Partial Purification and Characterization of Latent Polyphenol Oxidase in Iceberg Lettuce (*Lactuca sativa* L.)

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Polyphenol oxidase (EC 1.14.18.1), a thylakoid membrane-bound enzyme, was partially purified in iceberg lettuce (*Lactuca sativa* L.) in its latent form using a two-phase partitioning approach with Triton X-114. The enzyme thus obtained was practically free of phenolic compounds and clorophylls and showed a recovery of 70%. Polyphenol oxidase was kinetically characterized with two phenolic substrates (4-*tert*-butylcathechol and chlorogenic acid) in both the latent and activated enzyme forms. The latent form was activated by sodium dodecyl sulfate (SDS) so that characterization was carried out in the presence and absence of SDS.

Keywords: *Plant polyphenol oxidase; lettuce; latency; activation; Triton X-114*

INTRODUCTION

One of the most important physiological disorders of iceberg lettuce (*Lactuca sativa* L.) is russet spotting (RS), a postharvest disorder that can develop during transport and storage. It is characterized by the appearance of numerous small brown spots along both sides of the midrib which may spread over the leaf blade in severe cases (Link and Gardner, 1919). The lignification and cell wall thickening in lesions of RS-affected tissue are accompanied by the accumulation and oxidation by polyphenol oxidase (monophenol dihydroxy-L-phenylalanine:oxygen oxide reductase, EC 1.14.18.1) (PPO) of soluble phenolic compounds such as flavonoids and chlorogenic acid derivatives, resulting in the brown discoloration which is characteristic of RS tissue (Ke and Salveit, 1988).

In plants, PPO is located in the chloroplast thylakoid membranes and is often found in multiple forms. 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 Whitaker, 1984; Garcia-Carmona et al., 1988). One unusual and intriguing characteristic of the enzyme is its ability to exist in an inactive or latent state (Mayer and Harel, 1979). PPO can be released from latency, or activated, by a variety of treatments or agents including acid and base shock (Kenten, 1957), urea (Swain et al., 1966), polyamines (Jimenez et al., 1991), anionic detergent, such as SDS (Kenten, 1958; Flurkey, 1986; Golbeck and Cammarata, 1981; Sánchez-Ferrer et al., 1993), proteases (Golbeck and Cammarata, 1981; King and Flurkey, 1987; Soderhal and Soderhal, 1989; Sánchez-Ferrer et al., 1989), and fatty acids (Golbeck and Cammarata, 1981; Hutcheson and Buchanan, 1980). SDS is particularly interesting as an activating agent because few enzymes are known to be activated by it, in contrast to the many enzymes that are inactivated. Thus, PPO is activated at high SDS concentrations (Moore and Flurkey, 1990), which would denature many other enzymes.

Although plant PPO has been extensively studied (Mayer and Harel, 1979; Mayer, 1987) because browning decreases the market value of fresh fruits and vegetables, few studies refer to iceberg lettuce PPO. Thus, Sharples et al. (1963) pointed to the presence of PPO in lettuce, since lettuce extract turned dark brown upon addition of 10 g of pyrocatechol or pyrogallol L^{-1}, and Fujita et al. (1991) studied the properties of soluble lettuce PPO. However, surprisingly the latent form of enzyme has not so far been described in lettuce.

For the enzyme to be extracted in its latent form the extraction method must be very mild to prevent its activation or modification, as occurs with acetone powders and ammonium sulfate fractionation (Golbeck and Cammarata, 1981). The purification method based on the temperature-induced phase partitioning of Triton X-114 (TX-114), which produces an aqueous two-phase system, in which the soluble enzyme is separated from the particulate enzyme has been described by us (Sánchez-Ferrer et al., 1990) previously. In addition to enabling the particulate enzyme to be obtained in its latent form, this method has another important feature, in that it completely removes the clorophylls and phenols from the dark green extract, leaving an optically transparent extract which is poor in detergent and in which the PPO of lettuce can be found in latent state.

PPO in its latent state was characterized, and its activation and stability with SDS and different temperatures and pH values were studied.

