Novel Procedure for Extraction of a Latent Grape Polyphenoloxidase Using Temperature-Induced Phase Separation in Triton X-114[1]

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ABSTRACT

Polyphenoloxidase from grape berries is extracted only by nonionic detergents with a hydrophilic-lipophilic balance between 12.4 and 13.5. The enzyme was partially purified in latent form, free of phenolics and chlorophylls, by using temperature phase partitioning in a solution of Triton X-114. This method permits the purification of the enzyme with the same fold purification as the commonly used method, but with a yield three times higher and a 90% reduction in time needed. The latent enzyme can be activated by different treatments, including trypsin and cationic and anionic detergents. Cetyltrimethylamonium bromide was found to be the most effective detergent activator, followed by sodium dodecyl sulfate. Polyphenoloxidase in grape berries, in spite of being an integral membrane protein, had an anomalous interaction with Triton X-114, remaining in the detergent-poor phase after phase separation. This could be explained by its having a short hydrophobic tail that anchors it to the membrane.

In vitro, PPO[2] (EC 1.14.18.1) catalyzes the *o*-hydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity) (25). These quinones polymerize to brown pigments. In some plants, this enzyme exists in a latent form (10, 18), and is activated by different treatments such as trypsin (32), fatty acids (10), aging (22), detergents (31), association-dissociation phenomena (26) and cations (30). This kind of latent PPO has never been isolated in fruit.

The physiological function of PPO (33) and its intracellular location (16, 25) remain unknown. Perhaps one of the reasons for this is that it is difficult to purify. This may be due to two factors. First, is the interference of endogenous phenolic compounds during extraction which inactivates or modifies the enzyme (23). Second, is the interference of Chl released when detergents are used to extract the enzyme; these Chl give dark green supernatants.

[2] Abbreviations: PPO, polyphenoloxidase; HLB, hydrophilic-lipophilic balance; CMC, critical micelle concentration; TX-100, Triton X-100; TX-114, Triton X-114; CTAB, cetyltrimethylamonium bromide; Brij 96, poly-(10)-oxyethylene oleyl ether, $C_{12}E_9$, poly-(9)-oxyethylene lauryl ether; Aerosol OT, dioctyl sulfosuccinate.

To overcome the first problem, some phenol scavengers (PEG, insoluble PVP, Amberlite XAD-2) can be used. However, the best combination of these compounds has to be found for each plant, since all plants contain different types and quantities of phenols. The second problem is solved by the use of additional hydrophobic chromatography (Bio-Gel P6 column, Bio-Rad) or by the use of drastic methods, such as acetone powders, to eliminate the green pigments.

In this paper we report for the first time the extraction, partial purification and activation of a latent PPO from grape berries, using temperature-induced phase separation in TX-114. This method was developed by Bordier (3) to separate mixtures of hydrophilic and hydrophobic proteins in animal cells. The detergent TX-114 forms clear micellar solutions in water at low temperatures (4°C) but separates into two phases in equilibrium at temperatures above 20°C, one detergent-rich (20% [w/v]), where membrane proteins were found, and the other detergent-poor (0.03% [w/v]), where cytoplasmic and peripheral membrane proteins were found (3).

When we used this detergent to digest the chloroplast membrane, we found that Chl and phenols coaggregated with TX-114 micelles in the detergent-rich phase, after phase separation. This treatment gave a partially purified grape PPO in a latent form, free of phenolics and Chl.

MATERIALS AND METHODS

Plant Material

The ripe grape berries (*Vitis vinifera* L. cv Monastrell) used in this study were harvested in Jumilla, Murcia, Spain, and stored at −20°C until used.

Reagents

Biochemicals were purchased from Sigma Chemical Co. and used without further purification. TX-114 was obtained from Fluka AG (Bucks, Switzerland) and condensed as described by Bordier (3), but using 100 mM sodium phosphate buffer (pH 7.3) instead of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl. The detergent phase of the third condensation had a concentration of 25% TX-114 (w/v) and was used as the stock solution of detergent for all the experiments.

