50% activity loss after heating at 76.7 °C for 3 min (*49*) and lower thermostability than HRP C (RZ = 3.0) (*46*). Crude extracts have shown higher thermal resistance than that of the purified enzymes. This has been attributed to differences in the surrounding environment (*50*). Using dynamic light scattering, we have noted better enzyme stability for larger peroxidase aggregates, which have been observed for the more impure samples. Structural properties of the different isoenzymes are largely responsible for the observed variability in their heat inactivation. Adams (*51*) has reported that electrostatic interactions and hydrogen bonds significantly contribute to enzyme stability. The carbohydrate moieties of peroxidase also show a contribution to the enzyme stability as shown for turnip peroxidase (*38*) and HRP (*36*). BP had 16% carbohydrate content, which, in addition to structural properties, might explain its lower heat stability compared to that of commercial HRP isozyme C (type VI, RZ = 3.0) (*52*), having 22% carbohydrate (*34*).

However, the thermal stability of BP was lower than that of oil palm tree peroxidase, which lost 75% activity after heating at 65 °C for 10 min, pH 3 (*42*), whereas no activity was lost at 65 °C for 10 min, pH 10.5 (*53*). Royal palm tree peroxidase kept full activity after heating for 1 h at 70 °C (*23*). An unusual thermally stable soybean peroxidase lost 50% activity after heating at 85 °C for 2.5 h (*54*).

BP Kinetic Studies. Double-reciprocal plots according to eq 2, measured at pH 4.6, are shown in **Figure 5A**, where the parallel lines indicate that broccoli peroxidase follows a two-substrate ping-pong mechanisms as shown by Childs and Bardsley (*16*) for horseradish peroxidase. The slopes (eq 3) and *y*-intercepts (eq 4) from **Figure 5A** were determined, and the calculated intercepts were plotted against $1/A_0$. A plot of *y*-intercept against $1/A_0$ gave a straight line (**Figure 5B**). From the intercept of this figure the V_{max} value was calculated according to eq 5, to give a value of 130 μM ABTS consumed min^{-1} (mg of BP)$^{-1}$. From eq 3 K_m for ABTS was 0.2 mM, similar to that obtained for two Brussels sprouts peroxidase isozymes (0.2 mM) (*9*), about 50 times lower than that reported for marula fruit peroxidase (9.56 mM) (*55*) and 20 times lower than that reported for acidic HRP (A2, 4 mM) (*56*). This K_m value was lower than those reported for radish peroxidase (4.78 mM) (*57*) and anionic melon peroxidase (0.25 mM) (*58*). On the other hand, BP K_m was 12 times higher than that for

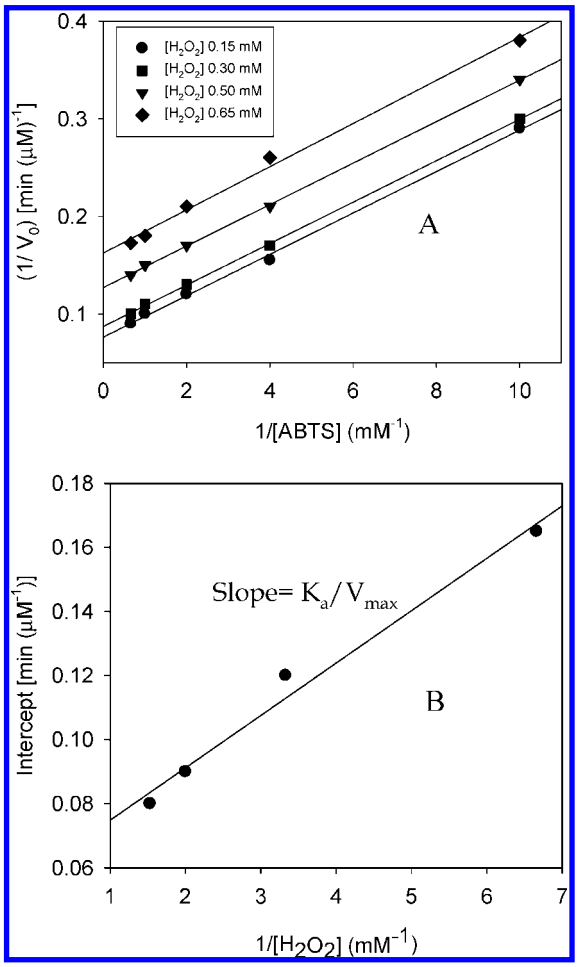

Figure 5. Kinetic behavior of the two-substrate reactions for purified BP (3 nM): (**A**) plot of the substrate−velocity relationship, according to eq 2; (**B**) plot of the *y*-intercepts of the lines of (**A**) versus ($1/H_2O_2$), according to eq 4. Means of triplicates, with standard deviation within 5% of the mean, are shown.

