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MONOPHENOLASE ACTIVITY OF POLYPHENOL OXIDASE FROM BLANQUILLA PEAR

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Key Word Index—*Pyrus communis*; Rosaceae; pear; tyrosinase; polyphenol oxidase; enzyme kinetics; monophenols; hydroxyphenyl propionic acid; MBTH.

Abstract—Pear polyphenol oxidase (PPO) has been isolated by using two sequential phase partitionings with Triton X-114 (TX-114). The enzyme showed monophenolase activity when assayed on *p*-hydroxyphenyl propionic acid (PHPPA) with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in a continuous spectrophotometric method, with high sensitivity and precision. The initial monophenolase activity showed a lag period (τ) prior to the attainment of the steady state rate (V_{ss}). Both kinetic parameters, V_{ss} and τ, depended on the enzyme and monophenol concentrations, as well as on the presence of catalytic amounts of *o*-diphenol. The enzyme showed an optimum pH of 4.3 and the value of K_m toward PHPPA was 0.5 mM. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Enzymic browning in fruits and vegetables is often an undesirable reaction which is responsible for unpleasant sensory qualities and losses in nutrient quality. The prevention of this reaction has always been a challenge to food scientists [1, 2]. The main enzyme involved in this reaction is tyrosinase or polyphenol oxidase (EC 1.14.18.1; PPO) which has been the subject of several reviews [3–9]. PPO is a copper enzyme which in the presence of oxygen catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red or black pigments [10-12].

Diphenolase activity of pear PPO [13–19] as well as the effect of many inhibitors on this activity [13–15, 19] has been studied. However, the monophenolase activity of this enzyme has never been reported in the literature. This lack of information stems from the lability of the enzyme during the purification process [2]. This phenomenon is well known in other plant PPOs [20] and results from changes in the structure of the protein during purification [21].

The aim of this paper is the detection and kinetic characterization of the monophenolase activity of pear PPO var. Blanquilla. The enzyme is extracted by using a two phase partitioning method in TX-114 [22] with some modifications [23]. The monophenolase activity is assayed by means of a continuous spectrophotometric method in the presence of the chromogenic nucleophile MBTH. The method is based on that previously proposed for mushroom and apple PPO [24–27]. The experiments were carried out in order to confirm whether pear PPO has both the diphenolase and monophenolase activities, as reported for other PPO sources previously studied [23, 27–30], and to investigate the possible applicability of the kinetic mechanism previously described for frog epidermis PPO [28, 30], mushroom PPO [29, 30], grape PPO [30] and apple PPO [23, 27].

RESULTS AND DISCUSSION

Preparation of PPO

Pear PPO was extracted by using two sequential phase partitionings with TX-114 [23]. The main advantage of this method lies in the preservation of both pear PPO monophenolase and diphenolase activities. Other more drastic methods lead to the loss of the monophenolase activity of PPO from other biological sources, such as fruits and vegetables [2, 20, 21]. The possibility that our preparation of PPO contains a mixture of isoenzymes should be considered, as described for other pear PPOs [14]. Maybe, our extraction procedure has produced a preparation enriched in a high monophenolase form.

The purification procedures used by other authors

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[13–19, 31, 32] might have provoked some changes in the structure of the enzyme with a consequent loss of the monophenolase activity. This activity has a catalytic power, V_m^M/K_m^M, around 100 times lower than the catalytic power of the diphenolase activity, V_m^D/K_m^D [29, 30]. Therefore, slight changes in the concentration and/or functionality of PPO may not affect the diphenolase activity, whereas the monophenolase activity may be seriously altered. In fact, if this occurs, the monophenolase activity cannot be detected with the most widely used techniques such as spectroscopy or polarography.

Catalytic activity of PPO

Pear PPO presented both diphenolase (Fig. 1, curve a) and monophenolase (Fig. 1, curves b–d) activities. The continuous spectrophotometric method for assaying both activities used DHPPA and PHPPA as substrates, respectively. The assays were performed in the presence of the chromogenic nucleophile MBTH so that the detectable species was the corresponding chromophoric MBTH-quinone adduct [23, 27]. The high molar absorptivity (ε) and the high catalytic power of PPO on DHPPA and PHPPA facilitated the detection of both catalytic activities of pear PPO. This method was sensitive, reliable and widely applicable to PPO from other biological sources [26, 27].

