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TRITON X-114 AS A TOOL FOR PURIFYING SPINACH POLYPHENOL OXIDASE

ALVARO SÁNCHEZ-FERRER, JOSÉ VILLALBA and FRANCISCO GARCÍA-CARMONA*

Departamento de Bioquímica, Facultad de Biología, Universidad de Murcia, E-30001 Murcia, Spain

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; polyphenol oxidase; Triton X-114; enzyme purification; cresolase; lag period.

Abstract—Spinach polyphenol oxidase was partially purified by using phase partitioning in a solution of Triton X-114 in a latent form, free of phenolics and chlorophylls, with a high recovery rate. The enzyme had both catecholase and cresolase activity. The latter, never before described, presented a lag period which was affected by enzyme and substrate concentration. The K_m for *p*-cresol was 2 mM. Substrate inhibition was seen for catecholase activity when 4-methylcatechol was used. The values found for V_{max}, K_m and K_{si} were 244 μM/min, 4.2 mM and 104 mM, respectively. The latent enzyme was activated by trypsin (4.5-fold), by SDS and 1-decanesulphonic acid at detergent concentration of 1 mM, but it was strongly inhibited by CTAB, frost, and SDS at a detergent concentration of 10 mM. Non-ionic detergents did not alter significantly the activity of latent enzyme.

INTRODUCTION

Extraction of polyphenol oxidase (EC 1.14.8.1) (PPO) from plant sources is complicated by the presence of endogenous phenolic compounds or their corresponding quinones which interact with the enzyme and change its characteristics or even inactivate it [1]. An extra problem appears when the enzyme is bound to the chloroplast membrane, as the detergents release the chlorophylls [2, 3]. Some methods have been developed to overcome these problems, such as the use of phenol-scavengers (like PVP, PEG, Amberlite XAD-2), ammonium sulphate fractionation and acetone powder. These last two methods are drastic because they can modify or activate the enzyme.

We describe here a new mild extraction method for latent spinach PPO that avoids the above problems, by using the special features of TX-114 described by Bordier [4] to separate mixtures of hydrophilic and hydrophobic proteins. Bordier found that the detergent TX-114 forms clear micellar solution in water at low temperature (4°) but separates into two phases in equilibrium above 20° (temperature phase partitioning), one detergent-rich [20% (w/v)], where membrane proteins are found, and the other detergent-poor [0.03% (w/v)] where cytoplasmic and peripheral membrane proteins are found [4]. The PPO thus obtained, had both catecholase (oxidation of *o*-diphenols to *o*-quinones) and cresolase (*o*-hydroxylation of monophenols to *o*-diphenols) activity. Some characteristics of the latter, never before reported are studied in this paper.

*Author to whom correspondence should be addressed.

Abbreviations: PPO, polyphenoloxidase; TX-100, Triton X-100; TX-114, Triton X-114; CTAB, cetyltrimethylammonium bromide; Brij 96, poly-(10)-oxyethylene oleyl ether, $C_{12}E_9$, poly-(9)-oxyethylene lauryl ether, 1-DS, 1-decanesulphonic acid.

RESULTS AND DISCUSSION

Isolation and purification of thylakoid-bound polyphenol oxidase

Polyphenol oxidase of spinach leaf cells is predominantly located in the thylakoid membranes of chloroplast in a latent state [5–10]. Chloroplasts were prepared and osmotically shocked using a modification of the method of ref. [10]. When the chloroplast membranes were digested by TX-114 at 4°, we found that the detergent was able to solubilize the chloroplast membrane as TX-100 did [2]; however it failed to maintain all the membrane components and chlorophylls in solution. This was used to advantage: after a few minutes, a dark precipitate containing membrane proteins, phospholipids and chlorophylls could be recovered by high speed centrifugation, giving a light green supernatant which when subjected to temperature-induced phase partitioning by addition of TX-114 at a final concentration of 8% (w/v), rendered a dark green detergent rich phase (with the remaining chlorophylls and phenolic compounds) and a colourless supernatant with PPO activity (see Experimental). The result of typical purification is summarized in Table 1.