Protein Determination and Enzyme Assay

Protein content was determined by the dye binding method of Bradford (4) using BSA as a standard, after precipitating

proteins with TCA. Chl were measured in 80% acetone (2). Phenolic compounds were determined spectophotometrically in 80% etanol (19). Catecholase activity toward 4-methyl catechol and cresolase activity towards p-cresol were determined spectophotometrically at 400 nm (29). One unit of enzyme was defined as the amount of the enzyme that produces 1 μmol of 4-methyl o-benzoquinone per min. Unless otherwise stated, the reaction media at 25°C contained 10 mM sodium phosphate buffer at pH 6.5 and 25 mM 4-methyl catechol in a final volume of 1.0 mL. After the system had been equilibrated, an aliquot of sample containing PPO was added. In the activation assays, the sample was preincubated with trypsin (1000 units/ml) or with detergent for 5 or 15 min, respectively.

Enzyme Extraction

Grape berries (25 g) were defrosted in 12.5 mL of 100 mM sodium phosphate buffer at pH 7.3, containing 10 mM sodium ascorbate, homogenized in a blender for 15 s, filtered through 8 layers of gauze and centrifuged at 4,000g for 15 min. The precipitate was extracted with sodium phosphate buffer of different ionic strengths or with different detergents for 30 min, and then centrifuged at 60,000g for 15 min. The supernatant was tested for activity with and without trypsin.

Purification of Latent PPO in TX-114

The precititate of 4000g for 15 min corresponding to 50 g of grape berries was extracted with 20 mL of 1.5% TX-114 (w/v) in 100 mM sodium phosphate buffer (pH 7.3) for 30 min at 4°C. After high speed centrifugation (60,000g for 15 min), this dark green extract yielded a slight green supernatant with PPO activity.

This supernatant was subjected to temperature phase partitioning by adding TX-114 at 4°C, so that the final detergent concentration was 4° (w/v). The mixture was kept at 4°C for 15 min and then warmed to 35°C. 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 the remaing Chl. This solution was centrifuged at 5000g for 10 min at room temperature. The clear supernatant was used as enzyme source and the dark green detergent-rich phase with no PPO activity was discarded.

This new method, which we named TX-114 method, was compared with the most cited method for purification of grape berry PPO (12). This latter method uses TX-100 for 30 min to digest the chloroplast membrane, and subsequently precipitates the enzyme with ammonium sulfate between 45 to 95% saturation at 4°C.

Electrophoresis

SDS-PAGE was carried out as described by Angleton and Flurkey (1), using the method of Laemmli (21). Samples were mixed with glycerol and bromphenol blue before being applied to 7.5% or 12.5% polyacrylamide gels. Electrophoresis was carried out for 6 h at room temperature. Gels were stained

for PPO activity in 100 mL of 10 mM acetate buffer (pH 5.0), containing 5 mM L-dopa.

RESULTS AND DISCUSSION

Extraction of the Latent Enzyme

The extraction of PPO from grape berries was carried out with different ionic strengths and detergents independently. The results of these treatments are shown in Table I. The amount of enzyme extracted without using any detergent did not exceed 1 to 2% of the total extracted. However, when the detergents were added, this percentage ranged between 1.3 and 100%, depending on the electrical charge of the detergent. Among the detergents used, the most efficient were the nonionic, with a hydrophile-lipophile balance (HLB) between 12.4 and 13.5. These values of hydrophile-lipophile balance are very close to those used in the solubilization of mitochondrial membrane proteins (6). Anionic and cationic detergents extracted PPO with the same low efficiency as buffer, with the exception of sodium deoxycholate, perhaps because of its low aggregation number.

The possible synergic effect of trypsin in the extraction of grape PPO was studied (Table IC), by combining it with different extraction media: 1.5% TX-100 (w/v), 0.75% CTAB (w/v) or 100 mM sodium phosphate buffer. The degree of extraction obtained was of 65%, 10%, and 10%, respectively. The results of Table IC show that trypsin itself was able to extract little enzyme, but its action was enhanced by the presence of detergents that digest the membrane. However, the activity obtained was greater when the enzyme was first extracted with TX-100 and later activated with trypsin (Table IB), than when the TX-100 and trypsin acted simultaneously (Table IC). This effect could be explained by the interference of other membrane components in the action of trypsin.

