Partial Purification of Latent Polyphenol Oxidase from Peach (*Prunus persica* L. Cv. Catherina). Molecular Properties and Kinetic Characterization of Soluble and Membrane-Bound Forms

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This paper analyzes the kinetic and structural characteristics of polyphenol oxidase (PPO) from peach cv. Catherina. The PPO was obtained in a latent state in both the soluble and membrane-bound forms, and both forms were activated by acid shock and the detergent SDS. Plant defense is the main function assigned to PPO, which would be activated by the acid environment resulting from tissue damage. On the other hand, it has been suggested that, physiologically, the role played by SDS may be fulfilled by lipids. Native isoelectric focusing identified two acid isoforms of p*I* 5.7 and 5.8 for the soluble form and one isoform with p*I* 5.7 for the membrane-bound form. A partially denaturing SDS-PAGE revealed two very close bands of activity in both cases, but the Western blot performed on a totally denaturing SDS-PAGE, using polyclonal antibodies against bean PPO, revealed a single band in the membrane-bound fraction with a molecular mass of 60 kDa.

INTRODUCTION

Peach (*Prunus persica* L. cv. Catherina), a member of the Rosaceae family, was first cultivated in China and revered as a symbol of longevity. According to FAO data, the main peach-producing countries of Europe are Italy ($\cong$1,700,000 tons/year), and Spain ($\cong$1,050,000 tons/year), whereas the leading producer in the world is China ($\cong$4,200,000 tons/year). Murcia is one of the most important peach-growing areas of Spain. There are a large number of varieties of peach, and in only the past decade more than 500 new varieties have reached the market (*1*). Among the most appreciated by consumers cv. Catherina has an excellent aroma and a fresh, juicy pulp. It is used both for fresh consumption and as a canned food.

The natural and health-promoting phenolic compounds present in peaches (*2–4*) can be oxidized by endogenous enzymatic activities, such as polyphenol oxidase (PPO) (*5–7*). Polyphenol oxidase, also known as tyrosinase (monophenol, *o*-diphenol: oxygen oxidoreductase EC 1.14.18.1), is a copper-containing enzyme that catalyzes two different reactions, using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) (*8–10*) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (*11–15*). PPO is widely distributed in plants. It is a very important enzyme in

the food industry because during the processing of fruits and vegetables any wounding may cause cell disruption and lead to the formation of quinones, and their interaction with amino acids and proteins will enhance the brown color produced (*16*). Not only may the appearance of food and beverages be affected but also the taste and nutritional value, often decreasing the quality of the final product (*17*) with considerable economic and nutritional loss. In Catherina peach, the oxidation of catechin by PPO has been confirmed (*5*).

One unusual and intriguing characteristic of the enzyme is its ability to exist in an inactive or latent state. Some authors have reported that the degree of latency is maintained throughout the purification procedure (*18*). In other cases it has been found that the enzyme is spontaneously activated as the degree of purification increases (*19*). On the other hand, it has also been observed that enzyme latency increases as the purity increases, and this provokes changes in the kinetic behavior of the enzyme, which shows hysteresis and positive cooperativity when purified (*11*). Latent PPO has been activated by a variety of treatments using agents such as proteases (*20, 21*), fatty acids (*19*), acid or basic shock (*22*), and SDS (*11–14, 23, 24*). The use of SDS as an activating agent is particularly interesting because PPO is active at high SDS concentrations (1 mM) (*23*), which would denature or inactivate many other enzymes. Moreover, physiologically, it has been suggested that lipids might fulfill this role and be the physiological counterpart of the SDS (*24*). On

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the other hand, SDS activation of tyrosinase has been demonstrated in vivo with *Terfezia claveryi* sections (*25*).

A wide variety of plant PPOs have been described and reviewed, and the overall results indicate a high heterogeneity in PPO biochemical characteristics (K_m, optimum pH, and latency), number of enzymatic forms, and molecular masses (*26*), even within one family, such as rosaceas (*27*). No study on the molecular and kinetics properties of Catherina peach PPO has been performed. Nevertheless, it is important to carry out such studies because it has been shown that this enzyme can oxidize natural phenolics from peaches (*5*). Because of the considerable economic and nutritional loss induced by enzymatic browning in the commercial production of fruits, any information on the molecular and catalytic properties of PPO is of great importance for regulation of tissue browning. Thus, the purpose of this paper is to analyze both the molecular and kinetic properties of soluble and membrane-bound latent PPO extracted and partially purified from peach.

