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Subcellular Localization and Isoenzyme Pattern of Peroxidase and Polyphenol Oxidase in Beet Root (*Beta vulgaris* L.)

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The two enzymes involved in enzymatic browning reactions, polyphenol oxidase (PPO) and peroxidase (PO), have been partially purified and extracted from different fractions of beet root. PPO is mainly located in the membrane fraction, and it was also found in the soluble fraction. In both cases PPO was in its latent state. However, PO activity was higher in the soluble fraction than in the membrane fraction. Nevertheless, the highest values of specific activity for PO were obtained from the solubilized enzyme from acetone powders. Under native isoelectric focusing (IEF), several PPO isoenzymes were present in the pH range of 4.8−5.8. All of these isoenzymes shared a single band with a similar apparent mass under sodium dodecyl sulfate−polyacrylamide gel electrophoresis. PO was also analyzed by IEF, showing a complex isoenzyme pattern in all fractions. The characteristic basic PO isoenzyme of high p*I* found in both the soluble fraction and the solubilized enzyme from acetone powders was not detected in the membrane fraction. The kinetic characterization of PPO and PO from all fractions was carried out.

KEYWORDS: Peroxidase; polyphenol oxidase; *Beta vulgaris* L.; isoenzyme pattern

INTRODUCTION

Fruits and vegetables are very susceptible to undesirable alterations as a consequence of injuries suffered during storage, handling, and processing (*1*). Among such alterations, changes in texture, flavor, and color, which decrease the market value of the product, can lead to substantial economic loss. From among the organoleptic properties that determine a food's acceptance by consumers, the appearance is the most important, and the color is the main characteristic of this property (*2*).

One of the most important causes of color alterations is due to either formation or degradation of pigmented compounds usually present in the produce. This phenomenon is mediated by endogenous enzymatic activities such as polyphenol oxidase (PPO) and peroxidase (PO). This process ultimately leads to the formation of dark brown polymers of a quinoidal nature (*3*).

Peroxidases (donor:H_2O_2 oxidoreductase; EC 1.11.1.7) constitute a group of glycoproteins the main function of which is the oxidation of different substrates at the expense of H_2O_2. Robinson (*4*) reviewed the physiological role of POs in postharvest fruits and vegetables and attributed many of the physiological functions to phenol oxidation. Thus, phenol oxidation mediated by PO is believed to be associated with deterioration in the flavor, color, texture, and nutritional qualities of processed foods and their products.

Polyphenol oxidase (monophenol, *o*-diphenol:oxygen oxidoreductase; EC 1.14.18.1) catalyzes 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) (*5*).

Enzymatic browning reactions resulting from injuries start when cells are damaged and phenolic compounds and one of these enzymes are mixed. The first event occurs when either PO or PPO reacts with a phenolic substrate to yield a colored quinone. Then, the reaction may continue, enzymatically or not, with the formation of products of increasing molecular weight and color density. There are data that support a direct cooperation between PO and PPO in the browning reaction and suggest the need of both enzymes for the browning reactions (*6*).

PPOs and POs do not exist as a single enzyme in fruits and vegetables; like many other plant enzymes, they are found in a number of enzymatic forms, which in turn can be detected by different electrophoretic methods. Although PPO and PO activities and the existence of their multiple forms are well documented in some fruits and vegetables (*4*, *7*), there are no comparative data on PPO and PO activities; neither are there data on their isoenzyme patterns in beet root.

POs consist of families of homologous isoenzymes that apparently have different substrates specificities and perform different physiological functions in plant cells (*8*). However, their exact relationship to developmental events is often obscured

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by their extensive polymorphism in a single plant species. Part of this polymorphism is introduced during post-translational processing, which can result in PO isoenzymes of different electrophoretic mobilities (*9*), some of which may be physiologically irrelevant (*10*). The problem is compounded by the apparent similar kinetic characteristics that different POs show with a variety of substrates in vitro (*11*), which therefore complicates the interpretation of the polymorphism observed. These problems complicate the selection of peroxidases associated with a specific function in plants for their purification and further studies.

