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Diphenol Activation of the Monophenolase and Diphenolase Activities of Field Bean (*Dolichos lablab*) Polyphenol Oxidase

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This paper reports a study on the hydroxylation of ferulic acid and tyrosine by field bean (*Dolichos lablab*) polyphenol oxidase, a reaction that does not take place without the addition of catechol. A lag period similar to the characteristic lag of tyrosinase activity was observed, the length of which decreased with increasing catechol concentration and increased with increasing ferulic acid concentration. The activation constant K_a of catechol for ferulic acid hydroxylation reaction was 5 mM. The kinetic parameters of field bean polyphenol oxidase toward ferulic acid and tyrosine were evaluated in the presence of catechol. 4-Methyl catechol, L-dihydroxyphenylalanine, pyrogallol, and 2,3,4-trihydroxybenzoic acid, substrates with high binding affinity to field bean polyphenol oxidase, could stimulate this hydroxylation reaction. In contrast, diphenols such as protocatechuic acid, gallic acid, chlorogenic acid, and caffeic acid, which were not substrates for the oxidation reaction, were unable to bring about this activation. It is most likely that only *o*-diphenols that are substrates for the diphenolase serve as cosubstrates by donating electrons at the active site for the monophenolase activity. The reaction mechanism for this activation is consistent with that proposed for tyrosinase (Sanchez-Ferrer, A.; Rodriguez-Lopez, J. N.; Garcia-Canovas, F.; Garcia-Carmona, F. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11). The presence of *o*-diphenols, viz. catechol, L-dihydroxyphenylalanine, and 4-methyl catechol, is also necessary for the oxidation of the diphenols, caffeic acid, and catechin to their quinones by the field bean polyphenol oxidase. This oxidation reaction occurs immediately with no lag period and does not occur without the addition of diphenol. The kinetic parameters for caffeic acid (K_m = 0.08 mM, V_{max} = 32440 u/mg) in the presence of catechol and the activation constant K_a of catechol (4.6 mM) for this reaction were enumerated. The absence of a lag period for this reaction indicates that the diphenol mechanism of diphenolase activation differs from the way in which the same *o*-diphenols activate the monophenolase activity.

KEYWORDS: ***o*-Diphenols; cosubstrate cresolase; catecholase; lag period**

INTRODUCTION

Tissue browning, a major cause of quality loss during harvesting, post-harvest handling/storage, and processing of fruits and vegetables (*1*), is attributed to the reaction catalyzed by the enzyme polyphenol oxidase (EC 1.10.3.1, PPO). PPO, which is ubiquitous in nature and widely distributed in higher plants, animals, and microorganisms, is a binuclear copper containing enzyme that catalyzes two ostensibly distinct reactions: (1) hydroxylation of monophenols to *o*-diphenols, the only specific reaction catalyzed by this enzyme (cresolase or monophenolase); and (2) oxidation of the self-generated *o*-diphenols to the corresponding *o*-quinones (catecholase or diphenolase). The second reaction can be achieved not only by PPO but also by peroxidases and laccases (*2*). The nascent *o*-quinones are usually unstable in aqueous solutions, and undergo cyclization, addition, and polymerization leading to the formation of colored pigments.

Isotopic studies using ^{18}O-labeled oxygen indicate that the source of oxygen of the new hydroxyl group in the hydroxylation process is molecular oxygen (*3*). This hydroxylation step shows a characteristic lag period before the maximum velocity is attained and thereby is rate limiting (*4*, *5*). In contrast, the catecholase activity shows no such slow transition phenomenon. Sanchez-Ferrar et al. (*6*) in their comprehensive review of the mechanism of mushroom tyrosinase activity, clearly explain the theoretical basis of both diphenolase activity and the existence of the lag period for the monophenolase. Chemical and spectroscopic studies indicate that the binuclear copper active site of tyrosinase can be prepared in any of the three forms: met, oxy and deoxy, which are interrelated (*7*). Only the oxy form acts on monophenols, whereas the met form can be converted to the oxy form by H_2O_2 that acts on monophenols (*8*). Cooksey et al. (*9*) explain the kinetics of the lag period of mushroom tyrosinase by an autocatalytic mechanism dependent

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on the generation of a dihydric phenol, which acts as an activator. Addition of small quantities of reducing agents or *o*-diphenols as cosubstrates (*4*, *10*) can reduce the characteristic lag period of the monophenolase activity.