MATERIALS AND METHODS

Plant Material. The peaches (*Prunus persica* L. cv. Catherina), grown in Cieza (FRUTAS ESTER, Murcia, Spain), were harvested by us directly from the trees in a state of commercial maturity and without any damage. The following day, the peaches were washed and hand-peeled, cut into small pieces, lyophilized, and kept at −80 °C until use.

Chemicals. 4-*tert*-Butylcatechol (4-TBC) was obtained from Fluka (Madrid, Spain). Bovine serum albumin (BSA), bicinchoninic acid solution, and copper(II) sulfate solution 4% (w/v) were obtained from Sigma (Madrid, Spain). Triton X-114 (TX-114) was obtained from Fluka (Madrid, Spain). All other reagents were of analytical grade and supplied by Sigma (Madrid, Spain). All buffers were prepared with water purified by a Milli-Q water purification system (Millipore Ibérica, Madrid, Spain). Polyclonal antibodies against broad bean leaf PPO were a gift from Dr. William H. Flurkey.

Enzyme Extraction. Soluble and membrane-bound fractions were obtained from 200 g of lyophilized peach, which was homogenized at maximum speed for 1 min in an Omnimixer 230 (Sorvall Inc., Norwalk, CT) with 100 mL of cold buffered 100 mM sodium phosphate, pH 7.3, containing 10 mM ascorbic acid and serine protease inhibitors [1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM benzamidine hydrochloride], both of which were added immediately before use. The homogenate was filtered through two layers of cheesecloth and centrifuged at 4000*g* for 10 min at 4 °C. The pellet, containing membrane fraction, was later used. The supernatant was centrifuged at 120000*g* for 30 min at 4 °C and considered to be the soluble fraction of PPO.

The pellet containing the membrane-bound fraction was resuspended with 10 mL of 10 mM sodium phosphate buffer, pH 7.3, with 6% (w/v) TX-114 and kept at 4 °C for 1 h under continuous stirring. This solution was warmed to 37 °C and subjected to temperature phase partitioning. After 15 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large micelles composed of detergent, hydrophobic proteins, and the remaining chlorophylls and phenolic compounds. This solution was centrifuged at 10000*g* for 15 min at 25 °C. The aqueous phase was used as a membrane-bound enzyme (*18*).

Soluble and membrane-bound fractions were brought to 25–80% saturation with $(NH_4)_2SO_4$, pH 7.0. The final solution was centrifuged at 120000*g* for 30 min at 4 °C. The precipitates obtained with 80% ammonium sulfate were collected and dissolved in 10 mM sodium phosphate buffer, pH 7.3. The salt content was removed by dialysis against the same buffer. These solutions were used as soluble and membrane-bound PPO fractions and stored at −80 °C until used.

Enzyme Assays. The diphenolase activity was determined using the substrate 4-TBC, which was used for the high stability of its enzymatic product, 4-(*tert*-butyl)benzo-1,2-quinone. The enzyme activity was determined spectrophotometrically at 25 °C by measuring the appear-

ance of the 4-(*tert*-butyl)benzo-1,2-quinone at 400 nm ($\varepsilon = 1150$ M^{-1} cm^{-1}). The international unit of enzyme activity (IU) was defined as the quantity of enzyme that produces 1 μmol of 4-(*tert*-butyl)benzo-1,2-quinone per minute at pH 6.0 and 25 °C. The enzyme obtained showed no monophenolase activity in the presence or absence of SDS when it was spectrophotometrically measured at 400 nm and 25 °C, using *p*-cresol as substrate ($\varepsilon = 1433$ M^{-1} cm^{-1}) or at 500 nm and 25 °C using *p*-hydroxyphenylpropionic acid as substrate with MBTH ($\varepsilon = 40000$ M^{-1} cm^{-1}) (*28*).

Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium phosphate buffer, pH 6.0, and 5 mM 4-TBC. The reaction was started by adding the enzyme. In activation assays, SDS was added at a routine concentration of 2 mM. pH studies were carried out using 50 mM sodium acetate and sodium phosphate buffers in a pH range from 3.5 to 7.5. The reaction time in each case was 4 min.

Spectrophotometric measurements were performed in a Kontron Uvikon 940 spectrophotometer (Kontron Instruments, Zurich, Switzerland). Experiments were performed in triplicate, and the mean and the standard deviation (SD) were plotted.

Kinetic Data Analysis. Kinetic data analysis was carried out using linear and nonlinear regression fitting, using the Sigma Plot Scientific Graphing for Windows version 8.0 (2001, SPSS Inc., Chicago, IL).

Protein Determination. Protein concentration was determined using the bicinchoninic acid (BCA) (*29*) protein assay using BSA as standard.

Denaturing SDS-PAGE. Electrophoresis was carried out using the method of Laemmli (*30*). Samples were applied to 10% polyacrylamide gels. The slab gels of 1.5 mm thickness were run in a Miniprotean II cell (Bio-Rad) at a constant current of 180 mV.

Partial Denaturing SDS-PAGE. The SDS-PAGE was performed using the method of Laemmli (*30*) but in the absence of β-mercaptoethanol and without heating in order to preserve enzymatic activity. Samples were applied as described previously.

Isoelectric Focusing (IEF). IEF was performed on 5% (w/v) polyacrylamide gels in 3.5–10.0 pH gradients using a MiniProtean II (Bio-Rad) electrophoresis kit and basically according to the method of O'Farrell (*31*). For this, slab gels consisted of 5% acrylamide/bis(acrylamide) (29:1), 5% glycerol, 2% ampholines (Pharmacia), and 0.06% ammonium persulfate. Samples were prepared by mixing identical amounts of enzymatic extract and a buffer that contained 30% glycerol and 15% ampholines in distilled water.

The slab gels of 1 mm thickness were run at a constant voltage (200 V) for 90 min and then at 400 V for 15 min at 4 °C using 20 mM acetic acid and 25 mM sodium hydroxide as electrode solutions.

Gel Staining. After electrophoresis and IEF, the gels were equilibrated into 50 mM sodium phosphate buffer, pH 6.0, used to detect the enzymatic activity, or prepared for protein transfer and Western blot. The staining for PPO activity was carried out with 50 mM sodium phosphate buffer, pH 6.0, 5 mM dopamine, 0.69 mM SDS, and 2 mM MBTH (*28*). The addition of 86 mM tropolone was used for PPO activity inhibition (*32*).

Western Blotting of SDS-PAGE. Electrophoretic transfer of the proteins from denaturing SDS-gels onto PVDF membranes (Bio-Rad) was performed using a Mini Trans-Blot apparatus (Bio-Rad). The transfer was carried out at 4 °C, under constant stirring in 25 mM Tris, 192 mM glycine, and 15% MeOH at pH 8.3 (as transfer solution) at 100 V for 1.5 h. Once the transfer was finished, the membranes were blocked in PBST (25 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl, and 0.15% Tween 20) containing 3% albumin at 25 °C for 1 h and then incubated overnight at 4 °C in PBST containing 1% albumin and polyclonal antibodies against PPO from broad bean leaves. The membranes were then incubated with goat anti-rabbit secondary antibodies conjugated with peroxidase for 1 h, under constant stirring, at 25 °C to detect the relevant proteins.

Finally, protein bands were detected on the membranes using a reaction medium for peroxidase activity staining, in 50 mM sodium acetate buffer, pH 5.0, 1 mM 4-methoxy-α-naphthol (4MN), and 0.45 mM H_2O_2. In all experiments after the transfer, a control was carried out without primary antibodies, and the resulting membrane was incubated with this staining solution.