MATERIALS AND METHODS

Reagents. Fresh iceberg lettuce was purchased from a local market in Murcia city. 4-*tert*-Butylcatechol (4tBC) from Aldrich Química S.A. (Madrid, Spain) was used without further purification. Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. TX-114 was obtained from Fluka AG (Buchs, Switzerland) and condensed three times as described by Bordier (1981) but using 100 mM sodium phosphate buffer (pH 7.3). The detergent phase of the third condensation had a TX-114 concentration of 25% (w/v) and was used as the stock solution of detergent for all of the experiments.

Enzyme Purification. Membrane-bound PPO of iceberg lettuce leaf cells in its latent state was partially

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Table 1. Purification of Iceberg Lettuce PPO

step	vol (mL)	total protein (mg)	act.[a] (units/mL)		sp act. (units/mg of protein)		total act. (units)		purifn (fold)		yield (%)	chlorophylls (μg/mL)	phenols (μg/mL)
			no SDS	SDS	no SDS	SDS	no SDS	SDS	no SDS	SDS	no SDS		
TX-114 extract	42	28.6	1.01	3.67	1.48	5.40	42.5	154.1	1	1	100	5.63	2.15
supernatant of TX-114 extract	35	25.6	1.06	5.59	1.50	7.65	37.1	195.7	1.01	1.42	87.3	4.59	1.74
supernatant of 8% of TX-114	26	4.7	1.15	5.55	6.39	30.83	29.9	144.3	4.29	5.71	70.4	ND[b]	0.68

[a] Assayed with 1 mM chlorogenic acid in 50 mM phosphate buffer (pH 6.6) with or without 0.66 mM SDS. [b] Not detected.

purified from osmotically shocked chloroplast prepared by using the method described by Sanchez-Ferrer et al. (1990). A 100 g sample of the outermost green leaves of iceberg lettuce was added to 50 mL of 100 mM phosphate buffer (pH 7.3) containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and serine protease inhibitors, which were added immediately before use (1 mM phenylmethanesulfonyl fluoride and 1 mM benzamidine hydrochloride). The mixture was homogenized in a mortar with 10 g of sand, filtered through eight layers of gauze, and centrifuged at 25*g* for 10 min. The precipitate was discarded and the supernatant centrifuged at 20000*g* for 30 min. The pellet obtained after the latter centrifugation was resuspended in a solution containing 25 mL of 10 mM sodium phosphate buffer (pH 7.3) and kept at 4 °C for 20 min. Then, the solution was centrifuged at 20000*g* for 20 min, pelleting chloroplast membranes. These membranes were resuspended with 40 mL of 1.5% (w/v) TX-114 in 100 mM phosphate buffer (pH 7.3) for 30 min at 4 °C (TX-114 extract). After high-speed centrifugation (60000*g* for 30 min), this dark green extract yielded a light green supernatant with PPO activity. This was subjected to temperature phase partitioning by adding TX-114 at 4 °C to give a final concentration of 8% (w/v). The mixture was kept at 4 °C for 15 min in a Tectron thermostatic bath and then warmed to 35 °C. After 15 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large micelles composed of detergent, hydrophobic proteins, and the remaining chlorophylls. This solution was centrifuged at 5000*g* for 10 min at room temperature. The clear supernatant was used as latent enzyme source.

Enzyme Assay. The catecholase activity of the enzyme was determined using two different substrates, 4tBC, which was used for the stability of the *o*-benzoquinone obtained as product of the enzymatic activity (Waite, 1976), and chlorogenic acid, since this seems to be the normal substrate, it being widely distributed in plant tissue and strongly oxidized by plant PPO (Schaller and Von Elbe, 1970; Paulson et al., 1980; Fujita and Tono, 1981). In both cases activity was determined at 25 °C by spectrophotometrically monitoring, at 400 nm, the appearance of the *o*-benzoquinone product of the reaction ($\epsilon = 1150$ and 1018 M^{-1} cm^{-1}, respectively). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of quinone from chlorogenic acid/min. Unless otherwise stated, the reaction medium (1 mL) contained 50 mM sodium acetate buffer (pH 4.4) or 50 mM sodium phosphate buffer (pH 6.6) and 5 mM 4-tBC (1 mM chlorogenic acid). For the experiments carried out in the presence of SDS, 0.66 mM of it was added to the reaction mixture.