The percentage of activation by trypsin with each treatment varied from 100 to 5700%. The highest values were found in the group of nonionic detergents (Table IB). The method used to purify TX-114 is important, since the use of TX-114 purified in Tris-HCl buffer, as described by Bordier (3), activates the enzyme twice as readily as the TX-114 purified in sodium phosphate buffer. This method then does not serve our purpose as we wanted to keep the enzyme in latent form. For this reason, the rest of this work was carried out with TX-114 purified in sodium phosphate buffer.

Based on the nomenclature used by Helenius and Simons (15), the results of Table I might permit classifying grape PPO as an integral membrane protein, since it needs to be extracted with detergents.

Purification of the Latent Enzyme

The results of Table I show that TX-114 was able to solubilize the proteins and chlorophylls with the same efficiency as TX-100 did. However, unlike TX-100, TX-114 fails to maintain all of the proteins and Chl in solution. This was used to advantage: after a few minutes a dark precipitate was formed by the aggregation of large mixed micelles of TX-114, which contained membrane proteins, phospholipids, phenols and Chl. After high speed centrifugation, the supernatant was

Table I. *Enzyme Extraction from Chloroplast Membrane*

Treatment	Total Units[a]		Activa-tion %	Extrac-tion[b] %
	−Trypsin	+Trypsin		
			%	%
A. Phosphate buffer (mM)				
100	1.3	1.3	100	0.7
200	1.9	3.1	163	1.6
500	1.2	1.5	125	0.8
B. Detergents[c]				
Cationic: CTAB 0.73% (w/v)	1.0	2.6	260	1.4
Anionic:				
Deoxycholate 0.24% (w/v)	6.0	6.8	113	3.6
Aerosol OT 0.089% (w/v)	1.2	2.5	208	1.3
Nonionic				
TX-100 1.5% (w/v)	3.3	189	5727	100
TX-114 1.5% (w/v) Tris-HCl	6.5	189	2907	100
TX-114 1.5% (w/v) phos-phate	3.3	189	5727	100
C12E9 1.5% (w/v)	3.6	191	5277	100
Brij 96 1.5% (w/v)	5.0	166	3320	88
C. Trypsin (1000 units/mL)				
Phosphate buffer	19			10
TX-100 1.5% (w/v)	123			65
CTAB 0.73% (w/v)	20			10

[a] Assayed with 4-methyl catechol as substrate. [b] Refers to the trypsin activated form. [c] All the detergents were dissolved in 100 mM phosphate buffer pH 7.3.

slightly green, clearly indicating the elimination of Chl and phenols from the original extract.

This property of TX-114, never before described, has permitted us to devise a new purification method. This method, which we named TX-114 method, involves two steps. The first is the extraction of proteins by TX-114, and the second is a temperature-induced phase partitioning. To carry out this latter step, it is necessary to increase the concentration of TX-114 to 4% in the supernatant of the extract (Table II).

This new purification procedure was compared with the method of Harel and Mayer (12), which is the most cited method used to purify grape berry PPO. The results, presented

in Table II, show that the purification fold was very close in both methods. However, the yield (%) was three times greater in the TX-114 method. This result could have been explained by the inactivation and precipitation of the enzyme by the high concentration of phenolics present in the Monastrell red grape berries. This phenomenon has been described in other grape enzymes (14), where dark black insoluble precipitates are produced by the reaction of phenolics with the proteins. These precipitates were never found in any step of the TX-114 method, even when the samples were stored at 4°C for longer than a week.

The PPO isolated by the TX-114 method was latent, and,

Table II. *Partial Purification of Grape PPO*

	Total Protein	Total Activity[a]		Specific Activity	Purification	Recovery	Activation	Chl	Phenolic Compounds
		−Trypsin	+Trypsin						
	mg	units		units/mg	-fold	%	%	μg/mL	mg/mL
Ammonium sulfate method									
Crude extract	15	500	500	33	1	100	100	11	42.5
TX-100 extract	7.8	30	370	47	1.5	74	1233	16	14.5
Supernatant of TX-100 extract	5.7	5.7	324	57	1.7	65	5684	13	12.1
45%–95% ammonium sulfate	0.6	50	101	168	5.1	20	200	ND[b]	0.5
TX-114 method									
Crude extract	15	500	500	33	1	100	100	11	42.2
TX-114 extract	7.8	10	270	47	1.4	74	3700	19	14.5
Supernatant of TX-114 extract	4.3	5.5	322	75	2.3	65	5854	4	3.5
Supernatant 4% of TX-114	1.9	5.0	320	168	5.1	64	6400	ND	0.6

[a] Assayed with 4 methyl catechol as substrate. [b] ND, not detected.

when activated by trypsin, produced a 65-fold increase in activity, whereas the enzyme obtained by the method of Harel and Mayer (12) produced only a twofold increase (Table II). This implies that the ammonium sulfate is an activator agent of the enzyme, as has been described before for other latent plant PPOs (10, 31).

