JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Characterization of a Latent Polyphenol Oxidase from Beet Root (*Beta vulgaris* L.)

FERNANDO GANDÍA-HERRERO, FRANCISCO GARCÍA-CARMONA, AND JOSEFA ESCRIBANO*

Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain

Polyphenol oxidase (PPO) has been extracted from beet root, in both soluble and membrane fractions. In both cases, the enzyme was in its latent state, and it was activated by sodium dodecyl sulfate. PPO was purified to apparent homogeneity. The soluble PPO purification was achieved by hydrophobic interaction chromatography and gel filtration chromatography, with apparent molecular mass of 55 kDa. The membrane PPO purification was achieved by anion exchange chromatography and gel filtration with apparent molecular mass of 54 kDa. A totally denaturing SDS-PAGE indicated the presence of a single polypeptide with an apparent molecular mass of 60 kDa for both fractions, with the band also revealed by Western blot. A partially denaturing SDS-PAGE stained a single active 36 kDa band for both fractions. Under native isoelectric focusing, a major acidic band of pH 5.2 was detected in both fractions. Kinetic characterization of PPO on the natural substrate L-dopa was carried out.

KEYWORDS: Polyphenol oxidase; *Beta vulgaris* L.; betalains; enzyme purification

INTRODUCTION

Polyphenol oxidase (PPO) (monophenol, *o*-diphenol, oxygen oxidoreductase; EC 1.14.18.1) is a copper-containing enzyme that catalyses two different reactions using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (*1*). This enzyme is widely distributed in microorganisms, animals, and plants, so that PPO is responsible not only for browning in plants but also for melanization in animals.

PPO is widely distributed in the plant kingdom and has been detected in most fruits and vegetables. In plants, PPO is predominantly located in the chloroplasts' thylakoid membranes (*2*−*5*). However, it is not an intrinsic membrane protein and can be released from the thylakoids by sonication, mild detergent treatment, or protease treatment (*6*). The enzyme has also been detected in soluble fractions in homogenates from different vegetables (*5*, *7*)

Due to their high reactivity, the quinone molecules can react with each other as well as with other substances in the wounded tissue, forming a variety of brown or black compounds, which are not the direct result of PPO activity (*8*). This makes PPO 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 quinone formation. Not only may the appearance of the product be affected but also the taste and its nutritional value, often decreasing the quality of the final product (*9*, *10*). Because of the considerable economic and nutritional loss induced by enzymatic browning in the commercial production of fruits and vegetables, numerous studies have been devoted to the biochemical and catalytic properties of PPO (*8*, *11*). Information on molecular and catalytic properties of PPO is of importance in studies of the regulation of tissue browning.

A wide variety of plant PPO behaviors have been described and reviewed (*8*, *12*). The overall results indicate a high heterogeneity in plant PPO biochemical characteristics (*K*m, optimum pH, and latency), number of enzymatic forms and molecular masses. PPO protein has been purified from several higher-plant species, including spinach (*2*), broad bean (*13*), tomato (*14*), banana (*15*), cabbage (*16*), and lettuce (*17*). The molecular mass has been determined as 40−45 kDa or 60−65 kDa. This variation in molecular mass may result from modification of PPO proteins during purification, both by proteolytic cleavage and by association with other compounds (*18*). Proteolytic processing of the enzyme may play a role in the heterogeneity of the observed forms as well as participate in activating the latent forms of the enzyme, which are observed in many species (*19*).

The physiological function of PPO in higher plants is yet to be fully determined, but it has been implicated in pigment formation (*20*) and scavenging molecular oxygen in the chloroplast (*21*). A role in plant defense has also been suggested (*22*). Recently, it has been proposed that PPO is involved in the biosynthesis of betalains of higher plants (*23*). The hy-

* To whom correspondence should be addressed. Tel.: +34 968 364762. Fax: +34 968 364147. E-mail: pepa@um.es.

10.1021/jf034381m CCC: $27.50
Published on Web 01/15/2004

droxylation of tyrosine to dopa and the oxidation of dopa, both catalyzed by PPO, are considered the first steps in the biogenesis of betalains. The enzyme has been partially purified from betacyanin-producing callus of *Portulaca grandiflora* (*23*) and *Amanita muscaria* (*24*). Betalains are also present in sugar beet, and PPO activity has been measured in both leaves (*5*, *23*, *25*) and root (*26*).

In the present study, PPO has been purified from beet root, and some molecular and kinetic properties have been investigated.

MATERIALS AND METHODS

Plant Material. *Beta vulgaris* L. roots (granadina variety) were grown in an organic plantation without adding any pesticide. The roots were harvested, sliced, and frozen in liquid nitrogen and stored at −80° C until use.

Chemicals. Superdex 200 HR 10/30, Resource-Q, and Resource-ISO columns and gel filtration markers were purchased from Amersham Biosciences (Barcelona, Spain). PPO substrates and SDS-PAGE molecular weight markers were obtained from Sigma (Barcelona, Spain). The reagents for electrophoresis were procured from Bio-Rad Laboratories (Barcelona, Spain). All other chemicals were of analytical grade.