Electrophoresis and Gel Staining. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was carried out using the method of Laemmli (1970) but without the addition of β-mercaptoethanol and without any heating in order to preserve any enzymatic activity. Two gels were run simultaneously with equal amounts of enzyme sample applied to replicate gels. Samples were mixed with glycerol and bromophenol blue before being applied to 7.5% polyacrylamide gels. The electrophoresis was carried out in a Mini-Protean II cell (Bio-Rad) for 45 min at 200 V. After electrophoresis, the gels were equilibrated in 50 mM acetate buffer (pH 5) and their activity was detected in this same buffer containing 5 mM dopamine, 2 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH), and 0.66 mM SDS supplemented or not with the PPO inhibitor 2-hydroxy-2,4,5-cycloheptatrien-1-one (tropolone) as described by Rodriguez-López et al. (1994), except that the tropolone concentration was 86 mM.

Optimum pH. pH studies were carried out using 50 mM sodium acetate and sodium phosphate buffers from pH 3.5 to pH 8. Enzyme assays were made in the presence and absence of 0.66 mM SDS at each pH. The pH of the assay solution was determined at room temperature, using a Crison micro pH 2002 meter and test tube electrode. After catalysis, the pH of the assay solution was again measured.

Optimal Activation by SDS. Activation studies were carried out in 50 mM sodium phosphate buffer (pH 6.6) and 5 mM 4tBC in a final volume of 1 mL. The concentration of SDS in the medium varied from 0 to 2.5 mM.

pH Stability. The enzyme was preincubated (1:10) in 0.1 M sodium acetate and sodium phosphate buffers from pH 3.5 to pH 8 at 4 °C for 15 h in the presence and absence of 0.66 mM SDS at each pH. Residual activity was determined at pH 6.6 with 0.66 mM SDS and at pH 4.4.

SDS Stability. Preincubation of the enzyme was carried out (1:10) in 0.1 M sodium acetate at pH 4.4 in the presence and absence of 1 mM SDS at 4 °C. The kinetic activity of the enzyme preincubated in the absence of SDS was measured at various times at pH 4.4 and at pH 6.6 with 0.66 mM of SDS as described in the enzyme assays. The activity of the enzyme preincubated in the presence of SDS was also measured at both pH values.

Thermal Stability. The enzyme was heated to various temperatures between 30 and 90 °C for 5 min. The residual activity was measured at pH 6.6 with 0.66 mM SDS and at pH 4.4.

Other Methods. The protein content was determined according to the Bradford Bio-Rad protein assay using bovine serum albumin as a standard (Bradford, 1976). Chlorophylls were measured in 80% acetone (Arnon, 1949), and total phenolic compounds were determined in 80% ethanol according to the Folin−Denis method (Horowitz, 1970).

RESULTS AND DISCUSSION

An outline of a typical purification of the enzyme from the chloroplastid extract is given in Table 1. Purification was about 5-fold with a recovery of 70%. The chlorophylls and phenols were eliminated by the TX-114. The PPO obtained by using this method was in its latent state and could be activated by acid pH (results not shown in Table 1) and SDS, while the PPO obtained by Fujita et al. (1991) and purified with ammonium sulfate was already active (Golbeck and Cammarata, 1981). Chlorogenic acid was used as substrate in the purification experiment because it produced an optically transparent solution in the presence of TX-114 (TX-114 extract, Table 1), while 4tBC under the same conditions resulted in scattering.

The partially purified latent iceberg lettuce PPO only appeared as a single activity band in PAGE when dopamine was used as substrate (Figure 1), which agrees with that found in PAGE for other lettuce PPO (Angleton and Flurkey, 1984; Flurkey, 1986; Fujita et al., 1991). This band did not appear when the stained solution contained 86 mM tropolone.