The activation of the enzyme by trypsin in the TX-114 method increased during purification, while in the ammonium sulfate method it decreased. However, it was the same in the supernatants of both TX-100 and TX-114 extracts (Table II).

In addition to purifying PPO, TX-114 was able to remove the chlorophylls and phenolic compounds presented in the extract (Table II). This elimination of Chl and phenols during the purification process is a new and remarkable feature of this detergent, never before described. TX-114 was able to remove 79% of the Chl and 75% of phenolics present in the TX-114 extract by centrifugation, compared to the 19% and 16%, respectively, removed by TX-100. In the next step, where TX-114 was at 4% (w/v), the Chl were completely removed and 97% of phenols were removed (Table II). These results open a new way for removing Chl and phenols from green plant extracts in order to clarify them, avoiding the use of drastic extraction methods, such as acetone powders or other organic solvents.

The grape PPO purified by the two methods presented in Table II was examined by SDS-PAGE (1). The Coomassie-stained gels revealed the presence of several other proteins. When this gel was stained with L-dopa to develop the PPO activity (Fig. 1), we found two clearly defined bands in the case of the PPO purified by the TX-114 method (Fig. 1, lane 1). However, in the grape PPO purified by the ammonium sulfate method (12), we found not only the same two bands but also a series of small associated bands arranged in the form of a dark trail over all the lane (Fig. 1, lane 2). This difference in the enzymatic pattern for the same enzyme, could be explained by the formation of the artefactual bands formed by the interactions between phenols, ammonium sulfate and the enzyme. In fact, these multiple forms have been described for other grape PPO (13) and for other plant PPO (17, 26), where some of the bands convert into others during isolation and storage (13, 17). In these cases, the TX-114 method might enable one to determine whether or not these bands are artificial interactions.

The mol wt of the two PPO bands obtained by the TX-114 method was determined using SDS-PAGE (21). The values of 38,000 and 39,000 were found, respectively (Fig. 2). These mol wt were very close to those described for other plant PPOs, ranging from 40,000 to 45,000 (7).

Attempts at further purification of the latent enzyme were made by using gel filtration in different kinds of gels. The result of this was the activation of the latent enzyme. The use of ion-exchange chromatography onto a DEAE-cellulose column (Pharmacia) decreases the latent enzyme to 60% and onto a CM-cellulose column (Pharmacia) to 30%. The use of hydrophobic chromatography onto a Phenyl Sepharose CL-4B column (Pharmacia) activates the enzyme due to the use of ammonium sulfate to elute the enzyme.

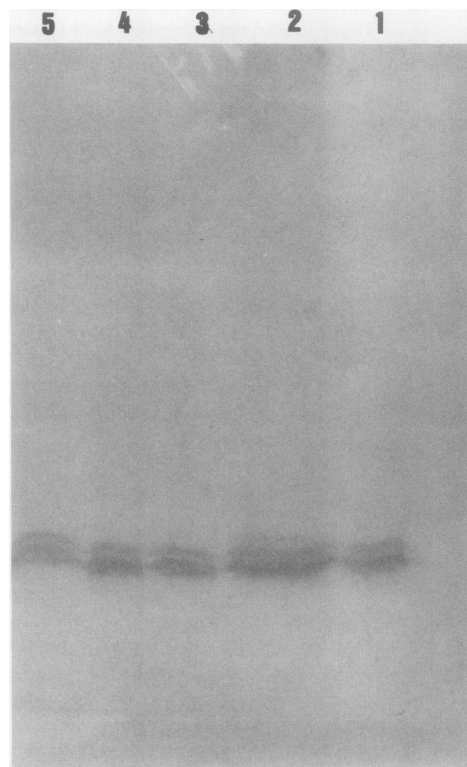Finally, we used affinity chromatography with Con A