The monophenolase activity of pear PPO (Fig. 1, curves b–d) showed a τ defined as the intercept on the abscissa axis obtained by extrapolation of the linear part of the product accumulation curve. The slope of this linear part was equivalent to V_{ss} of the set of coupled non-enzymic reactions [23, 27].

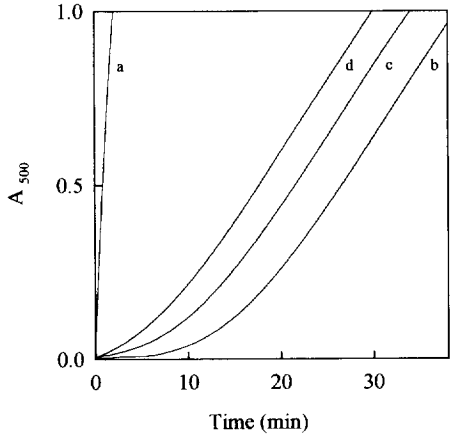


Fig. 1. Enzymic activities of soluble pear PPO. (a) Diphenolase activity; the reaction medium included 2.4 μg protein, 10 mM DHPPA, 1 mM MBTH and 2% DMF in 50 mM AB pH 4.3. (b–d) Monophenolase activity; the reaction medium contained: (b) 18 μg protein, 8 mM PHPPA, 1 mM MBTH and 2% DMF in 50 mM AB pH 4.3; (c, d) the same reaction medium as (b) except for (c) different monophenol concentration (5 mM PHPPA) and (d) the addition of the *o*-diphenol DHPPA to the reaction medium (1 μM).

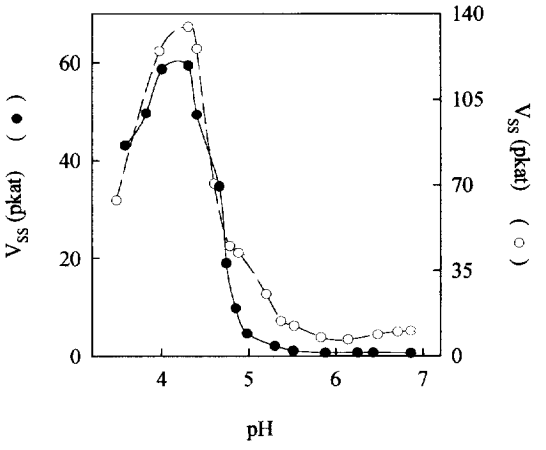
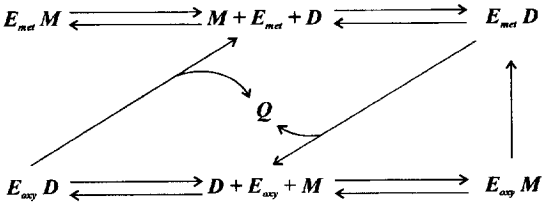


Fig. 2. Effect of pH on monophenolase activity (●); the reaction medium included 121 μg protein, 1 mM MBTH, 2% DMF and 0.5 mM PHPPA in 50 mM AB pH 3.6–5.6 and PB pH 5.8–7.0. Diphenolase activity (○); the reaction medium included 1.26 μg protein, 1 mM MBTH, 2% DMF and 3 mM DHPPA in 50 mM AB pH 3.6–5.6 and PB pH 5.8–7.0.

Effect of pH

The monophenolase and diphenolase activities of pear PPO toward PHPPA and DHPPA, showed bell-shaped profiles with a coincident optimum pH of 4.3 (Fig. 2). The assays were performed with a saturating MBTH concentration for the range of pH considered [26, 27]. In this way the coupled non-enzymic reactions [23, 27] did not vary significantly with pH, and the effect observed corresponded only to the enzymic reactions (Scheme 1). These results were in accordance with those previously obtained for PPO from other biological sources [23, 30]. In pear, the optimum pH described for the diphenolase activity ranges from pH 4 [14] to 7 [15] depending on the variety.

In other sources, different optimum pHs have been described for the monophenolase and diphenolase activities of PPO [33, 34]. These results may be due to



$$2Q + N \longrightarrow D + NQ$$

Scheme 1. Kinetic reaction mechanism proposed to explain the monophenolase and the diphenolase activities of pear PPO, in the presence of a chromogenic nucleophile. M, monophenol; D, *o*-diphenol; Q, *o*-quinone; N, chromogenic nucleophile; NQ, nucleophile-quinone chromophoric adduct; E_{met}, met-PPO; E_{oxy}, oxy-PPO [23, 26–30].

the method of assay used, where coupled non-enzymic reactions under assay conditions sensitive to the pH variations occurred. In this case, the rapid kinetic assays of the diphenolase activity and the typical slow kinetic assays of the monophenolase activity show apparently different behaviours vs pH.