Table 1. Partial Purification of PPO from 135 g of Peach Fruit Flesh

fraction	volume (mL)	protein (mg)	enzyme activity (units[a])	specific activity (units/mg)	yield (%)	purification fold	activation fold
PPO-S							
crude extract	108	375	23.6	0.063	100	1	
supernatant 120000*g*	97.5	279.6	22.0	0.079	93	1.25	
ammoniun sulfate 25–80%	7.5	29.7	13.4	0.45	56.6	7.1	2.8
PPO-B							
6% Triton-X-114	15.4	75.3	7.22	0.096	100	1	3.5
ammoniun sulfate 25–80%	2.1	12.8	2.82	0.22	39	2.3	3.5

[a] One unit of enzyme is defined as the amount of PPO that produces 1 μmol of quinine per minute at 25 °C and pH 6.0 using TBC as substrate.

PPO Activity Staining. After partially denaturing electrophoresis, proteins were transferred to PVDF membranes under the conditions indicated for the membrane PPO fraction in the Western blotting of SDS-PAGE. The PVDF membranes were equilibrated into the buffer used to detect the enzymatic activity. The staining for PPO activity was carried out with 50 mM sodium phosphate buffer, pH 6.0, 5 mM dopamine, 0.69 mM SDS, and 2 mM MBTH (*28*). Membrane transfer substantially improves band visualization for reproduction purposes in comparison to direct gel staining.

RESULTS AND DISCUSSION

Enzyme Extraction. PPO was extracted from Catherina peach flesh by subcellular fractionation. PPO activity was found to occur in both fractions of the crude extract, a soluble and a particulate PPO resulting from the supernatant and pellet, after 4000*g* centrifugation, respectively. The membrane-bound PPO was extracted by solubilization with the detergent Triton X-114 followed by temperature-induced phase separation (*18*). This detergent has a cloud point at 23 °C, which allows it to be used in a temperature range compatible with protein stability. After phase separation induced by incubation at 37 °C, most of the detergent, plastidic pigments, phospholipids, some phenolics, and very hydrophobic membrane proteins partition to the lower detergent-rich phase, whereas polar phenolics and many membrane proteins are found in the upper aqueous detergent-poor phase. The membrane-bound PPO activity was found in the upper detergent-depleted phase.

An outline of a partial purification of soluble and membrane-bound fractions of PPO is given in **Table 1**. In both fractions the enzyme was obtained in its latent state after ammonium sulfate fractionation, and it was activated by acid pH and by the detergent SDS. However, the degrees of activation were different for the soluble fraction (2.84-fold) and for the membrane fraction (3.55-fold). Different degrees of activation for both fractions have previously been described even for purified PPO (*13*).

Effect of pH. pH is a determining factor in the expression of enzymatic activity and, in the case of latent PPO, its activation by acid or basic shock has been described (*22*). On the other hand, plant PPO in its latent state can also be induced or activated by SDS (*23*). For this reason, the determination of the optimum pH of the enzyme together with its kinetic characterization was carried out in the presence and absence of SDS.

The enzyme activity toward 5 mM 4-TBC was determined in a range of pH from 3.5 to 7.5. When the pH was scanned in the absence of SDS, both soluble (PPO-S) and membrane-bound PPO (PPO-B) showed a high activity at pH between 3.5 and 4.5, the optimum pH being 4.5 (**Figure 1**). The latent enzyme was incubated from 1 to 24 h at pH 4.5 and then measured at pH 6.0, in which conditions the enzyme remained active, activation reaching 60% when the incubation had lasted 24 h (results not shown). Therefore, the low pH optimum is a result
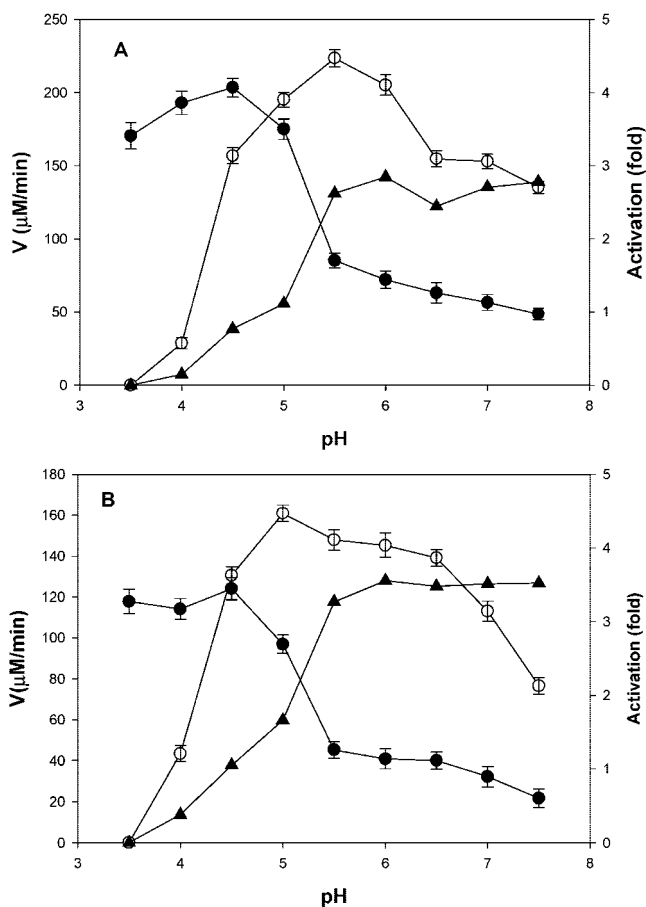

