Characterization of Monophenolase Activity of Polyphenol Oxidase from Iceberg Lettuce

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Polyphenol oxidase (EC 1.14.18.1), a thylakoid membrane-bound enzyme, was isolated by sonication of osmotically shocked chloroplasts from iceberg lettuce (*Lactuca sativa*). The enzyme showed monophenolase activity when assayed on (*p*-hydroxyphenyl)propionic acid with 3-methyl-2-benzothiazolinone hydrazone in a reliable continuous spectrophotometric method, with high sensitivity, accuracy, and precision. The monophenolase activity showed a lag period before the steady-state rate (V_{ss}) was reached. Both kinetic parameters, the lag period and the steady-state rate, depended on the pH, the enzyme and substrate concentrations, and the presence of catalytic amounts of *o*-diphenol. This activity shows inhibition by high substrate concentration. The experimental results correspond with the mechanism previously described for PPO from other sources. Kinetic constants K_m, V_{max}, and K_i were determined.

Keywords: *Plant polyphenol oxidase; lettuce; Lactuca sativa; monophenolase activity*

INTRODUCTION

Horticultural products suffer important losses, both quantitative and qualitative, from harvesting to consumption such as the loss of its nutritive value, due to the oxidation of the antioxidant phenolic compounds or the undesired enzymatic browning that develops after tissue injury. These alterations are mediated by endogenous enzymatic activities such as polyphenol oxidase (PPO), which decreases the market value of this product.

In plants, PPO (EC 1.14.18.1) is predominantly located in the chloroplast thylakoid membranes (Golbeck and Camarata, 1981; Chazarra et al., 1996; Jimenez and Garcia-Carmona, 1996a). However, it is not an intrinsic membrane protein and can be released from the thylakoids by sonication, mild detergent treatment, or protease treatment (Robinson and Dry, 1992). The enzyme has also been detected in soluble fractions in homogenates from different vegetables (Mayer and Friend, 1960; Fujita et al., 1991; Escribano et al., 1997a) and is often found in multiple forms.

In living tissues, the phenolic substrate and the enzyme are separated within the cells, but upon any cell-damaging treatment, the enzyme and substrates may come in contact, permitting rapid oxidation of phenols. The *o*-quinones formed during oxidation are highly reactive substances that normally react further with other quinones, amino acids, peptides, and proteins, thus altering not only the structural and functional properties of the proteins but also its nutritive value (Matheis and Whitaker, 1984; García-Carmona et al., 1988).

The most important postharvest disorder that can develop during transport and storage of iceberg lettuce is russet spotting (RS). 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 PPO of soluble phenolic compounds such as flavonoids and chlorogenic acid derivaties, resulting in the brown discoloration that is characteristic of RS tissue (Ke and Salveit, 1988).

PPO is a copper protein that catalyzes two different reactions, using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity).

Diphenolase activity of plant PPO has been widely studied, as has been one unusual and intriguing characteristic of the enzyme, which is its ability to exist in an inactive or latent state (Mayer and Harel, 1979; Jimenez and García-Carmona, 1996b). However, there are only a few works on monophenolase activity of plant PPO (Escribano et al., 1997b; Espín et al., 1995) maybe due to the lack of a highly sensitive and continuous method of measurement and the lability of the enzyme during the purification process (Matheis, 1987). This phenomenon is well-known in some plant PPOs (Mayer and Harel, 1979) and results from changes in the structure of the protein during purification (Walter and Purcell, 1980).

There are few studies that refer to iceberg lettuce PPO (Fujita et al., 1991; Sharples et al., 1963; Heimdal et al., 1994). The latent form has been described only by us (Chazarra et al., 1996) along with the fact that this PPO suffers suicide inactivation (Chazarra et al., 1997). With regard to monophenolase activity, it has not been detected in iceberg lettuce PPO. Thus, the aim of this study was the detection and kinetic characterization of the monophenolase activity of this enzyme. Monophenolase activity of PPO shows a characteristic lag period before the maximum velocity of the hydroxylation step is reached. The results obtained will be discussed

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broadly under Results and Discussion according to the enzyme mechanism proposed by Cabanes et al. (1987 b).

MATERIALS AND METHODS

Materials. Fresh iceberg lettuce was obtained from a local market in Murcia (Spain). Substrates and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma Chemical Co. (Madrid, Spain), and all other reagents were of analytical grade.

Enzyme Extraction. Membrane-bound PPO of iceberg lettuce leaf cells was extracted by sonication of osmotically shocked chloroplasts and partially purified by precipitation with $(NH_4)_2SO_4$.