The PPO obtained by this method was latent and could be activated by trypsin (~4.5-fold) as described before for spinach PPO [7], although the degree of activation with trypsin was less than the one obtained by Tolbert (3–30-fold) [7]. The difference was that Tolbert used spinach chloroplast suspension instead of partially purified PPO such as we used, and so the total activation found by him may have been the sum of PPO released from the membrane and the PPO activation by trypsin.

The purification achieved was 4.5-fold with 86% recovery for PPO activity (assayed at pH 6.5 using 4-methylcatechol as substrate with and without trypsin activation). The degree of purification was smaller than that found by Golbeck and Cammarata [10] (20.3-fold)

Table 1. Purification of spinach thylakoid polyphenol oxidase

Step	Volume (ml)	Total activity (units*) −trypsin	Total activity (units*) +trypsin	Specific activity (units/mg) −trypsin	Specific activity (units/mg) +trypsin	Purification (fold)	Recovery (%)	Chl. (mg)
TX-114 extract	40	240	1080	1.6	7.1	1	100	14.4
Supernatant TX-114 ext.	36	233	972	1.8	7.9	1.1	90	7.2
Supernatant after phase partitioning	32	208	928	7.2	32	4.5	86	N.D.

*Assayed at pH 6.5 with 4-methylcatechol as substrate.
Chl. = Chlorophylls.
N.D. = Not detected.

after subjecting the supernatant of sonicated chloroplast to ammonium sulphate 35–65% cut. The discrepancy with our results could be explained by the activation of the latent enzyme extracted by Golbeck and Cammarata [10] by ammonium sulphate during purification, as has been described by Kenten [11]. Therefore, in Table 1, if we consider the activity in the first step without trypsin and the last step with trypsin the fold achieved was the same as [10]; however the recovery was greater in our method.

Finally, the amount of PPO obtained by TX-114 method was greater than in the method of ref. [10], in the latter method 1140 units were obtained from 1 kg of spinach leaves, while with our method we obtained 928 units with only 50 g.

The chlorophyll elimination by TX-114 during the purification process is a new and remarkable feature of this detergent never before described. TX-114 was able to remove 50% of the chorophylls only by centrifugation. In the next step where X-114 was at 8% (w/v), the chlorophylls were completely removed (Table 1). This result opens a new way for removing chlorophylls and Triton detergents from enzyme chlorophyllase, which is at present a difficult task [12].

Characterization of PPO

Partially purified PPO has both catecholase and cresolase activity. The latter 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 polyphenol oxidases when cresolase activity was measured [13, 14]. The lag period and the steady-state rate (defined as the slope of the linear part of the accumulation product curve) are affected by substrate and enzyme concentration.

When enzyme concentration was varied in a range between 90 and 540 μg/ml, a linear dependence of steady-state rate was obtained for cresolase activity (data not shown) and an inverse dependence between enzyme concentration and the lag period was found (Fig. 1, a). This result was similar to the one obtained for polyphenol oxidase from other sources [13–16].

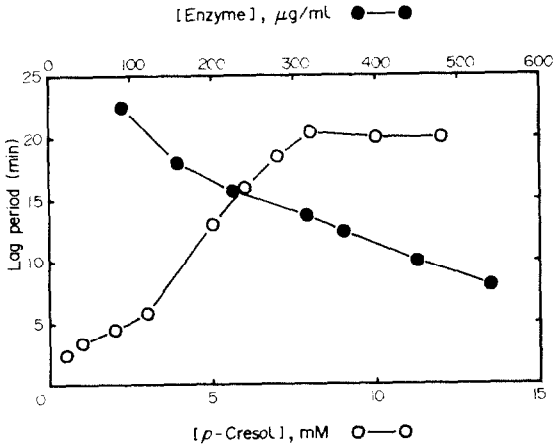

Fig. 1. (a) Effect of substrate (at enzyme concentration of 225 μg/ml) and (b) enzyme (at substrate concentration of 5 mM) concentration on the lag period of cresolase activity.