buckwheat peroxidase (0.016 mM) (*59*) and twice the value for basic HRP (C, 0.1 mM) (*56*). The K_m for H_2O_2 (78 μM) was similar to that reported for turnip neutral peroxidase (70 μM) (*8*), lower than those for marula fruit (1.77 mM) (*55*) and turnip peroxidase isoenzyme A1 (0.2 mM) (*27*), and higher than the values of 11.4 and 6.2 μM reported for Brussels sprouts peroxidase isozymes A1 and A2 (*9*). These studies used the same substrates (ABTS and hydrogen peroxide) and similar chemical concentration and therefore may be compared. The K_m values are important when peroxidase is used as a reporter enzyme. Thus, due to the relatively low K_m value for ABTS and H_2O_2, peroxidase obtained from largely available broccoli processing wastes could be suitable for applications requiring low ABTS and H_2O_2 thresholds.

To evaluate efficiency of peroxidases (which follow a two-substrate ping-pong mechanism) it is better to determine the constant of the rate-limiting step (k_3), here evaluated as k_{app}. From the experiments used to calculate the kinetic mechanism, the optimal conditions were obtained using 0.1 mM ABTS, which was used to calculate k_{app} from eq 7, giving 3.4×10^6 M^{-1} s^{-1}. This value is about 1 order of magnitude lower than that reported for leaves of royal palm tree peroxidase, one of the most active plant peroxidases reported to date. The k_{app} value was similar to that of sweet potato (*60*) and similar to those of horseradish, tobacco, and alfalfa peroxidases, whereas it was 10 times higher than that of soybean and peanut peroxidases

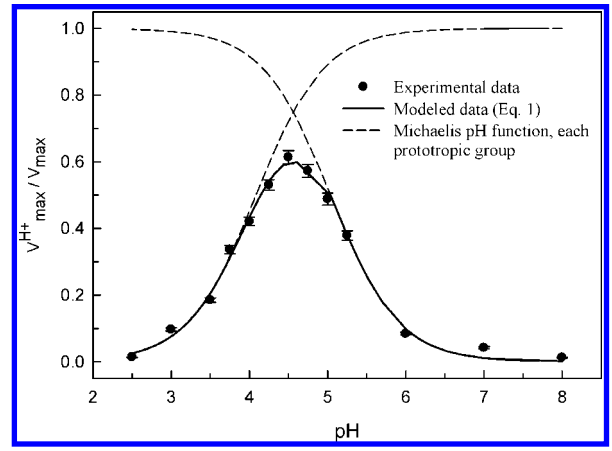

Figure 6. Optimum pH of purified BP (35 μg of protein), using ABTS as substrate. Buffers: 10 mM citrate, pH 2.5–6; 10 mM phosphate, pH 7.0; and 10 mM Tris-HCl, pH 8.0. A constant ionic strength of 430 mM, adjusted with KCl, was used. Means of triplicates, with error bars indicating standard deviation, are shown.

(*23*). There are few papers on k_{app} of peroxidases, and from the available literature it can be concluded that only one peroxidase showed a catalytic efficiency higher than that of BP.

Substrate Specificity. BP showed slight activity over substrates normally involved in lignin biosynthesis, such as caffeic and ferulic acids (**Table 1**). Some authors have stated that peroxidase has an important role in this process; however, the specific function of peroxidase remains unclear. BP showed important ascorbate peroxidase activity because an oxidizing effect was produced over ascorbate. BP also displayed a high oxidizing capacity of *o*-diphenols, such as catechol, in the presence of H_2O_2, which may be important for possible control of browning reactions.

Optimum pH and pH Stability of Purified BP. The optimum pH for any peroxidase depends on the hydrogen donor and buffer solutions used in the activity assay. Using ABTS as H donor, BP showed high activity in a narrow pH range (**Figure 6**). From eq 1, the calculated parameters for BP were $K_1 = 8.318 \times 10^{-5}$ M, $K_2 = 9.120 \times 10^{-6}$ M, and $V_{max} = 230$ μM ABTS consumed min^{-1} (mg of BP)$^{-1}$. The data fitted eq 1 with a correlation coefficient of 0.997, and the real V_{max} was calculated to be about 1.63 times the apparent V_{max}. The broken lines of **Figure 6** reaching V_{max} were calculated from the Michaelis pH function (eq 1), taking each prototropic group separate from the other.

From the mean value of the two p*K* values, the optimum pH was 4.6. Peroxidases purified from various sources have their optimum pH mostly in the region of 4.5–6.5. The pH optimum for turnip peroxidases ranged from 4.5 to 5.5 (*20*); that for Brussels sprouts was 4.5 (*9*), and that for strawberry was 6.0 (*61*).