Figure 1. Catecholase activity of (**A**) soluble and (**B**) membrane-bound peach PPO toward 4-*tert*-butylcatechol, at different pH values, without (●) SDS or with 2 mM SDS (○); (▲) degree of activation. The reaction medium at 25 °C contained 5 mM 4-*tert*-butylcatechol in 50 mM acetate (pH 3.5–5.0) or phosphate (pH 5.0–7.5) buffers and (**A**) 200 μg/mL PPO-S and (**B**) 325 μg/mL PPO-B.

of the enzyme being induced by acid shocking (*22*). The activation was higher in PPO-B, although both forms showed a significant fall in activity at more basic pH values (**Figure 1**). These results were similar to those obtained from lettuce, beet root, Dominga grape, loquat fruit, and Babygold peach PPO (*12−15, 33*). When the assays were performed in the presence of SDS, the low pH optimum obtained for both latent forms was abolished (**Figure 1**), but for values above pH 5 an activating effect was provoked by SDS. The greatest activation was obtained at pH 6.0, and so it was decided to perform kinetic studies in the presence and absence of SDS at pH 6.0, although the activity of the enzyme in the presence of SDS was quite high from pH 5.0 to 7.0. In this case the activations were similar in PPO-S and PPO-B. Moore and Flurkey (*23*) showed that a
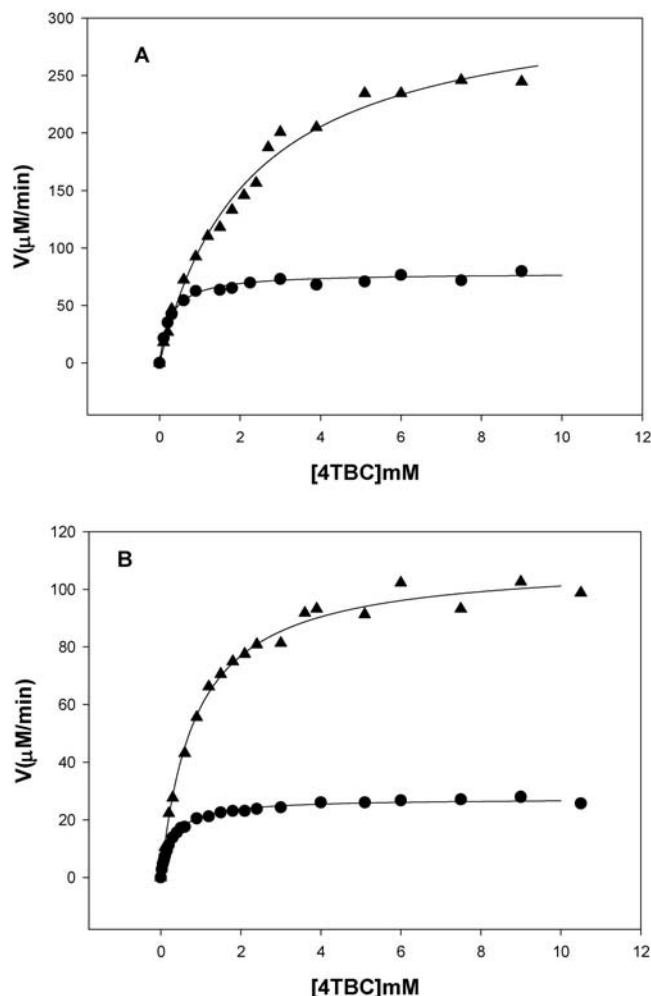
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Figure 2. Effect of 4-*tert*-butylcatechol concentration on catecholase activity of (**A**) soluble and (**B**) membrane-bound peach PPO. The reaction medium contained 4-*tert*-butylcatechol at the indicated concentration, in 50 mM sodium phosphate buffer, pH 6.0 (●) and 2 mM SDS (▲). (**A**) 200 μg/mL PPO-S; (**B**) 295 μg/mL PPO-B.

