Partial Purification of a Banana Polyphenol Oxidase Using Triton X-114 and PEG 8000 for Removal of Polyphenols

M. Mar Sojo, Estrella Nuñez-Delicado, Francisco García-Carmona, and Alvaro Sánchez-Ferrer*

Department of Biochemistry and Molecular Biology-A, Faculty of Biology, University of Murcia, Campus de Espinardo, E-30071 Murcia, Spain

Banana pulp polyphenol oxidase was partially purified in a latent form using sequential aqueous two-phase systems based on Triton X-114 and PEG 8000/phosphate. The purification achieved from a crude extract of banana pulp was 5-fold, with 50% recovery of the activity. The (poly)phenols, including tannins, were also reduced to 6% of the original, avoiding the postpurification tanning of the enzyme and rendering only one enzymatic form in contrast to the many previously shown for this enzyme in both electrophoretic and isoelectrofocusing methods. The enzyme was kinetically characterized with its natural substrate (dopamine) and *tert*-butylcatechol in the presence and in the absence of the main activating agent, sodium dodecyl sulfate. In addition, the effect of several inhibitors was also tested, and the K_{si} values of the two most effective substrate analogues (tropolone and kojic acid) were determined.

Keywords: *Aqueous two-phase system; polyphenol oxidase; banana; Triton X-114; polyethylene glycol; latent enzyme; diphenolase; SDS*

INTRODUCTION

In common with many fruits and vegetables, banana contains polyphenol oxidase (PPO; EC 1.14.18.1, monophenol dihydroxy-L-phenylalanine:oxygen oxidoreductase), which catalyzes two different reactions, each using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) [for review, see Sánchez-Ferrer et al. (1995)]. The quinones thus formed are highly reactive substances, which normally react further with other quinones, amino acids, or proteins to produce the colored compounds that are responsible for losses in nutrient qualities (Sánchez-Ferrer et al., 1993a). This is also a severe problem in the isolation of plant enzymes. The successful prevention of these reactions in the pulp, where most of the PPO occurs (Padron et al., 1975), involves a detailed study of the enzyme's biochemical properties before a technological process can be developed (Cano et al., 1990).

Banana PPO has been seen as a clear example of this problem since it was first described by Griffiths (1959) as the enzyme responsible for the browning of this fruit by oxidizing its naturally occurring *o*-diphenol, dopamine. Since then, this enzyme has been purified according to different drastic and time-consuming methods. The first was described by Palmer (1963) and included extraction in phosphate buffer (pH 7.0) containing 1% of a nonionic detergent followed by acetone precipitation and several chromatographic steps. Deacon and Marsh (1971) used a more complicated procedure to demonstrate the capacity of the enzyme to *o*-hydroxylate tyramine to dopamine. This procedure included homogenization in cold (−10 °C) 95% ethanol and precipitation with protamine sulfate and $CaCl_2$, followed by acetone precipitation, ammoniun sulfate fractionation, and storage at −10 °C for 2 days before the chromatografic steps were initiated.

At the beginning of the 1980's, 1% polyvinylpyrrolidone (PVP) was included to remove phenols, prior to acetone precipitation and reextraction with 0.5% Triton X-100, although the material still had to be stored in a freezer (−35 °C) for a minimum of 7 days before the chromatographic steps to remove any insoluble dark material. Since then, however, few changes have been introduced in the prechromatographic steps, except for the addition of 0.01% ascorbic acid and the use of $(NH_4)_2SO_4$ fractionation, which gave better results than the acetone precipitation (Thomas and Janave, 1986).

The above clearly indicates that banana PPO is difficult to purify without discoloration due to the presence of a large quantity of (poly)phenols and tannins, which covalently modify the enzyme during the first purification steps and give rise to a multiplicity of bands in gel electrophoresis, ranging from 2 (Padron et al., 1975) to 14 (Thomas and Janave, 1986).

Temperature-induced phase partitioning in Triton X-114 (TX-114 has been shown to be a suitable extraction method for plant enzymes with the above problems since it was first used by our group in 1989 [for reviews, see Sánchez-Ferrer et al. (1994a,b)]. TX-114 can extract plant enzyme in wild conditions as Triton X-100 can, but, at the same time, it is able to remove phenols and chlorophylls by centrifugation alone, rendering untanned latent enzymes (Sánchez-Ferrer et al., 1989a,b, 1990, 1993a,b; Nuñez-Delicado et al., 1996).

Preliminary experiments with banana pulp solubilized by using the TX-114 method showed that this detergent was able to remove phenols from the extract to render a clear enzyme, which, when stored, turned brown, indicating that the tanning of this enzyme was due not only to the oxidation of monomeric phenols oxidation but also to the presence of a high level of

* Author to whom correspondence should be addressed (fax +34 968 364147; e-mail alvaro@fcu.um.es).

