Evidence for a Tetrameric Form of Iceberg Lettuce (*Lactuca sativa* L.) Polyphenol Oxidase: Purification and Characterization

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Polyphenol oxidase from iceberg lettuce (*Lactuca sativa* L.) chloroplasts was released from the thylakoid-membrane by sonication, and it was extensively purified to homogeneity as judged by SDS−PAGE. Purification was achieved by ammonium sulfate fractionation, gel-filtration chromatography, and ion-exchange chromatography. Two molecular forms were separated by gel-filtration chromatography with apparent molecular masses of 188 and 49 kDa. Both forms were characterized by sedimentation analysis with $S_{20,W}$ values of 10.2 and 4.1 S, respectively. For the high-molecular-weight form purified to homogeneity, denaturing SDS−PAGE indicated a molecular mass of 60 kDa. Thus, from these data we suggest that lettuce polyphenol oxidase is a tetramer of identical subunits.

Keywords: *Polyphenol oxidase; lettuce; purification*

INTRODUCTION

Polyphenol oxidase (PPO, EC 1.14.18.1) is an enzyme widely distributed in the plant kingdom and has been detected in most fruits and vegetables. In plants, PPO is predominantly located in the chloroplast thylakoid membranes (*1*−*3*). However, it is not an intrinsic membrane protein and can be released from the thylakoids by sonication, mild detergent treatment, or protease treatment (*4*). The enzyme has also been detected in soluble fractions in homogenates from different vegetables (*5*−*8*).

PPO is a copper protein that catalyses two different reactions by using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity). Because its *o*-quinone products undergo subsequent reactions leading to dark-colored pigments (*9*, *10*), PPO has been held responsible for browning of damaged plant tissues. The enzyme is localized in chloroplast, and its phenolic substrates are mainly located in the vacuole, but upon any cell-damaging treatment, the enzyme and substrates may come into contact, permitting rapid oxidation of phenols. Because of the considerable economic and nutritional loss induced by enzymatic browning in the commercial production of fruit and vegetables, numerous studies have been devoted to the biochemical and catalytic properties of PPO (*11*, *12*). Information on molecular and catalytic properties of PPO is of importance in studies of the regulation of tissue browning.

Plant PPOs have been extensively studied since their initial discovery by Bertrand (*13*, *14*). Despite their long history, relatively little is known about their structure. The enzyme has often been found in multiple forms. Enzyme multiplicity has been attributed to one or more processes such as polymerization (*15*), glycosylation (*16*), proteolysis (*4*, *17*), and disulfide bonding (*18*). These PPO isoforms are a significant factor in the high and varying estimates of the number and size of the polypeptides which constitute higher plant PPO.

There are few studies that refer to iceberg lettuce PPO. The soluble enzyme has been studied by Fujita et al. (*7*) and Heimdal et al. (*19*). Fujita et al. (*7*) described a molecular mass of 56 kDa, determined by size-exclusion chromatography. However, little information is available about the thylakoid-bound enzyme. This form has been described only by us, along with the facts that this PPO is in latent state (*2*), suffers suicide inactivation (*20*), and displays monophenolase activity (*21*). The structure of this enzyme has not been described previously. Thus, the purpose of this study was the purification and structural characterization of thylakoid-bound PPO from iceberg lettuce. We report its purification to apparent homogeneity using several chromatographic procedures, and its characterization through electrophoresis, gel-filtration chromatography, and sedimentation analysis.

MATERIALS AND METHODS

Materials. Fresh iceberg lettuce (*Lactuca sativa* L.) was obtained from a local market in Murcia (Spain). SDS, Sephacryl HR S-200, SDS−PAGE molecular weight markers, and gel filtration markers were purchased from Sigma Chemical Co. (Madrid, Spain). The Bradford reactive, bovine serum albumin, and reagents for electrophoresis were procured from Bio-Rad Laboratories (Barcelona, Spain). 4-*tert*-Butylcatechol (4tBC) was obtained from Fluka (Madrid, Spain). All the other reagents were of analytical grade.