Similarly, a wide variety of plant PPO behaviors have been described and reviewed (*12*, *13*). 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. Some discrepancies on the estimation of molecular masses may be related to artifacts occurring during extraction (*14*). Thus, a factor leading to disparate estimations of molecular mass has been attributed to proteolysis during isolation. 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 (*15*).

Over the past few years considerable information has been accumulated regarding the subcellular localization of PO isoenzymes. Thus, different PO isoenzymes have been associated with the soluble and bound fractions in the same tissue (*16*). Moreover, most of the studied plant materials, both basic and acidic PO isoforms, have been located in the cell wall free spaces, probably in equilibrium with those bound to cell walls; basic isoenzymes of pI >9.0 are the only ones located in vacuoles (*17*).

The localization of PPO in the plant cell depends on the species, age, and—in fruits or vegetables—on maturity (*18*). In green leaves, a considerable part of PPO activity is localized in the chloroplasts (*13*). Furthermore, PPO has been detected in the soluble fraction in different fruits and vegetables (*12*). In some species, for example, lettuce, broad beans, and sugarbeet leaves, the enzyme was present in a latent form in the membrane fraction (*19*−*21*). However, the membrane fraction from other sources such as potatoes and mushrooms did not exhibit latency. Thus, latency does not seem to be related to the localization of the enzyme in the cell (*22*). It has been described that during ripening, the concentration of the membrane enzyme decreased with simultaneous appearance of a soluble fraction (*13*).

Recently, it has been proposed that PPO is involved in the biosynthesis of betalains of higher plants (*23*). Related to the degradation of these pigments, a protein fraction with PO activity from *Beta vulgaris* L. roots oxidized betanidin, suggesting the involvement of this enzyme in the oxidation of this pigment (*24*). For this reason, both PPO and PO could be integrated in the betalain metabolism.

For a better understanding of the complex interrelations between PPO and PO and the oxidation of phenols, in the present paper we have carried out some studies on the detection of PPO and PO isoenzymes in the different subcellular fractions and the kinetic characterization of these enzymes in beet root.

MATERIALS AND METHODS

Plant Material. *B. vulgaris* L. roots were purchased in a local market, sliced, frozen in liquid nitrogen, and stored at −80 °C until use.

Methods. *Subcellular Fractionation.* Soluble and membrane fractions were obtained from 100 g of beet root slices, which were homogenized twice at maximum speed for 10 s in a model 230 Omnimixer (Sorvall Inc., Norwalk, CT) with 200 mL of 0.1 M sodium phosphate buffer (pH 7) 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 120000*g* for 40 min. Thus, the resultant supernatant was considered to be the soluble fraction and the pellet, the membrane fraction.

The extraction of both PPO and PO enzymes from the membrane fraction was carried out by using two parallel treatments: (1) sonication for 10 min in 0.1 M sodium phosphate buffer (pH 7); (2) salted treatment, incubation for 60 min in 0.1 M sodium phosphate buffer containing 1 M NaCl. These fractions were centrifuged at 120000*g* for 40 min to obtain, in the supernatant, the solubilized enzyme.

Acetone Powders. Another extraction method of PPO and PO was carried out with 100 g of beet root slices, which were homogenized by using an Omnimixer with cold acetone (1w:2v). The homogenate was then filtered under pressure. The residue was washed three times with 200 mL of 80% cold acetone, 200 mL of 50% cold acetone, and 200 mL of cold acetone, respectively. The acetone powders obtained were dried overnight at room temperature to remove residual acetone and frozen at −80 °C until use.

PO and PPO activities were assayed from 1 g of acetone powders, which were resuspended in 60 mL of 0.1 M sodium phosphate buffer at pH 7.0 containing 1 M NaCl and 0.1 M $CaCl_2$ and stirred overnight at 4 °C. The supernatant obtained from the centrifugation at 120000*g* for 40 min was considered to be the enzymatic fraction solubilized from the acetone powders.