10.1021/jf980473d CCC: $15.00
Published on Web 12/01/1998

oxidable polymeric (poly)phenols, including tannins. Because polyethylene glycols have been demonstrated to bind banana latex (Jones, 1965) and banana pulp tannins (Badran and Jones, 1965) and, in the presence of high salt concentrations, they give rise to an aqueous two-phase system (ATPS), we investigated whether an additional phase partition in an aqueous two-phase system based on PEG 8000/phosphate system [for review, see Walter et al. (1991) and Walter and Johansson (1994)] could remove the tannins that discolored the enzyme.

The results described in this paper show for the first time not only that an additional PEG 8000/phosphate system was needed to obtain an unmodified enzyme but also that banana PPO was isolated in a latent form by using this new purification procedure that avoids the general use of acetone powders.

MATERIALS AND METHODS

Plant Material. Air-freighted green bananas (*Musa acuminata* subgr. Cavendish var. Spanish Pequeña Enana) produced in the Canary Islands (Spain) were obtained from a commercial source in Murcia (Coplaca, S.A.). After sitting for 24 h with ethylene gas in the dark, the samples were stored at 17 °C for 72 h until they were at stage 4 with the peel "more yellow than green" (Sunday and Dismas, 1994) and with a soluble solids content of 10%.

Reagents. Biochemicals were purchased from Sigma (Madrid, Spain) and used without further purification. TX-114 was obtained from Fluka (Madrid, Spain) and condensed three times as described by Bordier (1981). Thirty grams of TX-114 containing 16 mg of butylated hydroxytoluene was added to 980 mL of 100 mM sodium phosphate buffer (pH 7.3). After dissolution at 0 °C, the clear solution was incubated at 30 °C. The detergent condensed and the mixture separated overnight into a large detergent-depleted aqueous phase and a smaller detergent-rich phase. The aqueous phase was discarded and replaced by the same volume of 100 mM sodium phosphate buffer (pH 7.3). Buffer and detergent phase were mixed at 0 °C and condensed twice at 30 °C under the same condition. The detergent phase of the third condensation had a concentration of 25% TX-114 (w/v) and was used as the detergent stock solution for all of the experiments. The TX-114 concentration was estimated from the 278 nm absorption of dilute detergent solution ($A_{278} = 1.25$ for 0.05%, w/v) (Werck-Reichhart et al., 1991).

Partial Purification of Banana PPO. A 30 g pulp sample was homogenized with 45 mL of 4% (w/v) TX-114 in 100 mM sodium phosphate buffer (pH 7.3) for 1 min. The homogenate was centrifuged at 15000*g* for 15 min at 4 °C. The supernatant was subjected to temperature-induced phase partitioning by increasing the TX-114 concentration by an additional 6% (w/v) at 4 °C and then warming to 37 °C for 15 min. After 10 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large mixed micelles of detergent, which contained hydrophobic protein and phenolic compounds. This solution was centrifuged at 10000*g* for 15 min at 25 °C. The detergent-rich phase was discarded, and the clean supernatant containing the banana PPO was subjected to a different aqueous two-phase system based on PEG 8000 (5% w/w)/potassium phosphate buffer (pH 7.0). After the solution had been stirred for 15 min at room temperature, it was centrifuged at 10000*g* for 10 min at 25 °C. The upper PEG-rich phase was discarded, and the phosphate-rich phase containing the banana PPO was brought to 15% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60000*g* for 30 min at 4 °C, and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clean supernatant to give 35% saturation and stirred for 1 h at 4 °C. The precipitate obtained between 15 and 35% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 100 mM sodium phosphate buffer (pH 7.3). The enzyme was stored at −20 °C.

Enzyme Activity. Diphenolase activity was determined spectrophotometrically at 400 nm (Sánchez-Ferrer et al., 1989a) with *tert*-butylcatechol (TBC) ($\epsilon = 1150\ M^{-1}\ cm^{-1}$) and at 480 nm with dopamine ($\epsilon = 3300\ M^{-1}\ cm^{-1}$). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of the corresponding quinone/min.

The standard reaction medium at 25 °C contained 7 μg of partially purified banana PPO, 10 mM sodium phosphate buffer (pH 6.5), 2.5 mM dopamine, or 5 mM TBC in a final volume of 1 mL.

In the sodium dodecyl sulfate (SDS) standard assays the sample contained the above but with 1.4 μg of partially purified banana PPO and 2 mM SDS detergent in a cuvette. To determine the effect of the inhibitors, PPO activity in the steady state was measured according to standard reaction methods in the presence or absence of the stated concentration of inhibitors.

Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out as described by Angleton and Flurkey (1984). Samples were mixed with glycerol, SDS, and bromophenol blue before being applied to 7.5% polyacrylamide gels. Electrophoresis was carried out for 1 h at 25 °C in a Mini protein cell (Bio-Rad). The gels were stained for PPO activity in 100 mL of 10 mM sodium phosphate buffer (pH 6.5) containing 5 mM L-DOPA.

Isoelectric Focusing. The isoelectric pH was determined by isoelectric focusing on polyacrylamide gel following the technique of Robertson et al. (1987) in native conditions. The ampholine used has a pH of 3.0−10.0 from Fluka. The electrodes were connected to a power supply, and the current was maintained at 200 V for 1.5 h and at 400 V for 1.5 h.