Purification. *Enzyme Extraction.* Lettuce leaves (100 g) were homogenized in 50 mL of 100 mM sodium phosphate buffer, pH 7.3, containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and serine protease inhibitors (1 mM phenylmethyl sulfonylfluoride and 1 mM benzamidine). The serine protease inhibitors were added immediately before use. The homogenate was filtered through 8 layers of gauze and centrifuged at 20000*g* for 30 min. The pellet, consisting mainly of chloroplast, was resuspended and lysed with 40 mL of 100 mM sodium phosphate buffer pH 7.3 for 30 min and sonicated for 5 min.

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Table 1. Summary of Iceberg Lettuce PPO Purification[a]

purification step	protein (mg)	specific activity (units/mg protein)	total activity (units)	purification (fold)	yield (%)	active/latent PPO (%)
initial extract	7.602	29.02	220.62	1	100	11.1/88.9
$(NH_4)_2SO_4$	5.601	34.90	195.47	1.2	88.6	10.6/89.4
Sephacryl HR S200	0.618	157.15	97.12	5.4	44.0	5.8/94.2
first DEAE	0.053	1301.13	68.96	44.8	31.3	1.6/98.4

[a] Assayed with 5 mM 4tBC and 0.7 mM SDS in 50 mM sodium phosphate buffer pH 6.5.

The sonicated chloroplasts were centrifuged at 120000*g* for 30 min and the sediment was discarded.

Ammonium Sulfate Fractionation. The supernatant from enzyme extraction was brought to 30% saturation of $(NH_4)_2SO_4$ and kept at 4 °C under continuous agitation for 1 h. The solution was centrifuged at 120000*g* for 30 min. The resulting supernatant was then brought to 65% saturation of $(NH_4)_2SO_4$ and kept overnight at 4 °C under continuous stirring. The solution was centrifuged at 120000*g* for 30 min, and the precipitate was dissolved in 2 mL of 100 mM sodium phosphate buffer, pH 7.3.

Chromatography on Sephacryl HR S-200. The concentrate fraction was loaded onto a 2.5 × 100 cm column of Sephacryl HR S-200 equilibrated with 100 mM sodium phosphate buffer, pH 7.3, containing 150 mM KCl. PPO was eluted with the same buffer at a flow rate of 1 mL/min. The eluate was collected in 3-mL fractions and PPO activity was measured. The column was calibrated using cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Elution volume was determined for each protein by absorbance measured at 280 nm.

Anion Exchange Chromatography. The major peaks' fractions from the Sephacryl column were pooled, dialyzed overnight against 20 mM sodium phosphate buffer, pH 6, and then concentrated using a Millipore ultrafiltration membrane (Ultrafree 15, PM-10). This fraction was applied to a diethylaminoethyl (DEAE)-Mensep 1000 (prepacked chromatography cartridge with a bed volume of 1.4 mL of Millipore) equilibrated with 20 mM sodium phosphate buffer, pH 6. The column was washed with the same buffer, and the sample was eluted with different gradients of KCl in this buffer at a flow rate of 1 mL/min. Active fractions were concentrated by ultrafiltration and then diafiltered with the same membrane against three changes of 20 mM sodium phosphate buffer, pH 6. Each PPO form peak was collected separately.

The major peak's fractions were loaded onto a quaternarymethylamine (QMA)-Mensep 1000 (prepacked chromatography cartridge with a bed volume of 1.4 mL of Millipore) equilibrated with 20 mM sodium phosphate buffer, pH 6. The column was washed with the same buffer, and then PPO was eluted with 200 mM KCl in this buffer. The active fractions from QMA-Mensep column were pooled, concentrated by ultrafiltration, and also diafiltered with three changes of 20 mM sodium phosphate buffer, pH 6.