Enzyme Partial Purification. The membrane and acetone powders fractions were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) overnight before their partial purification with $(NH_4)_2SO_4$ to remove the salt content. Each fraction was brought to 45% saturation with $(NH_4)_2SO_4$ and kept overnight under continuous stirring at 4 °C. Then, the solution was centrifuged at 120000*g* for 40 min at 4 °C, and the pellet was discarded. The supernatant was brought up to 85% saturation with $(NH_4)_2SO_4$ and stirred for 1 h at the same temperature. The precipitate obtained between 45 and 85% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 10 mM sodium phosphate buffer (pH 7.0). The salt content was removed by dialysis against 10 mM sodium phosphate buffer (pH 7.0). The solution thus obtained for each extract was used as the different enzyme fraction sources. To avoid any possible activation of the PPO enzyme by endogenous proteases, PMSF and benzamidine hydrochloride were added before and after dialysis to give a final concentration of 1 mM.

Enzyme Assays. *PPO Activity.* The diphenolase activity was determined spectrophotometrically by measuring the appearance of dopaminechrome at 480 nm (ϵ = 3300 M^{-1} cm^{-1}) (*25*). In this work, the international unit of enzyme activity (IU) was defined as the quantity of enzyme that produces 1 μmol of dopaminechrome per minute at pH 6.8 and 25 °C.

Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium phosphate buffer (pH 6.8), 0.69 mM SDS, and 5 mM dopamine.

PO Activity. Peroxidase activity was determined by using 4-methoxy-α-naphthol (4MN) and H_2O_2 as substrates (*26*). The enzymatic activity was determined spectrophotometrically by measuring the appearance of the dye product at 593 nm (ϵ_{593} = 21000 M^{-1} cm^{-1}). In this work, the international unit of enzyme activity (IU) was defined as the quantity of enzyme that produces 1 μmol of the dye product per minute at pH 5.0 and 25 °C.

Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium acetate buffer (pH 5.0), 0.45 mM H_2O_2, and 1 mM 4MN.

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 (*27*).

Table 1. Fractionation and Partial Purification of PPO and PO from Beet Roots

fraction	total volume (mL)	total protein (mg)	total activity (units)	specific activity (units/mg)	recovery (%)	purification (fold)
PPO enzyme						
soluble	203	134	120	0.9	100	1
ammonium sulfate 45–85%	9.5	34.2	69.1	2.02	57.6	2.24
membrane NaCl	11	1.02	10.2	9.9	100	1
ammonium sulfate 45–85%	2	0.21	7.43	35.4	73.1	3.57
acetone powder	50	1.88	4.86	2.6	100	1
ammonium sulfate 45–85%	5.8	0.7	4.24	6.01	87.2	2.3
PO enzyme						
soluble	203	134	172.8	1.29	100	1
ammonium sulfate 45–85%	9.5	34.2	63.6	1.86	36.8	1.44
membrane NaCl	11	1.02	0.69	0.67	100	1
ammonium sulfate 45–85%	2	0.21	0.29	1.39	42.5	2.07
acetone powder	50	1.88	15.13	8.04	100	1
ammonium sulfate 45–85%	5.8	0.7	7.42	10.6	49.1	1.32

Data Analysis. Kinetic data analysis was carried out by using linear and nonlinear regression fitting (*28*), using the Sigma Plot 2.01 for Windows program (Jandel Scientific, 1994).

IEF. The partially purified PPO and PO isoenzymes were detected on pH 3.5−10 gradients, basically according to the method of O'Farrell (*29*). Cylindrical gels consisted of 7.5% acrylamide, 0.25% bisacrylamide, 10% glycerol, 1% ampholines, and 0.05% ammonium persulfate. Samples in 60% sucrose were applied to the top of the gels covered by a layer of protector ampholines and electrophoresed at constant voltage (200 V) for 12 h and then at 800 V for 1 h.

