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Purification and Partial Characterization of Broccoli (*Brassica oleracea* Var. *Italica*) Peroxidases

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Three peroxidase (POD) isoenzymes were purified from a soluble extract of broccoli stems. The acidic and neutral PODs were purified to homogeneity by using ion exchange and hydrophobic chromatography. The basic POD was purified by cation exchange and gel filtration chromatography. The neutral and basic PODs had molecular masses of ~43 kDa, and the acidic POD had a molecular mass of 48 kDa by SDS-PAGE. p*I* was approximately 4, 5, and 8 for acidic, neutral, and basic PODs, respectively. Optimum activity using guaiacol as the H donor was obtained at pH ~6 for both neutral and basic PODs and at pH ~4 for acidic POD. All three of the purified isoenzymes are glycosylated. Reaction rates with various substrates including guaiacol, guaiacol/MBTH, DMAB/MBTH, and ferulic acid/MBTH were different among the isoenzymes. K_m and amino acid composition were also determined.

KEYWORDS: Broccoli; peroxidases; purification

1. INTRODUCTION

Peroxidase (POD) is an oxidoreductase that catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms (*1*−*3*). Higher plants contain ferriprotoporphyrin peroxidases, which are one of the three major classes of peroxidases. The ferriprotoporphyrin peroxidases contain ferriprotoporphyrin IX (hematin or heme) as a prosthetic group (*1*−*3*). Heme is attached to the protein portion by an amino acid side chain bound to the fifth coordination position of the iron.

The enzyme is reported to exist in both soluble and membrane-bound forms (*4*). The enzyme can be found in vacuoles, tonoplast, plasmalemma, and inside and outside the cell wall and has a variety of functions. It is involved in plant hormone regulation (*5*), defense mechanisms (*6*), indoleacetic acid degradation during maturation and senescence of fruits and vegetables (*7*), and lignin biosynthesis (*8*). Because of its multiple functions, the enzyme is commonly found as several isoenzymes in plants.

POD is found in many plant-based foods. The enzyme is highly specific to its peroxide substrate, of which H_2O_2 is the most common, but it has low specificity toward its hydrogen donor substrate (*1*). In the presence of peroxide, PODs from plant tissues are able to oxidize a wide range of phenolic compounds, such as guaiacol, pyrogallol, chlorogenic acid, catechin, and catechol (*9*). Oxidation of a wide range of organic compounds has led to the speculation that the enzyme may be associated with losses in color, flavor, and nutritional value of raw and processed foods (*10*−*13*). The enzyme is also of concern to food processors because of its high thermostability. POD is commonly used as an index of the adequacy of fruit and vegetable blanching due to its high concentration in most plant tissues, its high thermal stability, and its ease of assay (*14*, *15*). Guaiacol is a common hydrogen donor substrate traditionally used to check the adequacy of the thermal treatment.

PODs from several plants have been purified and studied. These included, for example, oil palm leaf (*16*), sweet potato tubers (*17*), turnip (*18*, *19*), melon (*20*), Brussels sprouts (*21*, *22*), cabbage (*23*), barley (*24*), okra (*25*), oranges (*26*), tea leaves (*27*), pepper fruits (*28*), carrot roots (*29*), tobacco (*30*), wheat germ (*31*), mango (*32*), green pea (*33*, *34*), papaya fruit (*35*), spinach (*36*), Cox's apple pulp (*37*), rice (*38*), cotton (*39*), peanut (*40*), tomato (*41*, *42*), green asparagus (*43*), and strawberry (*44*). In all cases, multiple isoenzymes have been reported. Isoenzymes purified from these various plant sources differ with respect to molecular mass, thermal stability, pH optimum, substrate specificity, and physiological role.

Heat treatment is commonly used to inactivate enzymes. However, it is well-known that POD can recover its activity after heat treatment (*14*, *45*). Many studies have revealed that residual or reactivated POD can cause significant deterioration in the quality of various high-temperature−short-time-processed foods (*1*, *3*, *14*, *45*).

Broccoli is among the vegetables with the highest POD activity, compared to other rich sources such as horseradish (*46*), and it is an economically important vegetable for the food industry. This study describes the purification procedure and

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initial characterization of some properties of three POD isoenzymes from broccoli, the acidic, neutral, and basic forms of POD.

The purified isoenzymes will be a useful component for future investigation of the detailed mechanisms involved in the heat inactivation and reactivation of peroxidase.

2. MATERIALS AND METHODS

2.1. Materials. Fresh broccoli (*Brassica oleracea* var. *Italica*) was obtained from a local market and washed with distilled water. Broccoli stems and florets were separated. Only the stems were used for peroxidase purification due to their relatively higher activity of peroxidase as compared to the floret (*46*). Fresh prepared samples were frozen and stored at −20 °C until used.

2.2. Crude Extract. Broccoli stems were removed from frozen storage and homogenized at 4 °C using phosphate buffer, pH 7.0, in a ratio of 1:2 (milligrams of broccoli per milliliter of buffer). The extract was centrifuged, and the supernatant was used for further purification.

2.3. Protein Precipitation. Precipitation of protein was carried out using ammonium sulfate, first with 50% saturation and centrifugation, and then the saturation level was increased to 90% followed by centrifugation. The precipitate from the 90% ammonium sulfate solution was redissolved in 0.05 M Tris-HCl, pH 7.8, dialyzed overnight against the same buffer, and used in the purification steps.