This fraction was rechromatographed on the same column of DEAE-Mensep and run under conditions identical to those described above. The eluted proteins were monitored by measuring absorbance at 280 nm.

Enzyme Assay. The catecholase activity of the enzyme was determined at 25 °C by spectrophotometrically monitoring at 400 nm the appearance of the 4-*tert*-butyl-*o*-benzoquinone product of the reaction (1150 $M^{-1}cm^{-1}$) (*22*). The reaction medium contained 50 mM sodium phosphate buffer, pH 6.5, 5 mM 4tBC, and 0.7 mM SDS. In the latency assays SDS was not added. One unit of enzyme was defined as the amount of enzyme that produces 1 μmol *o*-benzoquinone/min.

Sedimentation Analyses. Sedimentation analyses of the molecular forms of PPO were performed on 5% to 20% (w/v) linear sucrose gradients prepared in 100 mM sodium phosphate buffer, pH 7.3, free of detergent, with added Triton X-100 (0.5% w/v) or with Brij-96 (0.5% w/v). Calculation of the sedimentation coefficient was made according to Martin and Ames (*23*) using catalase (11.4 $s_{20,W}$) and phosphatase alkaline (6.1 $s_{20,W}$) as internal markers. Samples and marker enzymes were layered on top of the gradients and centrifuged in a SW 41 Ti Beckman rotor (Fullerton, CA) at 165000*g* for 18 h at 4 °C. About 40 fractions were collected and assayed for PPO and enzyme marker activities.

Electrophoresis. Denaturing SDS−PAGE was carried out using the classical discontinuous gel system (*24*) in a Bio-Rad Miniprotean cell. The samples were diluted with a sample buffer containing 0.5 M Tris−HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) mercaptoethanol, and 0.01% (w/v) bromophenol blue, and heated to 95 °C for 10 min. Denatured proteins were run on 10% polyacrylamide gels. The gels were stained for protein using the sensitive silver stain. For molecular mass determination, we used the calibration kit of SDS−PAGE (SDS-6H) from Sigma.

Protein Determination. The protein content of the enzyme preparation was determined according to the Bradford Bio-Rad protein assay using bovine serum albumin as standard (*25*).

RESULTS AND DISCUSSION

Structure Determination. Chloroplasts were isolated from lettuce, lysed, and sonicated to release PPO. The enzyme was purified to homogeneity and was found latent. Activity measures were made in the presence of SDS. The existence of latent PPO forms has been shown in several species, including lettuce (*2*, *20*); in addition to activation by SDS, PPO can be released from latency by a variety of treatments or agents including acid and base shock (*26*), urea (*27*), polyamines (*28*), proteases (*29*), and fatty acids (*1*).

The results of the PPO purification are summarized in Table 1. The 30% to 65% ammonium sulfate fractionation resulted in an 88% recovery of PPO activity with a 1.2-fold purification, in agreement with literature data for PPO from other sources (*1*, *30, 31*). Thus, this procedure was principally used for concentration. The concentrated PPO was then purified by gel filtration chromatography on Sephacryl HR S-200, and two peaks of activity were eluted (Figure 1). Most of the PPO activity appeared in a high-molecular-weight fraction which contained 50% of the applied enzyme, whereas a small amount of activity eluted in a lower-molecular-weight fraction, which contained 3.5% of the applied enzyme. The apparent molecular masses of the eluted forms of lettuce PPO were found to be 188 kDa for the major peak and 49 kDa for the minor one (Figure 1; inset). These data suggest that lettuce PPO could be a tetrameric form. In the bibliography, evidence for a tetrameric plant PPO has been obtained only for spinach PPO (*1*, *32*). Golbeck and Cammarata (*1*) described both the tetrameric and the monomeric forms of spinach PPO by gel filtration on Sephacryl HR S-200, and suggested the existence of a slow monomer−multimer interconversion. To investigate this interconversion in lettuce PPO, the tetramer obtained by gel filtration was submitted to another chromatography on Sephacryl HR S-200, after being thawed from frozen, concentrated, dialyzed, and kept at 4 °C for 1 day. In these conditions, the tetrameric form was the only one identified. There-