SDS-PAGE of PPO. Partially denaturating polyacrylamide gel electrophoresis was carried out using the method of Laemmli (*30*) without the addition of β-mercaptoethanol and with no heating in order to preserve the enzymatic activity. Samples were applied to 10% polyacrylamide gels. The slab gels of 1 mm thickness were run in a Miniprotean II cell (Bio-Rad) at a constant current of 175 mV.

Cationic Native PAGE of PO. Peroxidase was visualized following the method described by Reisfeld and co-workers (*31*) with some modifications by using the Mini-Protean II. Slab gels (1 mm thick) consisted of 10% acrylamide/bisacrylamide (29:1), 0.05% ammonium persulfate, and 0.15% TEMED in 0.2 M potasium acetate (pH 4.3). Protein extracts were mixed with 10% glycerol and methyl green before being immersed in the electrode buffer [0.35 M β-alanine and 0.14 M acetic acid (pH 4.5)]. The slab gels were subjected to a constant current of 150 mV during 50 min at 4 °C.

Gel Staining. After electrophoresis and IEF, the gels were equilibrated into the buffer used to detect the enzymatic activity or prepared to be processed in the Western blot. PPO and PO activities were detected as described by Rodriguez-Lopez et al. (*25*) and Ferrer et al. (*26*), respectively. The PPO activity was stained with 50 mM sodium phosphate buffer (pH 6.8), 5 mM tyramine, 2 mM MBTH, and 0.69 mM SDS. PO activity was detected with 50 mM sodium acetate buffer (pH 5.0), 1 mM 4MN, 0.45 mM H_2O_2, and 5 mM tropolone to prevent the PPO-catalyzed hydroxylation of 4MN.

Western Blotting of Cationic Native PAGE of PO. Proteins were transferred to PVDF membranes (Bio-Rad) using a Mini Trans-Blot apparatus (Bio-Rad). Following the manufacturer's instructions, the transfer was carried out at 4 °C, under constant stirring in 0.7% acetic acid (as transfer solution), and electrophoresed at 100 V for 1 h.

Once the transfer was finished, the membranes were blocked overnight in PBST [0.025 M sodium phosphate buffer (pH 7), which contained 0.15 M NaCl and 0.1% Tween 20] containing 5% skimmed milk at 4 °C and then incubated for 1 h with anti-HRP (Sigma) in PBST containing 1% BSA. To detect the relevant proteins, the membranes were incubated with secondary antibodies conjugated with peroxidase for 1 h, under constant stirring, at 25 °C. Previously, the inactivation of endogenous peroxidase was carried out by immersing the membrane in boiling distilled water for 5 min.

Finally, protein bands were detected on the membranes using the reaction medium described previously for the PO activity staining.

RESULTS AND DISCUSSION

An outline of a partial purification of the several fractions of PPO and PO from *B. vulgaris* L. roots is given in **Table 1**.

As can be appreciated in the table, PPO is mainly located in the membrane fraction, being very effective the extraction with NaCl, whereas the PPO activity obtained by sonication was very low (results not shown). Steiner et al. (*23*) have also obtained a very high PPO activity from crude extracts and cell suspensions of *B. vulgaris* when they used high salt concentrations. These results seem to indicate that PPO is mainly localized ionically bound to the membranes. Besides, PPO was also found in the soluble fraction after centrifugation at 120000*g*. In both cases the enzyme obtained was in its latent state and could be activated by SDS. PPO has also been extracted from table beet leaves in both soluble and membrane-bound fractions, and in both cases the enzyme was also in its latent state (*21*, *32*).

Parallelly, PO was detected in both soluble and membrane fractions, the total activity values being higher in the soluble fraction than in the membrane fraction. Similarly to what occurred during the extraction process of PPO from the membrane fraction by sonication, the PO activity obtained from this was very low (data not shown).