A 1 kg sample of lettuce leaves was homogenized with 1 L of 50 mM Tris-HCl buffer (pH 7.8) containing 0.4 M sucrose and serine protease inhibitors (1 mM phenylmethanesulfonyl fluoride and 1 mM benzamidine hydrochloride). The serine protease inhibitors were added immediately before use. The homogenate was filtered through eight layers of gauze and centrifuged at 5000*g* for 25 min in a Kontron Centrikon H-401 centrifuge (Germany). The pellet consisting mainly of chloroplasts was washed by resuspension in 300 mL of extraction buffer and centrifugation for 25 min at 5000*g*. The final chloroplast sediment was resuspended with 40 mL of 67 mM sodium−phosphate buffer (pH 6.3) and sonicated for 5 min. The sonicated chloroplasts were centrifuged at 120000*g* for 30 min in a Kontron Centrikon T-2050 centrifuge. The clear supernatant containing PPO was acidified to pH 5 and kept for 1 h at 4 °C before being centrifuged at 120000*g* for 30 min. The supernatant was brought to 30% saturation of $(NH_4)SO_2$ and kept overnight at 4 °C under continuous stirring. The solution was centrifuged at 100000*g* for 30 min. The supernatant was then brought to 65% saturation of $(NH_4)SO_2$ and kept at 4 °C under continuous agitation for 1 h. The solution was centrifuged at 100000*g* for 30 min and the precipitate dissolved in a minimal volume. The salt content was removed by a desalting column of Sephadex G-50 from Sigma Chemical Co.

Enzyme Assay. The monophenolase activity was assayed by means of a reliable continuous spectrophotometric method with the presence of the nucleophile MBTH (Rodríguez-López et al., 1994). This method was based on previous works involving nonenzymatic reactions of the *o*-quinones produced by PPO in the absence (García-Carmona et al., 1982) and presence of nucleophiles (Cabanes et al., 1987a; García-Carmona et al., 1987, 1988; Valero et al., 1988).

Monophenolase activity was determined at 25 °C by spectrophotometrically monitoring, at 500 nm, the appearance of the MBTH−quinone adduct (ϵ = 40000 M^{-1} cm^{-1}), using *p*-hydroxyphenylpropionic acid (*p*-HPPA) as substrate with MBTH (Rodríguez-Lopez et al., 1994).

Unless otherwise stated, the reaction medium contained 1 mM MBTH, 2% *N*,*N*′-dimethylformamide (DMF), and 50 mM sodium acetate buffer (pH 4.5) as well as the different *p*-HPPA and PPO concentrations detailed below.

Diphenolase activity was assayed spectrophotometrically at 500 nm using as substrate 3,4-dihydroxyphenylpropionic acid (DHPPA) with MBTH (Winder and Harris, 1991). The standard reaction mixture contained 8 mM DHPPA, 1 mM MBTH, and 2% DMF in 50 mM sodium acetate buffer (pH 4.5).

The protein content was determined according to the Bradford Bio-Rad protein assay using bovine serum albumin as standard (Bradford, 1976).

Data Analysis. Kinetic data analysis was carried out by using linear and nonlinear regression fitting (Marquardt,1963), using the Sigma Plot for Windows program (Jandel Scientific, 1994, Sausalito, CA).

RESULTS AND DISCUSSION

The thylakoid-bound PPO from iceberg lettuce had both diphenolase (Figure 1, curve a) and monophenolase activities (Figure 1, curves b−d) when assayed on *p*-HPPA/DHPPA pair with MBTH in a reliable continuous spectrophotometric method, with high sensitivity, accuracy, and precision. The monophenolase activity was characterized by a lag period, defined as the intercept on the abscissa axis obtained by extrapolation of the linear part of the product accumulation curve. The steady-state rate (V_{ss}) was defined as the slope of the linear part of the product accumulation curve (see Figure 1). Both the lag period and the steady-state rate are affected by the pH and the enzyme and substrate concentrations. Finally, the presence of catalytic concentrations of *o*-diphenols affected the lag period but not the steady-state rate.