PPO purified from field bean (*Dolichos lablab*) of M_r ~120,000 Da is a tetramer and exists as a single isoform in the seed. The purified enzyme exhibited no activity toward the hydroxylation of monophenols (*11*). In this paper, we present the results on the oxidation of monophenols by field bean PPO in the presence of small quantities of diphenols as cosubstrates, ascertaining the existence of monophenolase activity. The most important characteristic of this activity is that only those diphenols that exhibit high binding affinity for the diphenolase activity act as cosubstrates. In addition, we demonstrate that these diphenols also activate diphenolase activity by an entirely different mechanism.

MATERIALS AND METHODS

Materials. Catechol, 4-methyl catechol, L-dihydroxy phenylalanine (L-DOPA), ferulic acid, caffeic acid, catechin, protocatechuic acid, chlorogenic acid, gallic acid, and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were obtained from Sigma Chemical Co. (St. Louis, MO). 2,3,4-Trihydroxy benzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). L-Tyrosine was from E-Merck, Germany. All other chemicals used were of analytical grade.

Enzyme Preparation. The enzyme extraction and purification of PPO of *Dolichos lablab* was performed as previously described (*11*). Protein concentration was determined by the method of Bradford (*12*). Bovine serum albumin was used as the standard.

Spectrophotometric Assays. Kinetic assays were performed by measuring the appearance of the quinone products in the reaction mixture using a Shimadzu UV−Vis spectrophotometer model UV-160A. The assay method was based on the spectrophotometric procedure of Cosetang and Lee (*13*). The assay mixture consisted of 0.9 mL of 0.05 M sodium acetate buffer pH 4.5, 0.1 mL of substrate, and 10−100 μg of enzyme. For catechol, 4-methyl catechol, and catechin, increase in the absorbance at 420 nm was monitored. For L-DOPA, tyrosine, ferulic acid, and caffeic acid the increase in absorbance at 480 nm was followed. One unit of enzyme activity is defined as the amount of enzyme that causes an increase in the absorbance of 0.001/min at 25 °C. The reference cuvette contained all the compounds except the enzyme in a final volume of 1 mL.

Cresolase activity is characterized by a lag period. Cresolase activity in the steady state was estimated by extrapolation of the linear zone of the product accumulation curve after the lag period.

Oxygen Measurements. Oxygen consumption was followed using a dissolved oxygen meter model FE 247 (EDT Instruments, UK) based on a Clark electrode. The sample cell contained 4.9 mL of 0.05 M sodium acetate buffer pH 4.5, 0.1 mL of substrate, and 50−500 μg of the enzyme. Oxygen was bubbled through the buffer to reach saturation levels. The reaction was initiated by addition of enzyme to the reaction mixture containing buffer, substrate, and cosubstrate.

RESULTS

PPO purified from field bean (*Dolichos lablab*) seeds showed no activity toward the monophenols (*11*) indicating the absence of hydroxylating activity (monophenolase). The absence of monophenolase activity of several plant PPOs has been attributed to the lability of the enzyme during the purification process used (*14*). Ferulic acid, a monohydroxyphenol, is not hydroxylated by field bean PPO. **Figure 1** shows the time course of ferulic acid oxidation under varying experimental conditions. When ferulic acid oxidation was attempted at saturating oxygen concentrations, but in the absence of catechol, there was no measurable activity (**Figure 1**, curve a). No monophenolase activity on ferulic acid could be detected when either MBTH, a potent nucleophile used to capture unstable *o*-quinones, was included in the reaction mixture, or when the reaction time was increased to 15 min (results not shown). In the presence of small concentrations of catechol, ferulic acid was oxidized to its corresponding quinone. Curves b and c (**Figure 1**) show the oxidation rate when catechol (2 and 20 mM) was included in the assay mixture. This activity was characterized by a lag period, defined as the abscissa obtained on the extrapolation of the linear zone of the oxidation product accumulation curve (steady-state rate curve, **Figure 1**, curve b). Such a lag period is common to several plants' PPOs (*8*, *15*−*21*). Catechol is oxidized by field bean PPO to *o*-quinone, whose absorption maximum is 420 nm (*11*). The absorption maximum of the ferulic acid oxidation products is 480 nm (**Figure 2**). At this wavelength, the contribution to the spectrum by the *o*-quinone of catechol oxidation is negligible (<2%). This demonstrates that the measured increase in absorbance at 480 nm is due to the formation of quinone from ferulic acid and not from catechol.