Subcellular Fractionation. Soluble and membrane fractions were obtained from 105 g of beet root slices, which were homogenized twice at maximum speed for 10 s in a Model 230 Omnimixer (Sorvall Imc. Norwalk, CT) with 210 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.33 M sorbitol, 10 mM ascorbic acid, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, and 1 mM benzamidine. All procedures were carried out at 4 °C. The homogenate was filtered through two layers of cheesecloth and centrifuged at 1000*g* for 10 min. The pellet, containing the wall fraction, was discarded, and the supernatant was centrifuged at 120 000*g* for 40 min. Thus, the resultant supernatant was considered to be the soluble fraction, and the pellet, the membrane fraction.

Soluble fraction was brought up to 35−85% $(NH_4)_2SO_4$. The salt content was removed by dialysis against 10 mM sodium phosphate buffer, pH 7.0.

The extraction of PPO from the membrane fraction was carried out by using three parallel treatments (*1*): sonication for 10 min in 0.1 M sodium phosphate buffer pH 7.0 (*2*); treatment with TritonX-114 as described by Escribano et al. (*5*) (Triton treatment-1, (Triton-1)) (*3*); and salted treatment, incubating for 60 min in 0.1 M sodium phosphate buffer containing 1M NaCl. These fractions were centrifuged at 120 000*g* for 40 min to obtain the solubilized enzyme in the supernatant and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) overnight to remove the salt content. The pellet obtained after the solubilization with NaCl was treated with TritonX-114, as indicated in ref 2 to solubilize the remaining proteins (Triton treatment-2, (Triton-2)).

To avoid any possible activation of the PPO enzyme by endogenous proteases, PMSF and benzamidine hydrochloride were added before and after the dialysis to give a final concentration of 1 mM.

Enzyme Purification. All steps were carried out at 4 °C except those using the Äkta purifier (Amersham Biosciences, Barcelona, Spain).

Protocol I, Soluble Enzyme Purification. Soluble PPO was purified by hydrophobic interaction chromatography and gel filtration using an automated liquid chromatography system (Äkta purifier, software Unicorn version 3.0). The concentrated fraction after 35−85% $(NH_4)_2SO_4$ fractionation was loaded onto a 1-mL Resource-ISO pre-packed column equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 1.5 M $(NH_4)_2SO_4$ (solvent A). PPO was eluted with a linear gradient of $(NH_4)_2SO_4$ from 1.5 to 0 M $(NH_4)_2SO_4$ in 20 mM sodium phosphate buffer (pH 7.0) (solvent B) within 20 mL (flow rate 2.0 mL min^{-1}). The fractions containing PPO activity were pooled, desalted, and concentrated by ultrafiltration with a YM 10 membrane (Millipore, Madrid, Spain). For gel filtration, the concentrated peak fractions were applied to a Superdex 200 HR 10/30 column equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The protein was eluted with the same buffer at a flow rate of 1 mL min^{-1}. The eluate was collected in fractions, and PPO activity was measured.

Protocol II, *Membrane-Bound Enzyme Purification*. Membrane-bound PPO was purified by anion exchange chromatography and gel filtration. The sample partially purified with Triton X-114 was loaded onto a 1-mL Resource-Q column equilibrated with 20 mM bis-tris (HCl) buffer (pH 6.0). PPO was eluted with a linear gradient from 0 to 0.7 M NaCl within 50 mL (flow rate 1.0 mL min^{-1}). The fractions containing PPO activity were pooled, desalted, and concentrated by ultrafiltration. For gel filtration, the same column and method as in protocol I were used.

For the molecular weight estimation on Superdex 200 HR 10/30 column, the calibration curve was determined using chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (66 kDa), albumin dimer (132 kDa), aldolase (158 kDa), and catalase (232 kDa).

Enzyme Assays. The diphenolase activity was determined spectrophotometrically by measuring the appearance of dopachrome at 475 nm ($\epsilon = 3700\ M^{-1}\ cm^{-1}$) or dopaminechrome at 480 nm ($\epsilon = 3300\ M^{-1}\ cm^{-1}$) (*7*). In this work, the international unit of enzyme activity (IU) was defined as the quantity of enzyme that produces 1 μmol of dopachrome or dopaminechrome per minute at pH 6.0 and 25 °C.

Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium phosphate buffer (pH 6.5), 0.69 mM SDS, and 5 mM dopamine.

Spectrophotometric measurements were performed in a Kontron Uvikon 940 spectrophotometer.

Protein Determination. Protein concentration was determined according to the Bradford Bio-Rad protein assay using serum albumin as standard (*28*).

Denaturing SDS-PAGE. Electrophoresis was carried out using the method of Laemmli (*29*). Samples were applied to 10% polyacrylamide gels. The slab gels of 1.5 mm thickness were run in a Miniprotean II cell (BioRad) at a constant current of 180 mV. Gels were stained for protein using a standard Coomassie Blue or silver staining method.

Partially Denaturing SDS-PAGE. The SDS-PAGE was performed as described above, but in the absence of β-mercaptoethanol and without heating, to preserve the enzymatic activity.

Isoelectric Focusing (IEF). IEF was performed on 5% (w/v) polyacrylamide gels in 3.5−10.0 pH gradients using a MiniProtean II (BioRad) eletrophoresis kit.

Gel Staining. After partially denaturing electrophoresis and IEF, the gels were equilibrated into the buffer used to detect the enzymatic activity. Staining for PPO activity was carried out with 50 mM sodium phosphate buffer (pH 6.0), 5 mM tyramine, and 2 mM MBTH (*30*).