Table 2. Kinetic Parameters of Soluble (PPO-S) and Membrane-Bound (PPO-B) Peach Polyphenol Oxidase

	4-*tert*-butylcatechol			
	V_m (μM/min)	K_m (mM)	V_m/K_m (min^{-1})	r
PPO-S without SDS	78.3 ± 1.5	0.263 ± 0.03	0.3	0.988
PPO-S with 2 mM SDS	293.5 ± 7.51	1.84 ± 0.15	0.16	0.998
PPO-B without SDS	27.44 ± 0.5	0.30 ± 0.027	0.09	0.988
PPO-B with 2 mM SDS	109.8 ± 1.77	0.85 ± 0.054	0.13	0.996

higher in the presence of SDS, an increase that agrees with previous results obtained for PPO from different sources (*11, 13, 33*). However, activation by SDS only increased the catalytic efficiency (V_m/K_m) in the membrane-bound fraction, which was more latent than the soluble one.

Electrophoretic and Electrofocusing Study. Partially denaturing SDS-PAGE was used to detect the PPO isoforms in the enzyme extract. As can be observed from **Figure 3**, both the membrane-bound (lane 1) and the soluble (lane 2) fractions showed a doublet (two active bands) when the gel was stained with dopamine and MBTH. This doublet has been described for the mature forms of PPO (*12, 33, 35*). To ascertain whether these bands correspond to PPO, the staining was also carried out in the presence of tropolone, a specific PPO inhibitor (*32*). As can be seen, no enzymatic activity was detected in either the membrane-bound (lane 3) or soluble (lane 4) fractions. This result, therefore, confirmed that those two bands corresponded to PPO. These bands could also be seen by blot transfer of this gel and further activity staining of the proteins transferred to the membrane (lanes 5 and 6, membrane-bound and soluble, respectively). The apparent M_r values obtained were 48 and 50 kDa. These values do not correspond to molecular masses because the proteins are not completely denatured.

IEF is a useful electrophoretic method for studying PPO isoenzymes. Several acidic isoenzymes have been detected in the soluble and membrane-bound fractions from beet root (*13*). When PPO from broad bean leaves was subjected to native IEF, several isoforms were present with apparent p*I* values from 4.9 to 5.9, and all of these isoforms shared a 65 kDa protein (*36*). Two acidic PPO isoforms were detected from beet hypocotyls (*37*), and two tyrosinase isoforms with p*I* values of 5.1 and 5.2 were isolated by Wichers et al. (*38*) from *A. bisporus*. Recently, two acidic PPO isoforms have also been detected in Babygold peaches with p*I* values of 5.7 and 5.8 in the soluble form and one more at p*I* 5.4 in the membrane-bound form (*33*). Given all these considerations, PPO from Catherina peach was analyzed by IEF (pH 3.5–10). As can be seen (**Figure 4**) in the soluble fractions (lane 3) two acidic bands of p*I* 5.7 and 5.8 were detected, whereas in the membrane-bound fraction only one band of p*I* 5.7 was detected (lanes 1and 2). No protein bands were detected (data not shown) when the staining was carried out in the presence of tropolone. Thus, Catherina peach PPO showed an acidic isoform pattern, which agrees with previous results obtained for PPO from different sources (*13, 33, 36–38*) but with only one isoform for the membrane-bound form.