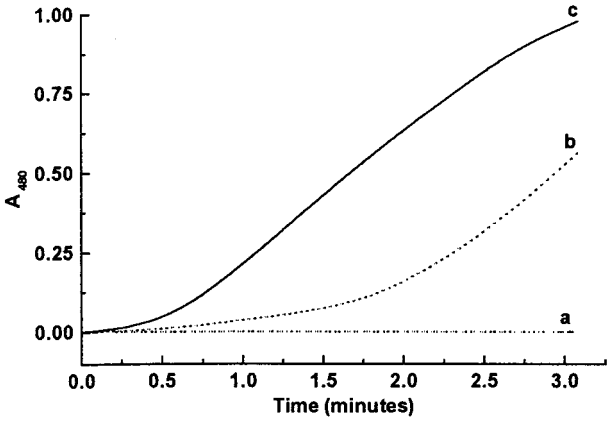


Figure 1. Time course of product formation in the oxidation of ferulic acid by field bean PPO. (a) Progress curve with the reaction medium containing 0.2 mM ferulic acid in 50 mM sodium acetate buffer pH 4.5, and 20 μg of enzyme. (b) and (c) Progress curves for the oxidation of 0.2 mM ferulic acid in 50 mM sodium acetate buffer pH 4.5, and 20 μg of enzyme in the initial presence of 2 mM catechol (b) or 20 mM catechol (c).

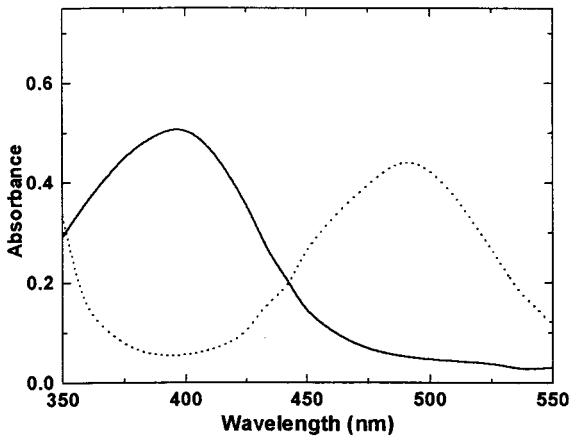


Figure 2. Absorbance spectra for the oxidation products of catechol and ferulic acid catalyzed by field bean PPO. Assay medium containing 50 mM sodium acetate buffer pH 4.5, 20 μg of purified PPO, and 50 mM catechol (—) or 0.2 mM ferulic acid in the presence of 10 mM catechol (- - -).

The lag period and the monophenolase steady-state rate were affected by the concentrations of both catechol and the substrate ferulic acid. The effect of varying concentrations of catechol on the lag period of the monophenolase activity at a fixed

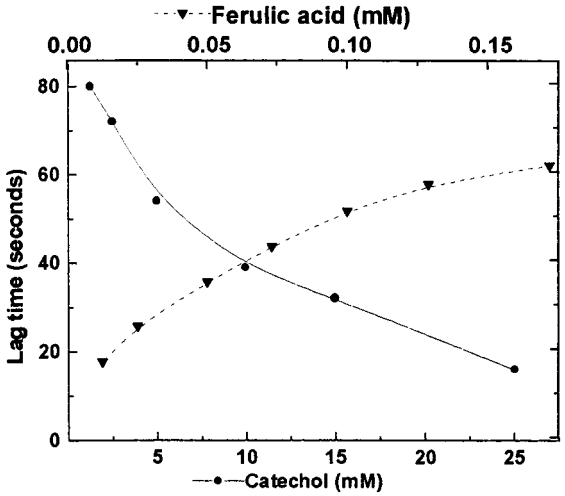


Figure 3. Effect of variation of ferulic acid and catechol concentrations on the lag period of monophenolase activity of field bean PPO. (▼) The reaction medium included 40 μg of purified field bean PPO, 10 mM catechol, and indicated concentrations of ferulic acid in 50 mM sodium acetate buffer, pH 4.5. (●) The reaction medium included 40 μg of purified field bean PPO, 0.2 mM ferulic acid, and indicated concentrations of catechol in 50 mM sodium acetate buffer, pH 4.5.