The gels were stained for PPO activity in 100 mL of 10 mM sodium phosphate buffer (pH 6.5) containing 5 mM L-DOPA and 2 mM of SDS. The pH gradient was determined by cutting an unstained gel into 0.5 cm fragments, eluting with distilled water, and reading in a pHmeter with a microelectrode.

Other Methods. The protein content was determined according to the Bradford (1976) dye binding method using bovine serum albumin (BSA) as a standard. Phenolic compounds were measured spectrophotometrically according to the Folin−Denis method in 80% ethanol using chlorogenic acid as standard (Kidron et al., 1978).

RESULTS AND DISCUSSION

The experiments carried out to extract PPO from the pulp tissue of Spanish banana (subgr. Cavendish var. Pequeña Enana) using buffer, or detergent in buffer, showed that the second method alone increased the activity extracted (data not shown), as is the case with PPO extraction from other bananas (Galeazzi et al., 1981). This fact, together with the large amount of rapidly oxidized substances observed in the pulp extract after homogenization, made it necessary to include a substantial quantity of TX-114 (4% w/v) in the extraction buffer to prevent browning, as first described for potato PPO (Sánchez-Ferrer et al., 1993a). After centrifugation, the supernatant gave a clear solution, termed crude extract in the purification table (Table 1). To take full advantage of TX-114 in the removal of phenols and hydrophobic proteins (Sánchez-Ferrer et al., 1989a), another 6% (w/v) of TX-114 was added and the solution was induced to phase separation by increasing the temperature to 37 °C and holding for 15 min. This two-phase step involved a loss of proteins, a slight loss of PPO, and a 22% reduction in the level of phenols. This clear enzyme solution became brown when ammonium sulfate fractionation was carried out. Because of this, a new alternative in PPO purification procedures

Table 1. Partial Purification of Latent Banana PPO

	vol (mL)	total activity[a] (units)	specific activity (units/mg of protein)	purification (-fold)	recovery (%)	phenolic compounds (mg/mL)	activation[b] (-fold)
crude extract	46.0	10213	40.4	1.0	100	20.0	3
supernatant 6% TX-114	46.0	9509	65	1.6	93	15.6	3
salt phase	59.2	5505	127.4	3.1	54	9.1	10
15−35% $(NH_4)_2SO_4$	2.5	5065	195	5.0	50	1.2	11

[a] Assayed at pH 6.5 with 5 mM TBC as substrate. [b] Activated with 2 mM SDS.

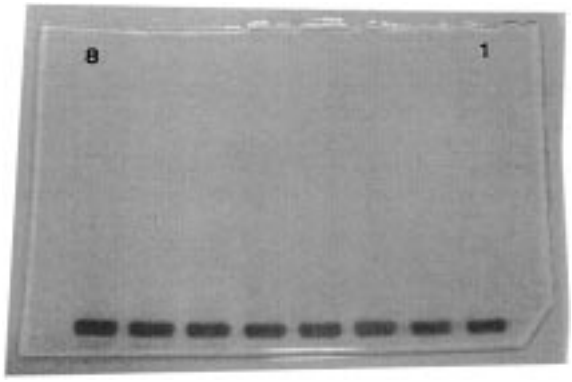

Figure 1. Electrophoresis (7.5% gel) of banana (*M. acuminata* subgr. Cavendish var. Pequeña Enana) PPO stained with 5 mM L-DOPA in 10 mM sodium phosphate (pH 6.5). The lanes contained different enzyme concentrations ranging from 5 μg (lane 1) to 15 μg (lane 8).

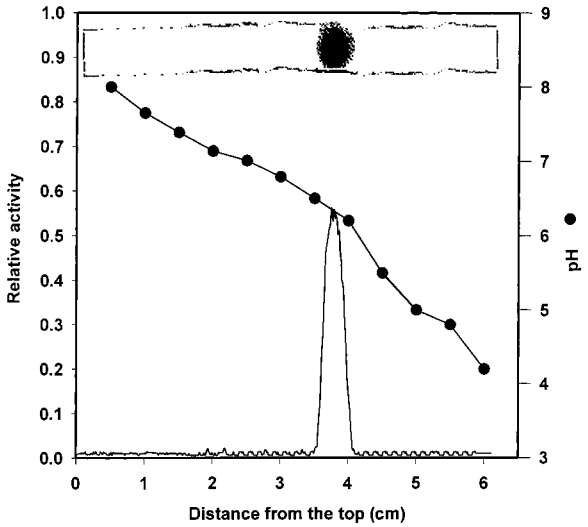

Figure 2. Isoelectric focusing of the partially purified banana PPO on polyacrylamide gel containing ampholines with a range of pH values between 3.0 and 10.0. Staining was done with 5 mM L-DOPA and 2 mM SDS in 10 mM sodium phosphate (pH 6.5). The lane contained 5 μg of enzyme purified according to the two-phase partition method.

was introduced to remove the oxidized substances present in the 6% (w/v) TX-114 supernatant. This step involved an additional phase partition in an aqueous two-phase system containing PEG 8000/phosphate salt. After phase partitioning, PPO mainly concentrated in the salt-rich phase and showed a 39% loss of enzyme activity and a 32% loss in phenol-like subtances, particularly tannins. This salt phase, when subjected to ammonium sulfate fractionation, rendered a clear and stable enzyme solution, with a 5-fold purification and 50% recovery.

