Partial Purification of Soluble Potato Polyphenol Oxidase by Partitioning in an Aqueous Two-Phase System

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Soluble potato polyphenol oxidase was partially purified using a two-phase partitioning approach with Triton X-114. The purification achieved was 5-fold from a crude extract of potato tubers, with an 18% recovery of activity. The phenols were also reduced to 3% of the original content, avoiding the postpurification tanning of the enzyme. The enzyme was kinetically characterized with two phenolic substrates (*tert*-butylcatechol and chlorogenic acid) at two pHs (4.5 and 6.5). The latter substrate presented inhibition at high substrate concentration with a K_{Si} of 5.5 mM. Selected inhibitor agents were also studied. Tropolone was found to be the most effective inhibitor and presented a mixed type of inhibition.

INTRODUCTION

Postharvest enzymatic browning is a serious problem in food technology, especially in the case of potato tubers which have a high phenol content (mostly chlorogenic acid) and a high level of polyphenol oxidase (monophenol dihydroxy-L-phenylalanine:oxygen oxidoreductase EC 1.14.18.1) (Matheis, 1987). The enzyme catalyzes two different reactions, each using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (catecholase activity). The quinones thus formed are highly reactive substances which normally react further with other quinones, amino acids, or proteins to produce colored compounds, which are responsible for losses in nutrient quality and undesirable sensory qualities (Matheis and Withaker, 1984; García-Carmona et al., 1988).

The commercial interest in avoiding these unwanted reactions catalyzed by polyphenol oxidase (PPO) has led to many studies of this enzyme in potato over the past 60 years (Kubowitz, 1937), although no easy nondrastic purification method has yet been developed. The preparation of an acetone powder has been used as the first step of potato PPO purification to remove as many endogenonous phenolic substrates from the enzyme as possible (Matheis, 1987). However, not all of them are extracted by acetone, and these substances, as well as the proteins themselves (Yasunobu et al., 1959; Cory and Frieden, 1967; Matheis and Whitaker, 1984), can serve as substrates for PPO in the acetone powder (Patil and Zucker, 1965). Consequently, considerable browning occurs during the initial fractionation of the enzyme by ammonium sulfate precipitations. If calcium acetate is added, many of the brown pigments are precipitated, although, unfortunately, there is a considerable loss of enzyme activity (Patil and Zucker, 1965). Alternative ways of preventing browning in the purification of potato PPO are the use of reducing agents, such as ascorbic acid, cysteine, dithiothreitol, metabisulfite, or synthetic resins [poly(vinylpyrrolidone), Amberlite, etc.], to adsorb phenols (Matheis, 1987). However, most of these act as PPO inhibitors and make the enzyme assay difficult.

The purpose of this work is to present an alternative method to those described above for the purification of soluble potato PPO and to study the kinetic characteristics of the enzyme. The method of purification is based on the temperature-induced phase partitioning of Triton X-114 (TX-114), which produces an aqueous two-phase system, where the soluble enzyme is separated from the particulate enzyme.

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MATERIALS AND METHODS

Plant Material. The potato (*Solanum tuberosum* cv. Desirée) tubers used in this study were manually harvested at maturity and carefully sorted and stored at constant temperature (10 °C) in the dark and with a relative humidity of 50–60%.

Reagents. Biochemicals were purchased from Sigma (Madrid, Spain) and used without further purification. Triton X-114 (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 200 mM sodium acetate buffer (pH 4.0) with 20 mM ethylenediaminetetraacetic acid (EDTA). After dissolution at 0 °C, the clear solution was incubated at 30°C. Condensation of the detergent occurred, and the mixture separated overnight into a large aqueous phase depleted of detergent and a smaller phase enriched in detergent. The aqueous phase was discarded and replaced by the same volume of 200 mM sodium acetate buffer (pH 4.0) with 20 mM EDTA. Buffer and detergent phase were mixed at 0 °C, and condensation was repeated twice at 30 °C under the same conditions. The detergent phase of third condensation had a concentration of 25% TX-114 (w/v) and was used as the detergent stock solution for of all the experiments. TX-114 concentration was estimated from the 278-nm absorption of dilute detergent solutions (A_{278} = 1.25 for 0.05%; w/v) (Werck-Reichart et al., 1991).