Acid values for the optimum pH have been determined for PPOs from different fruits and vegetables, whereas optimum pHs near neutrality have been reported for mushroom PPO, mouse melanoma and human melanoma [3–8]. In addition, the bell-shaped profile of V_{ss} vs pH (Fig. 2) indicated the substantial contribution to the catalysis of two protonable groups with $pK_a = 3.5$ and $pK_a = 4.7$. Note the pronounced decrease in V_{ss} when the pH is higher than the optimum (Fig. 2). This must be taken into account in the enzyme preparation procedures and when assaying the catalytic activity with special emphasis on the monophenolase activity of pear PPO.

Effect of $[PPO]_0$

An increase of enzyme concentration produced a linear increase in V_{ss} as well as a shortening in τ (Fig. 3). This behaviour had been widely described from several PPO sources [23, 28–30]. The kinetic analysis of the proposed reaction mechanism (Scheme 1) predicted a linearity between V_{ss} and PPO concentration [23, 27, 29, 30]. τ was proportional to the time that the enzyme needed to generate in the medium the *o*-diphenol (D) required to reach the steady state [23, 28–30]. The *o*-diphenol was generated through a set of non-enzymic reactions from the *o*-quinones generated by the enzyme. In this way, if the initial enzyme concentration is raised, more *o*-quinones are formed and the steady state level of D is reached more quickly with a shorter τ. The main feature of this model is the native state in which PPO is found: E_{met} ($Cu^{2+}Cu^{2+}$) and E_{oxy} ($Cu^{2+}Cu^{2+}O_2^{2-}$). The E_{met} form is inactive on monophenols and active on *o*-diphenols which compete for E_{met} and E_{oxy} forms (Scheme 1).

Effect of $[PHPPA]_0$

An increase in the initial PHPPA concentration ($[PHPPA]_0$) produced a hyperbolic increase in V_{ss} and a convex increase in τ (Fig. 4), similar to that described for other plant PPOs [23, 30, 33, 35, 36].

The dependence of V_{ss} coincided with the previously proposed reaction mechanism (Scheme 1; [23, 27, 29, 30]). The experimental data were fitted by non-linear regression to the Michaelis–Menten equation [37]. Initial estimations of V_{max} and K_m were obtained by linear regression to the Hanes–Woolf plot $[PHPPA]_0/V_{ss}$ vs. $[PHPPA]_0$ (results not shown). The values of the kinetic constants, V_{max} and K_m, at the optimum pH 4.3 were 46.67 pkat and 0.5 mM, respectively.

As was demonstrated for PPO from other sources [23, 29, 30] the steady state level of D ($[D]_{ss}$) was in direct proportion to the monophenol (M) concentration. Therefore, at constant enzyme concentration, an increase of M originated higher $[D]_{ss}$ and consequently the enzyme had to realise more turnovers resulting in a longer τ.

Effect of $[DHPPA]_0$

The standard assay of the monophenolase activity of pear PPO on PHPPA included no initial DHPPA concentration ($[DHPPA]_0$) (Fig. 5, curve a). The addition of different $[DHPPA]_0$ produced a shortening in τ (Fig. 5). This lag period could be abolished with a certain $[DHPPA]_0$, in which case the experimental recordings were linear from the beginning of the reaction (Fig. 5, curve c). Above a certain $[DHPPA]_0$ a burst in activity resulted (Fig. 5, curves d–e). However,

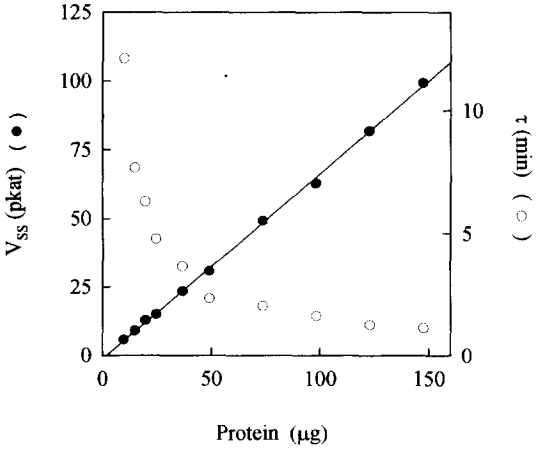


Fig. 3. Effect of enzyme concentration on monophenolase activity of PPO (●) and on its lag period (○). The reaction medium included 1 mM PHPPA, 2% DMF, 1 mM MBTH in 50 mM AB pH 4.3 with different protein quantities (10–147 μg).