Western Blotting of SDS-PAGE. Electrophoretic transfer of the proteins from a denaturing SDS-gel 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 for the soluble fraction) or in 0.7% acetic acid and 0.01% SDS (as transfer solution for the membrane fraction) 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 the polyclonal antibodies against PPO from broad bean leaf (a gift from Dr. William H. Flurkey). To detect the relevant proteins, the membranes were incubated with secondary antibodies conjugated with peroxidase for 1 h, under constant stirring, at 25 °C.

Finally, protein bands were detected on the membranes using the reaction medium described previously for the peroxidase activity staining (*26*), in 50 mM sodium acetate buffer (pH 5.0), 1 mM 4MN, 0.45 mM H_2O_2. In all experiments, after the transfer, a control was carried out incubating a PVDF membrane with this staining solution.

Estimations of the Mr values were based on nonprestained molecular mass markers stained with ponceau S. Prestained molecular mass markers were used to indicate approximate size and transfer efficiency.

Table 1. Summary of PPO Extraction and Purification from Beet Root

fraction	vol (mL)	prot (mg/mL)	prot (mg)	activ (u/mg)	total activ (u)	purif. fold	recovery (%)
			membrane				
Triton-1	8.52	1.24	10.56	6.22	65.60		
NaCl	11.83	0.33	3.93	4.19	16.46		
Triton-2	1.40	1.20	1.67	5.57	9.31		
			soluble				
crude extract	249	0.28	69.70	14.12	982.80	1	100
$(NH_4)_2SO_4$	11.2	5.0	56.0	14.4	806.4	1.02	82.1
hydrophobic C.	14.9	0.17	2.53	163.4	413.4	11.59	42.0

RESULTS AND DISCUSSION

Enzyme Extraction. PPO was extracted from beet root by subcellular fractionation. The enzyme was detected in both soluble and membrane fractions. The extraction of the membrane-bound enzyme was carried out with high ionic strength, detergent or sonication. The results of the different treatments are summarized in **Table 1**. The use of NaCl for PPO extraction has provided good yields (*23*, *26*); however, in this case, the extraction with Triton X-114 (Triton-1) was the most effective method to solubilize PPO, whereas the PPO activity obtained by sonication was very low (results not shown). To determine if the enzyme obtained with high salt concentration, and therefore ionically bound to the membrane, presented any difference to the one solubilized with the nonionic detergent, Triton X-114, an additional extraction from the remaining proteins (the pellet) after the extraction with NaCl was carried out with Triton X-114 (Triton-2). The results are shown in **Table 1**. PPO activity was solubilized after the second treatment with Triton X-114 (Triton-2). However, the yield of this treatment (14.2%) together with the one obtained with NaCl (25%) was lower than the one obtained with the first solubilization with Triton X-114 (Triton-1). These findings suggested that the enzyme was closely associated with membranes and detergent extraction can disrupt the membranes to release more enzyme. PPO was also found in the soluble fraction, with the PPO activity being higher in this fraction than that in the membrane fraction.

Effect of pH. pH is a determinant factor in the expression of enzymatic activity, and in the case of latent PPO, its activation by acid or basic shock has been described (*31*, *32*). On the other hand, plant PPO in its latent state can also be induced or activated by SDS (*3*, *5*, *33*). For this reason, the determination of the optimum pH and the kinetic characterization of the enzyme was carried out in the presence and absence of SDS.

The enzyme activity was determined toward 5 mM dopamine in a range of pH 3.5−7.5. In **Figure 1A**, the pH profile corresponding to the extraction only with Triton X-114 (Triton treatment-1) is represented, although the PPO solubilized by the other treatments showed the same behavior. In the absence of SDS, the optimum pH was 4.5, falling at higher pH values, and PPO activity was almost negligible from pH 5.5 to 7.5. These results were similar to the ones obtained from beet leaves extracts (*5*) but different from the ones found from beet root homogenates (*23*, *34*, *35*), which showed that the PPO activity was maximum at pH 7.0. When the activating effect of SDS with respect to pH was studied, the highest value in the activation process was obtained at pH 6.5, although the activity of the enzyme was quite high from 6.0 to 7.5 (**Figure 1A**). Quite similar results were obtained with the soluble enzyme (**Figure 1B**) in the presence of SDS. However, in the absence of SDS, soluble PPO showed an optimum pH in a range from 5.5 to 7.5, whereas the membrane PPO activity was almost negligible at these pH values. A similar optimum pH was obtained for the soluble fraction from beet leaves extracts (*5*). In all cases, the enzyme was totally activated in the presence of 0.69 mM SDS. However, the degree of activation was different for the soluble fraction (2.3-fold) than for the membrane fraction (∼13-fold in all cases). The latency is common in thylakoid-bound PPO and has been described for potato leaf (*36*), lettuce (*3*), broad bean (*37*), and table beet leaves (*5*) and root (*26*). The degree of activation depends on the plant material and the extraction method used (*36*). In this case, the use of a high salt concentration or a non ionic detergent to solubilize PPO from membrane did not modify the degree of activation. Therefore, all the kinetic assays were carried out at 0.69 mM SDS concentration, at which PPO was totally activated.