2.4. Anion Exchange Chromatography. A 2.5 × 50 cm Bio-Rad column packed to a height of 45 cm with DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO, capacity of 100−140 μequiv/mL gel volume) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.8. Broccoli extract was loaded onto the column and washed with the equilibrating buffer using a 86 mL/h flow rate. The retained protein was eluted at the same flow rate using a linear 1 L gradient of 0.0−0.5 M NaCl in the above buffer. Fractions of 6.5 mL were collected, the absorbance was read at 280 nm, and POD activity was measured.

All chromatographic steps were performed at temperatures of 4−5 °C.

2.5. Cation Exchange Chromatography. Fractions from the DEAE-Sephacel column eluted during washing with equilibrating buffer that showed POD activity were combined. These fractions were concentrated by ultrafiltration using Millipore stirred ultrafiltration cells with 10 kDa molecular weight cutoff membranes (Millipore) and dialyzed against 0.04 M sodium phosphate buffer, pH 6.0. The sample was loaded into a 2.5 × 50 cm Bio-Rad column packed to 45 cm height with SP-Sepharose (Sigma Chemical Co., capacity of 180−250 μequiv/mL gel volume), which was previously equilibrated with 0.04 M phosphate buffer, pH 6.0. The sample was then washed with the equilibrating buffer using a 49 mL/h flow rate. The retained protein was eluted at the same flow rate using a linear 1 L gradient of 0.0−0.5 M NaCl in the above buffer. Fractions of 6.5 mL were collected.

2.6. Gel Filtration Chromatography. Pooled fractions from both the DEAE-Sephacel and SP-Sepharose columns that were eluted using a linear salt gradient and showed POD activity were concentrated by ultrafiltration. These samples were called acidic, neutral, and basic fractions, respectively. Each sample was loaded onto a 2.5 × 50 cm column packed with Sephadex G-100 and equilibrated with 0.1 M sodium phosphate, pH 6.0. Elution of the protein was carried out at 22 mL/h flow rate with the equilibrating buffer. Fractions of 4.6 mL were collected.

2.7. Hydrophobic Chromatography. Fractions from the gel filtration chromatography column showing POD activity were combined, concentrated, and dialyzed against 1.7 M $(NH_4)_2SO_4$ in phosphate buffer, pH 6.0. The dialyzed samples were loaded onto a 2.5 × 20 cm column packed with Phenyl-Sepharose CL-4B and previously equilibrated with the dialyzed buffer. The loaded sample was washed with the equilibrating buffer using a 32 mL/h flow rate. The retained protein was eluted at the same rate by decreasing the ammonium sulfate concentration in a linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ in phosphate buffer, pH 6.0. Fractions of 4.6 mL were collected. Fractions showing POD activity were combined and dialyzed against distilled water and freeze-dried.

2.8. Protein and Peroxidase Activity Determination. Protein was quantified by using the dye-binding method of Bradford with bovine serum albumin (BSA) as a standard. During the purification process, protein was measured by absorbance at 280 nm.

POD activity was determined by monitoring the time course of the change in absorbance at 420 nm upon oxidation of the substrate catalyzed by the enzyme. Guaiacol (Sigma Chemical Co.) was used as substrate. The final reaction mixture contained 50 mM guaiacol, 50 μL of enzyme, 10 mM H_2O_2, and 50 mM Tris−acetate buffer, pH 6.0, in a volume of 1.5 mL (*14*). The assay was performed at 25 °C using a UV−vis scanning spectrophotometer (UV-2101PC Shimadzu) connected to a temperature controller. The absorbance increase at 420 nm was monitored for up to 3 min with the slope of the linear portion of the curve used to determine activity. Enzyme activity was calculated using an extinction coefficient of 25.5 mM^{-1} cm^{-1} for tetraguaiacol. One unit of enzyme activity was defined as the amount of guaiacol consumed in 1 min.

2.9. Enzyme Characterization. *2.9.1. SDS-PAGE.* Purity and molecular weight of the different enzyme fractions were analyzed using SDS-PAGE under reducing conditions. This was conducted using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gels were prepared according to the method of Bollag et al. (*68*) with some modifications. The stacking gel had 4% T and 2.6% C, whereas the separating gel had 12% T and 2.6% C. %T refers to the total acrylamide content (w/v), whereas %C is the ratio of cross-linking reagent (bisacrylamide) to acrylamide monomer (w/w). Runs were performed at constant current (10 mA/plate). The following molecular weight markers used for electrophoresis were obtained from Bio-Rad: myosine (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5kDa). The Coomassie blue staining technique was used.

2.9.2. Isoelectric Focusing (IEF). IEF for neutral POD was conducted in a Mini-Protean II Cell (Bio-Rad Laboratories) using precast IEF gel 2.6% C with pH 3−10 and a 10-well comb (Bio-Rad Laboratories). Sample was prepared by mixing with 2× sample buffer containing 60% glycerol and 4% ampholytes, pH 3.5−10. IEF for basic POD was performed in a vertical electrophoresis system 10 × 10 cm model FB-VE10-1 (Fisher Scientific, Fair Lawn, NJ). Denaturing IEF gel was composed of 5% T and 3.3% C acrylamide gel, 12.9 M urea (Bio-Rad), 3.7% ampholyte solution, pH 3.5−10 (Bio-Rad Laboratories), 0.32% of 10% ammonium persulfate, and 0.26% TEMED. Sample buffer contained 8 M urea, 4.7% ampholyte solution, pH 3.5−10, 19.45% of 20% Triton X-100, 1.9% 2-mercaptoethanol, and 7.8% 1% bromophenol blue.