The degree of purification obtained with this method before the start of the chromatographic steps was similar to those described for banana pulp PPO by Galeazzi et al. (1981) (4.9-fold) and Deacon and Marsh (1971) (5.5-fold). However, the recovery was the same as in the latter method but lower than that obtained by Galeazzi et al. (1981).

In addition, the method reduced the phenol and tannin content to only 6% of the original without the aid of special polymeric resins or acetone, which means that when the level of oxidizing compounds is high and combined with a high tannin content, an additional phase partitioning in a PEG system after phase partitioning in TX-114 is necessary. PEG8000/phosphate was found to be the most suitable and cheapest for banana pulp PPO, avoiding the use of expensive dextran or other starch derivates. Moreover, phosphate had an additional stabilizing effect, increasing the degree of activation of the latent enzyme, which is in agreement with the previously described stabilizing effect of phosphate ions in banana PPO (Galeazzi et al., 1981). The removal of phenols with these two different two-phase systems was sufficient to avoid any browning of the enzyme solution even after many cycles of freezing and thawing or after months of storage at −20 °C.

The partially purified banana pulp PPO appeared as a single band in PAGE (Figure 1) only when L-DOPA was used as substrate, even though this substrate gives more activity bands with other banana PPOs (Montgomery and Sgarberi, 1975). This result contrasted with the two (Padron et al., 1975), four (Galeazzi et al., 1981), and nine (Montgomery and Sgarberi, 1975) bands found in other banana pulp PPOs. This multiplicity confirms that in the other methods used, (poly)phenols and tannins are oxidized by the enzyme during purification, resulting in a covalent modification of the enzymes, as has been previously described for potato PPO (Sánchez-Ferrer et al., 1993a), when conjugation of the enzyme with its own products produces some new potato PPO forms with a yellow-white fluorescence (Patil and Zucker, 1965). The efficiency of the new method in avoiding this multiplicity was also seen when the enzyme was subjected to isoelectric focusing in the range of pH 3.0−10.0; the zymogram reflected the existence of only one band with a p*I* value of 6.2 (Figure 2). This is clearly in contrast with the eight peaks with a p*I* in the range of 3.82−9.25 described for Valery bananas by Mowlah et al. (1982) and the 14 isoenzymes for Dwarf Cavendish bananas (Thomas and Janave, 1986) and the 10 isoenzymes for Red Skin bananas (Thomas and Janave, 1986). Galeazzi et al. (1981) also reported that banana pulp PPO from Dwarf Cavendish has a broad range of isoenzymes between pH 4.0 and 6.0, with a predominant band at 5.2.

The above-described results, that is, latency and only one isoenzyme, would suggest the existence of a well-defined pH profile with a clear maximum. However, the experiments showed a flat pH profile in the absence of SDS and only a slight increase at pH 5.5 in the presence of SDS (Figure 3). This behavior contrasts both with the generally accepted optimum pH values

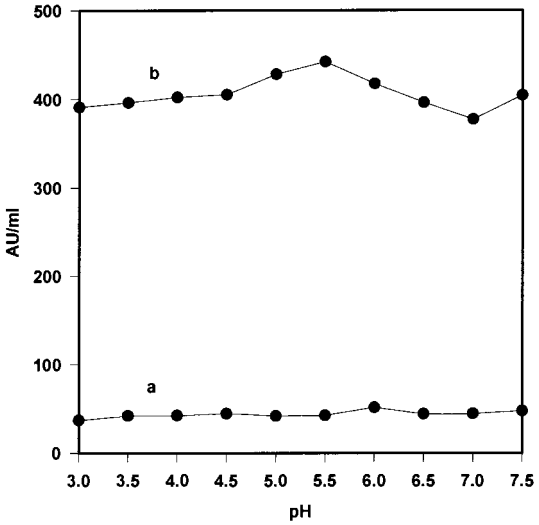

Figure 3. Effect of pH on the diphenolase activity of banana PPO in 10 mM sodium acetate (pH 3.0−5.5) and sodium phosphate (pH 5.5−7.5) expressed as enzymatic units per milliliter. The reaction medium contained 7 μg/mL PPO, 5 mM TBC, without SDS (a) and 2 mM SDS (b).