The effect of substrate concentration in the lag period was studied using *p*-cresol. There was an increase in the lag period when substrate concentration was increased (Fig 1, b). Although the whole effect of substrate concentration was similar to other PPO [13–16], this kind of sigmoid-shape plot has not been described previously, being a special characteristic of spinach PPO cresolase activity. The apparent K_m for *p*-cresol, calculated by the Hanes plot, was 2 mM. This result cannot be compared with any published data, because spinach cresolase activity has not been reported up to now. This may be due to loss of cresolase activity during extraction and purification, as has been described for banana PPO when it is purified from acetone powder [17]. Also, in general, the optimal conditions for measuring this activity have not been sought: for example if the same enzyme concentration was used in the catecholase activity the lag period can last more than one hour [13, 14].

The substrate specificity and kinetic constants were evaluated for catecholase activity using as *o*-diphenols, L-DOPA and 4-methylcatechol. The apparent K_m for L-DOPA was 3.3 mM. This value was smaller than those found in refs [7, 10].

When 4-methylcatechol was studied as substrate, and the rates were plotted against the diphenol concentration, a substrate inhibition was found above 25 mM (Fig. 2). The apparent V_{max}, K_m and K_{si} was calculated using equation 1 by means of the Marquardt algorithm [18],

$$[v = \frac{V_{max}\,[S]}{K_m + [S] + [S]^2/K_{si}} \quad (1)$$

with the values of 244 μM/min, 4.2 mM and 104 mM, being found respectively. The percentage of inhibition as a function of substrate concentration was calculated as the difference between the theoretical and experimental rate values at a given concentration, being 36% at 60 mM. This value is lower when compared with the one given in ref. [19] for wheat PPO, where 50.8% of inhibition was reached at 20 mM of 4-methylcatechol.

Electrophoretic analysis

Polyacrylamide gel electrophoresis (PAGE) of the partially purified PPO was run in order to reveal the different forms present in the thylakoid membrane of spinach chloroplast. SDS-electrophoresis could not be perfomed, because PPO was totally inhibited during the run. This result was in agreement with Meyer and Biehl [20] but contrasted with the four PPO forms by Angleton and Flurkey [21] when the gel was run in the presence of 0.1% SDS. Three bands were observed using L-DOPA as substrate when the PAGE was performed without SDS (Fig. 3, b). However, when 0.5% (v/v) TX-100 was added to the electrode buffer, interconversion of PPO form occurred (Fig. 3, a), which suggests association–dissociation of active subunits. This phenomenon has been reported by Satô and Hasegawa [5], but until now it could not be shown by electrophoresis [22]. The two PPO bands observed when TX-100 was used (Fig. 3, a) are in agreement with the membrane forms IV and V, extracted Meyer and Biehl [20].

Activation of latent PPO

Latent spinach PPO can be activated by storage of membrane containing fractions [6, 8], frost [6], various detergents [5, 8] and trypsin [7]. The effect of some of the above activator agents was studied with spinach PPO purified by temperature-induced phase partitioning in TX-114.

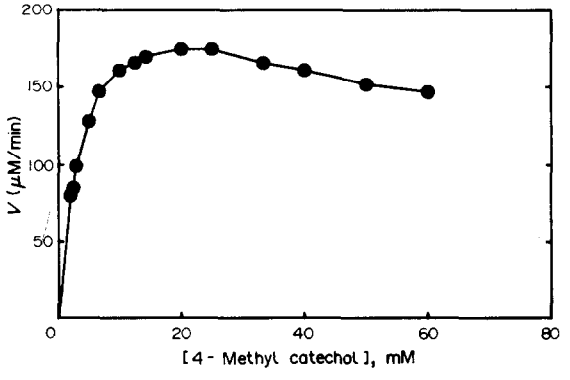

Fig. 2. Effect of 4-methyl catechol concentration on initial rate of spinach PPO. The apparent K_m, V_{max} and K_{si} was calculated after fitting the experimental data to the equation (1) (see text).