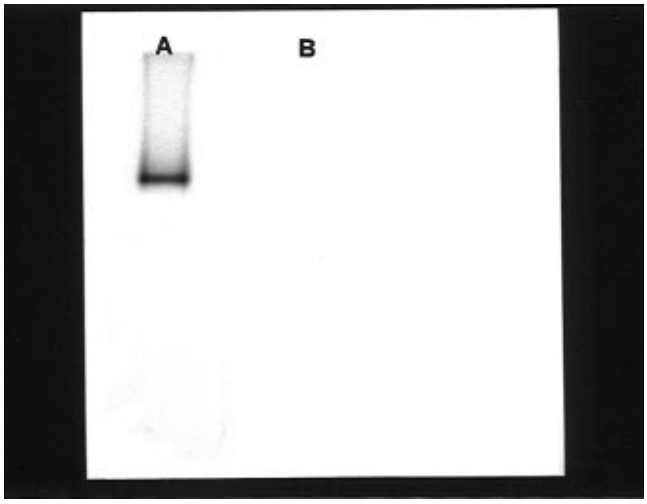

Figure 1. Electrophoresis of latent iceberg lettuce PPO stained with 5 mM dopamine in 50 mM sodium acetate (pH 5) in the presence of 0.66 mM SDS and 2 mM MBTH: (A) in the absence of tropolone; (B) in the presence of 86 mM tropolone. The lane contained 4.8 μg of purified enzyme.

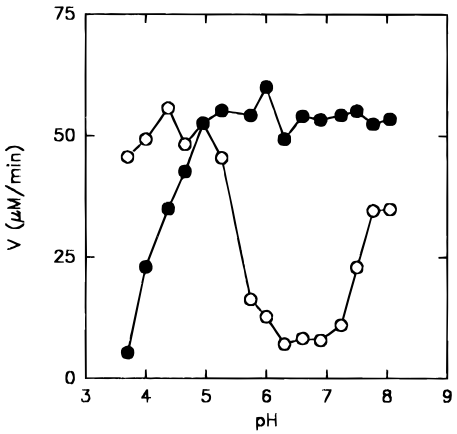

Figure 2. Effect of pH on the enzyme in 50 mM sodium acetate and sodium phosphate (pH 3.5−8) in the presence (●) or absence (○) of 0.66 mM SDS. The reaction medium at 25 °C contained 20 μg/mL PPO and 5 mM 4tBC.

pH is a determining factor in the expression of enzymatic activity and, in the case of latent PPO, its activation by acid or basic shock has been described (Kenten, 1957). On the other hand, plant PPO in its latent state can also be induced or activated by SDS (Moore and Flurkey, 1990). For this reason, the determination of the optimum pH and studies of the enzyme's stability at different pH values and temperatures, together with its kinetic characterization, were carried out in the presence and absence of SDS.

When the pH was scanned in the absence of SDS, at least two pH optimum zones were observed, one of them above pH 7.5 and a peak at pH 4.4 (Figure 2). The low pH optimum is a result of the enzyme being induced by acid shocking. Interestingly, this low pH optimum was abolished when the assays were conducted in the presence of SDS, being displaced to pH values >5. It remains to be seen whether the acid-shocking activation process and the SDS effect are related through a common mechanism. Similar results were obtained when chlorogenic acid was used as substrate (results not shown).

The pH scan in the presence and absence of SDS (Figure 2) reveals that the highest value in the activation process is obtained between pH 6 and 7, a value more basic than the optimum pH. It was therefore decided to study activation by detergent at pH 6.6, although assays in the absence of SDS were carried out at pH 4.4.