Partial Purification of Soluble Potato PPO. Tubers were washed, hand-peeled, and halved longitudinally. A 100-g sample was homogenized with 150 mL of cold buffered 200 mM sodium acetate (pH 4.0), 20 mM EDTA, and 6% (w/v) TX-114 for 1 min. The homogenate was filtered through four layers of cheesecloth and kept at 4 °C for 90 min before being centrifuged at 100000*g* for 30 min at 4 °C. The supernatant was collected and used as a crude enzyme extract (step 1). This supernatant was subjected to temperature-induced phase partitioning by increasing the TX-114 concentration by an additional 4% (w/v) at 4 °C and then warming to 35 °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 proteins 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 soluble potato PPO brought to 30% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the

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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 60% saturation and stirred for 1 h at 4 °C. The precipitate obtained between 30 and 60% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of water. The salt content was removed by a desalting column of Sephadex G-25. The enzyme was stored at −20 °C, with a 30% loss of the original activity after 6 months and no discoloration.

Enzyme Activity. Catecholase activity was determined spectrophotometrically at 400 nm (Sánchez-Ferrer et al., 1989a) with *tert*-butylcatechol (TBC) (ϵ = 1682 M^{-1} cm^{-1}) and chlorogenic acid (ϵ = 1018 M^{-1} cm^{-1}). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of the corresponding quinone/min.

Unless otherwise stated, the standard reaction medium at 25 °C contained 0.2 μg/mL partially purified PPO, 50 mM sodium acetate (pH 4.5) or sodium phosphate buffer (pH 6.5), and 3 mM TBC in a final volume of 1 mL. After the system had been equilibrated, an aliquot of sample containing PPO was added. Each sample was assayed in triplicate and the mean and standard deviation plotted. In the activation assays, the sample was preincubated with trypsin or with detergent for 5 or 15 min, respectively.

To determine the effect of the inhibitors, PPO activity was measured in the standard reaction medium in the presence or absence of the stated concentration of inhibitor. The activity was determined from the slope of the reaction curve after any delay produced by the addition of the inhibitors.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was carried out as described by Angleton and Flurkey (1984). Samples were mixed with glycerol 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 acetate buffer (pH 6.5) containing 5 mM L-DOPA.

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

RESULTS AND DISCUSSION

Enzyme purification in plant extracts is made difficult by the presence of a large variety and quantity of secondary products that can bind tightly to the enzymes and change their characteristics (Loomis, 1974). To overcome this problem, different methods have been developed such as the use of acetone powders, ammonium sulfate fractionation, salts, insoluble polymers, and detergents. Among the last, TX-114 shows the special feature of forming clear solutions in buffers at 4 °C, whereas it separates into two phases at 20 °C due to the formation of large micellar aggregates. This fact has been used to separate integral proteins from hydrophilic proteins, since the former remain in the detergent-rich phase (Bordier, 1981; Pryde and Phillips, 1986). Recently, TX-114 has found another use in plant biochemistry to remove phenolic compounds from grape berries and broad bean leaves (Sánchez-Ferrer et al., 1989a, 1990), although no data referring to other plant tissues with a high phenolic content, such as potato tuber, have been presented.

The method described in this paper used the combination of the detergent TX-114 and ammonium sulfate fractionation to avoid enzymatic browning in potato tuber during PPO purification. The main difference from the other methods already published lies in step 1, which mainly consists of adding 6% (w/v) TX-114 instead of acetone, Ca^{2+} ions, or reducing agents such as metabisulfite, ascorbic acid, or cysteine. These reducing agents usually act as PPO inhibitors.