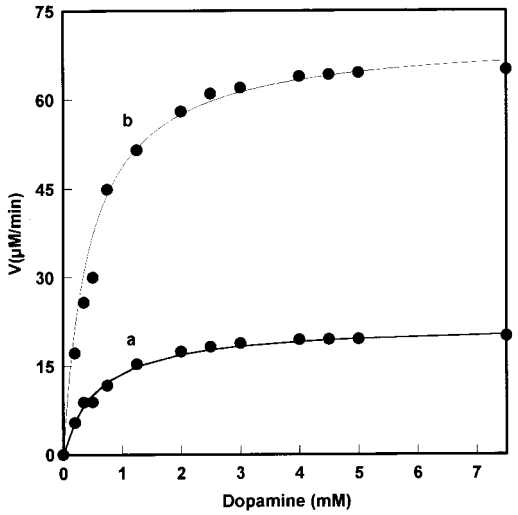

Figure 4. Effect of dopamine concentration on the initial rate. The reaction medium contained (a) 7 μg/mL PPO or (b) 1.4 μg/mL PPO and 2 mM SDS both, with dopamine concentration ranging from 0 to 8 mM.

of 6.5−7.0 described for banana pulp PPO (Palmer, 1963) and 6.5 for plantain PPO (Ngalani et al., 1993) and with the pH profile of other latent PPOs, because they are easily activated at acid pH (Sánchez-Ferrer, 1989a,b, 1990, 1993a,b; Nuñez-Delicado et al., 1996) and give rise to hysteretic responses (Valero and García-Carmona, 1992). However, this flat pH profile was not due to the PEG8000/phosphate step, because a similar pH profile was obtained with the 6% (w/v) TX-114 (data not shown).

The study of the kinetic parameters (V_m and K_m) was carried out at pH 6.5 to compare them with the previously published results. When dopamine, the natural substrate of banana (Palmer, 1963) was used, the same K_m (0.57 mM) (Figure 4) was obtained in the presence and in the absence of SDS, whereas a higher V_m (7.3 versus 72.7 μM) was obtained. This increase in V_m and same K_m are in agreement with previously described behavior with and without SDS in latent potato leaf PPO (Sánchez-Ferrer et al., 1993b) and

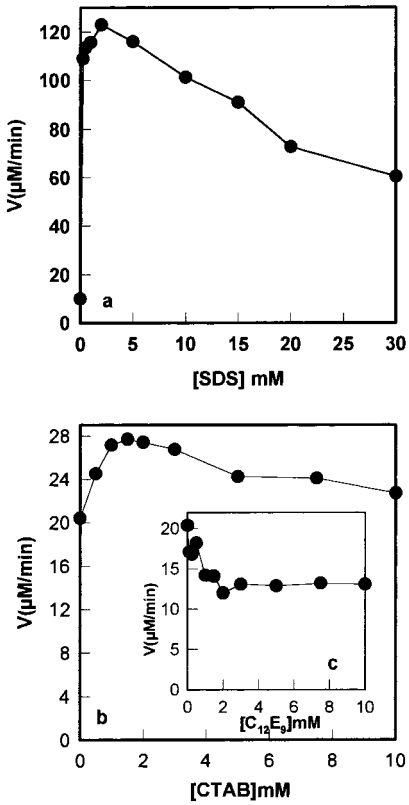

Figure 5. Effect of detergents on the diphenolase activity of banana PPO. (a) Effect of SDS concentration. The reaction medium contained 2.5 mM dopamine, 2.8 μg/mL PPO, and the appropiate SDS concentration. (b) Effect of CTAB concentration. The reaction medium contained 2.5 mM dopamine, 7 μg/mL PPO, and the appropiate CTAB concentration. (c) Effect of $C_{12}E_9$ concentration. The reaction medium contained 2.5 mM dopamine, 7 μg/mL PPO, and the appropiate $C_{12}E_9$ concentration.

latent broad bean PPO (Jiménez and García-Carmona, 1996). The presence of SDS resulted in a 10-fold increase in the catalytic efficiency of the activated enzyme, which was similar to that described for broad bean PPO (Sánchez-Ferrer et al., 1990) and lower than that described for grape berry PPO (Sánchez-Ferrer et al., 1989a). The K_m value of 0.57 mM was similar to that described by Palmer (1963) (0.63 mM) and in the middle of the values described for banana pulp PPO, which range from 0.17 mM for the enzyme extracted from *M. cavendishii* (Galeazzi and Sgarbieri, 1981) to 1.3 mM for the enzyme extracted from *M. sapientum* (Mowlah et al., 1982).

To further characterize the partially purified enzyme, a detailed study of the activation and inhibition of the enzyme was carried out. Normally, latent plant PPO can be activated by different treatments that include trypsin (Tolbert, 1973; Sánchez-Ferrer et al., 1989b), pH (Valero and García-Carmona, 1992; Sánchez-Ferrer et al., 1993b), and detergents (King and Flurkey, 1987; Sánchez-Ferrer et al., 1989a,b, 1990, 1993b; Nuñez-Delicado et al., 1996). However, latent banana pulp PPO was significantly activated by SDS (~10-fold) (Table 1) only at its optimum SDS concentration (2 mM) (Figure 5a). The presence of cationic detergents, such as CTAB, activated the latent enzyme 2-fold only at its optimum concentration (Figure 5b). This degree of activation is lower than the 50−60-fold activation in grapes (Sánchez-Ferrer et al., 1989a) and in broad bean (Jiménez and García-Carmona, 1996) and similar to

Table 2. Inhibition (Percent) of Partially Purified Plantain PPO by Reducing Agents[a]

	without SDS			with SDS		
	1 mM	0.1 mM	10 μM	1 mM	0.1 mM	10 μM
ascorbic acid	100	18	0	100	6	0
DEDTC	18	6	0	20	17	0
metabisulfite	100	58	25	100	72	21
L-cysteine	92	37	18	100	45	23

[a] Standard assay conditions were used with the appropriate concentration of inhibitor and TBC as substrate.