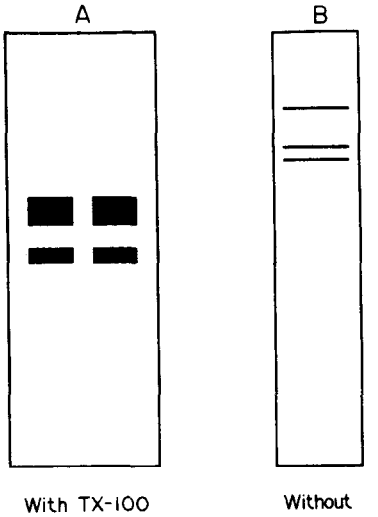

Fig. 3. Electrophoresis (7.5% gel) and enzyme staining of spinach PPO. (a) The electrophoresis was carried out with 0.5% (v/v) TX-100 in the electrode buffer. (b) The same electrophoresis without 0.5% (v/v) TX-100 in the electrode buffer. Each lane contained 80 μg of protein.

Trypsin was the most effective activator. The activation achieved was 4.5-fold in freshly prepared PPO (Table 1). The ability of trypsin to activate PPO decreased with time when the enzyme was stored at 4°, because the enzyme itself achieved full activation in two days, as can be seen in the control of Table 2, a and 2, c. But when the enzyme was stored at −20° for one month the activation obtained was 2.5-fold. This desensitization to trypsin when stored at 4° could be due to conformational changes in the protein that prevent the proteolytic attack and that fully activate the enzyme.

The modulation of activity by detergent was studied using anionic (SDS and 1-decanesulphonic acid), cationic (CTAB) and non-ionic ($E_{12}C_9$ and Brij 96) detergents. Table 2a shows the interaction of the above detergents at a final concentration of 1 mM. Anionic detergents were the most effective activators among the detergents used, while the cationic detergent acted as an inhibitor. Non-ionic detergents at this concentration had little effect on the activity. When the samples previously treated with detergent were activated with trypsin, all samples reached more or less the same activity level, except in the case of CTAB, showing that at this low concentration CTAB still acted as denaturing agent.

The stability of the enzyme with detergent was checked 24 hr later (Table 2, b), showing that SDS had lost its activating effect while 1-decanesulphonic acid retained it; the denaturing effect of CTAB was more marked, and $C_{12}E_9$ inhibited the activity by 30%.

When the detergent concentration was increased to 10 mM (Table 2, c), the modulation of the catecholase activity changed in the case of SDS, which became an inhibitor; this effect was in agreement with the result found for SDS by Meyer and Biehl [20], where 1% SDS inactivated PPO rapidly, while 0.1% SDS activated it within the first 0.5–5 hr, but strongly inactivated it later on. CTAB and $E_{12}C_9$ presented the same inhibitory response as that which showed after 24 hr at a detergent concentration of 1 mM. 1-Decanesulphonic acid and Brij 96 did not affect the activity. When activation with

Table 2. Activation of latent spinach PPO by detergents.

	−Trypsin		+Trypsin	
	Units/ml	% of control	Units/ml	% of control
a) Detergent concentration = 1 mM				
None	5.3	100	7.1	134
E12C9	4.3	81	6.6	124
Brij 96	5.7	103	6.6	124
CTAB	2.8	53	2.8	53
SDS	7.5	142	8.7	164
1-DS	7.1	134	8.7	164
b) Detergent concentration = 1 mM after 24 hr				
None	4.4	100	—	—
E12C9	3.0	67	—	—
Brij 96	4.4	100	—	—
CTAB	1.4	30	—	—
SDS	4.0	90	—	—
1-DS	7.8	178	—	—
c) Detergent concentration = 10 mM				
None	4.4	100	4.4	100
E12C9	3.0	68	4.8	109
Brij 96	4.4	100	5.1	116
CTAB	0.9	20	1.2	27
SDS	0.7	16	0.7	16
1-DS	3.5	80	4.6	104

