Study of Stereospecificity in Pear and Strawberry Polyphenol Oxidases

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The stereospecificity in pear and strawberry polyphenol oxidases (PPO) is studied in this paper. For this purpose several enantiomorphs of *o*-diphenols were assayed: L-Dopa, DL-Dopa, D-Dopa; L-α-methyl-Dopa, DL-α-methyl-Dopa; L-isoproterenol and DL-isoproterenol. V^{D}_{max} values obtained for the stereoisomers within each series of *o*-diphenol were the same. This lack of stereospecificity in the transformation steps of the catalytic mechanism could be explained on the basis of the nucleophilic power of each *o*-diphenol, which was related to δ_3 and δ_4 values. The effect of the nucleophilic power of each *o*-diphenol and the different sizes of the side substituents could explain the different V^{D}_{max} values observed for different *o*-diphenols. However, K^{D}_{m} values were lower for L-isomers than those for D-isomers, which revealed the existence of stereospecificity in the affinity of PPO toward its substrates. The kinetic behaviors were interpreted in the light of a previously proposed reaction mechanism for the diphenolase activity of PPO from different fruits and vegetables.

Keywords: *Enzyme kinetics; MBTH; NMR; pear; PPO; stereospecificity; strawberry*

INTRODUCTION

Tyrosinase or polyphenol oxidase (PPO) (monophenol, *o*-diphenol-oxygen oxidoreductase, EC 1.14.18.1) is a copper enzyme that is of central importance in such processes as vertebrate pigmentation and browning of fruits and vegetables (Prota et al., 1988). Different PPOs obtained from several biological sources have similar structural and functional characteristics (Robb, 1984).

The active site of PPO consists of two copper atoms and three states: "met", "deoxy", and "oxy" (Jolley et al., 1972; Schoot-Uiterkamp and Mason, 1973, 1976; Makino and Mason, 1973; Lerch, 1981). Structural models for the active site of these three forms have been proposed (Himmelwright, 1980; Solomon, 1981; Wilcox et al., 1985; Solomon and Lowery, 1993; Solomon et al., 1996). This enzyme catalyzes the *ortho*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Robb, 1984; Mayer and Harel, 1991; Martínez and Whitaker, 1995). The monophenolase activity is coupled to its diphenolase activity and to the nonenzymatic reactions from the corresponding *o*-quinones (García-Cánovas et al., 1982; García-Carmona et al., 1982; Tudela et al., 1987; Rodríguez-López et al., 1991, 1992, 1994; Rodríguez-López et al., 1992; García-Moreno et al., 1994; Rodríguez-López et al., 1994; Sánchez-Ferrer et al., 1995; Espín et al., 1995a,b, 1996, 1997a−e).

Several *o*-diphenols are chiral substrates of PPO (L-, DL-, and D-enantiomers). Some works reported the stereoselective action of several PPOs on these enantiomers (Winder and Harris, 1991; Sonesson et al., 1995), although this phenomenon was not systematically characterized. Wilcox et al. (1985) reported stereospecificity for PPO from *Neurospora crassa*. L-Tyrosine, D-tyrosine, L-Dopa, and D-Dopa were assayed, and their kinetic constants were obtained. The results did not show a determined sequence in the K_m and V_{max} values when the different isomers were compared. Khan and Pomerantz (1980) reported the same V_{max} values for L-tyrosine and D-tyrosine, as well as for L-Dopa and D-Dopa using avocado PPO. The same pattern was obtained for *V. tyrosinaticus* tyrosinase (Pomerantz and Murthy, 1974). Different results were obtained for mammalian (Pomerantz, 1963) and *Pseudomonas* tyrosinase (Yoshida et al., 1974). All of these contradictory results may arise from the assay methods, which could have not rendered reliable and precise results.

Catalytic stereospecificity has been reported in other enzymes such as horseradish peroxidase and lactoperoxidase. This phenomenon was approached with ESR spin stabilization techniques combined with optical methods by Ferrari et al. (1993) using the substrates L-Dopa and D-Dopa. The kinetic parameters K_m and V_{max} were different for these enantiomers. The peroxidase reaction mechanism could justify these results.