In a wide range of woody and nonwoody plant species PO activity is located in the cell wall free spaces probably in equilibrium with those bound to the cell walls, as may be expected from its well-established role in lignin biosynthesis (*33*−*35*). However, the amount of PO ionically bound to the cell walls, in beet root, was negligible (result not shown).

Different techniques have been used to protect PO and PPO activities obtained from crude extracts, as well as to avoid artifacts that may appear in the electrophoretic assays. Between them, the addition of protective agents during the process of enzymatic extraction turned out to be the most effective method to prevent either the binding of phenolic compounds to the proteins and/or the oxidation of these compounds present in the homogenates. To establish an optimization of the extraction conditions of both enzymes, we have also made a solubilization of these proteins from an acetone powder.

The highest values of specific activity for PO were those obtained from the enzymatic solubilization from acetone powders, being 6 times higher than those recovered from the soluble fraction and almost 8 times higher than those recovered from the membrane fraction (**Table 1**).

When the PPO extraction from acetone powders was carried out, the values of specific activity were 6 times lower than those obtained in the membrane fraction. Although the values of specific activity obtained with this extraction method were 3

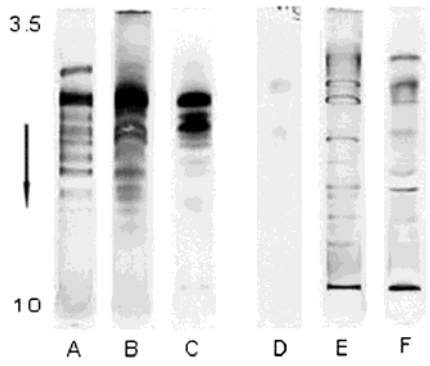

Figure 1. Isoenzymatic patterns of the different PPO and PO fractions separated by IEF in pH 3.5–10 gradients: membrane fraction of PPO (A) and PO (D); soluble fraction of PPO (B) and PO (E); enzyme-solubilized from acetone powders of PPO (C) and PO (F). The staining of the gels was carried out as indicated under Materials and Methods.

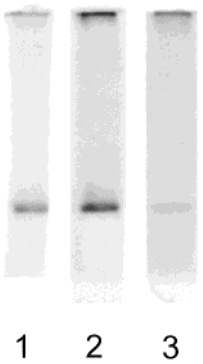

Figure 2. Partially denaturing SDS-PAGE of PPO: lane 1, membrane fraction; lane 2, soluble fraction; lane 3, PPO solubilized from acetone powders. The PPO activity was detected by the MBTH method according to the procedure described under Materials and Methods.

times higher than those obtained in the soluble fraction (**Table 1**), this PPO extraction method was not as effective as the subcellular fraction.

In all fractions, including acetone powders, PPO was obtained in its latent state and it was activated by SDS. The highest value of PPO activity in the activation process was obtained at pH 6.8 when the enzymatic activity was measured in the presence of 0.69 mM SDS. Therefore, all of the kinetic assays were carried out at this SDS concentration at which PPO was totally activated.

All different fractions including the acetone powders were subjected to ammonium sulfate fractionation (45−85%) (**Table 1**). The highest PPO purification was found in the membrane fraction, being almost 4-fold with a recovery of 73%, although the highest recovery was found in the acetone powders at 87%. However, in the case of PO, the recovery was under 50% in the three different extraction processes, and the purification was ∼2-fold. Although this treatment did not provide a high degree of purification, it allows us to obtain a clarified and concentrated enzymatic solution, which was appropriate for further electrophoretic studies.