The lower chamber contained 10 mM phosphoric acid, and the upper chamber contained 20 mM sodium hydroxide. Samples were loaded into the sample wells and run for 30 min at 150 V (constant voltage) followed by 200 V for 2.5 h (constant voltage).

After running, gels were treated with 10% trichloroacetic acid (TCA) for 10 min and in 1% TCA overnight. After the gel had been rinsed twice with deionized water, it was silver stained (silver stain kit, Sigma Chemical Co.). The pH gradient was determined along the length of the gel by cutting a strip of gel into 1 cm slices. Before fixing with TCA, each slice was suspended in 1 mL of 10 mM KCl for ∼30 min and the pH determined from KCl solutions.

2.9.3. Peroxidase Assay Using Other Substrates. The activity of broccoli and horseradish PODs on various substrates was calculated by following the change in absorption maxima. Substrates used included mixtures of 0.25 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 20 mM guaiacol, 0.07 mM MBTH and 3.3 mM 3-(dimethylamino)-benzoic acid (DMAB), and 20 mM ferulic acid and 0.25 mM MBTH. The H_2O_2 concentration for all assays was 10 mM. All substrates were prepared in 0.1 M Tris−acetate buffer, pH 6.0. Activity was measured from the change in absorbance at 500 nm for MBTH/guaiacol, at 590 nm for MBTH/DMAB, and at 500 nm for ferulic acid/MBTH.

2.9.4. Optimum pH for Activity. Peroxidase activity was analyzed in the range of pH 3−9 using the following buffers: 0.05 M citrate buffer, pH 3−6, 0.05 M phosphate buffer, pH 7, 0.05 M Tris-HCl buffer, pH 8, and 0.05 M borate buffer, pH 9. The activity assay

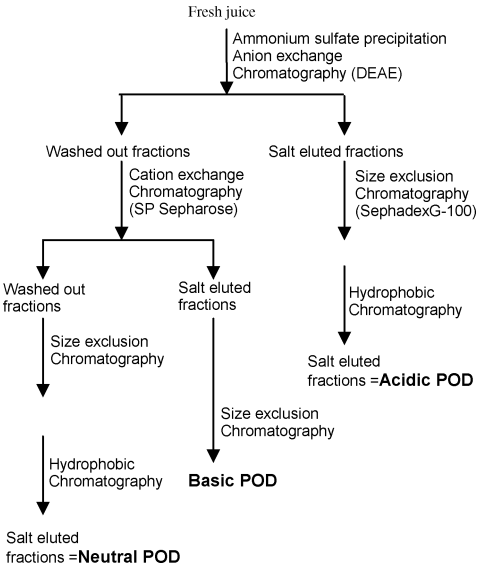

Figure 1. Diagram for the purification of acidic, neutral, and basic PODs from broccoli.

included H_2O_2 (10 mM), 20 mM guaiacol, and 50 μL purified fractions of enzyme. Ionic strength was kept constant at 0.27 M by adjustment with NaCl.

2.9.5. Identification of POD as a Glycoprotein. The glycosylated nature of the peroxidase isoenzyme was evaluated by staining after SDS-PAGE, using a glycoprotein detection kit (Sigma Chemical Co.). This test is based on the oxidation of the oligosaccharides by periodic acid and staining with Schiff reagent. The aldehyde groups (e.g., from 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 (*48*).

2.9.6. K_m Determination and Kinetic Mechanism. K_m values were determined using the Lineweaver−Burk reciprocal plot graphic method for the two-substrate ping-pong mechanism followed by POD. Individual experiments for each H_2O_2 concentration were performed at final concentrations of guaiacol of 1, 2, 5, 10, and 15 mM for acidic, neutral, and basic PODs. The following H_2O_2 concentrations (final concentrations) were used: 0.1, 0.2, 0.5, 0.75, and 1 mM for acidic and neutral PODs and 0.2, 0.3, 0.4, 0.5, and 1 mM for basic POD. The reaction volume was 1 mL. The reciprocal plot of the initial rate and the substrate concentrate follow a ping-pong mechanism. A replot of *y*-intercepts versus reciprocal of guaiacol concentration will produce a strength line with a slope and intercept that can be used to calculate V_{max} (maximum rate) and K_m for guaiacol and H_2O_2. More detail on the calculation can be found in ref *19*.

2.9.7. Amino Acid Analysis. The amino acid analysis was performed by molecular structure facility at the University of California at Davis using an amino acid analyzer (Hitachi L-8800). The Hitachi L-8800 utilizes a sodium citrate buffer system and is optimized for hydrolyzed proteins/peptides. The analyzer uses ion exchange chromatography to separate amino acids followed by a postcolumn ninhydrin reaction detection system. Samples were first oxidized with performic acid, yielding the acid stable forms, cysteic acid and methionine sulfone, prior to the standard acid hydrolysis, which was employed by 6 N HCl for 24 h at 110 °C. After dried hydrolyzed samples had been dissolved in the dilution buffer, 50 μL of the sample was loaded into the analyzer.

3. RESULTS AND DISCUSSION

3.1. Purification of Acidic, Basic, and Neutral POD Isoenzymes. A summary of the purification procedure and specific information on the degree of purification obtained at each step appears in **Figure 1** and **Table 1**. Ammonium sulfate precipitation helped to improve peroxidase purification and concentrate the crude extract. The specific activity and purification-fold following ammonium sulfate treatment were twice those of the crude extract.