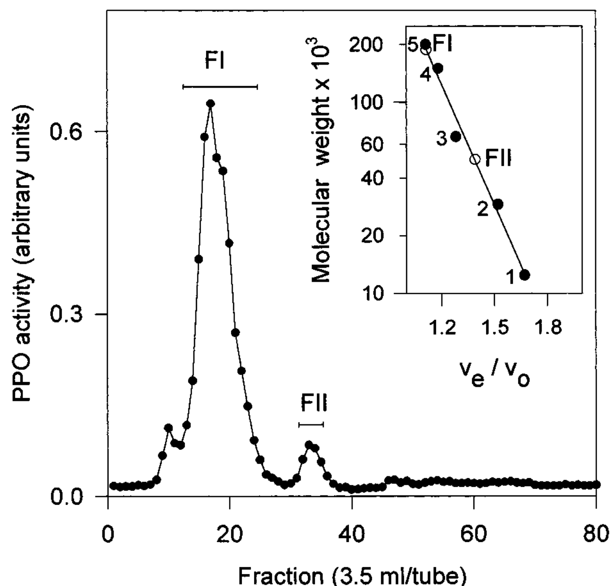

Figure 1. Elution profile of PPO activity on Sephacryl HR S-200. Inset: Molecular weight estimation on Sephacryl HR S-200. The calibration curve was determined using cytochrome C (12.4 kDa, **1**), carbonic anhydrase (29 kDa, **2**), bovine albumin (66 kDa, **3**), alcohol dehydrogenase (150 kDa, **4**), and β-amylase (200 kDa, **5**). The closed circles indicate protein markers and the open ones indicate PPO.

fore, it appears that lettuce PPO does not undergo considerable monomer−multimer interconversions.

To support and complete these results, the tetrameric and monomeric forms, separated initially by the chromatography on Sephacryl HR S-200, were characterized by sedimentation analysis in sucrose gradients. Figure 2 A shows the sedimentation profile obtained for the high-molecular-weight form, which revealed the occurrence of a single component of 10.2 S. This $S_{20,W}$ value, under nondissociating conditions, would be compatible with a protein of molecular weight around 188. Similarly, the value of 4.1 S obtained for the low-molecular-weight form (Figure 2 B) would be in agreement with the native molecular mass of this form, estimated by gel filtration. Furthermore, the sedimentation coefficient of 4.1 S obtained for the low-molecular-weight form was very similar to that obtained for PPO from broad bean (*27*) and a little higher than the $S_{20,W}$ value described for spinach beet PPO (*33*), both enzymes having been described as monomers. Therefore, these results are in accordance with previous data obtained in experiments performed by gel filtration chromatography, and they support the tetrameric character suggested for lettuce PPO. Furthermore, in these sedimentation experiments no monomer−multimer interconversions have been observed.

The sedimentation studies were performed in gradients made without detergent, with added Triton X-114 (0.5% w/v), or with Brij 96 (0.5% w/v). A comparison of the sedimentation profiles obtained revealed the absence of differences in the sedimentation values in the gradients (results not shown), which is a property of hydrophilic form and suggests that PPO forms are indeed hydrophilic molecules. These results support that PPO is not an intrinsic membrane protein (*4*, *34*), and they are in accordance with the feature that PPO can be purified by temperature-induced phase separation with the detergent Triton X-114 (*34*); with the enzyme