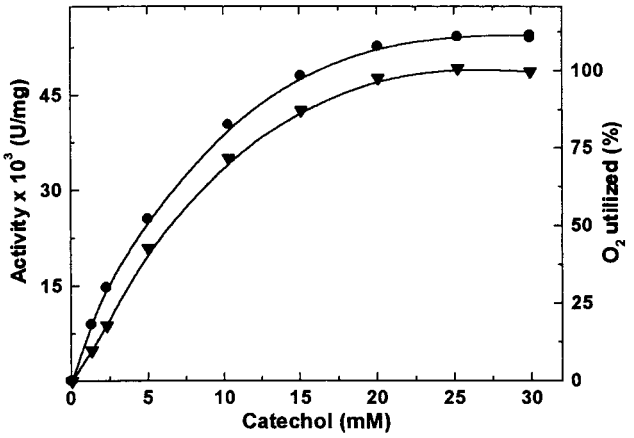


Figure 4. Effect of catechol concentration on the rate of ferulic acid oxidation by field bean PPO. The assay medium consisted of 50 mM sodium acetate buffer pH 4.5, 50 μg of field bean PPO, catechol at indicated concentrations, and 0.2 mM ferulic acid. (▼) Oxygen consumption; (●) quinone product formed.

concentration ferulic acid is shown in **Figure 3**. The length of the lag period decreases with increasing catechol concentrations and almost disappears at ~25 mM. Increasing the ferulic acid concentration from 0.0125 mM to 0.0175 mM resulted in an increase in the lag period (**Figure 3**) similar to that observed for other plant PPOs (*16*−*18*). **Figure 1** shows that, unless the enzyme was primed with small quantities of catechol, no monophenolase activity could be measured. This induced monophenolase activity was 5-fold greater when MBTH was included in the assay medium.

To characterize this catechol-dependent monophenolase activity toward ferulic acid, the effect of varying catechol concentrations, at a fixed ferulic acid concentration, on the steady-state rate was measured at 480 nm (**Figure 4**). The initial rate showed a hyperbolic response to increasing catechol concentrations, when ferulic acid oxidation was measured at 480 nm. As observed, an increase in catechol concentration resulted in increased enzyme activity and reached a plateau at concentrations >12.5 mM catechol. The initial rates of oxygen consumption at a fixed concentration of ferulic acid, but variable catechol concentration, showed a similar response (**Figure 4**) with the oxygen consumption increasing with increasing concentrations of catechol. The maximum reaction rate measured occurred at a catechol concentration ≥12.5 mM, irrespective of the method used to measure the reaction rate. The enzyme activation constant K_a, was evaluated from triplicate measurements of the steady-state rate V, for each initial catechol concentration [A_0]. The K_a was calculated from the linear plot ($1/V$ vs $1/A$) according to the method of Cornish-Bowden (*22*). K_a for catechol was 5 mM, lower than the K_m of catechol for the diphenolase activity (*11*) (**Table 1**).

Table 1. Kinetic Parameters for the Monophenolase and Diphenolase Activities of Field Bean PPO

substrate	V_{max} (u/mg)	K_m (mM)	K_a of catechol (mM)
catechol	67,347	10.5	–
4-methyl catechol	94,285	4.00	–
L-DOPA[a]	15,220	1.18	–
pyrogallol	16,163	12.50	–
2,3,4-trihydroxybenzoic acid	2,491	5.20	–
tyrosine[b]	19,841	3.13	0.58
ferulic acid[b]	56,687	0.09	5.00
caffeic acid[b]	32,440	0.08	4.60

[a] Assay buffer, sodium acetate, 50mM, pH 5.0. [b] Activity measured in the presence of 20 mM catechol as cofactor. K_ms are apparent.

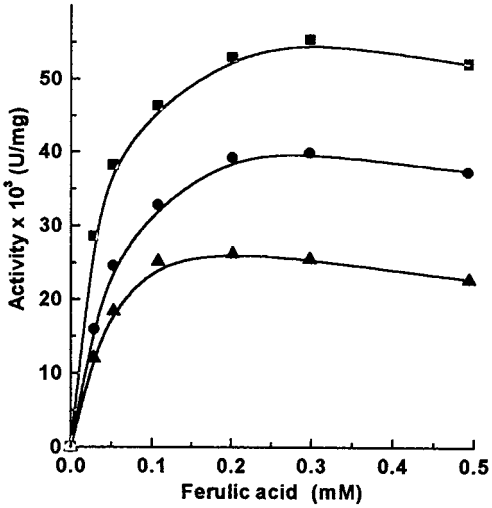


Figure 5. Effect of the variation of catechol concentrations on monophenolase activity at different concentrations of ferulic acid. Assay medium contained 50 mM sodium acetate, 50 μg of field bean PPO, ferulic acid at the indicated concentrations, and catechol at (▲) 2 mM, (●) 10 mM, and (■) 20 mM.