Table 1. Summary of the Purification of Acidic, Neutral, and Basic PODs

sample	total protein (mg)	total activity (units)	specific activity (units/mg)	fold	recovery (%)
fresh juice	47.5	240	5.1	1	100
$(NH_4)_2SO_4$ precipitation	10.1	83.2	8.3	1.6	34.7
neutral POD	0.05	23.6	472	92.5	9.8
acidic POD	0.05	48.2	882	173	20.1
basic POD	0.04	6.2	156	30.6	2.6

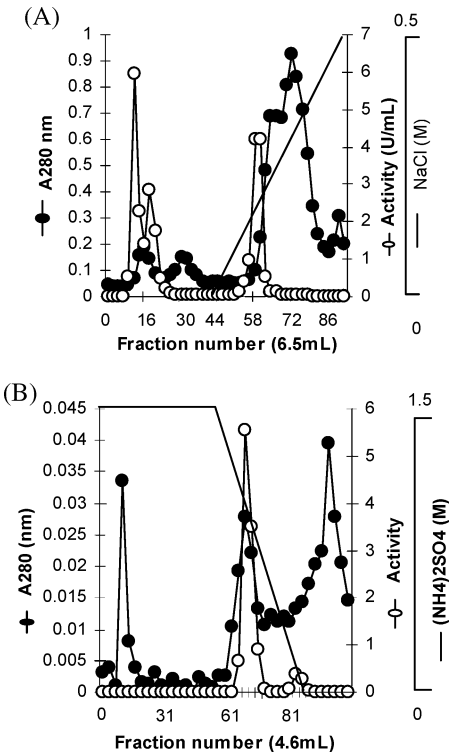

Figure 2. (**A**) Anion exchange chromatography (DEAE-Sephacel) of broccoli POD (Tris-HCl 0.05 M buffer, pH 7.8; the same buffer with added 0.5 M NaCl was used for 1 L gradient elution). (**B**) Hydrophobic interaction chromatography of pooled acidic POD fractions using Phenyl-Sepharose CL-4B (phosphate buffer, pH 6.0, with 1.7 M ammonium sulfate; the same buffer without added salt was used for 500 mL gradient elution).

After anion exchange chromatography (AEC), peroxidase was distributed into two peaks, the first of which was eluted during the washing step and the second eluted with the salt gradient (**Figure 2A**). The specific activity of the nonretained pooled fractions was greater than that of the pooled fraction eluted by the gradient. The bound protein with activity fractions was eluted at ~0.1 M NaCl in phosphate buffer, pH 7.8. The use of AEC resulted in an increase of 2.6 times in both the specific activity and the purification-fold during the eluting step. This relatively small increase in specific activity may be associated with the large amount of absorbing materials eluted along with the enzyme.

The fractions eluted by the salt gradient were pooled, concentrated, and then further purified by gel filtration chromatography. Gel filtration chromatography separated out some contaminating materials and increased the specific activity from 42 to 82 units/mg. However, it was not sufficient to purify the enzyme to homogeneity, as indicated by the presence of more than one protein band on SDS-PAGE (data not shown).

Further purification was obtained by using hydrophobic interaction chromatography (HIC) (**Figure 2B**). This technique has been used with good success in the the purification of tomato POD and in the separation of lipoxygenase and peroxidases in soybean (*41*). The chromatogram obtained from the HIC column shows three protein peaks. One of these was eluted during the washing step. Some polyphenol compounds, free sugars or amino acids, or some proteins with low hydrophobicity absorbed light at 280 nm but were not bound to the hydrophobic resin and were therefore eluted during the washing step. The other two peaks were eluted by decreasing the ammonium sulfate concentration in a linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ in phosphate buffer, pH 6.0 (**Figure 2B**). Peroxidase was eluted in the first peak of the salt gradient elution [$\sim$1 M $(NH_4)_2SO_4$]. The other eluting component was more tightly bound to the hydrophobic resin and could be eluted only with buffer that did not contain ammonium sulfate. The pooled fractions of the activity peak gave a single band on SDS-PAGE and will be referred to as the acidic POD, which has a negative charge at pH $\sim$7.8, a specific activity of 1309 units/mg, a purification-fold of 173, and an RZ of 1.69. The RZ value is the absorbance ratio, A_{403}/A_{280}, and has been commonly used as an indication of purity. However, Shannon et al. (*47*) reported that this ratio for isoenzymes varies and is influenced by buffer and pH. The reported RZ of purified POD from several sources varies from 1 to 3.

Unretained fractions from the AEC column indicated that positive charges predominate at the surface of the enzyme, which prevented electrostatic interaction with the anion exchanger. Therefore, these fractions were pooled and further purified using a cation exchange chromatography (CEC) column. During the washing step, one protein peak with POD activity was eluted. Elution of the retained protein by a linear gradient of 0.0−0.5 M NaCl in phosphate buffer, pH 6.0, gave several overlapping protein peaks with one major and three minor POD activity peaks (**Figure 3A**). This elution profile suggested that these POD isoenzymes are basic enzymes that possess positive net surface charges, whice allowed them to be adsorbed onto a negatively charged surface on SP-Sepharose. Fractions with the highest activity as a result of the salt elution (e.g., fractions 170−180) were pooled and referred to as the basic POD, which was positively charged at pH $\sim$6. Basic PODs have been isolated from other plant sources. Similar elution characteristics were reported from PODs from soga palm when loaded onto CM-Toyopearl 650 M (*9*) and for POD from strawberry fruit eluted from CM-cellulose (*44*).