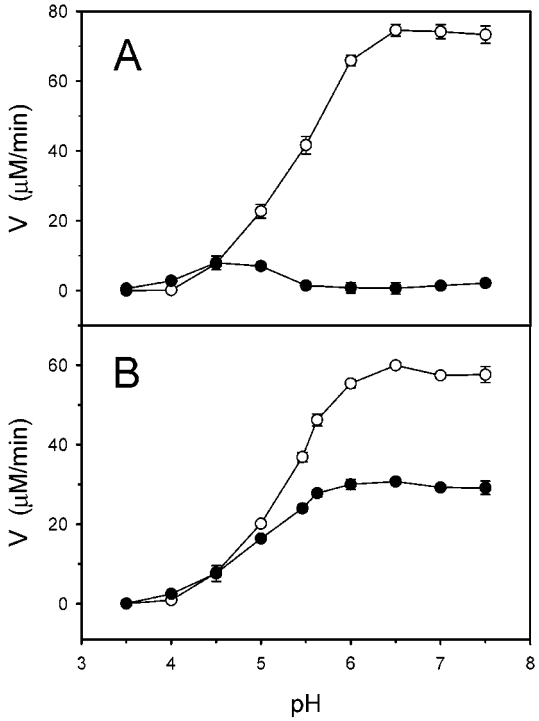

Figure 1. Effect of pH on the enzyme in 50 mM sodium acetate and sodium phosphate (pH 3.5–7.5) in the presence (○) or absence (●) of 0.69 mM SDS. The reaction medium at 25 °C contained 5 mM dopamine and 12.4 μg mL^{-1} PPO extracted with (A) Triton X-114, or (B) 5 μg mL^{-1} soluble PPO. Experiments were performed in triplicate, and the mean and standard deviation were plotted.

Because the extraction with Triton X-114 (Triton treatment-1) was the most effective method to solubilize the membrane PPO, the enzyme purification was carried out using this fraction and the soluble fraction.

Purification of the Enzyme. PPO was purified to apparent homogeneity from both soluble and membrane fractions. The results of PPO purification are shown in **Figure 2**.

The soluble fraction was subjected to ammonium sulfate fractionation (35−85%), resulting in an 82% recovery of PPO activity with a 1.02-fold purification (**Table 1**), in agreement with literature data for PPO from other sources (*2*, *3*, *38*). Thus, this procedure was principally used for concentration. The degree of activation of PPO by SDS was the same (2.3-fold) after being subjected to this treatment. The concentrated PPO was then purified by hydrophobic interaction chromatography on a 1-mL Resource-ISO column, and the elution profile is shown in **Figure 2A**. A single peak of PPO activity was eluted