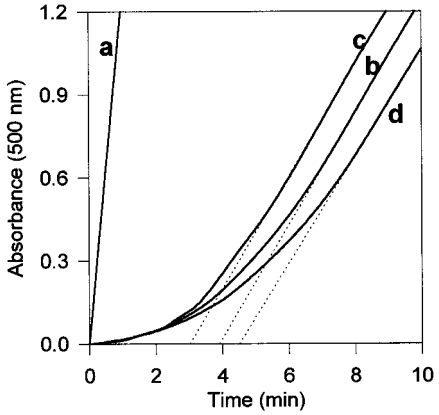


Figure 1. Enzymatic activities of PPO: **(**a**)** diphenolase activity [the reaction medium contained 1 μg/mL PPO, 8 mM DHPPA, 1 mM MBTH, and 2% DMF in 50 mM sodium acetate buffer (pH 4.5)]; **(**b−d**)** monophenolase activity [the reaction medium, at 25 °C, contained **(**b**)** 26 μg/mL PPO, 8 mM *p*-HPPA, 1 mM MBTH, and 2% DMF in 50 mM sodium acetate buffer (pH 4.5)]; **(**c, d**)** same as **(**b**)** except that in **(**c**)** the enzyme concentration was 31 μg/mL and in **(**d**)** the substrate concentration was 12 mM. The broken lines represent the extrapolation of the linear part of the product acumulation curve used for calculating the lag period by the intercept on the abscissa.

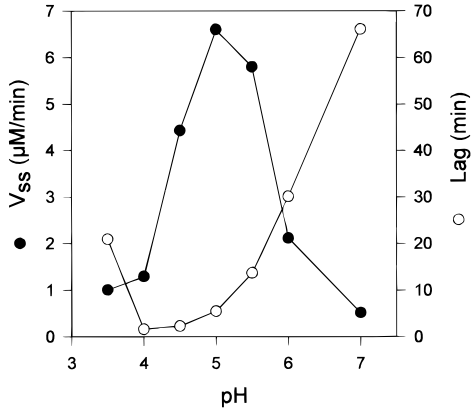


Figure 2. Effect of pH on monophenolase activity (●) and on its lag period (○). The reaction medium, at 25 °C, included 32 μg/mL PPO, 8 mM *p*-HPPA, 1 mM MBTH, and 2% DMF in 50 mM sodium acetate (pH 3.5−5) or sodium phosphate (pH 5.5−7) buffer.

Effect of pH. Monophenolase activity toward *p*-HPPA increased as the pH was increased from pH 3.6 and showed a maximum at pH 5 (Figure 2). The pH affected not only the enzyme activity but also the lag period (Figure 2); the shortest lag period occurred at pH 4.

Effect of Enzyme Concentration. An increase of enzyme concentration produced a linear increase in V_{ss}

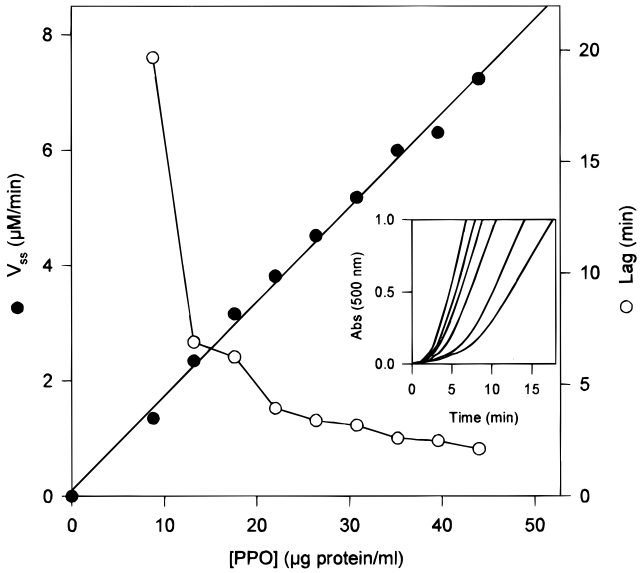


Figure 3. Effect of enzyme concentration on monophenolase activity of PPO (●) and on its lag period (○). The reaction medium, at 25 °C, contained 8 mM *p*-HPPA, 1 mM MBTH, 2% DMF, and different PPO concentrations (9−44 μg/mL) in 50 mM sodium acetate buffer (pH 4.5). (Inset) Spectrophotometric recordings of monophenolase activity of PPO for different enzyme concentrations.

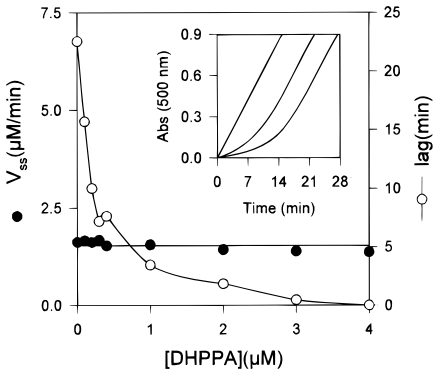


Figure 4. Effect of diphenol addition on monophenolase activity (●) and on its lag period (○). The reaction medium, at 25 °C, contained 9 μg/mL PPO, 8 mM *p*-HPPA, 1 mM MBTH, and 2% DMF with different DHPPA concentrations (0−4 μM) in 50 mM sodium acetate buffer (pH 4.5). (Inset) Spectrophotometric recordings of monophenolase activity of PPO for different concentrations of DHPPA.