Table I. Partial Purification of Soluble Potato Polyphenol Oxidase

	vol, mL	total act.,[a] units	specific act., units/mg of protein	purifn, *x*-fold	yield, %	phenols, mg
crude extract	175	285	4	1	100	34
4% (w/v) TX-114 after phase partitioning	160	70	4	1	24	20
30–60% $(NH_4)_2SO_4$	7	53	20	5	18	0.7

[a] Assayed at pH 6.5 with 3 mM *tert*-butylcatechol as substrate.

This combination of detergent and EDTA prevented the crude extract from browning at 4 °C and permitted the precipitation of the starch when the extract was stored for 1 h at 4 °C. After centrifugation, the supernatant gave a clear solution that is called crude extract in the purification table (Table I). To take full advantage of TX-114 in the removal of phenols and hydrophobic proteins, another 4% (w/v) of TX-114 was added and the solution phase-partitioned by increasing the temperature to 37 °C and holding for 15 min. This two-phase step involved a loss of proteins, including particulate PPO (Table I, step 2), although a soluble PPO with slightly more than half of the phenol content was obtained. In the following step, the ammonium sulfate fractionation gave a 5-fold purification with 18% recovery.

Up to the ammonium fractionation step, this degree of purification is 5 times higher than that found for potato tubers, var. Tebere, purified with 1 mM L-cysteine and Amberlite IRA 938 (Batistuti and Lourenço, 1985), and 6 times higher than that obtained with the method using acetone powder and calcium acetate (Patil and Zucker, 1965). However, the recovery is greater in the first method (62%) (Batistuti and Lourenço, 1985) than in the TX-114 method (Table I), which might be due to the partition of hydrophobic forms of PPO in the detergent-rich phase. The presence of these particulate forms of PPO in potato tubers has been clearly demonstrated (Alberghina, 1964) and evaluated as representing over half of the enzyme activity (Ruis, 1972).

Finally, the method reduced the phenol content to only 3% of the original without the aid of special polymeric resins. The remaining amount of phenolic compounds was greater than that obtained by the same detergents in broad bean leaves (Sánchez-Ferrer et al., 1990) and in grape berries (Sánchez-Ferrer et al., 1989a). However, it is clear that the amount of phenols present in tubers is also greater than in the other two plant materials (Sánchez-Ferrer et al., 1989a, 1990) and that some of the remaining phenols can be highly hydrophilic since they are attached to sugars. The removal of phenols by TX-114 was sufficient to avoid browning of the enzyme solution even after many cycles of freezing and thawing or after months of storage at −20 °C. When ammonium sulfate was used without a previous phase-partitioning step, the partially purified enzyme became discolored.

The partially purified soluble PPO was fully active at pH 4.5 and 6.5 and it was not necessary to activate any latent form, as has been described for other plant PPO (Tolbert, 1973; King and Flurkey, 1987; Sánchez-Ferrer et al., 1989a, 1990). These latent enzymes can be activated by several treatments that include trypsin (Tolbert, 1973), pH (Valero and Garcia-Carmona, 1992), and detergent (King and Flurkey, 1987). Attempts to activate PPO with 0–25 μg/mL trypsin produced a decrease in enzymatic activity (data not shown). The same effect was found with anionic (SDS) and cationic (CTAB) detergents,

Figure 1. Electrophoresis (7.5% gel) of soluble potato PPO stained with 5 mM L-DOPA in 50 mM sodium acetate (pH 4.5). The lane contained 5 μg of enzyme purified by the two-phase partition method.

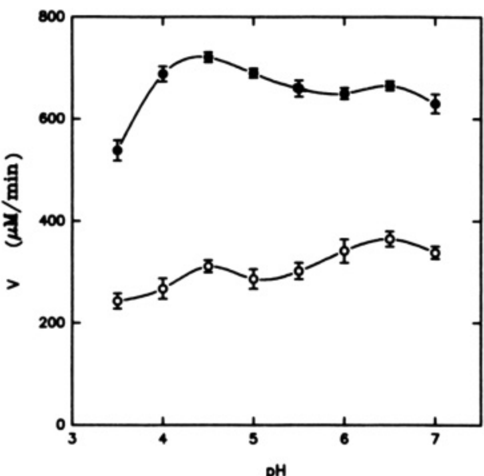

Figure 2. Effect of pH on the catecholase activity of soluble potato PPO in 50 mM sodium acetate (pH 3.5–5.5) and sodium phosphate (pH 5.5–7). The reaction medium contained 2 μg/mL PPO and 3 mM TBC (○) or 2 mM chlorogenic acid (●).

whereas no effect was found in nonionic detergents (Brij 96, $C_{12}E_9$). This lack of activation is in agreement with Sâto (1982), who found latency in spinach leaf PPO but not in spinach tuber PPO.