The partially purified PPO was analyzed by IEF (**Figure 1**) and presented a major acidic band of p*I* 5.2 in both the membrane (A) and the soluble (B) fractions, as well as the solubilized enzyme from acetone powders (C). Furthermore, a number of active PPO isoforms were detected in the pH range of 5.2−5.7. It should be pointed out that the isoenzymatic pattern of membrane PPO showed an acidic band of p*I* 4.7 that was almost undetectable in either soluble fraction or acetone powder, and so it seemed to be one of the most important PPO isoenzymes in this fraction. The high variety of isoforms for PPO has been described by other authors. Flurkey identified several isoenzymes of PPO in mushrooms in a p*I* range from 4 to 4.8 (*36*). When PPO was purified from chloroplasts of broad bean leaves to apparent homogeneity and 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 (*38*). Two acidic PPO isoforms were detected from red beet hypocotyls (*23*).

Peroxidases are highly polymorphic enzymes, and the functionality of each isoenzyme depends on its nature (acidic or basic) and its subcellular localization (*17*). IEF is a useful electrophoretic method for studying PO isoenzymes. Many researchers have identified multiple PO isoenzymes using this technique, that is, 42 in horseradish (*38*), 18 in pea (*39*), and 2 in spring cabbage (*16*). For these reasons, the protein was also analyzed by IEF (pH 3.5−10) and showed a complex isoenzyme pattern (**Figure 1**). The staining of PO activity on the gels revealed the presence of several acidic isoenzymes with p*I* in a range between 4.7 and 5.7, a few isoenzymes of a neutral and slightly basic nature, and, finally, a main strongly basic isoenzyme of p$I > 9$, in both the soluble (E) and the solubilized enzyme from acetone powders (F). On the other hand, this basic isoenzyme was not detected in the membrane fraction (D). Similar results have been found by other authors. Thus, Wang et al. (*40*) isolated from Chinese cabbage roots two anionic peroxidases, which were found to have p*I* values of 4.83 and 4.78. In this way, Dean et al. (*41*) have also found upon IEF a predominant PO isoenzyme that migrated to a p*I* of 3.6 in sycamore maple cell suspension culture.

It should be pointed out that basic peroxidases of high p*I* have been found in both cell walls and vacuoles of etiolated *Lupinus albus* hypocotyls, in particular, ionically bound to the cell wall and to the tonoplast membrane (*34*). The double localization of these strongly basic isoenzymes has also been found in other plant materials [*Catharanthus roseus* (*42*), *Capsicum annuum* (*43*), *Vitis vinifera* (*44*), and *Lupinus polyphyllus* (*45*)]. The presence of basic peroxidases of high p*I* in the soluble fraction seems to suggest that their localization could be in vacuoles so that they were free or slightly bound to the tonoplast in such a way that during the homogenization process, the vacuoles could have broken and all their content could be solubilized and extracted in the soluble fraction.

A partially denaturing SDS-PAGE was used to detect PPO isoform composition because a native electrophoresis did not reveal any bands (results not shown). Because PPO resists SDS, PPO activity may be detected by tyramine staining of such an SDS-PAGE. The electrophoretic mobilities do not correspond to molecular masses because the proteins are not completely denatured but allow us the detection of PPO isoforms. The results obtained from the SDS-PAGE for PPO are shown in **Figure 2**. As can be observed, all of the enzymatic fractions showed the presence of a single 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, and it avoided the interference with PO (*25*).

On the contrary, the partially denaturing SDS-PAGE did not work for detecting the isoform pattern of PO in any of the fractions. Instead of this, a cationic native PAGE was used to

Figure 3. Cationic native PAGE (A) and immunoblot detection (B) of PO from soluble fraction. The PO activity was detected by using H_2O_2 and 4MN as substrates (see Materials and Methods). The Western blot was carried out from a cationic native PAGE as described in the text. In both assays 3.6 μg of protein was used.