The latent enzyme (supernatant after phase partitioning) was incubated with detergents for 15 min and assayed for catecholase activity of pH 6.5 with and without trypsin activation using 4-methylcatechol as substrate.

trypsin was measured, only the non-ionic detergent and 1-decanesulphonic acid were able to recover the initial activity, showing that these three detergents did not change the structure of the protein as drastically as CTAB and SDS, thus allowing the proteolytic attack of trypsin. These results were in accordance with Lieberei *et al.* [22], since these authors found no loss of antigenic capacity after a non-ionic detergent treatment of spinach PPO, showing that the structure of the protein did not change drastically. A similar activating behaviour by detergents has been described for broad bean PPO [23, 24].

The partially purified PPO was very sensitive to frost, being inactivated by the process of frost-thawing, as has been described by Satô and Hasegawa [5] and Satô [9]; however, this inactivation seems to be due to a frost-dependent modification apparently brought about by conformational changes in the protein, rather than to a low M_r inhibitor [5, 9]. This was supported by the diminution in the degree of activation found in the samples stored at $-20°$ when they were treated with trypsin. This frost inactivation contrasted with the results of Lieberei and Biehl [6] who found activation. However, the experimental conditions were different, because they used extracts of washed chloroplast membranes while we used enzyme released from the thylakoid membrane.

Spinach PPO presented an anomalous interaction with TX-114 after the phase separation took place, remaining in the detergent-poor phase (colourless supernatant) instead of in the detergent-rich phase, as might be expected for a membrane bound protein. This fact together with the modulation with detergent support the idea that spinach PPO has a short hydrophobic anchor in the membrane. This short anchor might explain the spontaneous and easy release of the enzyme from the thylakoids during homogenization and leaf ageing described by Meyer and Biehl [25].

In conclusion, the results presented in this paper show that extraction and purification of enzymes by using temperature induced phase partitioning in TX-114 does not alter the native enzyme characteristics, saves time in purification with respect to other methods (such as ammonium sulphate fractionation) and removes phenolic compounds and chlorophylls in only one step. These features make TX-114 a useful tool for extracting plant enzymes.

EXPERIMENTAL

Reagents. Biochemicals were purchased from Sigma and used without further purification. TX-114 was obtained from Fluka and condensed × 3, dissolving TX-114 in buffer at 4°, warming it later to 35° and storing the soln at room temp. until the mixture separates into a large aq. phase depleted in detergent and a smaller phase enriched in detergent as described in ref. [4] but

using 100 mM Na-Pi 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 concn of 25% TX-114 (w/v) and was used as the stock soln of detergent for all the experiments.

Enzyme purification. Chloroplasts were prepared by homogenizing 50 g of non-senescent leaves of spinach (*Spinacia oleracea L.*) as described in ref. [10] in a modified buffer containing: 100 mM Na-Pi buffer pH 7.3, 0.33 M sorbitol, 2 mM EDTA and 1 mM $MgCl_2$ for 15 sec. They were then filtered through 8 layers of gauze and centrifuged at 1000 *g* for 2 min. The pellet was discarded and the supernatant was centrifuged at 3000 *g* for 25 min. Chloroplasts were osmotically shocked by resuspension of the pellet in 250 ml of 10 mM Na-Pi Buffer pH 7.3 for 20 min. and collected by centrifugation at 20000 *g* for 20 min. Pelleted chloroplasts were resuspended in 30 ml of 1.5% (w/v) TX-114 in 100 mM Na-Pi buffer pH 7.3 during 30 min. at 4° (TX-114 extract) and then centrifuged at 60000 *g* for 15 min. The light green supernant, with PPO activity, was subjected to temp. phase partitioning by adding TX-114 at 4°, so that the final detergent concn was 8% (w/v). The mixture was kept at 4° for 15 min and then warmed to 35°. After 10 min the soln became spontaneously turbid due to the formation, aggregation and pptn of large mixed micelles between detergent, hydrophobic proteins and the remaining chlorophylls. This soln was centrifuged at 5000 *g* for 10 min at room temp. The clear supernatant was used as enzyme source and the dark green detergent-rich phase without PPO activity was discarded.