BP remained stable over a wide pH range, between pH 4 and 9, where it retained ≥70% of its original activity (results not shown). However, as the pH was lowered below 3.8, a sharp decline in stability occurred, and at pH 2.5 the enzyme lost all of its activity. This loss has been attributed to the dissociation of the heme prosthetic group from the polypeptide chain (*62*).

Purification Using Reverse Micelles. Chromatographic separation of proteins, which can be applied to peroxidase purification, involves processing costs that can dramatically increase depending on the number of purification stages and type of chromatography (*63*). In addition, chromatographic procedures are limited in many cases by the scale of operation

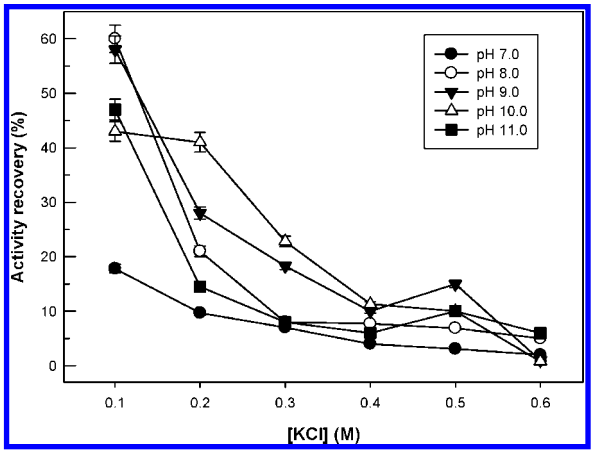

Figure 7. Effect of pH and ionic strength (KCl concentration) on reverse micellar solubilization of broccoli peroxidase (0.5 mg/mL). Means of three replicates are shown.

and the cost of resins. It seems that liquid–liquid extraction processes being potentially scalable to large-scale biotechnological separations show one of the most economically feasible alternatives for plant peroxidase purification. One purification scheme is reverse micellar extraction, which is selective, with high recovery and activity yields, and can be adequately modeled.

Preliminary studies using the anionic surfactant AOT resulted in a low broccoli peroxidase solubilization, and therefore the amount of re-extracted protein was small. Using CTAB as cationic surfactant a cosurfactant is needed for complete dissolution, and we tested butanol, *n*-pentanol, hexanol, and isooctanol. The combination producing the highest protein extraction was isooctane/*n*-pentanol 9:1 (v/v). The size of reverse micelles may be affected by the type and concentration of aqueous phase anions, such as KBr, KF, and KCl. Protein solubilization was poor when KBr and KF were used as anions at all concentrations tested. When broccoli peroxidase was solubilized at pH 8, using 0.1 M KCl in the aqueous phase, after re-extraction, the specific activity increased from 6 to 39 units/mg, the activity yield was 60%, and the purification factor was 7. This compares favorably with the specific activity of 4.19 units/mg and purification factor of 1.6 (**Table 2**), obtained using acetone precipitation. The effect of aqueous phase pH and IS during protein solubilization is shown in **Figure 7**, where the best activity recovery was obtained by using pH 8–9 and 0.1 M IS. A purification scheme similar to that shown in **Table 2** was conducted for reverse micellar prepurified BP, and the final specific activity was 1230 units/mg of protein, a purification fold of 480 and an activity yield of 3.0. These values are better than those obtained using acetone precipitation, with the advantage of recycling of the reverse micellar solution up to three times. Thus, reverse micellar liquid–liquid extraction may be used for the large-scale prepurification of broccoli peroxidase. Furthermore, the huge amounts of wastes produced by broccoli processing industries and the relatively easy purification procedure make BP an ideal enzyme for many industrial and clinical applications, given its pH stability and relatively high ABTS catalytic efficiency. BP peroxidase was freeze-dried with insignificant activity losses, and the dry enzyme has been stored under refrigeration, keeping essentially all activity for up to two years.

Due to the importance and availability of broccoli stems as byproduct, other purification schemes have been developed by our group, such as immunoaffinity chromatographic purification, by which a broccoli isoenzyme was purified to homogeneity following this scheme (*64*). Regarding applications of peroxidase, studies are being conducted on phenolic compound removal from model aqueous systems using immobilized and chemically modified peroxidase for catalyst recycle and to improve its thermal stability.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); AEC, anion exchange chromatography; BP, purified broccoli waste peroxidase; CD, circular dichroism; CTAB, cetyltrimethylammonium bromide; HRP, horseradish peroxidase; IS, ionic strength; RZ, ratio of A_{403}/A_{280} nm; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; T_m, midpoint inactivation temperature.

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