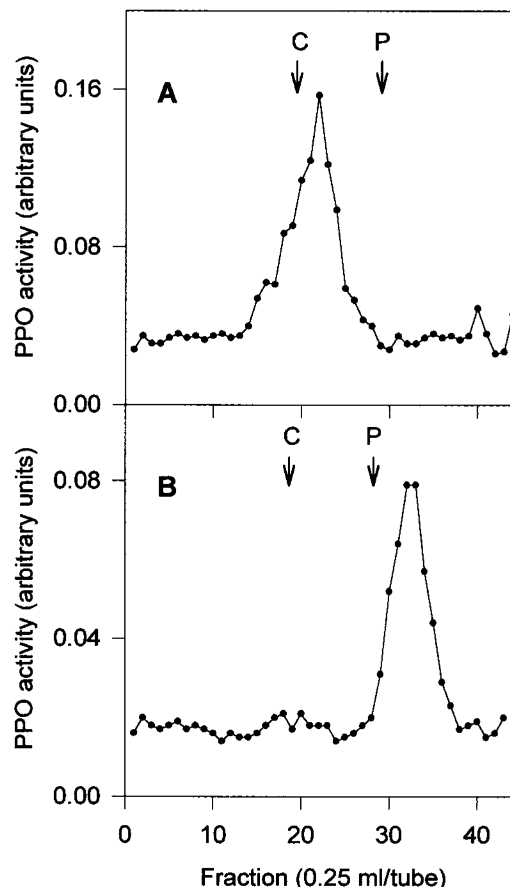

Figure 2. Sedimentation profiles of molecular weight forms obtained by chromatography on Sephacryl HR S-200. (A) High-molecular-weight form; (B) low-molecular-weight form. Samples were layered onto a continuous sucrose gradient (5−20%). Internal markers were catalase (C) and phosphatase alkaline (P).

being recovered from the aqueous upper phase, after phase partitioning, together with the hydrophilic molecules.

Purification to Homogeneity. Assuming that the amount of monomeric form recovered after the gel filtration chromatography was rather low, only the tetrameric form was purified to homogeneity. In the first place, the sample was fractionated by anion exchange chromatography on DEAE-Mensep which resolved two peaks of PPO activity (Figure 3 A): a small one eluted at approximately 250 mM KCl and a larger one near 150 mM KCl which contained 70% of the applied enzyme. Similar results were described by Golbeck and Cammarata (*1*) for spinach PPO.

For the major peak, eluted at 150 mM KCl, the chromatography on DEAE-Mensep improved the purification factor to a larger extent. The specific activity of this PPO fraction was 1301 U/mg protein with a 45-fold purification (Table 1). However, for the minor peak, eluted at 250 mM KCl, the purification was smaller (Figure 3 A) and resulted in very low yields, recovering only 7.5% of PPO activity with respect to the applied enzyme.

To determine the achieved purity, the two peaks of PPO activity obtained by chromatography on DEAE-Mensep were analyzed by denaturing SDS−PAGE. When the fractions eluted at 150 mM KCl were combined and dialyzed, they showed two major bands of protein on SDS−PAGE (Figure 4 A) (one in the range