Table 3. Inhibition (Percent) of Partially Purified Plantain PPO by Substrate Analogues[a]

	without SDS			with SDS		
	1 mM	0.1 mM	10 μM	1 mM	0.1 mM	10 μM
cinnamic acid	8	6	0	23	14	0
tropolone	80	40	20	95	78	50
L-mimosine	21	2	0	30	20	13
kojic acid	10	8	5	60	30	10

[a] Assayed under the standard reaction condition with the appropriate concentration of inhibitor and TBC as substrate.

those described for latent mushroom PPO (Nuñez-Delicado et al., 1996) and higher than those described for potato leaf PPO (Sánchez-Ferrer et al., 1993b) and spinach PPO (Sánchez-Ferrer et al., 1989b). The latent enzyme could not be activated in the presence of nonionic detergents (Figure 5b, inset), which is normal with latent PPOs (Sánchez-Ferrer et al., 1989a, 1993b).

Tables 2 and 3 show the effects of various inhibitors on the diphenolase activity of latent banana pulp PPO in the presence and absence of 2 mM SDS, using *tert*-butylcatechol as substrate to avoid interferences between the inhibitors and dopamine. All of the inhibitors, reducing agents, and substrate analogues used in this study inhibited PPO activity, with the extent of the inhibition being dependent on the concentration of the compound used and the presence or absence of SDS. Among the reducing agents (Table 2), ascorbic acid, L-cysteine, and metabisulfite appeared to be the most effective inhibitors in the presence of SDS, whereas, in its absence, diethyldithiocarbamate (DEDTC) and metabisulfite were the most efficient (Table 2). Similar results was obtained for the inhibition of plantain PPO (Ngalani et al., 1993) and Dwarf Cavendish banana pulp PPO (Galeazzi et al., 1981). The inhibition by thiol compounds may be due to an addition reaction with the quinones to form stable colorless products (Ikediobi and Obasuyi, 1982) and/or a binding to the active center of the enzyme as in the case of metabisulfite (Valero et al., 1992). Ascorbate acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions which lead to browning (Whitaker, 1972). Ascorbic acid has also been reported to cause irreversible inhibition (Golan-Goldhirsh and Whitaker, 1984). Finally, DEDTC possibly acts by complexing the copper prosthetic group of the enzyme, as has been found for other plant PPO (Anosike and Ayaebene, 1981).

Tropolone was the most effective inhibitor among the substrate analogues (Table 3), almost completely inhibiting enzyme at 1 mM in the presence of SDS. On the other hand, neither cinnamic acid nor L-mimosine had much of an inhibiting effect with or without SDS. The inhibition by kojic acid was SDS-dependent and was more effective in the presence of SDS. To further

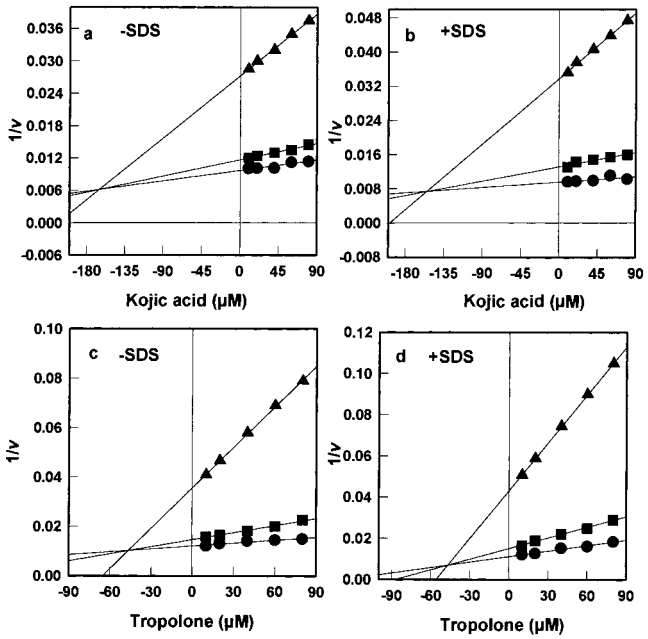

Figure 6. Dixon plot (1/*v* versus I) for the competitive type of inhibition presented by kojic acid (a, b) and tropolone (c, d). (a,c) The reaction medium contained 7 μg/mL banana PPO, kojic acid or tropolone (10−80 μM), and three different concentrations of TBC: 0.5 (●), 2.5 (■), or 5 (▲) μg/mL. (b, d) The reaction medium contained 2 mM SDS, tropolone or kojic acid (10−80 μM), and 1.4 μg/mL banana PPO at the above three TBC concentrations.

characterize this SDS-dependent inhibition, a kinetic analysis of the inhibition of tropolone and kojic acid was carried out. The inhibition was determined by Lineweaver−Burk plots of 1/*v* versus 1/*S* at three inhibitor concentration and confirmed by a Dixon plot of 1/*v* versus *I*. Straight lines were obtained for both inhibitor with or without SDS (Figure 6). The inhibition constant, K_i, of each compound was deduced from the points of interception of the plots. Both inhibitors showed a competitive inhibition with K_i values of 46 and 164 μM for tropolone and kojic acid, respectively. The same values were obtained with SDS.