The basic fractions were concentrated using ultrafiltration and further purified using a gel filtration chromatography column. This column was able to separate out some contaminating proteins and resulted in an increase of 2 times in the specific activity. The resulting chromatogram is illustrated in **Figure 3B**. However, when we attempted to use the HIC column following gel filtration, it failed to further purify the pooled activity fractions. The linear gradient made the enzyme spread in a wide range of fractions and resulted in a significant dilution of the enzyme. The basic POD used in further studies had a specific activity of 174 units/mg, a purification-fold of 23, and an RZ of 0.55.

The unretained fractions from the CEC column were pooled and loaded onto an anion exchange column using the same conditions previously described. The fractions containing activity were again eluted during washing with the equilibrating buffer. This confirmed that these fractions were peroxidase that did not attach to either the AEC or CEC columns, and these fractions

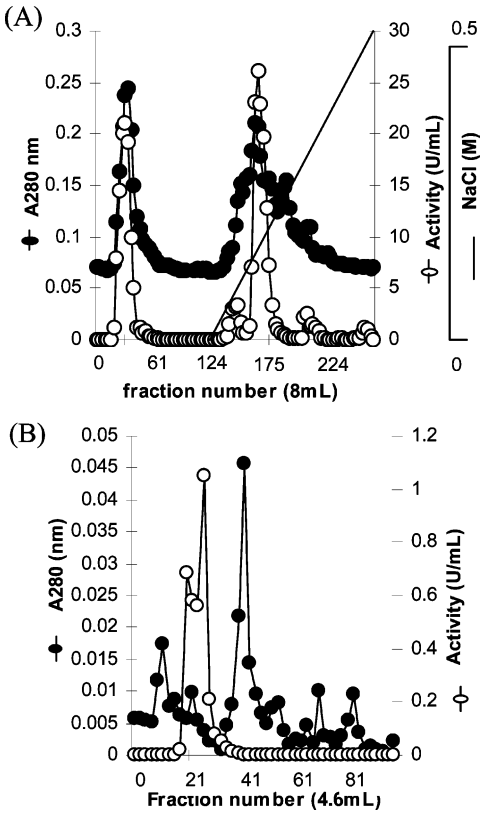

Figure 3. (**A**) Cation exchange chromatography (SP-Sepharose) of broccoli POD (0.04 M phosphate buffer, pH 6.0; the same buffer with added 0.5 M NaCl was used for gradient elution). (**B**) Gel filtration (Sephadex G-100) of salt-eluting fractions from cation exchange column (0.1 M phosphate, pH 6.0).

will be referred to as the neutral form. Further purification of the neutral fractions was performed by gel filtration and hydrophobic interaction chromatography. The chromatograms are shown in **Figure 4**. The elution profile from the HIC shows one POD activity peak, which has a specific activity of 486 units/mg, a purification-fold of 64, and an RZ of 2.15. The acidic, neutral, and basic fractions were dialyzed against distilled water, freeze-dried, and stored for future studies.

The chromatographic pattern of broccoli POD is similar to that of purified turnip POD. Turnip POD consists of acidic, basic, and neutral isoenzymes. After AEC, turnip POD activities distributed into two peaks: basic (unretained) and acidic (eluted with salt gradient) isoenzymes. The elution profile of unretained pooled fractions from AEC or CEC showed mainly three peaks having POD activity. One was unretained, and the other two were eluted with salt gradient (*19*, *48*). These results are similar to our own with broccoli POD.

Peroxidases are secretory proteins localized mainly in the plant cell walls, cytoplasm, and vacuole, depending on the nature of the cell and its development. Plant POD is present as multiple isoenzymes differing in molecular and catalytic properties (*49*). Due to the variety of reactions they catalyze in vitro and the large number of isoenzymes found, it has not yet been possible to assign an in vivo function for a particular isoenzyme (*16*). However, the basic PODs may be secreted into the vacuoles and the acidic ones into the cell wall and free intercellular spaces (*16*, *50*). Acidic POD was the major soluble POD found in broccoli stems in the present study, followed by neutral and basic POD. According to the literature, acidic POD isoenzymes are most commonly associated with the cell wall and may therefore be involved in lignifications. Specific isoenzymes of

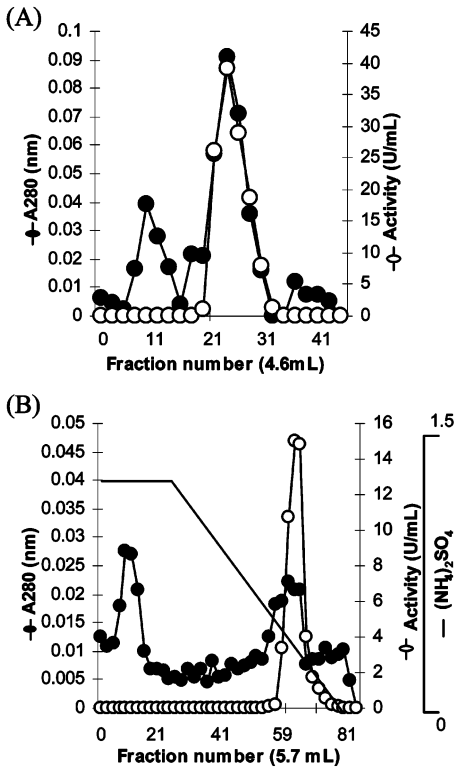

Figure 4. (**A**) Gel filtration chromatography (Sephadex G-100) of neutral broccoli POD (0.1 M phosphate, pH 6.0). (**B**) Hydrophic interaction chromatography of neutral broccoli POD using Phenyl-Sepharose CL-4B (phosphate buffer, pH 6.0, with 1.7 M ammonium sulfate; the same buffer without added salt was used for gradient elution).