The partially purified potato PPO only appeared as a single activity band in PAGE when L-DOPA was used as substrate (Figure 1). This is in contrast to the multiplicity found in PAGE for other soluble potato PPO (up to 18 bands), which may be due to covalent reactions of the enzyme with its generated *o*-quinones (Matheis, 1987). These reactions produce enzyme molecules with different isoelectric and electrophoretic properties (Matheis, 1987). In the case of potato PPO, conjugation of the enzyme with its own products produces some forms of PPO with a yellow-white fluorescence (Patil and Zucker, 1965).

The activity curves for the two different substrates showed a maximum at pH 4.5–5 and a secondary maximum or slight shoulder near pH 6–6.5 (Figure 2). The pH curves for chlorogenic acid are similar in shape to those found by Alberghina (1964) and Patil and Zucker (1965). The curve for TBC cannot be compared because it has never been studied as a substrate for potato PPO. The decreased activity below pH 4.0 and above pH 7.0 does not result from the enzyme inactivation by acid or alkaline conditions since incubation at these pHs for 30 min prior to assay at pH 4.5 produced no loss in activity. This result (one activity band and two pH maxima) might be explained

Table II. Kinetic Constants of Partially Purified Potato Polyphenol Oxidase[a]

	tert-butylcatechol		chlorogenic acid	
	pH 4.5	pH 6.5	pH 4.5	pH 6.5
V_m, mM/min	0.85	0.7	7.2	3.85
K_m, mM	5.6	2.3	1.4	0.9
K_{si}, mM			5.5	5.6

[a] Standard assay conditions were used except that substrate concentrations ranged from 0.1 to 20 mM.

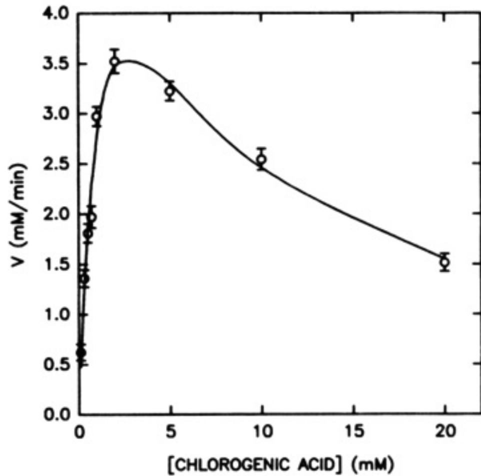

Figure 3. Effect of chlorogenic acid concentration on the initial rate. The reaction medium contained 10 μg/mL partially purified potato PPO and different substrate concentrations in 50 mM sodium acetate buffer) (pH 4.5) at 25 °C.

either by the existence of two protonic states of the ES complex to yield product (Tripton and Dixon, 1983) or by the existence of two different p*K*s in the system (Patil and Zucker, 1965), one related to the localization of histidine in the enzyme active sites at pHs around pH 5.0 and the other produced by the p*K* for the ionization of the first phenolic hydroxyl group of the substrates at pHs around pH 7.0.

The study of the kinetic parameters (V_{max} and K_m) was carried out at the two pHs (4.5 and 6.5). Table II summarizes the values found for TBC and chlorogenic acid. The latter produces inhibition at a substrate concentration above 2 mM (Figure 3). The apparent V_{max}, K_m, and substrate inhibition constant (K_{Si}) was calculated using the following equation (Cornish-Bowden, 1979) by means of Maquardt algorithm (Maquardt, 1967):

$$V = V_{max}[S]/\left(k_m + [S] + \frac{[S]^2}{K_{Si}}\right)$$

The percentage of inhibition as a function of substrate concentration was calculated as the difference between theoretical and experimental rate values at a given concentration and was found to be 77% at 20 mM at both pHs. This value is greater when compared with the inhibition of wheat PPO (50%) (Interesse et al., 1983) and spinach PPO (38%, K_{Si} = 104 mM) (Sánchez-Ferrer et al., 1989b).