Enzyme assay. Catecholase activity towards 4-methylcatechol and cresolase activity towards *p*-cresol were determined spectrophotometrically at 400 nm [13]. 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° contained 10 mM Na-Pi buffer pH 6.5 and 25 mM 4-methylcatechol in a final vol. of 1 ml. After the system had been equilibrated, an aliquot of polyphenoloxidase-containing sample was added. In the activation assays, the sample was preincubated with trypsin (1000 U/ml) or with detergent for 5 and 15 min respectively.

Electrophoresis. PAGE was carried out as described in ref. [26] without SDS, and by adding 0.5% (v/v) TX-100 in the electrode buffer. SDS PAGE was run as described in ref [21]. Gels were stained for PPO activity in 100 ml of 10 mM NaOAc buffer pH 5.0, containing 5 mM L-β-3,4-dihydroxyphenylalanine (L-DOPA).

Other methods. Protein content was determined by the dye binding method of ref. [27] using BSA as a standard. Chlorophylls were measured in 80% Me_2CO [28].

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REFERENCES

1. Smith, D. M. and Montgomery, M. W. (1985) *Phytochemistry* **24**, 901.
2. Harel, E., Mayer, A. M. and Shain, Y. (1965) *Phytochemistry* **4**, 783.
3. Vaughan, P. F. T., Eason, R., Paton, J. Y. and Ritchie, G. A. (1975) *Phytochemistry* **14**, 2383.
4. Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604.
5. Satô, M. and Hasegawa, M. (1976) *Phytochemistry* **15**, 61.
6. Lieberei, R. and Biehl, B. (1978) *Phytochemistry* **17**, 1427.
7. Tolbert, N. E. (1973) *Plant Physiol*, **51**, 234.
8. Lieberei, R. and Biehl, B. (1976) *Ber. Dtsch. Bot. Ges.* **89**, 663.
9. Satô, M. (1977) *Phytochemistry* **16**, 1523.
10. Golbeck, J. H. and Cammarata, K. V. (1981) *Plant Physiol.* **67**, 977.
11. Kenten, R. H. (1957) *Biochem. J.* **67**, 300.
12. Michalski, T. J., Bradsshaw, C., Hunt, J. E., Norris, J. R. and Katz, J. J. (1987) *FEBS Letters* **226**, 72.
13. Sánchez-Ferrer, A., Bru, R., Cabanes, J. and García-Carmona, F. (1988) *Phytochemistry* **27**, 319.
14. García-Carmona, F., Cabanes, J. and García-Cánovas, F. (1987) *Biochim. Biophys. Acta* **914**, 198.
15. García-Carmona, F., García-Cánovas, F. and Lozano, J. A. (1980) *Int. J. Biochem.* **11**, 325.
16. García-Cánovas, F., García-Carmona, F. and Lozano, J. A. (1981) *Phytochemistry* **20**, 1215.
17. Thomas, P. and Janave, M. T. (1986) *J. Food Sci.* **51**, 384.
18. Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431.
19. Interesse, F. S., Ruggiero, P., D'Avella, G. and Lamparelli, F. (1983) *Phytochemistry* **22**, 1885.
20. Meyer, H.-U. and Biehl, B. (1981) *Phytochemistry* **20**, 955.
21. Angleton, E. A. and Flurkey, W. H. (1984) *Phytochemistry* **23**, 2723.
22. Lieberei, R., Biehl, B. and Voigt, J. (1981) *Phytochemistry* **20**, 2109.
23. Kenten, R. H. (1958) *Biochem J.* **68**, 244.
24. Swain, T., Mapson, L. W. and Robb, D. A. (1966) *Phytochemistry* **5**, 469.
25. Meyer, H.-U. and Biehl, B. (1980) *Phytochemistry* **19**, 2267.
26. Laemmli, U. K. (1970) *Nature* **227**, 680.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.
28. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.