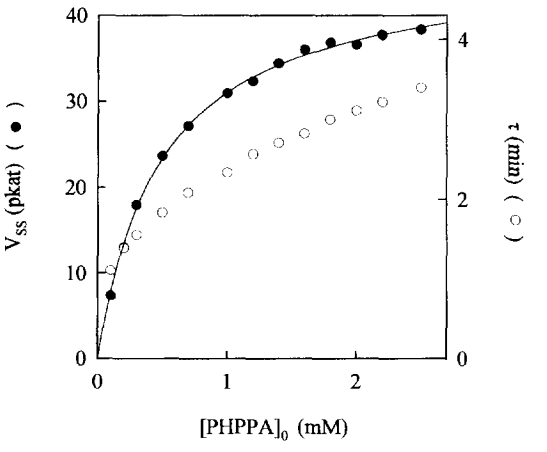


Fig. 4. Effect of monophenol concentration on monophenolase activity of PPO (●) and on its lag period (○). The reaction medium included 49 μg ml^{-1} of pear PPO, 1 mM MBTH, 2% DMF in 50 mM AB pH 4.3 with different PHPPA concentrations (0.1–2.5 mM).

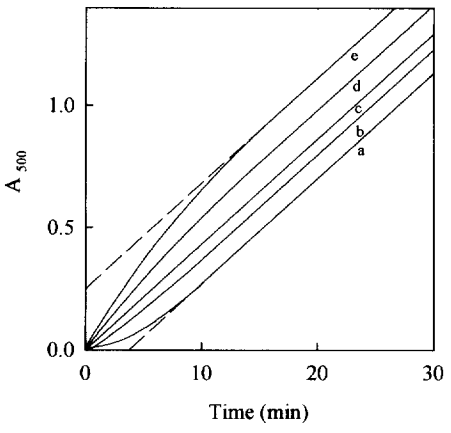


Fig. 5. Influence of DHPPA on the lag period of monophenolase activity. The standard reaction included 30.2 μg protein, 1 mM PHPPA, 1 mM MBTH, 2% DMF and 50 mM AB pH 4.3. The concentrations of DHPPA (μM) were: (a) 0; (b) 1; (c) 5; (d) 10; and (e) 20.

whatever the $[DHPPA]_0$ added, the monophenolase activity evolved toward final linear portions which appeared as a mass of parallel linear curves (Fig. 5). These qualitative changes were confirmed by determining the corresponding V_{ss} and τ values (Fig. 6) in complementary assays. These observations were similar to those previously made for PPO from other sources [23, 29, 30].

The independence of V_{ss} vs $[DHPPA]_0$ was in accordance with the kinetic analysis of the proposed reaction mechanism of PPO from other sources (Scheme 1; [23, 27, 29, 30]. The characteristic V_{ss} value of each assay of the monophenolase activity of pear PPO was only determined from the values of the initial PPO concentration ($[PPO]_0$) and $[PHPPA]_0$ of the corresponding assay medium.

The dependence of τ could also be interpreted on the basis of a $[D]_{ss}$, corresponding to the values of $[PPO]_0$ and $[PHPPA]_0$ in the considered assay medium

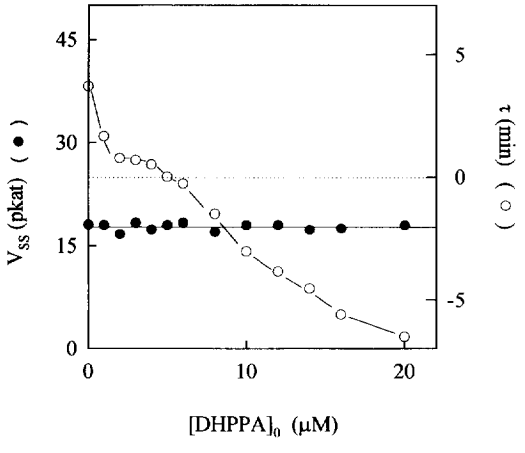


Fig. 6. Effect of catalytic amounts of DHPPA on the steady state rate of monophenolase activity (●) and on the lag period (○). The conditions are the same as in Fig. 5.