The aim of this paper is the systematic study of the stereospecificity in pear and strawberry PPOs in their action on several *o*-diphenolic enantiomers with different sizes of side substituents in the aromatic ring. The diphenolase activity will be determined spectrophotometrically by using a sensitive method with the chromogenic nucleophile MBTH (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996, 1997a−e). Moreover, NMR studies on these *o*-diphenols will be made to estimate their nucleophilic power to predict their catalytic efficiency as substrates of PPO. The results obtained for the enzymatic activity will be compared with the NMR predictions and fit to a previ-

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ously proposed reaction mechanism for PPO from different fruits and vegetables (Rodríguez-López et al., 1992; Ros et al., 1994; Espín et al., 1995b, 1997a,c−e).

MATERIALS AND METHODS

Reagents. L-Dopa, DL-Dopa, D-Dopa, L-α-methyl-Dopa, DL-α-methyl-Dopa, L-isoproterenol, DL-isoproterenol, and MBTH were purchased from Sigma (Madrid, Spain). All other chemicals were of analytical grade and supplied by Fluka (Madrid, Spain). Stock solutions of the phenolic substrates were prepared in 0.15 mM phosphoric acid to prevent their autoxidation. The acidic character of MBTH required the use of 50 mM buffer in the assay medium.

Triton X-114 (TX-114) was obtained from Fluka and condensed three times prior to use as described by Bordier (1981) but using 0.1 M PB, pH 7.3, containing EDTA 20 mM. The detergent phase of the third condensation had a concentration of 23% TX-114 (w/v).

To dissolve the MBTH-quinone adducts 2% (v/v) *N,N′*-dimethylformamide (DMF) was added to the assay medium (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996, 1997a−e). Milli-Q system (Millipore Corp.) ultrapure water was used throughout this research.

Preparation of PPO. Pear and strawberry PPOs were extracted by using the method with TX-114 (Espín et al., 1995a,b, 1996, 1997a,c,d). Protein concentration was determined according to the method of Bradford (1976).

Enzymatic Assays. Kinetic assays were carried out by measuring the appearance of the product in the reaction medium in an ultraviolet−visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced with an IBM PC 486 DX microcomputer (Madrid, Spain). Temperature was controlled at 25 °C using a Haake D1G circulating water bath (Berlin, Germany) with a heater/cooler and checked using a precision of ±0.1 °C. Reference cuvettes contained all of the components except the substrate, with a final volume of 1 mL.

Diphenolase activity of pear and strawberry PPOs was determined spectrophotometrically by using MBTH, which is a potent nucleophile through its amino group which realizes the nucleophilic attack on the enzyme-generated *o*-quinones at position 6 (Winder and Harris, 1991; Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996, 1997a−e). MBTH traps the enzyme-generated *o*-quinone, rendering a soluble and stable MBTH−quinone adduct with high molar absorptivity. The stability of MBTH−quinone adducts and the rapid assays provide a reliable assay method to determine the diphenolase activity of PPO from several sources (Rodríguez-López et al., 1994; Espín et al., 1995a,b, 1996, 1997a−e).

Kinetic Data Analysis. The K_m^D and V_{max}^D values on different *o*-diphenols were calculated from triplicate measurements of the steady-state rate, V_{ss}, for each initial substrate concentration, $[S]_0$. The reciprocals of the variances of V_{ss} were used as weighting factors in the nonlinear regression fitting of V_{ss} versus $[S]_0$ data to the Michaelis equation (Wilkinson, 1961; Endrenyi, 1981). The fitting was carried out by using the Marquardt's algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994). Initial estimations of K_m^D and V_{max}^D were obtained from the Hanes−Woolf equation, a linear transformation of the Michaelis equation (Wilkinson, 1961).

NMR Assays. ^{13}C NMR spectra of the different *o*-diphenols tested were obtained in a Varian Unity spectrometer of 300 MHz. The spectra were obtained at the optimum pH values for pear and strawberry PPOs (Espín et al., 1997a,d) using D_2O as solvent for the substrates.