The K_m value for chlorogenic acid was less than that obtained by Macrae and Duggleby (1968) and higher than that obtained by Batistuti and Lourenço (1985) for the variety Tebere and by Abukharma and Woolhouse (1966) for the varieties King Edward and Orion. The K_m values for TBC cannot be compared with other potato PPO, although it was similar to that described for other plant PPO (Bru et al., 1990; Sánchez-Ferrer et al., 1992).

TBC was used for the inhibition studies since it produced no substrate inhibition. In addition, minor differences

Table III. Inhibition (Percent) of Partially Purified Potato Polyphenol Oxidase by Reducing Agents[a]

	pH 4.5			pH 6.5		
	1 mM	0.1 mM	10 μM	1 mM	0.1 mM	10 μM
ascorbic acid	100	50	3	100	27	0
diethyldithio-carbamate	100	98	47	87	53	19
metabisulfite	100	82	0	100	90	18
L-cysteine	100	53	4	99	65	18

[a] Assayed under the standard reaction conditions with the appropriate concentration of inhibitor.

were observed between V_{max} values at pH 4.5 and 6.5, and the quinone derived from TBC is more stable than the corresponding quinone of chlorogenic (Kahn, 1990; Richard-Forget et al., 1992). Tables III and IV show the effects of various inhibitors on catecholase activity with TBC as substrate. All inhibitors used in this study inhibited PPO activity, the extent of inhibition being dependent on the concentration of the compound used and the pH. Among reducing agents (Table III), ascorbic acid, L-cysteine, and metabisulfite appeared to be most effective inhibitors at pH 6.5, whereas at pH 4.5 diethyldithiocarbamate was the most efficient since 98% inhibition was found at 0.1 mM (Table III). The action mechanism differs according to the reducing agent used. 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 since it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions which lead to browning (Whitaker, 1972). Additionally, it has been described that ascorbic acid caused irreversible inhibition (Golan-Goldhirsh and Whitaker, 1984). Finally, diethyldithiocarbamate possibly acts by complexing the copper prosthetic group of the enzyme, as has been found for other plant PPO (Anosike and Ayaebene, 1981).

With regard to substrate analogs (Table IV), tropolone was the most effective inhibitor, completely inhibiting enzyme at 1 mM. In addition, it was the most effective of the inhibitors used in Tables III and IV.

On the other hand, mimosine had little inhibiting effect at either pH, whereas inhibition by kojic and cinnamic acids were pH-dependent, the former being more effective at pH 6.5 and the latter at 4.5.

The kinetic analysis of the inhibition was carried out only with tropolone and cinnamic acid, since the former was the more effective inhibitor of the 2-hydroxy-1-one compounds (Table IV) and the latter is a structural analog to chlorogenic acid. The inhibition was determined by Lineweaver–Burk plots of $1/v$ vs $1/S$ at two inhibitor concentrations and confirmed by a Dixon plot of $1/v$ vs I. The inhibition constant, K_i, of each compound was deduced from the points of interception of the plots. Straight lines were obtained for both inhibitors (data not shown) at both pHs. The type of inhibition and K_i values for each inhibitor are shown in Table V. Cinnamic acid has previously been identified as a competitive inhibitor of potato PPO (Batistuti and Lourenço, 1985), with a K_i value of 20 μM when chlorogenic acid is used as substrate. This discrepancy stems from the fact that the K_i values and the type of inhibition depend on the substrate used to carry out the experiment (Macrae and Duggleby, 1968).