Analysis of the same samples by SDS-PAGE under fully denaturing conditions and a Western blot from the totally denaturing SDS gels was carried out using polyclonal antibodies against a broad bean leaf PPO (**Figure 5**). No band was recognized on the immunoblotting by the antibodies in the PPO soluble-bound fraction (lane not shown), whereas a band was recognized by the antibodies in the membrane-bound fraction, which corresponded to a molecular weight of 60 kDa. Similar results have also been described for PPO from other sources (*13, 33*). Therefore, the

limited conformational change due to the binding of small amounts of SDS may induce or initiate the activation of the latent enzyme. They also reported that the low pH optimum (below 4) obtained for the latent enzyme was abolished in the presence of SDS. It remains, however, to be seen whether the acid-shocking activation process and the SDS effect are related through a common mechanism, as has been shown in the case of the trypsin-measured activation and SDS (*34*).

Kinetics Studies. The kinetic characterization of Catherina peach PPO from both the soluble (**Figure 2A**) and the membrane-bound (**Figure 2B**) fractions was carried out after determination of the optimal conditions for measuring peach PPO in its latent state. Kinetic parameters (K_m and V_m) were studied at pH 6.0 using 4-TBC as substrate. As can be seen in **Figure 2**, the initial rate showed a hyperbolic dependence with respect to the 4-TBC concentration in the presence and absence of 2 mM SDS. The SDS concentration used was 2 mM because at this detergent concentration the enzyme was fully active and the critical micelle concentration (CMC) for the detergent was not reached (*23*).

K_m and V_m were determined by fitting the experimental points to the Michaelis–Menten equation. The solid line between the experimental points represents this fitting. **Table 2** shows the V_m and K_m values for both soluble and membrane-bound enzymatic fractions. As can be seen, the V_m and K_m values were

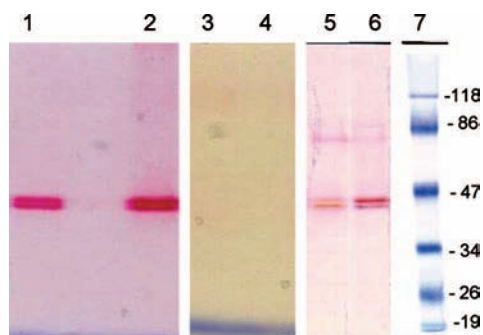**10450** *J. Agric. Food Chem.,* Vol. 55, No. 25, 2007

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Figure 3. Partially denaturing SDS-PAGE of the membrane-bound (lanes 1 and 3) and soluble (lanes 2 and 4) peach PPO. Lanes 5 and 6 show activity staining for membrane-bound and soluble PPO, respectively, after blotting to the gel on a PVDF membrane. Lanes 1, 2, 5, and 6 were stained with dopamine and MBTH. Lanes 3 and 4 were stained with dopamine, MBTH, and tropolone. Amounts of 19.25 μg of membrane-bound fraction protein and 13 μg of soluble fraction protein were applied respectively in each well.
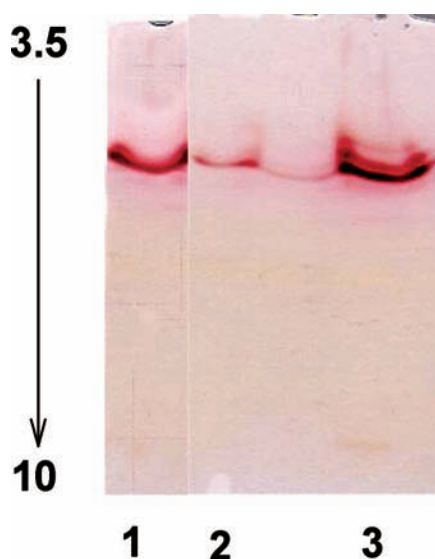