Figure 1. Electrophoresis (7.5% gel) and enzyme staining of grape PPO. Lane 1, 4% TX-114 enzyme; lane 2, ammonium sulfate enzyme; lane 3, DEAE-cellulose treated enzyme; lane 4, CM-cellulose treated-enzyme; lane 5, Con A treated-enzyme. Each lane contained 20 μg of protein.
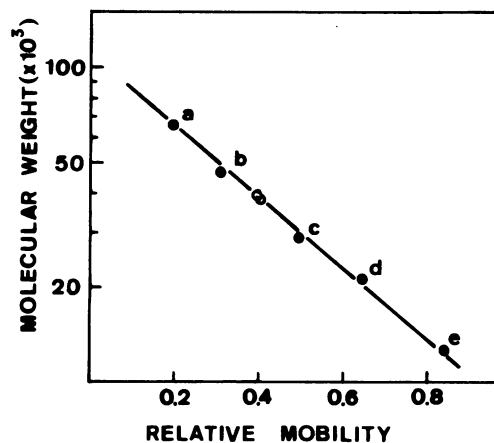

Figure 2. Estimation of mol wt of the two PPO bands by SDS polyacrylamide gel electrophoresis (12.5% gel). The calibration curve was determined using the following protein markers: (a) Bobine serum albumin, (b) ovoalbumin, (c) carbonic anhydrase, (d) soybean trypsin inhibitor, and (e) Cyt *C*. (O) indicates the grape PPO bands.

bound to Sepharose 4B (Sigma), since other plant PPOs contain carbohydrate (7). The gel was unable to bind the grape PPO, suggesting that this enzyme has little or no carbohydrate moiety that binds Con A.

The enzyme obtained after use of the above mentioned

types of chromatography was subjected to SDS-PAGE (1) and compared with the latent enzyme. No changes in their electrophoretic patterns were found (Fig. 1, lanes 1, 3, 4, and 5).

Activation of Latent Enzyme

The latent enzyme obtained after phase separation in TX-114 can be activated by several agents as has been mentioned throughout the above section. Among these are Tris-HCl buffer, ammonium sulfate, PVP, DEAE groups of DEAE-cellulose and carboxymethyl groups of CM-cellulose. To these agents it is necessary to add trypsin and detergents, which we shall now proceed to discuss.

Activation by Trypsin

The activation of latent grape PPO by trypsin is the result of tryptic action. This activation capacity was lost when the trypsin was boiled for 30 min. This result is in accordance with those reported recently by King and Flurkey (20) and contrary to Tolbert's findings (32).

The process of activation by trypsin was completed in five minutes at 37°C, using a concentration of 1000 enzyme units/mL. This procedure is quick and can be used to activate small samples of latent enzyme. However, it has one serious disadvantage in that the trypsin remains in solution with the active enzyme, so reducing its activity over a period of time. This disadvantage can be avoided by the use of immobilized trypsin on a CNBr activated Sepharose column (Pharmacia) (9).

To prove that both activating methods (trypsin in solution and immobilized trypsin) render the same enzyme, its activated enzymatic forms were subjected to SDS-PAGE (Fig. 3, lanes 2 and 3). The enzymatic pattern of trypsin-activated samples was similar to the one shown by the latent enzyme (Fig. 3, lane 1).

Characteristics of Trypsin Activated Enzyme

The column-activated enzyme is a real PPO, since it has both catecholase and cresolase activity. The latter (Fig. 4,b) is characterized by a lag period, defined as the intercept on the abscissa obtained by extrapolation of the linear part of the product accumulation curve. This lag period has been reported for other PPOs (5, 8). This activity has not been found for other plant latent PPOs (10, 29, 30), even after the addition of a small amount of catechol to eliminate the lag period in the hydroxylation reaction (10). The reason for this could be that the conditions used to measure cresolase activity are the same as those used to assay catecholase activity. Furthermore, it is possible that the assays were not carried out over a long enough period of time for cresolase activity to leave its lag period. This can be more than one hour if the same enzyme concentration as in the catecholase activity is present (29).