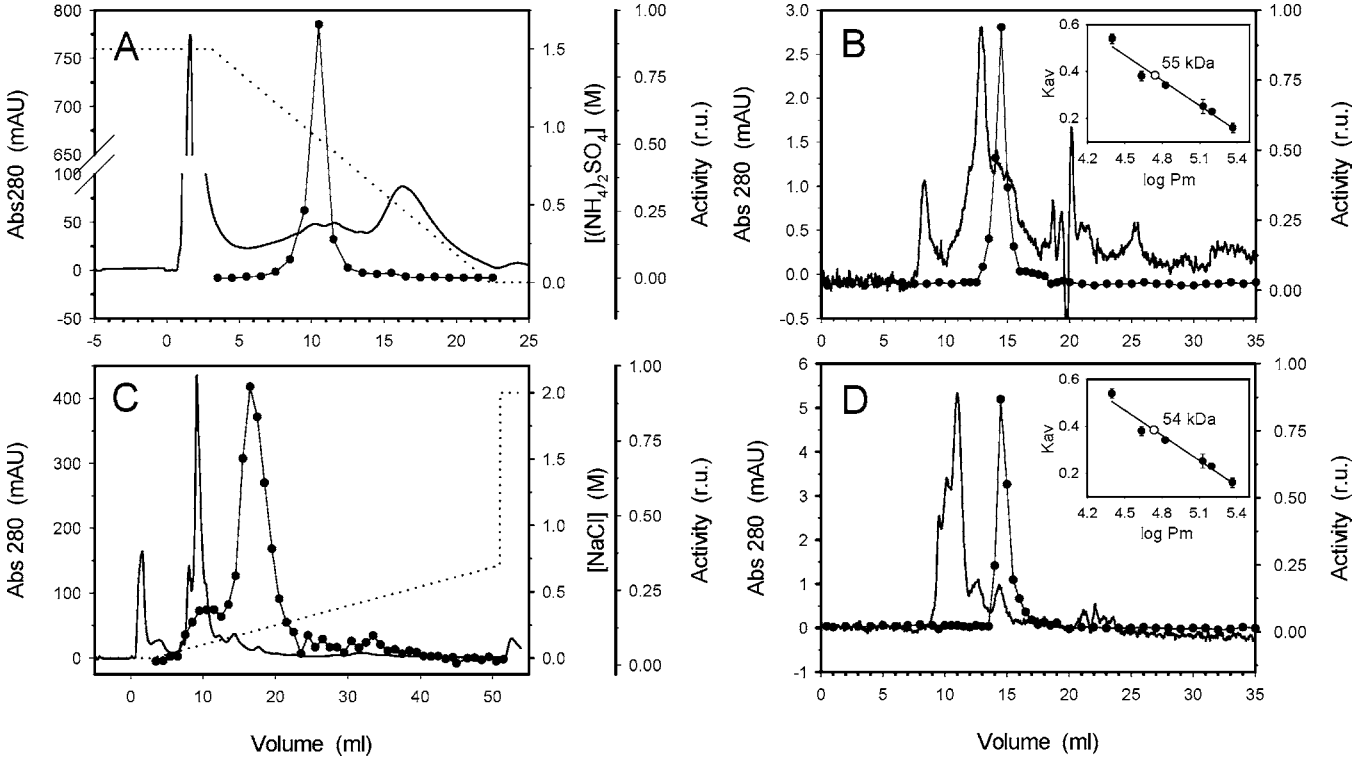

Figure 2. PPO purification from soluble (A and B) and membrane (C and D) fractions. Elution profiles of PPO activity on Resource-ISO (A), Superdex 200 HR (B), Resource-Q (C), and Superdex 200 HR (D). (–) Absorbance at 280 nm; (-●-) PPO activity; (...) salt concentration. Insets: Molecular weight estimation on Superdex 200 HR. The calibration curve was determined using the protein markers described in Materials and Methods and data are plotted as the mean and standard deviation of three independent experiments.

at 0.92 M $(NH_4)_2SO_4$, and it resulted in a 42% recovery of the enzyme with an 11.6-fold purification (**Table 1**).

Another PPO purification protocol was checked by using anion exchange chromatography with a Resource-Q column. Any attempt to use it as a second purification step resulted in a great loss of enzyme activity (results not shown). Furthermore, if the procedure was inverted, using first anion exchange chromatography and then the hydrophobic interaction chromatography, the PPO activity was eluted from the first step, but it was completely lost after the second. We can conclude that the enzyme turned out to be very labile after the first step of purification, independently of the procedure used. Other authors have also described this behavior for PPO from betacyanin-producing callus (*23*) and carrot (*39*). Finally, the anion exchange chromatography was discarded because it gave a lower yield (results not shown).

To achieve a purification to homogeneity, the peak eluted from the isopropyl column was concentrated and submitted to gel filtration on a Superdex 200 HR column. The obtained chromatography profile is shown in **Figure 2B**, and as can be seen, a single peak with PPO activity was eluted. The apparent molecular mass of the peak was estimated to be about 55 kDa (**Figure 2B**, inset).

The purification of PPO obtained from the membrane fraction using Triton X-114 was optimized and carried out as described in Protocol II under Materials and Methods. The results of the purification are not summarized in **Table 1**, because the protein concentration of the elutes was negligible, and it was not possible to determine it. The problem of low yields has also been described as a frequent occurrence in PPO purification by other authors (*2*, *6*, *17*, *23*). The membrane PPO was purified by anion exchange chromatography with a Resource-Q column. The elution profile is shown in **Figure 2C**. Most of the PPO was eluted in a major peak at 0.198 M NaCl, where few other proteins were eluted, and a previous smaller peak of PPO activity was detected but discarded because it was coeluted with most of the proteins. The fractions of the major peak were concentrated, dialyzed, and submitted to gel filtration, because any other attempt resulted in a great loss of enzyme activity (results not shown). The obtained chromatography profile is shown in **Figure 2D**, and as can be seen, a single peak with PPO activity was eluted. The apparent molecular mass of the peak was estimated to be about 54 kDa (**Figure 2D**, inset).

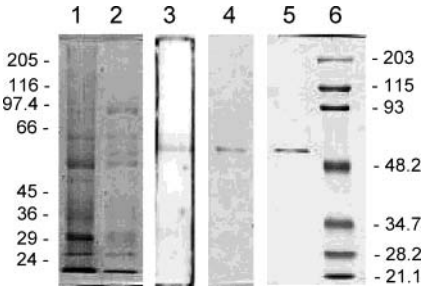

Figure 3. Denaturing SDS-PAGE and Western blotting of samples from the purification of soluble PPO. Lane 1, proteins after $(NH_4)_2SO_4$ fractionation; lane 2, PPO fractions from Resource-ISO; lane 3, PPO fraction from Superdex 200 HR. Proteins of lanes 1 (8 μg) and 2 (3 μg) were stained with Coomassie Blue, lane 3 was stained with silver. Lanes 4 and 5 correspond to Western blotting of proteins (2 μg) from lanes 2 and 1, respectively. Lane 6 is prestained molecular mass markers.

Electrophoretic Study. To determine the achieved purity, the peaks of PPO activity obtained by the different chromatography steps were analyzed by denaturing SDS-PAGE.

In the case of soluble PPO (**Figure 3**), homogeneity was reached according to silver-stained SDS-PAGE, that indicated the presence of a single polypeptide with an apparent molecular mass of 60 kDa (lane 3). A Western blot was carried out to

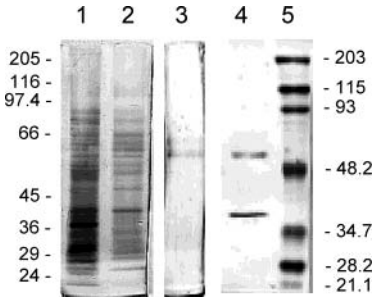

Figure 4. Denaturing SDS-PAGE and Western blotting of samples from the purification of membrane PPO. Lane 1, proteins extraction with Triton X-114; lane 2, PPO fractions from Resource-Q; lane 3, PPO fraction from Superdex 200 HR. Proteins of lanes 1 (1 μg), 2, and 3 were silver-stained. Lane 4 corresponds to Western blotting of proteins from lane 1. Lane 5 is prestained molecular mass markers.