Table 2. K_m and V_m Values for PPO and PO Activities[a]

enzyme fraction		K_m (mM)	V_m (mM/min)	V_m/K_m (min^{-1})
soluble PPO		0.45	0.10	0.22
membrane PPO		0.31	0.56	1.81
acetone powder PPO		0.29	0.12	0.41
soluble PO	(4MN)	0.10	0.02	0.20
	(H_2O_2)	0.14	0.05	0.36
acetone powder PO	(4MN)	0.06	0.30	5.00
	(H_2O_2)	0.14	0.44	3.14

[a] The reaction medium used to evaluate the kinetic parameters of PPO and PO is indicated under Materials and Methods. The V_m values are normalized with the same protein concentration (0.018 mg/mL).

analyze the soluble fraction. As can be observed in **Figure 3A**, only one isoform of the protein was stained for PO, so that it could be deduced that the main strongly basic PO isoenzyme detected by IEF is resolved by cationic native PAGE in only one band. To ascertain if other PO isoenzymes were present in the gel (even in trace amounts), a Western blot from it was carried out (**Figure 3B**). As can be seen on the immunoblotting, only a single band was also found by using a commercial preparation of anti-horseradish PO antibodies, which had a cross-reaction with several polypeptides from the major cationic peroxidase. The minor basic PO isoforms were not detected on the PVDF membranes either. These results supported the assumption that the PO activity shown on the gel by the basic PO of high p*I* represents a true isoenzyme that could correspond to the protein detected by immunoblotting.

The kinetic characterization of PO from the soluble fraction and the enzyme solubilized from acetone powders, using 4MN and H_2O_2 as substrates, led to the determination of the V_m and K_m values for both substrates. As can be seen in **Table 2**, the K_m values for H_2O_2 in both soluble fraction and acetone powders were identical and, so, the affinities of the enzyme for this substrate in both reactions were similar. Although the K_m values were equal, the catalytic efficiency (V_m/K_m) of PO on H_2O_2 was higher for the enzyme solubilized from acetone powders than for the soluble PO, a V_m value increase being the only factor that contributed to this difference in the catalytic efficiency.

On the other hand, the K_m values for 4MN were appreciably different in the two enzymatic fractions (**Table 2**). In fact, the K_m value for the soluble PO turned out to be almost twice as much as in acetone powders. These results together with the V_m value being 10 times lower than in acetone powders indicated that the catalytic efficiency of PO on 4MN was markedly lower (20 times) for the soluble PO than for that solubilized from acetone powders.

Kinetic characterization of the PPO for both soluble and membrane fractions was carried out. The evaluation of the kinetic parameters using dopamine as substrate is shown in **Table 2**. The K_m value for the soluble PPO was similar to the one obtained using beet leaves instead of beet roots, whereas for membrane-bound PPO, the K_m value was slightly lower than the one obtained using beet leaves (*32*). The catalytic efficiency of PPO on dopamine was much higher (8 times) for the membrane-bound enzyme than for the soluble PPO. This result for the PPO from beet root contrasts with the one obtained using beet leaves, for which the catalytic efficiencies for the PPO of both fractions were similar.

The evaluation of the kinetic parameters of PPO solubilized from acetone powders showed a K_m value quite similar to the one for the membrane fraction. However, the catalytic efficiency on dopamine was not as high as the one obtained for the membrane fraction (4 times lower). On the other hand, the catalytic efficiency for acetone powders turned out to be 2 times higher than for the soluble PPO due to the lower K_m value for the acetone powders PPO.

Nicolas (*13*) has pointed out that the solubilization of an enzyme after preparation of an acetone powder may result in modification of the enzyme properties. However, we have not found marked differences in the isoenzymatic pattern by IEF of PPO or PO between the soluble fraction and the enzyme solubilized from acetone powders. Only one isoform of PPO was detected by SDS-PAGE in both fractions. It is necessary to point out that the PPO solubilized from acetone powders was also obtained in its latent state and its catalytic efficiency was higher than for the soluble PPO. However, the catalytic efficiency for the soluble PO was much higher than for the PO solubilized from acetone powders, which, in turn, was free of phenolic compounds, and so this extraction method was very useful for electrophoretic assays.

ABBREVIATIONS USED

PPO, polyphenol oxidase; PO, peroxidase; 4MN, 4-methoxy-α-naphthol; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PMSF, phenylmethanesulfonyl fluoride.

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