Activation by Detergents

Detergent activation has been described for other plant PPO, concentrating mainly on anionic detergents, such as SDS and Aerosol OT (18, 31). The effect of detergents on the activity of latent grape PPO was studied using anionic (SDS),

cationic (CTAB) and nonionic (Brij 96 and $C_{12}E_9$). In addition to the above detergents, we included another anionic detergent (1-decanesulfonic acid) never before tested in the activation of a latent PPO. The results obtained with these detergents at two different concentrations are shown in Table III.

At low concentration of detergent (0.4 mM), the cationic detergent CTAB was the most effective activator, followed by SDS (Table III). The nonionic detergents have little effect on the activity. After treatment by detergents, the samples were incubated with trypsin, and in all cases the same specific activity was obtained. The results show that the detergent at this low concentration did not significantly affect the conformational structure of the protein, thus permitting the proteolytic attack of trypsin.

The activation effect of SDS and CTAB increased when detergent concentration was increased to 10 mM (Table III). This effect was higher than the one obtained with the nonionic detergents at this concentration. When the samples previously treated with detergent were activated with trypsin, only the samples treated with nonionic detergents reached the same specific activity as the control. The most marked decrease in trypsin activition was observed in the case of SDS and CTAB. This suggests that some changes in the structure of the protein could have been induced by these detergents, so limiting the proteolytic action of trypsin.

In fact, these two detergents (SDS and CTAB) are described as "denaturing" detergents, although some membrane proteins are resistant to their actions at room temperature (15). Among these proteins, we can include grape PPO.

The effect of detergents on the enzymatic pattern of latent enzyme was tested by SDS-PAGE (1). When the gel was developed with L-dopa (Fig. 3, lanes 4–9), no significant changes with respect to the bands shown by the latent enzyme were found in the samples treated with detergent. This suggests that no noticeable denaturing effect occurs even at high detergent concentration.

The modulation of the PPO activity by detergents could be explained by their dual effect on the protein structure. The detergents do not alter the protein structure or the activity at concentrations below or very close to its critical micelle concentration (Table III). However, when the concentration is above their critical micelle concentration (Table III), the binding of the detergent molecules to the protein changes its structure. In general, these changes are responsible for the loss of biological activity (34).

Anomalous Phase Partitioning Properties of Grape PPO

Grape PPO presents an anomalous interaction with TX-114. After the phase separation takes place, the enzyme remains in the detergent-poor phase instead of in the detergent-rich phase as might be expected for an integral membrane protein. This anomalous behavior has also been reported in other integral membrane proteins, such as the acetylcholine receptor (24) and the glycoprotein III from chromaffin granules (28). The possible reasons given in the bibliography for this behavior were as follows: (a) the protein has a strong association with peripheral membrane proteins (24); (b) the protein is a channel-forming membrane protein (24); and (c) the protein is a membrane glycoprotein (28).
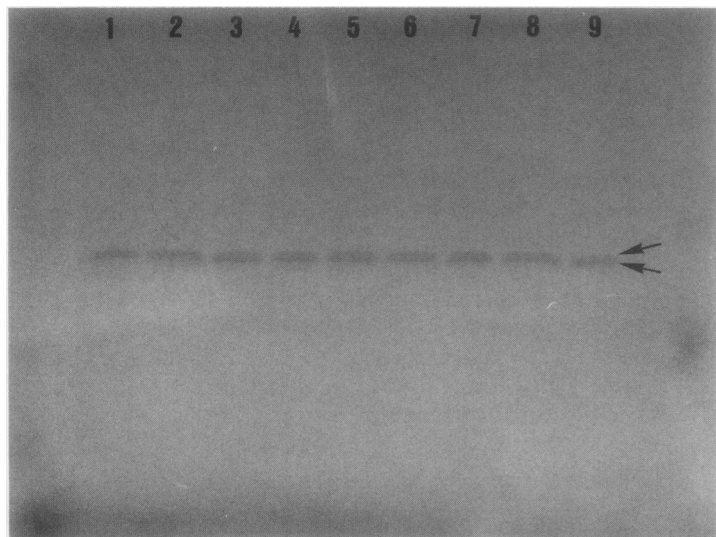
Figure 3. Activation of latent grape PPO with detergents and trypsin. Activation with trypsin: Lane 1, PPO passed through the trypsin column; lane 2, PPO activated with trypsin in solution (1000 units/mL); lane 3, control (4% TX-114 enzyme). Activation by detergents: (a) [detergent] = 0.4 mM; lane 4, SDS; lane 5, CTAB; lane 6, $C_{12}E_9$; (b) [detergent] = 10 mM; lane 7, SDS; lane 8, CTAB; lane 9, $C_{12}E_9$. Each lane contained 10 μg of protein.
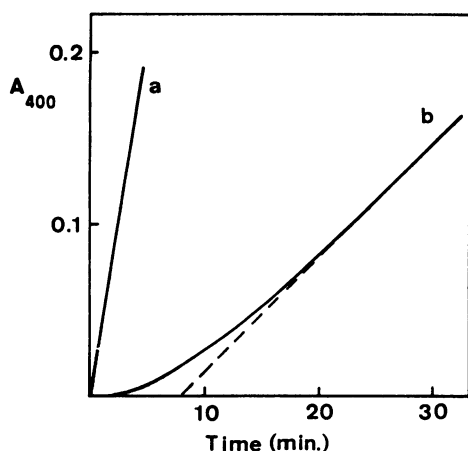