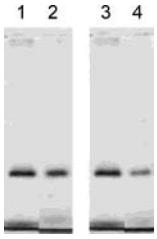

Figure 5. Partially denaturing SDS-PAGE of the soluble fraction (lanes 1and 2) and the membrane fraction (lanes 3 and 4) stained with tyramine and MBTH. Lane 1 (12.5 μg), proteins after ammonium sulfate fractionation; lane 2 (3 μg), PPO fraction eluted after hydrophobic interaction chromatography; lane 3 (21.7 μg), proteins extraction with Triton X-114; lane 4, PPO fraction eluted after anion exchange chromatography. The specific conditions are described under Materials and Methods.

detect the presence of PPO using polyclonal antibodies against broad bean leaf (a gift from Dr. W. Flurkey), and for the proteins of the different chromatography steps (lanes 4 and 5), only a single band was revealed with an Mr of 60 kDa, corresponding to the same Mr obtained by silver staining (lane 3). This molecular mass has also been described for PPO from other sources (*31*, *39*).

For the membrane fraction (**Figure 4**), the partially purified extract with Triton X-114 (lane 1) and proteins from the anion exchange chromatography (lane 2) and gel filtration chromatography (lane 3) were analyzed by denaturing SDS-PAGE and silver-stained. As can be observed, homogeneity was reached after the gel filtration chromatography according to silver-stained SDS-PAGE (lane 3) that revealed a band with an apparent molecular mass of 60 kDa and an additional faint band of higher molecular mass. This doublet has been described for the mature forms of PPO (40). The Western blot from the extract with Triton X-114 (lane 4) revealed two bands with 60 and 36 kDa. The 60 kDa corresponded to the same Mr obtained by silver staining (lane 3), and the 36 kDa band could correspond to either to a proteolytic form of PPO or a cross-reaction with a protein of the extract with Triton X-114.

A partially denaturing SDS-PAGE was also used to detect the active PPO isoform composition. As can be observed in **Figure 5**, all the enzymatic fractions showed the presence of a single active 36 kDa band when the gel was stained with tyramine as substrate. This monophenol together with MBTH was used for the electrophoretic staining instead of dopamine with MBTH, because it gave a nondiffusible product suitable for electrophoretic procedures with less background and a more clearly defined band. Furthermore, this staining turned out to be specific for the monophenolase activity of PPO (*30*). Previous

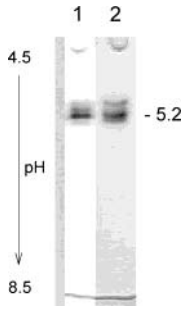

Figure 6. IEF of the soluble fraction (12.5 μg) (lane 1) and the membrane fraction (21.7 μg) (lane 2). The gel staining to detect PPO isoforms was carried out with tyramine and MBTH as indicated under Materials and Methods.

Table 2. Kinetic Parameters for PPO from Beet Root[a]

Fraction	SDS	*K*m (mM)	Vm (mM/min)	Vm/*K*m (min^{-1})
soluble	+[b]	2.01	0.23	0.114
	–[b]	2.45	0.11	0.0476
membrane	+[b]	1.96	0.085	0.0434
	–[c]	6.18	0.050	0.00809

[a–c] All assays were performed in a reaction media containing different L-dopa concentrations and 12.4 μg/mL of protein in 50 mM sodium phosphate buffer, pH 6.0 [b] or sodium acetate buffer, pH 4.5 [c], at 25 °C. When SDS was present in the medium, the final concentration was 0.69 mM.

results have also shown a single active band from beet root (*26*). Söderhäll (*39*), using carrot PPO, also detected a 36 kDa band showing PPO activity, while under denaturing conditions, a band at 59 kDa was revealed.

The partially purified PPO was analyzed by IEF (**Figure 6**) and presented a major acidic band of pH 5.2 in both the soluble (lane 1) and the membrane (lane 2) fractions. Furthermore, two active PPO isoforms were detected in the pH range of 5.0 and 5.5. The high variety of isoforms for PPO has been described by other authors. Several acidic PPO isoenzymes have also been detected from soluble and membrane fractions from beet root (*26*). Flurkey identified several isoenzymes of PPO in mushrooms in a pH range from 4 to 4.8 (*41*). When PPO was purified from chloroplasts of broad bean leaves to apparent homogeneity and it was subjected to native IEF, several isoforms were present with apparent pH values from 4.9 to 5.9, and all of these isoforms shared a 65 kDa protein (*42*). Two acidic PPO isoforms were detected from red beet hypocotyls (*23*) and two tyrosinase isoforms with pHvalues of 5.1 and 5.2 were isolated by Wichers et al. (*43*) from A. *Bisporus*.