On the other hand, inhibition by tropolone has never been kinetically characterized for potato PPO. This compound showed mixed-type inhibition at both pHs and was analyzed by plotting S/v against I at two substrate concentrations (Cornish-Bowden, 1974); this plot intersects the axis, where $I = -K_i'$ (Figure 4). In the case of mixed-type inhibition, the inhibitor can bind both to the free enzyme (to give the complex EI with the dissociation constant K_i) and to the ES complex (to give an unreactive EIS complex with dissociation constant K_i'), both inhibitor binding reactions being dead-end reactions.

This mixed type of inhibition found with tropolone is surprising because inhibition by this compound is time-dependent for crude grape PPO (Valero et al., 1991) and generates a progressive decrease in the initial activity, followed by a constant rate. Both parameters, initial and steady-state rates, are dependent on the inhibitor concentration.

CONCLUSIONS

Triton X-114 was used to remove phenols from potato tubers to an extent where no browning was produced during the partial purification of PPO. This avoids the use of methods using acetone powder or reducing agents, which inhibit the enzyme. However, it was only useful for

Table IV. Inhibition (Percent) of Partially Purified Potato Polyphenol Oxidase by Substrate Analogs[a]

	pH 4.5			pH 6.5		
	1 mM	0.1 mM	10 μM	1 mM	0.1 mM	10 μM
cinnamic acid	84	0	0	19	12	9
tropolone	99	96	88	93	91	76
L-mimosine	3	1	0	13	10	0
kojic acid	53	2	0	63	25	12

[a] Assayed under the standard reaction conditions with the appropriate concentration of inhibitor.

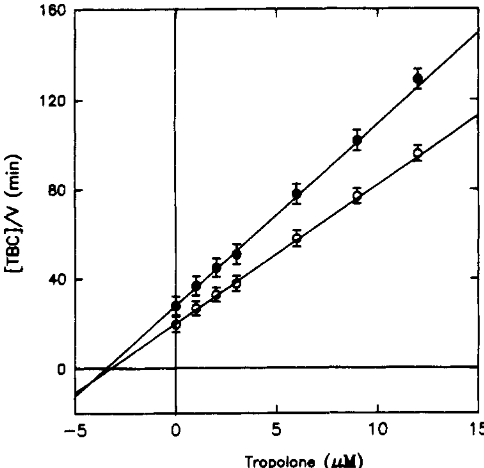

Figure 4. Cornish-Bowden plot (S/v vs I) for the mixed type of inhibition presented by tropolone. The reaction medium contained 2 μg/mL soluble PPO, tropolone (0–15 μM), and two different substrate concentrations of TBC, (○) 3 and (●) 6 mM.

Table V. Inhibition Kinetic Constants of Partially Purified Potato Polyphenol Oxidase[a]

	cinnamic acid (competitive inhibitor)		tropolone (mixed-type inhibitor)	
	pH 4.5	pH 6.5	pH 4.5	pH 6.5
V_m, mM/min	1.6	0.3	218	42.5
K_m, mM			4.7	1.8
K_i, μM	930	550	1.8	2.3
K_i', μM			3.2	4.0

[a] Assayed under the standard reaction conditions with different inhibitor concentrations ranging from 0 to 2 mM for cinnamic acid and from 0 to 15 μM for tropolone.

purifying soluble enzyme, since particulate (hydrophobic) forms of the enzyme were retained in the detergent-rich phase.

The removal of phenols also avoids the postpurification browning usually found with this enzyme and the appearance of artifactual isoenzymes caused by polymerization with o-quinones (Matheis, 1987).

The results are in agreement with those previously published for phenol removal by TX-114 in other aerial plant material (Sánchez-Ferrer et al., 1989a,b, 1990) and emphasize the usefulness of this detergent as a basic tool in enzyme purification in plant biochemistry.

ACKNOWLEDGMENT

This work was partially supported by CICYT (Proyecto BIO91-0790) and Comunidad Autónoma de Murcia (Ayudas a graduados para ampliación de estudios, 1992). A.S.F. is a holder of a Reincorporación grant (MEC).

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Received for review December 21, 1992. Revised manuscript received April 29, 1993. Accepted May 10, 1993.