To further study the ferulic acid oxidation by field bean PPO, the kinetic parameters V_{max} and apparent K_m in the presence of catechol as an activator were studied at pH 4.5 using sodium acetate buffer. The enzyme showed Michaelis−Menten kinetics (**Figure 5**), irrespective of whether the oxidation product was measured or whether the oxygen consumption rate (data not shown) was measured. Reciprocal plots for the kinetic data of ferulic acid oxidation resulted in linear relationships (data not shown) for all catechol concentrations. The kinetic parameter K_m for ferulic acid evaluated from these data was 0.09 mM.

The monophenolase activity of field bean PPO in the presence of catechol was also dependent on the enzyme concentrations. The steady state rate in the presence of a fixed concentration

Table 2. Effect of Cosubstrates on Monophenolase and Diphenolase Activity of Field Bean PPO[a]

		monophenolase (u/mg)		diphenolase (u/mg)	
no.	cosubstrate	ferulic acid (0.2mM)	tyrosine (5mM)	caffeic acid (0.2mM)	catechin (5mM)
1	catechol	56687	19841	32440	6021
2	4-methyl catechol	54248	19281	31340	5924
3	L-DOPA	52340	19841	30130	5897
4	pyrogallol	32450	10980	17894	0
5	2,3,4-trihydroxybenzoic acid	6876	1537	3452	0
6	protocatechuic acid	0	0	0	0
7	caffeic acid	0	0	0	0
8	catechin	0	0	0	0
9	chlorogenic acid	0	0	0	0
10	gallic acid	0	0	0	0

[a] Assay medium contained 50 mM sodium acetate buffer, pH 4.5, substrate concentration as indicated, and 50 μg of the enzyme. The cosubstrates were used at a final concentration of 20 mM.

of catechol increased linearly with the increase in enzyme concentration and the lag period decreased with increasing enzyme concentrations (results not shown). Ferulic acid was not oxidized in the absence of enzyme and in the presence of catechol. It can be concluded that the presence of an active enzyme is essential for ferulic acid hydroxylation and further oxidation.

When this catechol dependent monophenolase activity of field bean PPO was extended to tyrosine, a similar activation with a lag period was observed (results not shown). Tyrosine was not hydroxylated to DOPA by field bean PPO (*11*). However, in the presence of small quantities of catechol, tyrosine was oxidized to dopaquinone, with a specific activity of 19,841 u/mg (**Table 1**). The apparent K_m for tyrosine was 3.13 mM. The catechol activation constant, K_a, was 10-fold lower than that for ferulic acid.

Several diphenols and substituted diphenols have been reported to be excellent substrates of plant PPOs. **Table 2** reports some of the compounds investigated for activation toward the monophenolase activity of purified field bean PPO. Each of the compounds **2**−**10** differs from catechol only with respect to one substitution on the phenyl ring. The results show that among the diphenols, other than catechol, 4-methyl catechol, L-DOPA, the triphenols, pyrogallol, and 2,3,4-trihydroxybenzoic acid stimulate the monophenolase activity. These di- and triphenols are efficiently oxidized by field bean PPO to their respective quinones (*11*). In the absence of these compounds the monophenolase activity toward both ferulic acid and tyrosine was absent. The stimulation of the monophenolase activity by these compounds was also characterized by a lag period (results not shown). Among the substituted hydroxy benzoic acids, 2,3,4-trihydroxybenzoic acid stimulated this activity, whereas 3,4-dihydroxybenzoic acid (protocatechuic acid) and 3,4,5-trihydroxybenzoic acid (gallic acid) did not have the same effect. The latter two are not substrates for the enzyme but rather inhibit the activity (unpublished result). 2,3,4-Trihydroxybenzoic acid is oxidized by field bean PPO, and the kinetic parameters are V_{max} = 2513 u/mg and K_m = 5.2 mM. From these results it appears that the stimulation of monophenolase activity is restricted to those *o*-diphenols that are oxidized by field bean PPO.

Caffeic acid (3,4-dihydroxycinnamic acid) and catechin did not activate this monophenolase activity (**Table 2**). These two compounds are unreactive toward field bean PPO (curve a, **Figure 7** results shown for caffeic acid only). Inclusion of MBTH in the reaction mixture to complex any unstable *o*-quinones if any formed did not show the oxidation of caffeic acid. However, if catechol is present, these compounds react rapidly and the corresponding quinones are formed (Curves b and c, **Figure 6**). This diphenolase activity was measured both by polarographic-oxygen utilization technique and by changes in the absorption spectrum at 480 nm for caffeic acid. The steady