CONCLUSIONS

TX-114 has been extensively used to purify purification plant enzymes since it was first used by our group in 1989 (Sánchez-Ferrer et al., 1989a,b; Werck-Reichhart et al., 1991; Espín et al., 1995, 1997). This nonionic detergent avoids the use of drastic methods, such us acetate powder or insoluble polymers, which inhibit or activate latent enzymes [for a review, see Sánchez-Ferrer et al. (1994a,b)]. However, in some plant material it is difficult to prevent browning and modification of the enzymes by endogenous oxidizing compounds. Such is the case with banana pulp, in which large quantities of highly hydrophilic (poly)phenols and especially highly hydrophilic tannins are present. In this case, as has been demostrated in this paper, a different aqueous two-phase system based on PEG has to be used as a complement to TX-114. For banana pulp PPO, the system PEG8000/phosphate was optimum because it removed oxidizing tannins and the high phosphate concentration stabilized the enzyme so that it remained latent after ammonium sulfate fractionation. This new scheme for purifying plant enzymes will help in purify-

ing latent enzymes from other fruits and plant materials that contain high quantities of tannins.

ABBREVIATIONS USED

TX-114, Triton X-114; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; PEG 8000, polyethylene glycol Pm. 8000 Da; CTAB, hexadecyltrimethylammonium bromide; $C_{12}E_9$, nonaethylene glycol monododecyl ether; TBC, *tert*-butylcatechol; DEDTC, diethyldithiocarbamate.

LITERATURE CITED

Angleton, E. A.; Flurkey, W. H. Activation and alteration of plant and fungal polyphenol oxidase isoenzymes in sodium dodecyl sulfate electrophoresis. *Phytochemistry* **1984**, *23*, 2723−2725.

Anosike, E. O.; Ayaebene, A. O. Purification and some properties of Polyphenol oxidase from the yam tuber, *Dioscorea bulbiphera*. *Phytochemistry* **1981**, *20*, 2625−2628.

Badran, A. M.; Jones, D. E. Polyethylene glycols-tannins interaction in extracting enzymes. *Nature* **1965**, *206*, 622−624.

Bordier, C. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem*. **1981**, *256*, 1604−1607.

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.

Cano, P.; Marín, M. A.; Fúster, C. Effects of some thermal treatments on polyphenol oxidase and peroxidase activities of banana (*Musa cavendishii*, var. Enana). *J. Sci. Food Agric.* **1990**, *51*, 223−231.

Deacon, W.; Marsh, H. V. Properties of an enzyme from bananas (*Musa sapientum*) which hydroxilates tyramine to dopamine. *Phytochemistry* **1971**, *10*, 2915−2924.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Verdoncella apple. *J. Agric. Food Chem*. **1995**, *43*, 2807−2812.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Haas avocado. *J. Agric. Food Chem*. **1997**, *45*, 1091−1096.

Galeazzi, M. A.; Sgarbieri, V. C. Substrate specificity and inhibition of polyphenol oxidase (PPO) from a Dwarf variety of banana (*Musa cavendishii*, L.). *J. Food Sci*. **1981**, *46*, 1404−1406.

Galeazzi, M. A.; Sgarbieri, V. C.; Constantinides, S. M. Isolation, purification and physicochemical characterization of polyphenol oxidases (PPO) from a Dwarf variety of banana (*Musa cavendishii*, L.). *J. Food Sci*. **1981**, *46*, 150−155.

Golan-Goldhirsh, A.; Whitaker, J. R. Effect of ascorbic acid, sodium bisulfite and thiol compounds on mushroom polyphenol oxidase. *J. Agric. Food Chem*. **1984**, *32*, 1003−1009.

Griffiths, L. A. Detection and identification of the polyphenol oxidase substrate of the banana. *Nature* **1959**, *184*, 58−59.

Ikediobi, C. O.; Obasuyi, H. N. Purification and same properties of *o*-diphenolase from white yam tubers. *Phytochemistry* **1982**, *21*, 2815−2820.

Jiménez, M.; García-Carmona, F. The effect of sodium dodecyl sulphate on polyphenol oxidase. *Phytochemistry* **1996**, *42*, 1503−1509.

Jones, D. E. Banana tannin and its reaction with polyethylene glycols. *Nature* **1965**, *206*, 299−300.

Kidron, M.; Harel, M.; Mayer, A. M. Catechol oxidase activity in grapes and wine. *Am. J. Enol. Vitic.* **1978**, 30−35.

King, R. S.; Flurkey, W. H. Effects of limited proteolysis on broad been polyphenol oxidase. *J. Sci. Food Agric*. **1987**, *41*, 231−240.