(Figure 3) as well as shortening in the lag period (Figure 3). This behavior has been described for several PPO sources (Espin et al., 1995; Escribano et al., 1997b).

Effect of *o*-Diphenol Addition. The lag period observed in the monophenolase activity can be modified by the addition of catalytic concentrations of *o*-diphenol (Figure 4). Because the lag period is the time required to reach the *o*-diphenol concentration in steady state, the addition of catalytic amounts of *o*-diphenol to the enzyme assay shortens the lag. An increase in the DHPPA concentration shortened the lag period. It should be noted that the V_{ss} is the same for every *o*-diphenol concentration chosen. The above shortening in the lag period led to a null value (Figure 4). In other words, a rise in the initial *o*-diphenol concentration ($[D]_0$) shortened the time required for the steady-state level of *o*-diphenol ($[D]_{ss}$) to be reached. When $[D]_0 = [D]_{ss}$, the value of $[D]_{ss}$ was reached quickly and no lag period was detected in the initial monophenolase activity.

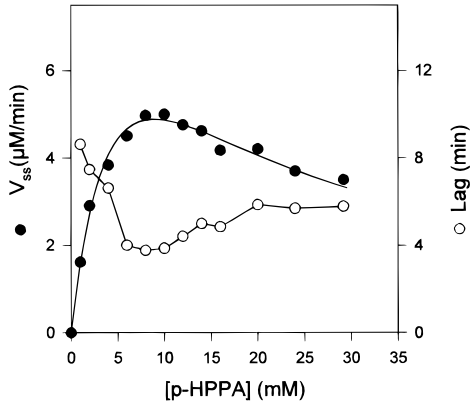
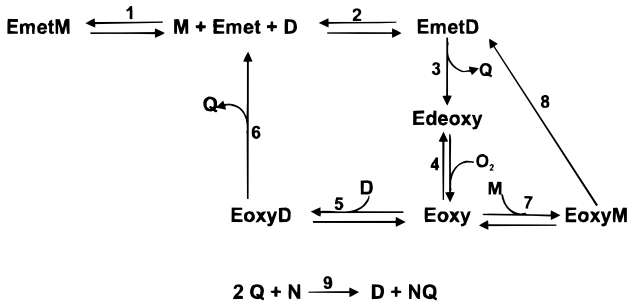


Figure 5. Effect of substrate concentration on monophenolase activity (●) and on its lag period (○). The reaction medium, at 25 °C, contained 26 μg/mL PPO, 1 mM MBTH, and 2% DMF with different *p*-HPPA concentrations (1−29 mM) in 50 mM sodium acetate buffer (pH 4.5).

Scheme 1. Reaction Mechanism for the Monophenolase and Diphenolase Activities of PPO in the Presence of a Nucleophile[a]



[a] M, monophenol; D, *o*-diphenol; Q, *o*-quinone; N, nucleophile; NQ, nucleophile−quinone adduct; Emet, met-PPO or oxidized form of PPO with Cu^{2+}−Cu^{2+} in the active site; Eoxy, oxy-PPO or the oxidized and oxygenated form with Cu^{2+}−Cu^{2+} in the active site; Edeoxy, desoxy-PPO or reduced form with Cu^{+}−Cu^{+} in the active site (Cabanes et al., 1987a; García-Carmona et al., 1987).

Effect of Substrate Concentration. The effect of low concentrations of *p*-HPPA (<8 mM) causing a shortening in the lag period (Figure 5) has not been described in the bibliography, but it was observed by us in other PPOs (results not shown). *p*-HPPA concentrations >8 mM cause an increase in the lag toward a maximum value, similar to the effects described for other PPOs (Cabanes et al., 1987b; Escribano et al., 1997b).

The steady-state rate increases with monophenol concentration, but inhibition by high substrate concentration can be observed (Figure 5).

Kinetic constants were evaluated from the data in Figure 5 (V_{max} = 11.9 μM/min; K_m = 6.3 mM and K_i = 12.5 mM) by using nonlinear regression fitting (Marquardt, 1963).