(Scheme 1; [23, 29, 30]). When the initial *o*-diphenol concentration ($[D]_0$) was lower than $[D]_{ss}$, a shorter τ was observed (Fig. 5, curves a–b and Fig. 6). In other words, a rise in $[D]_0$ shortened the time required for the level of $[D]_{ss}$ 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 (Fig. 5, curve c and Fig. 6). However, when $[D]_0 > [D]_{ss}$ the system must first consume the excess of D and then gradually consume M and D before the steady state is finally reached. In these conditions, there was a burst in activity, which was characterized by negative values of τ (Fig. 5, curves d–e and Fig. 6). Therefore, the physical meaning of the increase of the absolute value of τ was equivalent to a longer pre-steady state transient phase. The physical meaning of the sign $+\tau$ or $-\tau$ corresponded to the type of assay conditions with sub-steady state or over-steady state level of $[D]_0$, respectively.

Determination of PPO, expressed as protein concentration

Consideration of the above factors allows for the determination of the optimal monophenolase assay conditions on PHPPA for the determination of the initial pear PPO, expressed in terms of protein concentration. The method was applied with triplicate assays and a linear regression fitting was obtained. The precision of the method was checked by repeating ten times the estimation of the V_{ss} for three samples at three levels of $[PPO]_0$, expressed in terms of protein concentration, 0.49 μg ml^{-1}, 2.45 μg ml^{-1} and 4.42 μg ml^{-1}, the corresponding coefficients of variation (CV) being 5.3, 2.3 and 1.4%, respectively. The sensitivity of the method was checked by determining the values of the limit of detection (LOD) = 32 ng ml^{-1} and the limit of quantitation (LOQ) = 41 ng ml^{-1}. Therefore, this new continuous spectrophotometric method for measuring the monophenolase activity of pear PPO on PHPPA with MBTH was reliable and highly sensitive.

EXPERIMENTAL

Chemicals. Pears from the variety Blanquilla picked in Murcia, Spain, at commercial maturity and immediately stored at 5° were used as enzyme source. Substrates and MBTH were purchased from Sigma and all other reagents were of analytical grade and supplied by Fluka. TX-114 was obtained from Fluka and condensed three times prior to use as described in ref. [22] but using Na-P_i buffer (PB) pH 7.3 containing 20 mM EDTA. The detergent phase of the third condensation had a concn of 22% TX-114 (w/v).

Preparation of PPO. Pear PPO was extracted by using the method of ref. [23] which was previously applied to extract apple PPO.

Other methods. Protein was determined according to the method of ref. [38] using BSA as standard.

Enzymic assays. The use of MBTH as a nucleophilic

agent on some *o*-quinones generated by PPO had previously been described for the measurement of diphenolase [39] and monophenolase [26, 27] activities of PPO. The diphenolase activity of pear PPO was assayed at 500 nm using as substrates DHPPA with MBTH and was reported here for the first time. The standard reaction mixt. contained 10 mM DHPPA, 1 mM MBTH, 2% *N,N'*-dimethylformamide (DMF), 50 mM NaOAc buffer (AB) pH 4.3 and 2.4 μg of protein. The monophenolase activity of pear PPO was also determined at 500 nm using PHPPA with MBTH. The standard reaction mixt. included 1 mM MBTH, 2% DMF, 50 mM AB pH 4.3 as well as different PHPPA concns and protein quantities as detailed below. One unit of enzyme was taken as the amount that produced 1 μmol of the adduct per min. The final vol. of the assay mixture was 1 ml. Experiments were performed at 25° in triplicate and the corresponding mean values were plotted. The assays were performed with a spectrophotometer interfaced with a computer. All the assays were carried out at 25° with a Haake D1G circulating water-bath equipped with a heater/cooler and controlled by a Cole-Parmer digital thermometer with a precision of ±0.1°.

Data analysis. Kinetic data analysis was carried out by using linear and non-linear regression fittings [37], using the Sigma Plot 5.0 program [40]. PPO activity expressed in terms of protein concn was determined by lowering the concn to a level where V_{ss} was 10-fold higher than the rate of non-enzymic oxidation of the PHPPA substrate (blank rate). The cuvette contained a saturating concn of monophenol substrate and enough MBTH to trap all the *o*-quinone generated. Ten blank cuvettes were assayed for the determination of LOD and LOQ of the method. The precision of the method was also evaluated from 10 activity assays at each one of 3 protein concns used [41].

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