PODs are believed to be responsible for the final enzymatic step in lignificaton (*16*). The acidic POD is probably released when the cell wall is broken during the extraction process. The neutral and basic isoenzymes may be present in vacuoles and participate in other physiological functions. Basic PODs have been found to be effective in indole-3-acetic acid catabolism and ethylene biosynthesis, both of which are plant hormones (*51*).

3.2. Molecular Weight and Purity. The molecular weight and purity of the three peroxidase isoenzymes were analyzed by SDS-PAGE. After Coomassie blue staining, a single band was detected for the acidic and neutral forms after elution from the HIC column (**Figure 5A**). The acidic and neutral forms had molecular masses of approximately 48 and 43 kDa, respectively. In contrast, the basic POD was not brought to homogeneity by the CEC and HIC steps. Indeed, SDS gel electrophoresis showed small amounts of contaminants even after gel filtration (**Figure 5B**). The most intense band on the SDS-PAGE represents the basic fraction with a molecular mass of ~43 kDa, which is very close to that of the neutral form.

Our results indicate that broccoli PODs have molecular weights similar to those reported for horseradish POD (40−46 kDa) (*52*), oil palm leaf POD (48 kDa) (*16*), rice (48 kDa) (*38*), cotton POD (48 kDa) (*39*), peanut POD (42 kDa) (*40*), and tomato POD (43 kDa) (*41*). Quite different molecular weights have been reported for PODs purified from, for example, green asparagus (34 kDa) (*43*) and basic strawberry PODs (58 and 65 kDa) (*44*). Molecular weights of PODs from various sources have been reported to range from 30 to 60 kDa, and the differences observed are attributed to post-translational modifications of the polypeptide chain including the number and composition of glycan chains present in plant PODs (*19*, *53*).

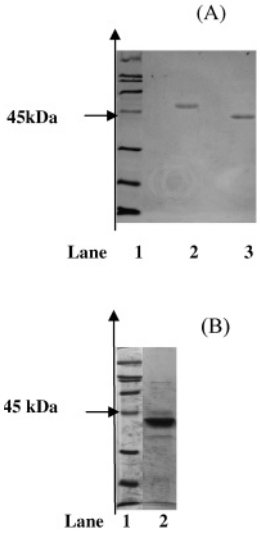

Figure 5. Gel electrophoresis (SDS-PAGE) of purified fractions having POD activity: (**A**) (lane 1) molecular weight standards, (lane 2) acidic POD from hydrophobic interaction chromatography, and (lane 3) neutral POD from hydrophobic interaction chromatography; (**B**) (lane 1) molecular weight standards and (lane 2) basic POD from gel filtration.

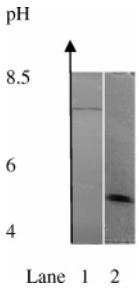

Figure 6. Isoelectric focusing electrophoresis of basic POD (lane 1) and neutral POD (lane 2).

3.3. Isoelectric Focusing. The purified broccoli PODs were submitted to IEF on polyacrylamide gels containing wide-range ampholytes (pH 3−10). Neutral POD appeared as a single band, which migrated to the anode of the gel. The isoelectric point value was ~5 according to the measured pH gradient along the gel (**Figure 6**). Basic POD migrated to the cathode, and the isoelectric point was ~8 (**Figure 6**). The acidic POD did not enter the focusing part of the gel, but it can be postulated that the enzyme migrated to the anode (opposite from the basic POD) and has a p*I* >4.

Peroxidases found in higher plants include basic, neutral, or acidic p*I*, and a single vegetable may contain several isoenzymes having wide range of p*I* values. Acidic peroxidases have been found in turnip roots, p*I* 3 (*48*); pepper fruits, p*I* 3.8 (*54*); the soluble fraction of potato tuber sprouts, p*I* 3 (*55*); and the salt extract of tomato, p*I* 3.5 (*56*). Basic peoxidases have been found in turnip roots, p*I* 8.5 (*48*), and the soluble fraction of potato tuber sprouts, p*I* 10 (*55*). The p*I* of neutral turnip POD has been found to be 7.2 (*19*).