Figure 4. Isoenzymatic patterns of the different PPO fractions separated by IEF in a pH 3.5–10 gradient: lanes 1 and 2, membrane-bound PPO (the amounts of protein used were 15 and 5 μg, respectively); lane 3, soluble PPO (the amount of protein used was 5 μg).

values of the M_r estimated by denaturing and by partially denaturing electrophoresis differ. As previously mentioned, in partially denaturing SDS-PAGE, the electrophoretic mobilities do not correspond to molecular masses because the proteins are not completely denatured. Thus, previous studies using Babygold peach detected 48 and 50 kDa bands showing PPO activity, whereas under denaturing condition a band at 60 kDa was revealed (*33*). From carrot PPO, a 36 kDa band was detected showing PPO activity, whereas under denaturing condition a band at 59 kDa was revealed (*39*). Using beet root PPO, a 36 kDa band was also detected showing PPO activity, whereas a band at 60 kDa was revealed under denaturing condition (*13*).

In conclusion, this paper has analyzed the characteristics of catecholase activity of PPO from Catherina peach. The enzyme was in latent state in both the membrane-bound and soluble fractions and could be activated by acid shock and by the anionic detergent SDS. However, when used jointly, both agents inhibited the enzyme. This inhibiting effect of SDS at low acid pH has been observed in other fruits, such as apple and loquat (*21, 26*). In apple, Marquès et al. (*26*) reported that SDS modified PPO behavior with
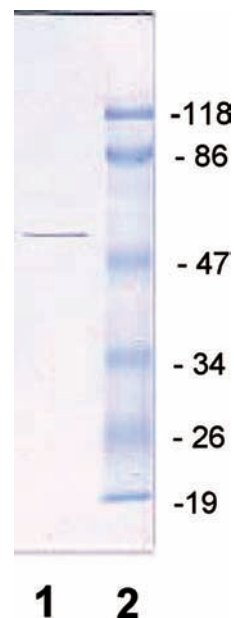

Figure 5. Western blotting of totally denaturing SDS-PAGE of peach PPO. Lane 1 contained 13.75 μg of the membrane-bound PPO, and lane 2 contained prestained molecular mass markers.

respect to pH instead of causing a general activation of the enzyme. The study of the effect of SDS on the pH profile and experiments of intrinsic fluorescence of a loquat fruit latent pure PPO (*21*) lend weight to the hypothesis concerning the regulation of PPO activity based on the binding of SDS. SDS would induce a conformational change in the protein that leads to a pH-profile shift (*21, 23*). Sellés et al. have observed that some unsaturated fatty acids such as linolenic acid have a similar effect to SDS on PPO activity, causing inhibition at pH 4.5 (*21*). An activatory effect of fatty acid on PPO activity at pH 7 has also been observed (*24*); thus, the fatty acid effect is qualitatively comparable to that of SDS (*21*). A hypothetical mechanism of PPO activity regulation based on a fatty acid-induced pH profile shift has been proposed (*21*). Because of the low acid pH within the thylakoidal lumen, PPO would express a high activity, but its ability to bind to fatty acid or to incorporate into a negatively charged amphiphilic surface at acid pH, such as the thylakoidal membrane, might keep the enzyme inactivated. Thus, in physiological conditions, enzymatic browning would be prevented not only by compartmentalization but also by direct control of the enzyme activity.

On the other hand, so far, no natural activator has been found, although SDS activation of tyrosinase has been demonstrated in vivo with *T. claveryi* sections (*25*), and other anionic detergents have also been seen to have a similar effect, underscoring the importance of a negative charge in the headgroup for activation occur at pH 6.0 or higher (*21*).

The only natural condition for latent PPO, both soluble and membrane-bound forms, activation demonstrated to date is an acid environment, which could be mediated by tissue damage or disruption. Thus, enzymatic browning occurs quickly only when fruit tissues become disorganized due to wounding, over-ripening, or senescence. Thus, it is very important for PPO to exist in the plant in a latent form and for its activity to be regulated to carry out its function in defending the plant.

ABBREVIATIONS USED

PPO, polyphenol oxidase; FAO, Food and Agriculture Organization; 4-TBC, 4-*tert* butylcatechol; MBTH, 3-methyl-2-benzothiazolinone hydrazone.

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