Kinetic Studies. Kinetic characterization of PPO was carried out on the natural substrate, L-dopa, that is found in beet root and has been proposed as the substrate of PPO in the betalains biosynthesis (*44*). To study whether the activation with SDS introduces a change in K_m and V_m values, the kinetic parameters were determined in the absence and presence of SDS at the pH value (pH 6.0) in which the degree of activation was higher. The results are summarized in **Table 2**. As can be observed for the soluble fraction, the activation with SDS introduces a change in V_m values, but the K_m value is quite similar, increasing the catalytic power (V_m/ K_m) almost three times. For the membrane fraction, the kinetic parameters were determined in the presence of SDS at pH 6.0. As can be observed in **Table 2**, under these conditions, the K_m value was quite similar to the one evaluated for the soluble fraction, but the catalytic power was 2.6-fold higher for the soluble PPO than for the membrane fraction. For the membrane fraction, PPO activity was almost undetectable

in the absence of SDS at pH 6.0; therefore, the kinetic parameters could not be evaluated at these conditions. Therefore, in the absence of SDS, the kinetic constants were evaluated at pH 4.5, at which the enzyme is activated by acidic pH. The K_m value (6.18 mM) obtained at pH 4.5, in absence of SDS, was much higher than the one obtained for the enzyme activated with SDS at pH 6.0 (1.9 mM). Steiner et al.[23] determined a similar K_m value (2.4 mM) for L-dopa from betacyanin-producing callus of *P. grandiflora*.

ABBREVIATIONS USED

PPO, polyphenol oxidase; 4MN, 4-methoxy-α-naphthol; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PMSF, phenylmethanesulfonyl fluoride; MeOH, methanol; ISO, isopropyl

ACKNOWLEDGMENT

We are indebted to Dr. William H. Flurkey (Indiana State University) for generous gifts of polyclonal antibodies against a broad bean polyphenol oxidase.

LITERATURE CITED

(1) Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.

(2) Golbeck, J. H.; Cammarata, K. V. Spinach thylakoid polyphenoloxidase. Isolation, activation, and properties of native chloroplast enzyme. *Plant Physiol.* **1981**, *67*, 977−984.

(3) Chazarra, S.; Cabanes, J.; Escribano, J.; García-Carmona, F. Partial purification and characterization of latent polyphenol oxidase in iceberg lettuce (*Lactuca sativa* L.) *J. Agric. Food Chem.* **1996**, *44*, 984−988.

(4) Jimenez, M.; García-Carmona, F. The effect of sodium dodecyl sulphate on polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1503−1509.

(5) Escribano, J.; Cabanes, J.; García-Carmona, F. Characterization of latent polyphenol oxidase in table beet: effect of sodium dodecyl sulphate. *J. Sci. Food Agric*. **1997a**, *73*, 34−38.

(6) Robinson, S. P.; Dry, I. B. Broad bean leaf polyphenol oxidase is a 60-kDa protein susceptible to proteolytic cleavage. *Plant Physiol.* **1992**, *99*, 317−323.

(7) Mayer, A. M.; Friend, J. Localization and nature of phenolase in sugar-beet leaves. *J. Exp. Bot.* **1960**, *11*, 141−150.

(8) Mayer, A. M.; Harel E. Phenoloxidases and their significance in fruit and vegetables. In *Food Enzymology*; Fox P. F., Ed.; Elsevier: London, 1991; Vol. 1, pp 373−398.

(9) Martinez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.

(10) Whitaker, J. R. Polyphenol oxidase. In *Food Enzymes-Structure and Mechanism*; Wong, D. W. S., Ed.; Chapman and Hall: New York, 1995; pp 271−307.

(11) Zawistowski, J.; Biliaderis, C. G.; Eskin, N. A. M. Polyphenol oxidase. In *Oxidative Enzymes in Foods*; Robinson, D. S., Eskin, N. A. M., Eds.; Elsevier: London, 1991; pp 217−273.

(12) Nicolas, J. J.; Richard-Forget, F. C.; Goupy P. M.; Amiot M. J.; Aubert S. Y. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 109−157.

(13) Flurkey, W. H. In vitro biosynthesis of *Vicia Faba* polyphenol oxidase. *Plant Physiol.* **1985**, *79*, 564−567.

(14) Yu, H.; Kowalski, S. P.; Steffens, J. C. Comparison of polyphenol oxidase expression in glandular trichomes of Solanum and Lycopersicon species. *Plant Physiol.* **1992**, *100*, 1885−1890.

(15) Yang C. P.; Fujita, S.; Ashrafuzzaman, MD.; Nakamura, N.; Hayashi, N. Purification and characterization of polyhenol oxidase from banana (*Musa sapientum* L.) pulp. *J. Agric. Food Chem.* **2000**, *48*, 2732−2735.

(16) Fujita, S.; Saari, N.; Maegawa, M.; Tetsuka, T.; Hayashi, N.; Tono, T. Purification and properties of polyphenol oxidase from cabbage (*Brassica oleracea* L.). *J. Agric. Food Chem.* **2000**, *43*, 1138−1142.

(17) Chazarra, S.; García-Carmona, F.; Cabanes, J. Evidence for a tetrameric form of Iceberg Lettuce (*Lactuca Sativa* L.) polyphenol oxidase: Purification and Characterization. *J. Agric. Food Chem.* **2001**, *49*, 4870−4875.

(18) Gooding, P. S.; Bird, C.; Robinson, S. P. Molecular cloning and characterization of banana fruit polyphenol oxidase. *Planta* **2001**, *213*, 748−757.

(19) Lax, A. R.; Cary, J. W. Biology and molecular biology of polyphenoloxidase. In *Enzymatic Browning and Its Prevention*; Lee, Ch., Whitaker, J. R., Eds.; ACS Symposium Series 600, American Chemical Society: Washington, DC, 1995; pp 120−128.