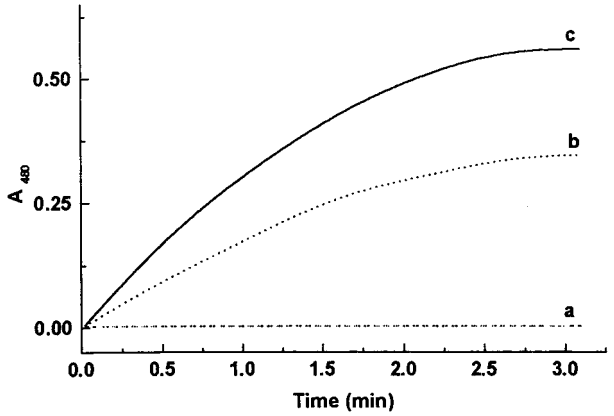


Figure 6. Time course of product formation in the oxidation of caffeic acid by field bean PPO. (a) Progress curve with the reaction medium containing 0.2 mM caffeic acid in 50 mM sodium acetate buffer pH 4.5, and 20 μg of enzyme. (b) and (c) Progress curves for the oxidation of 0.2 mM caffeic in 50 mM sodium acetate buffer pH 4.5, and 20 μg of enzyme in the initial presence of 2 mM catechol (b) or 10 mM catechol (c).

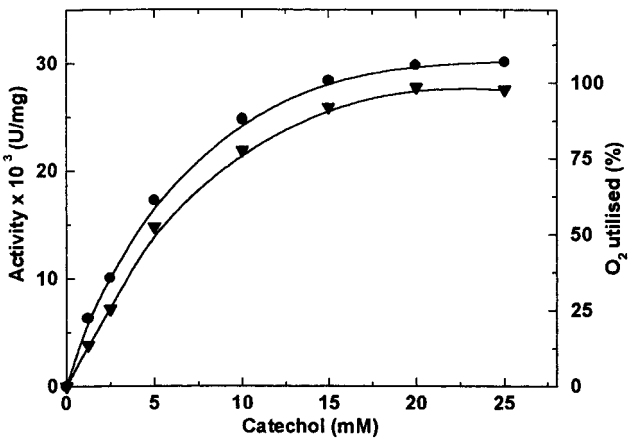


Figure 7. Effect of catechol concentration on the rate of caffeic acid oxidation by field bean PPO. The assay medium consisted of 50 mM sodium acetate buffer pH 4.5, 50 μg of field bean PPO, catechol at indicated concentrations, and 0.2 mM caffeic acid. (▼) Oxygen consumption; (●) quinone product formed.

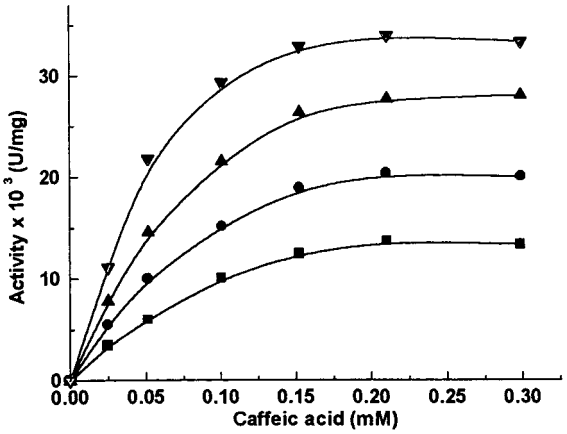


Figure 8. Effect of catechol concentration on diphenolase activity at variable concentrations of caffeic acid. Assay medium contained 50 mM sodium acetate pH 4.5, 50 μg of field bean PPO, caffeic acid at indicated concentrations, and catechol concentrations of (■) 2.5 mM, (●) 5 mM, (▲) 10 mM, and (▼) 25 mM.

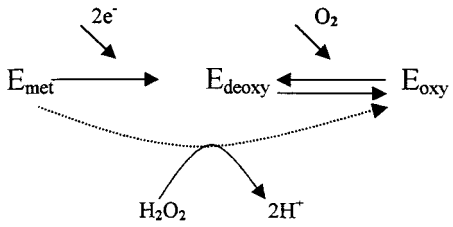
state occurs immediately with no lag period. The initial rates of oxygen consumption and quinone formation, at fixed concentrations of caffeic acid but variable concentrations of catechol, are shown in **Figure 7**. Oxygen consumption and quinone formation increased concomitantly with increasing levels of catechol up to ≥12.5 mM after which no further increase was observed (only curves for caffeic acid shown). The activation constant K_a for catechol evaluated from Cornish−Bowden plots of these data was 4.6 mM, very close to the catechol activation constant for the monophenolase activation.