The results presented for the monophenolase activity of partially purified iceberg lettuce PPO are in agreement with the mechanism previously described for PPO from other sources (Cabanes et al., 1987a,b) (Scheme 1). This model takes into account the occurrence of the three forms of the enzyme (indicated as Emet, Eoxy, Edeoxy) and the chemical recycling of the adduct by the *o*-quinone generated by the enzyme (step 9 in Scheme 1). The mechanism included both monophenolase and diphenolase activities. During diphenolase activity, *o*-diphenol (D) binds both to Eoxy (step 5) and Emet

(step2), rendering EoxyD and EmetD intermediates, which give rise to two *o*-quinones (Q) (steps 3 and 6). These two *o*-quinones recycle to regenerate one *o*-diphenol (D) and one nucleophile−quinone (NQ) (chromophoric adduct) (step 9).

During monophenolase activity, the binding of monophenols (M) to the Eoxy form renders EmetD (step 8), which gives rise to *o*-quinone (step 3), but the binding of monophenol to Emet (with no catalytic activity on monophenols) (step1) scavenges a portion of PPO from the catalytic turnover as a dead-end complex EmetM. The enzyme slowly re-enters the catalytic cycle (step 2), being transformed into the oxy form (steps 2−4), by means of the diphenol obtained by recycling in the subsequent chemical reactions (step 9). The increment of the *o*-diphenol level produces a greater transformation of enzyme into the catalytic active form and therefore a higher transformation of monophenol into *o*-quinone. This process continues until the rate of transformation from *o*-diphenol to *o*-quinone by Eoxy is the same as the rate of *o*-diphenol regeneration by chemical steps. The time necessary for the *o*-diphenol generation (step 9) and consumption (step3) rates to be equal is the lag period (Figure 1). When these identical rates are reached, the steady state for the system will also be obtained. Therefore, the lag period can only be interpreted as a dynamic equilibrium between the enzymatic and chemical steps to obtain the steady-state diphenol concentration. To reach such a concentration, the presence of a small amount of Eoxy form in the native PPO and the recycling steps is necessary. The former requirement stems from the fact that Eoxy is the only enzyme form able to transform monophenol into EmetD, which subsequently renders *o*-quinone. Thus, there are extracts from some PPO sources that show apparent low or null monophenolase activity, and this occurs when the native enzyme has a low proportion of the Eoxy form. This causes an increase in the lag period and a lower steady-state rate. This fact cannot affect the diphenolase activity, whereas the monophenolase activity may be seriously altered and cannot be detected with the most widely used techniques such as spectroscopy or polarography.

The profile of lag period variation with regard to pH (Figure 2) depends on the affinity of the enzyme (Emet and Eoxy) toward the monophenol at each pH. On the one hand, its binding to the Eoxy form accelerates the reaching of the steady state and, on the other hand, its binding to Emet, inactive on monophenols, delays the reaching of the steady state.

Increasing PPO concentration produces a proportional increase of the Eoxy form in the native state, which means that there is more enzymatic activity and so the level of *o*-diphenol in the steady state is reached more quickly; and therefore, the lag period diminishes (Figure 3).

Because the lag period is the time necessary to reach the steady-state level of *o*-diphenol, the addition of small amounts of *o*-diphenol concentrations at the start of the reaction leads to a shortening of the lag period (Figure 4), due to the lower time required for the attainment of the corresponding level of *o*-diphenol. It is completely abolised when the amount of *o*-diphenol added is the steady-state level.

The lag period dependence with regard to monophenol concentration (Figure 5) can present different profiles according to both PPO and substrate used. This is possible if one takes into account that the lag period is provoked by a dynamic equilibrium that implies Emet and Eoxy forms of the enzyme. These two forms of different PPOs can present different affinities for every monophenol and so (steps 1 and 7) reach the steady state at different times. Generally, an increase of the level of monophenol means that a higher level of *o*-diphenol is required for the steady state to be reached. This can be understood because both ligands, monophenol and *o*-diphenol, compete for Emet and Eoxy forms.

In conclusion, the kinetic characterization of monophenolase activity from membrane-bound iceberg lettuce PPO has been carried out, and all results obtained can be explained by the mechanism presented in Scheme 1. Therefore, one must keep in mind the complexity of this mechanism which implies two enzymatic forms presenting different affinities depending on the source and the substrate that has been used, the competition of the monophenol with the *o*-diphenol for the same enzymatic form, and the chemical steps to obtain the steady-state *o*-diphenol concentration. All of this affects the lag period of this activity, which can only be interpreted as a dynamic equilibrium between them.

ABBREVIATIONS USED

PPO, polyphenol oxidase; MBTH, 3-methyl-2-benzothiazolinone hydrazone; *p*-HPPA, *p*-hydroxyphenylpropionic acid; DHPPA, 3,4-dihidroxyphenylpropionic acid; DMF, *N*,*N*′-dimethylformamide.

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