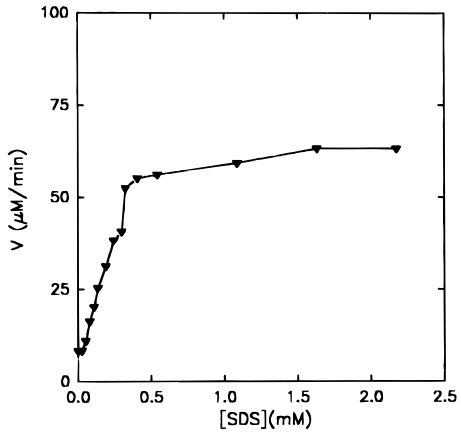

Figure 3. Activation of latent iceberg lettuce PPO by SDS. The reaction medium at 25 °C contained 20 μg/mL enzyme and 5 mM 4tBC in 50 mM phosphate buffer (pH 6.6).

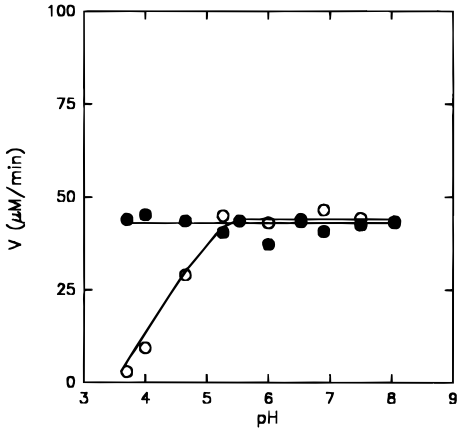

Figure 4. Effect of enzyme incubation in 100 mM acetate and phosphate buffers from pH 3.5 to pH 8 at 25 °C for 15 h with (○) or without (●) 0.66 mM SDS. The reaction medium at 25 °C contained 20 μg/mL enzyme and 5 mM 4tBC in 50 mM acetate buffer (pH 4.4).

The scan of the SDS concentration carried out at pH 6.6 showed that the activation process is saturable (Figure 3). Thus, the optimum SDS concentration was taken as 0.66 mM to avoid undesirable effects (micelle formation) due to an excess of detergent in the reaction medium. Similar results were obtained when chlorogenic acid was used as substrate (results not shown).

Incubating the enzyme for 15 h at pH values between 3.5 and 8 did not modify its enzymatic activity, which was always measured at pH 4.4 without the addition of 0.66 mM SDS to the reaction medium (Figure 4). However, incubation in the same conditions but in the presence of 0.66 mM SDS (Figure 4) showed that SDS inactivated the enzyme at values below pH 5 but not at higher values when the measurements were carried out at pH 4.4 and without the addition of 0.66 mM SDS. Similar results were obtained when, after 15 h of incubation in both the presence and absence of SDS, the enzymatic activity was assayed at pH 6.6 in the presence of 0.66 mM SDS (results not shown).

Thus, the pH activated enzyme can be inactivated by the SDS, although it has no effect on the enzyme at neutral pH values, even after 15 h of incubation. These results differ from those obtained with broad bean PPO

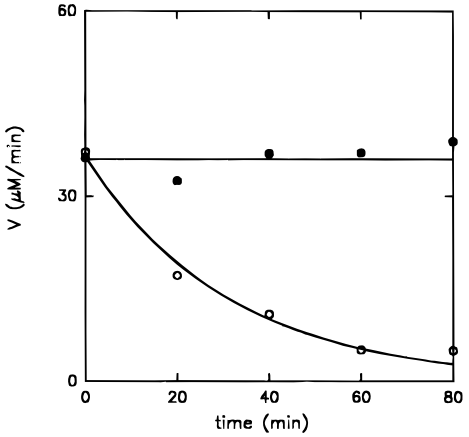

Figure 5. Effect of enzyme incubation in 100 mM acetate buffer (pH 4.4) at 4 °C, with time in the presence (○) or absence (●) of 1 mM SDS. The reaction medium at 25 °C included 20 μg/mL enzyme and 5 mM 4tBC in 50 mM acetate buffer (pH 4.4).