The dependence of this oxidation rate by field bean PPO on caffeic acid concentration was examined at pH 4.5. The enzyme showed Michaelis−Menten kinetics (**Figure 8**). Reciprocal plots for caffeic acid showed no important differences when kinetic parameters were calculated from the data obtained with variable catechol concentration from 2.5 to 15 mM. These data were averaged. The K_m for caffeic acid in the presence of catechol was 0.08 mM with a V_{max} of 32,440 u/mg. It is evident from these results that catechol, a diphenol, is an essential activator for caffeic acid oxidation by field bean PPO. In addition to catechol, among the several diphenols and substituted diphenols examined, 4-methyl catechol and L-DOPA stimulated the diphenolase-mediated oxidation of caffeic acid and catechin (**Table 2**).

DISCUSSION

Plant PPOs are nuclear encoded chloroplast proteins that generally occur in plastids, although their occurrence in other cell compartments has been reported (*23*). In higher plants, the enzyme is mostly membrane bound, but solubilizes as the fruit matures (*24*). A definitive way to confirm that an active PPO is present in plant material is by determining the monophenolase activity (*16*). The field bean PPO did not exhibit any monophenolase activity toward tyrosine and *p*-cresol (*11*). This phenomenon is well-known in other PPOs resulting from structural changes during purification (*25*). The absence of monophenolase activity in purified plant PPOs is attributed to (1) the lability of monophenolase activity during the purification process; and (2) the assay methods used to follow this activity (*6, 16*−*18, 26, 27*). Temperature-induced phase partitioning in Triton X-114 and other mild extraction methods that avoid the loss of monophenolase activity have been used to purify PPOs (*25, 27*−*31*). It has long been recognized that the monophenolase activity of PPOs requires a reducing agent for its initiation (*4, 32*).

Ferulic acid and tyrosine were not oxidized by field bean PPO (**Figure 1**, curve a). Addition of MBTH, used to capture unstable *o*-quinones, also did not show any monophenolase activity indicating that no *o*-quinones are formed. In the presence of small quantities of catechol (**Figure 1**) and other diphenols (**Tables 1** and **2**) it was oxidized to the *o*-quinone. This induced monophenolase activity of field bean PPO is characterized by a lag period that is common to PPOs from various sources when the monophenolase activity was measured (*25, 27, 28, 30*). The lag period and the steady-state rate of field bean PPO were affected by the substrate concentration, concentration of the diphenol, and the enzyme concentration (**Figures 3**−**5**). The monophenolase lag period is shortened by the increase in catechol concentration (**Figure 3**) and increased with increasing ferulic acid concentration. This peculiar characteristic existence of a lag period in monophenolases has been explained earlier (*6, 33*). The binuclear copper active site prepared in various forms; E_{met}, E_{deoxy} and E_{oxy} (*7*) are interconvertible as shown below.



The oxygenated form (E_{oxy}) is the form that binds and acts on monophenols. The E_{met} (resting form) cannot act upon monophenols, but it can be converted to E_{oxy} form (*8, 34*) and can bind diphenols (*14*). The monophenolase reaction requires that a minimum quantity of enzyme be present as E_{oxy} (*33, 35*). Mushroom tyrosinase acts on L-tyrosine, reaches a first pseudo-steady state with an initial concentration of the diphenol (part of which is provided by the monophenolase activity), and then evolves toward a true steady state with a much higher concentration of the diphenols, which is responsible for the lag period (*36*). Purified field bean PPO is unable to catalyze the oxidation of the monophenol tyrosine and ferulic acid suggesting that the purified PPO is in the E_{met} (resting) form that is incapable of binding monophenols. However, addition of catechol, 4-methyl catechol, L-DOPA, and pyrogallol serve as $2e^-$ donors that convert E_{met} to E_{deoxy}, which is then capable of binding oxygen reversibly to form E_{oxy}. The E_{oxy} thus formed binds the monophenols, ferulic acid, and tyrosine, hydroxylates them and further oxidizes them to the quinones (**Figure 1**, curve b). The K_m values for ferulic acid and tyrosine are lower than the K_m values of the diphenols tested (**Table 1**), indicating the higher binding affinity of the field bean PPO to monophenols. Increasing catechol concentrations produces a greater transformation of the E_{met} field bean PPO to E_{oxy} leading to an increase in the rate of ferulic acid oxidation (**Figure 4**). The oxygen consumption also increases concomitantly leading to an increased turnover of the active form. However, increasing monophenol concentrations in the reaction medium leads to an increase in the lag period (**Figure 3**) implying that the turnover to the E_{oxy} form is insufficient and therefore the time required to attain steady state is longer.