3.4. Other Peroxidase Assays. A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions based on the oxidative coupling of MBTH and DMAB was reported by Ngo and Lehnoff (*57*). The mechanism of the reaction was also described by the same authors. Setti et al. (*58*) described a peroxidase method based on an increase in the absorbance at 502 nm due to the formation of a red azo compound resulting from the peroxidase-catalyzed oxidative coupling of MBTH and guaiacol. Here, we report HRP and

Table 2. Substrate Specificity of HRP and Purified Broccoli POD

substrate	change in absorbance/min/μg of enzyme			
	HRP	acidic POD	neutral POD	basic POD
guaiacol	38.9	3.87	10.6	18.4
guaiacol/MBTH	53.5	13.2	43.8	36.8
DMAB/MBTH	57.5	6.82	17.2	1.31
ferulic acid/MBTH	87.0	23.5	21.7	2.48

purified broccoli POD activities with several substrates, and the results are shown in **Table 2**. For all substrates used, the rate of product formation catalyzed by HRP was higher than that of broccoli POD. In all cases, the coupling reaction between MBTH and guaiacol improved the sensitivity of the POD assay compared with when guaiacol was used alone. Results agreed with the study with HRP by Setti et al. (*58*), in which the rate of red color formation due to the oxidative coupling of MBTH and guaiacol during the initial phase of the enzymatic reaction, having absorbance maxima around 502 nm, was higher than that of formation of the brown dimeric oxidative product in the absence of MBTH. Using DMAB/MBTH as substrates lowered the sensitivity of the assay for broccoli POD compared to the guaiacol/MBTH assay, but showed no difference for HRP. An oxidative coupling reaction of MBTH and DMAB improved the sensitivity of the assay more than using guaiacol alone, except for basic POD.

Although the highest activity of neutral and basic PODs was expressed toward guaiacol/MBTH, the highest activity of acidic POD and HRP was expressed toward ferulic acid/MBTH. Ferulic acid is a compound belonging to the lignin formation pathway. Acidic PODs in plants have been shown to participate in lignin biosynthesis (*59*). This is in agreement with our result that acidic POD (pI <3) was shown to react more with ferulic acid.

3.5. Determination of Glycosylation. All soluble peroxidases purified from broccoli stems showed pink bands on SDS-PAGE staining with the glycoprotein detecting kit. This characteristic of glycoproteins is similar to what has been reported for many PODs from higher plants, which have also been characterized as glycoprotein having oligosacharide chains linked to asparagine. Horseradish (HRP), turnip, Japanese radish, and oil palm leaf PODs have all been reported to contain ca. 18, 12−18, 20, and 37% carbohydrate bound to the protein moiety, respectively (*16*, *60*). However, there is still no particular glycan function reported for PODs. For cationic peanut peroxidase, studies have shown that the glycans of this enzyme may play a role in secretion. The loss of the secretion function was later explained as degradation of the protein within the cell when it is not glycosylated (*61*).

3.6. pH Optimum for Activity. Using guaiacol as the H donor, broccoli POD had maximum activity at approximately pH 4−5 for the acidic POD and pH 6 for both the neutral and basic PODs (**Figure 7**). The basic POD had a narrow pH optimum range, showing a specific maximum at pH 6. A rapid decrease in activity was found on either the basic or acidic side of this pH optimum, whereas the neutral POD isoenzyme showed a broader range of maximum activity (pH ~4.5−7) around its optimum. Likewise, the optimum pH for activity of the acidic POD was also fairly broad.

It is well-known that the release of the heme group from the active site of the enzyme is pH dependent, occurring more rapidly below pH 4 and leading to a loss of POD activity. Neutral and basic PODs are completely inactivated at pH 3, whereas <20% of the activity of acidic POD is left under such

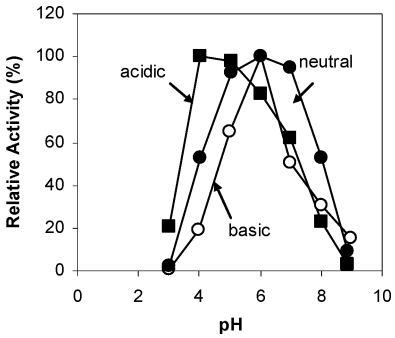

Figure 7. Effect of pH on activity of broccoli POD isoenzymes, with guaiacol as the H donor. The ordinate represents relative activity, which is the ratio of the activity to the maximum activity expressed as a percentage.

Table 3. K_m Values for Acidic, Neutral, and Basic Broccoli PODs

broccoli POD isoenzyme	*K*m (mM)	
	guaiacol	H_2O_2
acidic	0.305	0.042
neutral	0.711	0.128
basic	8.789	9.731

conditions. The activity of acidic POD decreases ~20% at the optimum activity of the neutral and basic PODs. Differences in pH optima suggested that POD isoenzymes may be synthesized by specific tissues in response to the cellular environment or availability of substrates. For tomatoes, for example, the tomato fruit provides an acidic environment and, of the 12 PODs found in the whole tomato plant, only 1 isoenzyme is found in the fruit (*62*).

PODs purified from various sources have been reported to have their pH optima mostly in the region of 4.5−6.5. The optimum pH for acidic turnip PODs was reported to be between 5 and 5.5 with ABTS as H donor (*48*). The POD isoenzyme from tomato juice was reported to have an optimum pH of 5.5 with guaiacol as H donor (*41*). The optimum pH for strawberry POD was found to be at pH 6 (*44*), and the optimum for POD from potato sprouts and tubers was 4−4.5 (*55*).

3.7. K_m Determination and Kinetic Mechanism. Lineweaver−Burk double-reciprocal plots for acidic, neutral, and basic PODs showed parallel lines, indicating that all POD isoenzymes follow ping-pong mechanisms. **Figure 8A** shows this plot for basic POD. The slopes and *y*-intercepts from **Figure 8A** were determined and the calculated *y*-intercept values were plotted against the reciprocal of H_2O_2 concentration, giving a straight line (**Figure 8B**). From the intercept of **Figure 8B**, V_{max} values for H_2O_2 can be determined (5.7 mM/min for acidic, 11.7 mM/min for neutral, and 438 mM/min). The K_m for H_2O_2 can be calculated from the slope. If V_{max} is known, the K_m for guaiacol can be calculated using the slope of **Figure 8A**. The K_m values for the acidic and neutral PODs were calculated in the same way. A summary of K_m values for the three broccoli PODs is given in **Table 3**.