Montgomery, M. W.; Sgarbieri, V. C. Isoenzymes of banana polyphenol oxidase. *Phytochemistry* **1975**, *14*, 1245−1249.

Mowlah, G.; Takano, K.; Kamoi, I.; Obara, T. Kinetics of heat inactivation and some physicochemical properties of banana polyphenol oxidases (PPO). *J. Agric. Sci*. (*Tokyo*) **1982**, *26*, 311−317.

Ngalani, J. A.; Signoret, A.; Crouzet, J. Partial purification and properties of plantain polyphenol oxidase. *Food Chem*. **1993**, *48*, 341−347.

Nuñez-Delicado, E.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. Triton X-114-aided purification of latent tyrosinase. *J. Chromatogr. B* **1996**, *680*, 105−112.

Padron, M. P.; Lozano, J. A.; González, A. G. Properties of *o*-diphenol: O_2 oxidoreductase from *Musa cavendishii*. *Phytochemistry* **1975**, *14*, 1959−1963.

Palmer, J. K. Banana polyphenol oxidase. Preparation and Properties. *Plant Physiol*. **1963**, *38*, 508−513.

Patil, S. S.; Zucker, M. Potato phenolases: purification and properties. *J. Biol. Chem*. **1965**, *210*, 3938−3943.

Robertson, E. F.; Dannelly, H. K.; Malloy, P. J.; Reeves, H. C. Rapid isoelectric focusing in a vertical polyacrylamide minigel system. *Anal. Biochem*. **1987**, *167*, 290−294.

Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Novel procedure for extraction of a latent grape polyphenol oxidase using temperature-induced phase separation in Triton X-114. *Plant Physiol*. **1989a**, *91*, 1481−1487.

Sánchez-Ferrer, A.; Villalba, J.; García-Carmona, F. Triton X-114 as a tool for purifying spinach polyphenol oxidase. *Phytochemistry* **1989b**, *28*, 1321−1325.

Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Partial purification of a thylakoid-bound enzyme using temperature-induced phase partitioning. *Anal. Biochem*. **1990**, *184*, 279−282.

Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. Partial purification of soluble potato polyphenol oxidase by partitioning in an aqueous two-phase system. *J. Agric. Food Chem*. **1993a**, *41*, 1583−1586.

Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. Substrate-dependent activation of latent potato leaf polyphenol oxidase by anionic surfactants. *J. Agric. Food Chem*. **1993b**, *41*, 1219−1223.

Sánchez-Ferrer, A.; Pérez-Gilabert, M.; Nuñez-Delicado, E.; Bru, R.; García-Carmona, F. Triton X-114 phase partitioning in plant protein purification. *J. Chromatogr. A* **1994a**, *668*, 75−83.

Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Phase separation of biomolecules in polyoxyethylene glycol nonoionic detergents. *Crit. Rev. Biochem. Mol. Biol*. **1994b**, *29*, 275−313.

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

Sunday, Y. G.; Dismas, A. A. Changes in composition and certain functional properties of ripening plantain (*Musa spp*., AAB group) pulp. *Food Chem*. **1994**, *50*, 137−140.

Thomas, P.; Janave, M. T. Isoelectric focusing evidence for banana isoenzymes with mono and diphenolase activity. *J. Food Sci*. **1986**, *51*, 384−387.

Tolbert, N. E. Activation of polyphenol oxidase of chloroplasts. *Plant Physiol*. **1973**, *51*, 234−244.

Valero, E.; García-Carmona, F. Hysteresis and cooperative behavior of a latent plant polyphenol oxidase. *Plant Physiol*. **1992a**, *98*, 774−776.

Valero, E.; Varón, R.; García-Carmona, F. Kinetic study of the effect of metabisulfite on polyphenol oxidase. *J. Agric. Food Chem*. **1992b**, *40*, 904−908.

Walter, H.; Johansson, G. *Methods in Enzymology, Vol. 228, Aqueous Two Phase Systems*; Academic Press: San Diego, CA, 1994.

Walter, H.; Johansson, G.; Brooks, D. E. Partitioning in aqueous two phase systems: recent results. *Anal. Biochem*. **1991**, *197*, 1−15.

Werck-Reichhart, D.; Benveniste, I.; Teutsch, H.; Gabriac, B. Glycerol allows low-temperature phase separation of membrane proteins solubilized in Triton X-114; application to the purification of plant cytochromes $P\text{-}_{450}$ and b_5. *Anal. Biochem*. **1991**, *197*, 125−131.

Whitaker, J. R. In *Principles of Enzymology for the Food Science*; Dekker: New York, 1972.

Received for review May 11, 1998. Revised manuscript received September 22, 1998. Accepted September 23, 1998. This work was partially supported by DGES (PB97-1032) and Programa ALFA ("TECMUSA" Project 6.0259.8). M.S. has a fellowship from the Programa Nacional de Formación del Personal Investigador, Ministerio de Educación y Ciencia (Spain), reference PN94. E.N.D. is a holder of a research grant from Caja Murcia (Murcia, Spain)

JF980473D