Among all the diphenols and substituted diphenols tested, only those that are oxidized by field bean PPO catalyze this unusual activation phenomenon. Catechol, 4-methyl catechol, L-DOPA, pyrogallol, and 2,3,4-trihydroxybenzoic acid exhibit a high binding affinity to field bean PPO (**Table 1**). Therefore,

it is most likely that only those compounds that bind to the enzyme serve as electron donors at the active site for the conversion of the binuclear copper from E_{met} to E_{oxy}. Protocatechuic acid, caffeic acid, chlorogenic acid, and gallic acid, compounds that show no substrate binding affinity to field bean PPO (*11*), do not catalyze this activation. The observation that the K_a for catechol is less than the K_m of catechol suggests its function as a cofactor ($2e^-$ donor). McIntyre and Vaughan (*37*) demonstrate that the diphenol caffeic acid acts as an electron donor at the active site of spinach-beet phenolase, whereas other reducing agents such as ascorbic acid and NADH function mainly to recycle cofactor amounts of caffeic acid, rather than to donate electrons. Ascorbic acid, hydroxylamine, and dithionite (*38*) also produce oxytyrosinase via this mechanism. In the presence of excess peroxide, oxytyrosinase (*8, 34*) is formed. For the first time in the literature Rodriguez-Lopez et al. (*33*) demonstrated the direct enzymatic release of the diphenol 4-*tert*-butyl catechol during the action of mushroom tyrosinase on 4-*tert*-butylphenol, a monophenol. These results confirmed their earlier proposed mechanism (*39*) that E_{oxy} tyrosinase was the only form capable of catalyzing the transformation of monophenols to diphenols.

Field bean PPO showed no activity toward caffeic acid (**Table 2**) even in the presence of MBTH. With catechol present in the incubation media, caffeic acid was oxidized rapidly (**Figure 6**, curves b and c). Both the oxygen consumption and *o*-quinone formation measurements show the absence of a lag period, earlier observed with monophenols (**Figure 1**, curve b). During the diphenolase activity, *o*-diphenols can bind to both the E_{oxy} and E_{met} forms, which give rise to the product quinones (*5*). Catechol activation of the monophenolase suggests that a large proportion of the purified PPO was in the E_{met} form, yet it did not bind caffeic acid or catechin. The absence of a lag period (**Figure 6**) indicates that this activation does not require the conversion of E_{met} to E_{oxy}. The inability of caffeic acid to stimulate monophenolase activity indicates that it is not a $2e^-$ donor for the conversion of E_{met} to E_{oxy} (**Table 2**). L-DOPA has been shown to promote tyrosinase activity by eliciting a conformational change (*40*). Depending on the catechol concentration, gallic acid behaves both as an activator and a competitive inhibitor of catechol oxidation by field bean PPO (unpublished results). It has been experimentally demonstrated that addition of small quantities of an auxiliary *o*-diphenol (e.g., catechol) considerably increases the accumulation of 4-*tert*-butylcatechol, which then competes with catechol to bind to the tyrosinase (*33*). Electron withdrawing side substituents in the aromatic ring of diphenols cause poor oxidation by diphenolases (*41*). A substituent with a high capacity to donate electrons increases this PPO-catalyzed reaction (*42*). The lower electron donor capability of the side chain of catechin and caffeic acid renders them unoxidizable by field bean PPO. The presence of catechol, 4-methyl catechol, and L-DOPA, which are oxidized at a very high rate (**Table 1**) leads to the co-oxidation of caffeic acid and catechin. The activation constants, K_as, of catechol activation of monophenolase and diphenolase are almost similar. However, the mechanism by which it stimulates these two distinct activities differs: the former characterized by a lag period and the latter attaining the steady-state immediately. The K_m for caffeic acid in the presence of catechol is severalfold lower than the K_m of catechol as the substrate, indicating the higher binding affinity of the enzyme to caffeic acid.

In conclusion, the lack of monophenolase activity observed in field bean PPO is probably due to the loss of the endogenous natural diphenols that occur during the drastic methods used to purify the enzyme, resulting only in the E_{met} (resting) form. The results presented are in agreement with the mechanism described (*6, 33*) that *o*-diphenols are needed in the medium before the enzymatic reaction starts. In parallel, diphenols also activate the diphenolase by a different mechanism. The presence of endogenous diphenols in the intact fruit or vegetable, which can activate both the monophenolase and diphenolase reactions, probably explains the instantaneous and severe browning reactions that occur upon mechanical damage during harvesting, handling, storage, and processing.

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