(20) Vaughn, K. C.; Duke, S. O. Function of polyphenol oxidase in higher plants. *Physiol. Plant.* **1984**, *60*, 106−112.

(21) Vaughn, K. C.; Lax, A. R.; Duke, S. O. Polyphenol oxidase: the chloroplast oxidase with no established function. *Physiol. Plant.* **1988**, *72*, 659−665.

(22) Constabel, C. P.; Bergey, D. R.; Ryan, C. A. Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, 407−411.

(23) Steiner, U.; Schliemann, W.; Böhm, H.; Strack, D. Tyrosinase involved in betalain biosynthesis of higher plants. *Planta* **1999**, *208*, 114−124.

(24) Mueller, L. A.; Hinz, U.; Zrÿd, J. P. Characterization of a tyrosinase from *Amanita muscaria* involved in betalain biosynthesis. *Phytochemistry* **1996**, *42*, 1511−1515.

(25) Escribano, J.; Cabanes, J.; Chazarra, S.; García-Carmona, F. Characterization of monophenolase activity of table beet Polyphenol Oxidase. Determination of kinetic parameters on the tyramine/dopamine pair. *J. Agric. Food Chem.* **1997b**, *45*, 4209−4214.

(26) Escribano, J.; Gandía-Herrero, F.; Caballero, N.; Pedreño, M. A. Subcellular localization and isoenzyme pattern of peroxidase and polyphenol oxidase in beet root (*Beta vulgaris* L.). *J. Agric. Food Chem.* **2002**, *50*, 6123−6129.

(27) Cabanes, J.; García-Canovas, F.; Lozano, J. A.; García-Carmona, F. A kinetic study of the melanization pathway between L-tyrosine and dopachrome. *Biochim. Biophys. Acta* **1987**, *790*, 101−107.

(28) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(29) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(30) Rodríguez-López, J. N.; Escribano, J.; García-Cánovas, F. A continuous spectrophotometric method for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. *Anal. Biochem.* **1994**, *216*, 205−212.

(31) Kenten, R. H. Latent phenolase in extracts of broad bean (*Vicia faba* L.) 1. Activation by acid and alkali. *Biochem. J.* **1957**, *67*, 300−307.

(32) Valero, E.; García-Carmona, F. pH-induced kinetic cooperativity of a thylakoid-bound polyphenol oxidase. *Biochem J.* **1992**, *286*, 623−626.

(33) Moore, B. M.; Flurkey, W. H. Sodium Dodecyl Sulfate Activation of a Plant Polyphenoloxidase. Effect of sodium dodecyl sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. *J. Biol. Chem.* **1990**, *265*, 4982−4990.

(34) Lee, C. Y.; Smith, N. L. Blanching effect on polyphenol oxidase activity in table beets. *J. Food Sci*. **1979**, *44*, 82−86.

(35) Im, J.; Parkin, K. L.; von Elbe, J. H. Endogenous polyphenoloxidase activity associated with the "black ring" defect in canned beet (*Beta vulgaris* L.) root slices. *J. Food Sci*. **1990**, *55*, 1042−1059.

(36) Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. Substrate-dependent activation of latent potato leaf polyphenol oxidase by anionic surfactants. *J. Agric. Food Chem.* **1993**, *41*, 1583−1586.
(37) Jimenez, M.; García-Carmona, F. Kinetics of the slow pH-mediated transition of polyphenol oxidase. *Arch. Biochem. Biophys.* **1996**, *331*, 15−22.
(38) Sojo, M. M.; Núñez-Delicado, E.; García-Carmona, F.; Sánchez-Ferrer, A. Partial purification of a Banana Polyphenol Oxidase using Triton X-114 and PEG 8000 for Removal of Polyphenols. *J. Agric. Food Chem.* **1998**, *46*, 4924−4930.
(39) Söderhäll, I. Properties of carrot polyphenoloxidase. *Phytochemistry* **1995**, *39*, 33−38.
(40) Shin, R.; Froderman, T.; Flurkey, W. H. Isolation and characterization of a mung bean leaf polyphenol oxidase. *Phytochemistry* **1997**, *45*, 15−21.
(41) Flurkey, W. H. Identification of tyrosinase in mushrooms by isoelectric focusing. *J. Food Sci.* **1991**, *56(1)*, 93−95.
(42) Ganesa, Ch.; Fox, M. T.; Flurkey, W. H. Microheterogeneity in purified broad bean polyphenol oxidase. *Plant Physiol.* **1992**, 472−479.
(43) Wichers, H.; Gerritsen, Y.; Chapelon, C. Tyrosinase isoforms from the fruitbodies of *Agaricus bisporus*. *Phytochemistry* **1996**, *43*, 333−337.
(44) Strack, D.; Vogt, T.; Schliemann, W. Recent advances in betalain research. *Phytochemistry* **2003**, *62*, 247−269.

Received for review April 14, 2003. Revised manuscript received December 2, 2003. Accepted December 8, 2003. Financial support provided in part by two grants from MCYT (AGL2003-05647) and the Fundación Séneca, Consejería de Agricultura, Agua y Medio Ambiente y FEDER (Project AGR/11/FS/02), Spain. F. Gandía holds a fellowship from the Fundación Séneca (Spain).

JF034381M