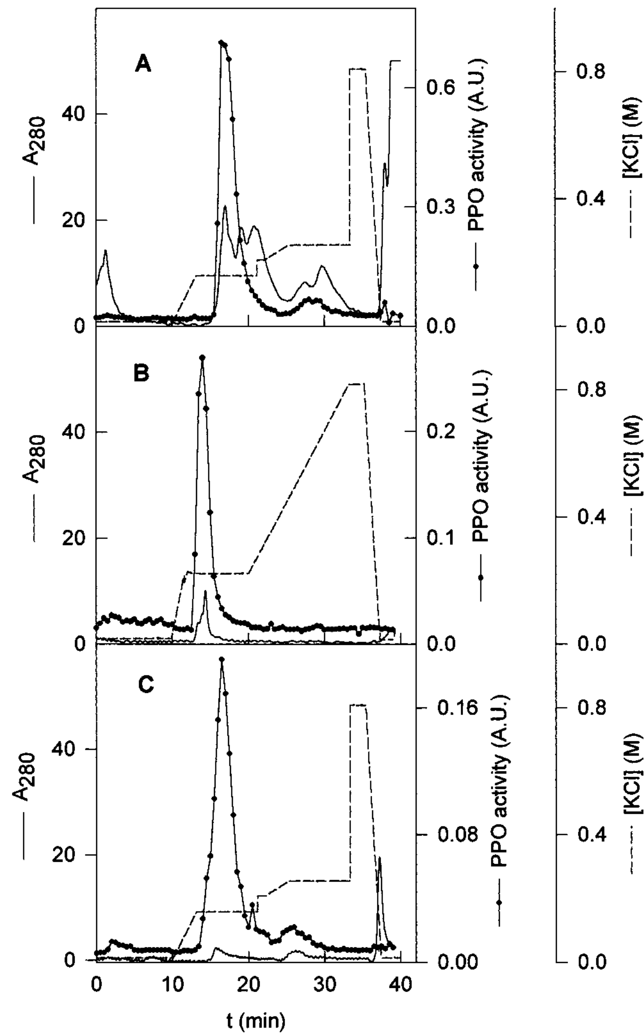

Figure 3. Purification of iceberg lettuce PPO by anion-exchange chromatography. (A) First DEAE chromatography. (B) Chromatography on QMA of PPO fraction eluted at 150 mM KCl from the first DEAE chromatography. (C) DEAE chromatography of PPO fraction eluted from the QMA column. (−) Absorbance at 280 nm; (-●-) PPO activity; (- - -) KCl concentration.

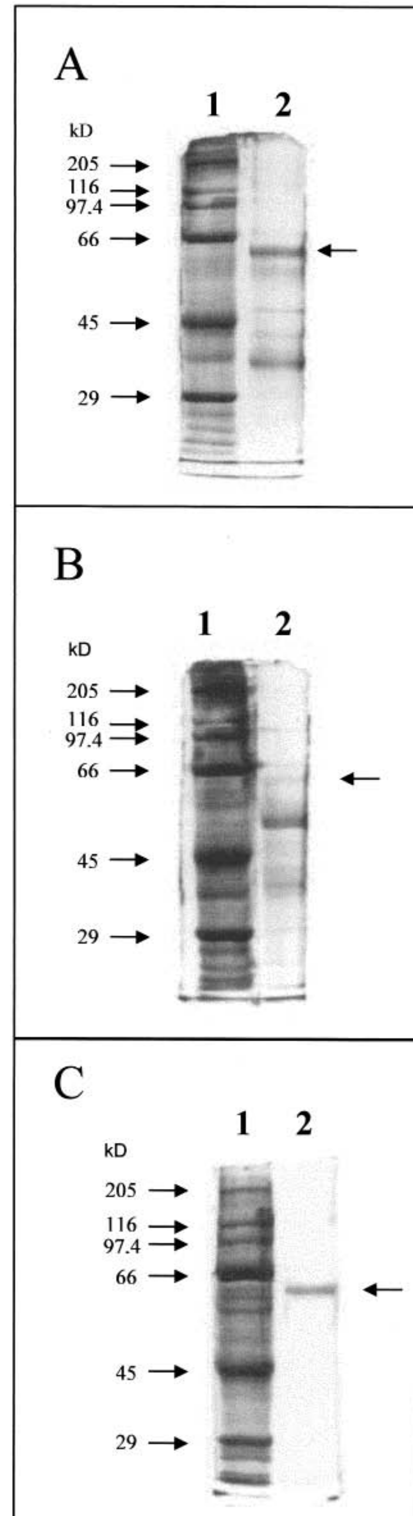

Figure 4. Denaturing SDS−PAGE of samples from the purification of PPO. (A) PPO fraction eluted at 150 mM KCl from the first DEAE separation. (B) PPO fraction eluted at 250 mM KCl from the first DEAE separation. (C) Purified PPO (PPO fraction eluted at 150 mM KCl from the second DEAE separation). Lane 1 contained molecular mass markers of carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa). Lane 2, arrows indicate the PPO position (C) or the band of protein that appears in the range of molecular masses corresponding to PPO (around 60 kDa) (A, B).