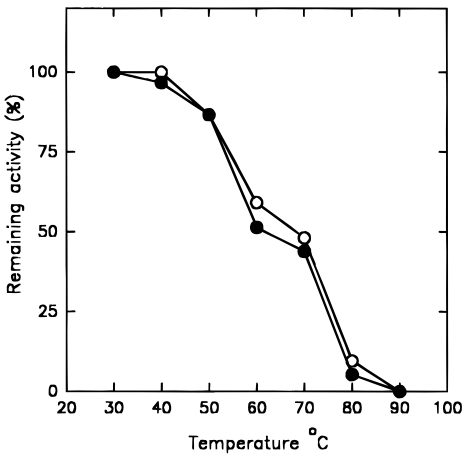

Figure 6. Thermal stability of the enzyme after heating for 5 min at temperatures between 30 and 90 °C as determined by assay for activity, at 25 °C, at pH 6.6 with 0.66 mM SDS (●) and at pH 4.4 (○).

by Kenten (1958), who showed that the activation process was reversible and that prolonged incubation with SDS resulted in a decrease in activity.

Since the enzyme was inactivated by SDS at pH values below 5, we carried out a study of this inactivation caused by SDS over a period of time. The assays were performed at pH 4.4, and the results obtained are shown in Figure 5. Inactivation followed first-order kinetics, and the inactivation constant calculated for this process was 0.032 min^{-1}. When the enzymatic activity was determined at pH 6 in the presence of 0.66 mM SDS, a similar value was obtained (0.024 min^{-1}) (results not shown).

With regard to enzyme stability with temperature, the results obtained at pH 4.4 and 6.6 with SDS are shown in Figure 6. Stability was relatively high in both conditions, as was observed in soluble lettuce PPO by Fujita et al. (1991).

After the optimal conditions for measuring PPO in its latent state in iceberg lettuce, and its stability versus pH and SDS were determined, the kinetic parameters (K_M and V_{max}) using 4-tBC and chlorogenic acid as substrates were determined at pH 4.4 in the absence of SDS and at pH 6.6 in the absence and presence of 0.66 mM SDS (Table 2).

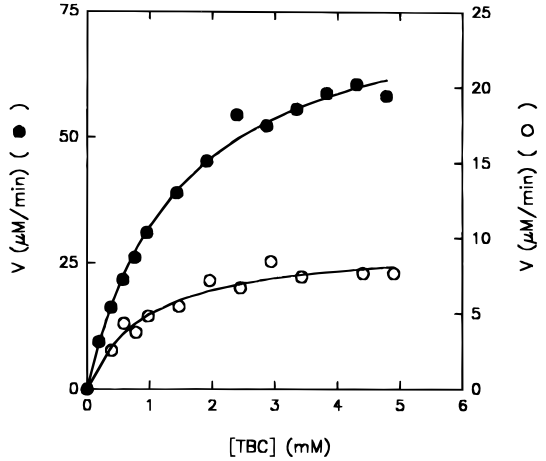

Figure 7. Effect of 4tBC concentration on the initial rate. The reaction medium contained 20 μg/mL PPO and different substrate concentrations in 50 mM sodium phosphate buffer (pH 6.6) at 25 °C with (●) or without (○) 0.66 mM SDS.

Table 2. Effect of SDS in the Kinetic Parameters of Latent Iceberg Lettuce PPO

	K_M at pH 4.4 (mM)	K_M at pH 6.6 (mM)	K_M with SDS (mM)	V_{max}^{SDS}/V_{max} at pH 6.6
chlorogenic acid	0.95	1.36	0.84	6.5
tert-butylcatechol	0.98	1.13	1.23	7.1

Figure 7 shows fitted curves for the variation in initial velocity with substrate concentration in the presence and absence of SDS. The value obtained in the fitting for each of the maximum velocities showed that the activation increased 7-fold with SDS.

ABBREVIATIONS USED

PPO, polyphenol oxidase; 4tBC, 4-*tert*-butylcatechol; SDS, sodium dodecyl sulfate; TX-114, Triton X-114; tropolone, 2-hydroxy-2,4,5-cycloheptatrien-1-one; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PAGE, polyacrylamide gel electrophoresis.

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