K_m is a constant for a given enzyme; its numerical value provides a means of comparing different enzymes. Clearly, K_m values for both substrates shown in **Table 3** differ among the isoenzymes, with the K_m of the basic POD being the highest, followed by those of neutral and acidic PODs. The low K_m value for acidic POD suggests that among the three isoenzymes isolated, this enzyme has the highest apparent affinity toward guaiacol and H_2O_2. The various values of K_m may suggest that

Table 4. Amino Acid Composition Comparison of Peroxidase Isoenzymes Purified from Broccoli with Other Plant Peroxidases

	mol %			no. of residues					
amino acid[a]	acidic POD	neutral POD	basic POD	acidic POD[b]	neutral POD[b]	basic POD[b]	tobacco POD (anionic)	potato POD (anionic)	horseradish POD (cationic)
Ser	8.79	12.53	11.28	32	40	36	21	22	18
Asx	16.69	13.21	11.03	60	43	35	48	48	43
Glx	8.78	7.82	4.30	31	25	14	22	25	16
Gly	10.17	9.07	10.75	37	29	35	30	27	13
Thr	8.30	10.42	7.82	30	34	25	29	23	20
Ala	9.69	12.03	10.46	35	39	34	22	30	19
Met	nd[c]	1.26	0.31	nd	4	1	4	4	2
Val	6.46	6.04	8.07	23	19	26	18	24	14
Arg	2.95	2.52	4.33	10	8	14	13	10	18
Ile	5.37	4.58	5.27	19	14	17	17	14	11
Leu	12.48	9.32	7.63	45	30	24	25	28	30
Lys	3.09	3.62	8.16	11	11	26	8	11	5
His	1.28	1.30	2.30	4	4	7	3	4	2
Tyr	0.80	1.85	2.64	2	6	8	3	6	4
total residues				339	306	302	302	315	251

[a] Cys and Trp were not determined. [b] Calculated using M_r of 48000 kDa for acidic POD and 43000 kDa for neutral and basic POD with 15% carbohydrate. Tobacco, potato, and horseradish POD data are from Mellon (*66*). [c] Not detectable.

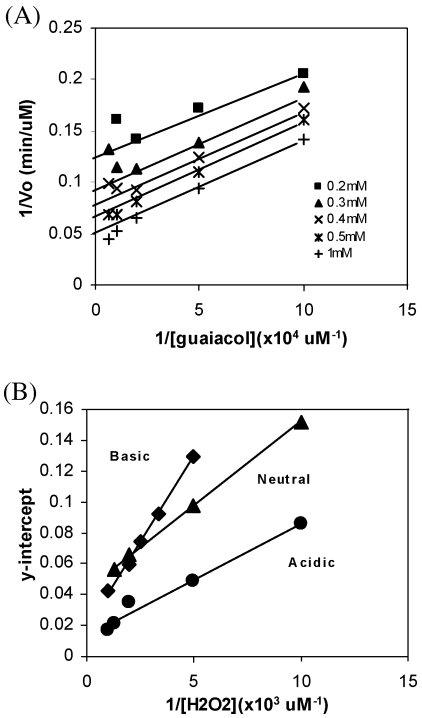

Figure 8. Kinetic behavior of the two-substrate reactions for broccoli POD: (**A**) plot of the substrate–velocity relationship of basic POD; (**B**) plot of the *y*-intercepts of the lines of (**A**) versus $1/H_2O_2$ for basic POD. Lines for acidic and neutral were obtained similarly to the basic POD.

these isoenzymes function differently in plant tissues or are located in different parts of the tissue.

The guaiacol K_m values for neutral and acidic PODs were lower than those found for guaiacol oxidation by peroxidases from turnip (3.7 mM) (*19*), Korean radish roots (6.7−13.8 mM) (*63*), green peas (10.2 mM) (*64*), and tomato (5−10 mM) (*65*). However, the K_m of basic POD falls within these ranges. The affinity of interaction with H_2O_2 reported from other plants also varied, depending on the individual enzyme.

3.8. Amino Acid Analysis. A comparison of amino acid composition of POD isoenzymes from broccoli and other plant PODs is presented in **Table 4**. Higher levels of serine were found in broccoli POD than in PODs from other sources. Acidic POD contained a higher level of charged residues (Asx, Glx) than neutral and basic PODs and those of other plant PODs. The basic POD and HRPc contained lower levels of Asx than acidic and neutral POD. The number of histidine residues (three to four) appeared to be conserved within the acidic and neutral PODs. A similar observation was reported by Mellon (*66*) and Sanchez-Romero et al. (*67*). At least one His is presumed to function as a ligand for the iron in the heme cofactor. The histidine residue of basic POD was unusually high compared to other peroxidases, probably due to a minor level of contaminating proteins. In addition, the amount of His may vary within the basic POD family. When hydrophobic residue numbers for Ile and Leu are summed, it appears that acidic POD contained a higher level of hydrophobic residues than those of other peroxidases, which seemed to be at a similar level. Whereas acidic and basic broccoli PODs showed fairly distinct amino acid residue patterns, a high degree of similarity was observed between that of neutral broccoli POD and acidic potato POD.

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