of the molecular mass obtained for the enzyme purified to apparent homogeneity, Figure 4 C). The fraction eluted at 250 mM KCl was also combined, dialyzed, and separated by SDS−PAGE, but in this case the band corresponding to a molecular mass around 60 kDa was very faint (Figure 4 B). These results would be in accordance with the profiles observed for each peak in Figure 3 A, where a PPO peak of high specific activity eluted at 150 mM KCl and a peak of lower specific activity eluted at 250 mM KCl.

A relevant result not described in the bibliography was observed: the diminution during the purification process of the percentage of the active form of PPO, while the percentage of the latent form increased (Table 1). These results suggest that the enzyme could occur in two conformational states. These are not due to enzyme association−dissociation, as both the monomer and the tetramer showed latency. Thus, the purification process would probably eliminate an isoform or a small percentage of the same enzyme that could be being modified during the extraction process.

To achieve a purification to homogeneity, the peak eluted at 150 mM KCl was concentrated and submitted to anion exchange chromatography on QMA-Mensep. The obtained chromatography profile is shown in

Figure 3 B, and as can be seen, a single peak with PPO activity was eluted at 200 mM KCl. Analysis of this peak by denaturing SDS−PAGE revealed that the peak consisted of more than a single band of protein (results not shown). So this peak was then purified by another chromatography on DEAE-Mensep. As in the first DEAE-Mensep chromatography, two peaks with PPO activity were obtained: most of the PPO activity appeared at approximately 150 mM KCl and a small amount of activity eluted near 250 mM KCl (Figure 3 C).

Because of the problem of low yields, a frequent occurrence described in the PPO purification (*1*, *4*, *30*, *35*), the two last chromatographies are not summarized in Table 1, and were made only to achieve purification to homogeneity in order to determine the molecular mass.

The enzyme eluted at 150 mM KCl was subjected to denaturing SDS−PAGE. 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 (Figure 4 C).

The native molecular masses of the PPO forms, obtained by gel filtration on Sephacryl HR S-200 column, were 188 and 49 kDa, respectively. However, the apparent molecular mass, determined by SDS−PAGE, was 60 kDa. Results have shown that the value of molecular mass for the subunit, revealed by SDS−PAGE (60 kDa), was higher than the one obtained by gel filtration chromatography (49 kDa).

It has been shown that PPO is synthesized as a 60 to 65 kDa protein (*4*, *36*−*39*), which can be converted to a 40−45 kDa form by proteolysis as has been found in peaches, apricots, almonds, plums, and cherries (*17*). Thus, PPO has been purified to apparent homogeneity from several species and a molecular mass of 45 kDa has been frequently reported (*1*, *32*, *33*, *40*, *41*).

On the other hand, Robinson and Dry (*4*) and Cary et al. (*37*) observed a 45 kDa faba bean PPO form under partially denaturing conditions, and they reported that this form was converted to a 63 kDa one with full denaturation. It appears that reduction of the protein sample before electrophoresis was inadequate, leaving presumed intramolecular disulfide bridges intact and thereby preventing an accurate molecular mass estimation. Thus, it is possible that the observed difference in the values of molecular mass for the subunit of lettuce PPO in this work (60 kDa and 49 kDa) could be explained for this last hypothesis. Therefore, the 49-kDa form observed by gel filtration could be the non-denatured form.

The purified enzyme was in its latent state, and once activated by SDS or by acid shock, its kinetic behavior was similar to that observed for nonpurified iceberg lettuce PPO (*2*).

ABBREVIATIONS USED

Brij 96, polyoxyethylene-10-oleyl ether; DEAE, diethylaminoethyl; QMA, quaternarymethylamine; PPO, polyphenol oxidase; 4tBC, 4-*tert*-butylcatechol.

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