Figure 4. Enzyme activities of PPO activated by trypsin column. (a) catecholase activity towards 25 mM 4-methyl catechol (1 μg of enzyme). (b) cresolase activity towards 2.1 mM *p*-cresol (100 μg of enzyme).

The activation studies with trypsin and detergents presented in this paper permit us to suggest the following possible explanation for the anomalous interaction of latent grape PPO with TX-114. This enzyme seems to belong to a fourth group of proteins, which have only a short hydrophobic tail to anchor them to the membrane. This hydrophobic chain is not big enough to bind a sufficient number of detergent molecules to be precipitated with the larger micelles of TX-114, when the temperature-induced phase partitioning takes place. This hypothesis is based on the following: (a) the little

Table III. *Activation of Latent Grape PPO by Detergents*

The latent enzyme (4% TX-114 supernatant) was preincubated with detergents for 15 min and assayed for catecholase activity at pH 6.5 with or without trypsin activation (5 min), using 4-methyl catechol as substrate.

Treatment	Specific Activity		Trypsin Activation	Detergent Activation
	−Trypsin	+Trypsin		
	units/mg		%	%
[Detergent] = 0.4 mM				
None	2.5	166	6640	100
SDS	5.8	180	3103	232
1-Decanesulfonic acid	3.1	174	5612	124
$C_{12}E_9$	3.7	170	4594	148
Brij 96	4.0	178	4450	160
CTAB	17.0	174	1023	680
[Detergent] = 10 mM				
None	2.5	166	6640	100
SDS	48.0	56	116	1920
1-Decanesulfonic acid	8.5	94	1105	340
$C_{12}E_9$	2.0	131	6550	80
Brij 96	3.5	135	3857	140
CTAB	57.0	90	157	2280

difference between the latent and trypsin-activated enzyme; (b) the lack of detergent modulation observed after activating the enzyme with trypsin; (c) the absence of association phenomena with other proteins during purification; (d) the absence of sufficient carbohydrate moietie to be considered as a glycoprotein; and (e) the enzyme has never been described as a channel-forming membrane protein.

CONCLUSIONS

This paper shows the advantages of purifying grape berry PPO by using phase partitioning in TX-114. Some of these advantages are: (a) the method isolates a latent form of grape berry PPO, which contrasts with an almost fully active form obtained using the commonly used method (12); (b) it saves time in purification. The commonly used method (12) take 2 to 3 d to obtain the enzyme, whereas the TX-114 method only takes 3 h to obtain the same degree of purification and a yield three times higher; (c) it removes phenolics and Chl in only one step without chromatography and avoids aggregations, inactivation and multiplicity of the enzyme during purification; and (d) the reproducibility of the entire procedure is excellent and the latent enzyme can be stored at −20°C without any loss of its activity for at least 2 months.

This procedure using TX-114 opens up many new avenues for plant research. It is especially useful in the study of plant plasma membrane proteins since there are as many as 100 polypeptides visible on two dimensional gels of plant plasma membrane proteins and only a few of these (such as H⁺-ATPase and β-glucan synthase) have been characterized (11). In addition, it can be used to separate Chl from the enzyme chlorophyllase (27). And finally, the method permits the elimination of phenolics and Chl in plant crude extracts.

ACKNOWLEDGMENTS

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