RESULTS AND DISCUSSION

Kinetic Assays. V_{max}^D and K_m^D values were obtained for the different isomers within each series of *o*-diphenol. V_{max}^D values were the same, whereas K_m^D values were different for the members within each series of *o*-diphenol at the same enzyme concentration for each PPO assayed. The sequence of K_m^D values was D-isomers > DL-isomers > L-isomers. This sequence of results was the same with both PPOs assayed (Tables 1 and 2). Moreover, it should be noted that the V_{max}^D values decreased and K_m^D values increased when the molecular weight of the side-chain substituent of different *o*-diphenols increased (Tables 1 and 2).

Table 1. Kinetic Constants for the Diphenolase Activity of Pear PPO on Several *o*-Diphenolic Enantiomorphs[a]

o-diphenol	V_{max}^D (μM/min)	K_m^D (mM)	V_{max}^D/K_m^D (min^{-1})
L-Dopa	2.63 ± 0.11	1.75 ± 0.06	$1.5 \times 10^{-3} \pm 1.1 \times 10^{-4}$
DL-Dopa	2.61 ± 0.11	2.41 ± 0.11	$1.1 \times 10^{-3} \pm 9.6 \times 10^{-5}$
D-Dopa	2.65 ± 0.12	4.51 ± 0.17	$5.9 \times 10^{-4} \pm 4.9 \times 10^{-5}$
L-α-methyl-Dopa	1.52 ± 0.08	2.91 ± 0.12	$5.2 \times 10^{-4} \pm 4.9 \times 10^{-5}$
DL-α-methyl-Dopa	1.51 ± 0.08	4.92 ± 0.18	$3.1 \times 10^{-4} \pm 2.8 \times 10^{-5}$
L-isoproterenol	1.21 ± 0.05	3.21 ± 0.13	$3.8 \times 10^{-4} \pm 3.1 \times 10^{-5}$
DL-isoproterenol	1.22 ± 0.05	5.62 ± 0.19	$2.2 \times 10^{-4} \pm 1.6 \times 10^{-5}$

[a] Conditions were AB 50 mM, pH 4.3, DMF 2%, saturating MBTH concentration, different *o*-diphenol concentrations, and 4 μg/mL pear PPO.

Table 2. Kinetic Constants for the Diphenolase Activity of Strawberry PPO on Several *o*-Diphenolic Enantiomorphs[a]

o-diphenol	V_{max}^D (μM/min)	K_m^D (mM)	V_{max}^D/K_m^D (min^{-1})
L-Dopa	2.58 ± 0.09	2.40 ± 0.07	$1.1 \times 10^{-3} \pm 7.0 \times 10^{-5}$
DL-Dopa	2.60 ± 0.10	3.81 ± 0.10	$6.8 \times 10^{-4} \pm 4.4 \times 10^{-5}$
D-Dopa	2.58 ± 0.09	6.11 ± 0.21	$4.2 \times 10^{-4} \pm 2.9 \times 10^{-5}$
L-α-methyl-Dopa	2.10 ± 0.08	7.15 ± 0.33	$2.9 \times 10^{-4} \pm 2.4 \times 10^{-5}$
DL-α-methyl-Dopa	2.08 ± 0.08	9.22 ± 0.45	$2.2 \times 10^{-4} \pm 1.9 \times 10^{-5}$
L-isoproterenol	0.88 ± 0.04	8.14 ± 0.36	$1.1 \times 10^{-4} \pm 9.9 \times 10^{-6}$
DL-isoproterenol	0.90 ± 0.05	9.45 ± 0.45	$9.5 \times 10^{-5} \pm 9.8 \times 10^{-6}$

[a] Conditions were AB 50 mM, pH 4.3, DMF 2%, saturating MBTH concentration, different *o*-diphenol concentrations, and 12 μg/mL strawberry PPO.

Table 3. δ Values for the Carbon Atoms in *p*- and *m*-Positions from the Phenolic Hydroxyl Groups of Several *o*-Diphenols at pH 4.3[a]

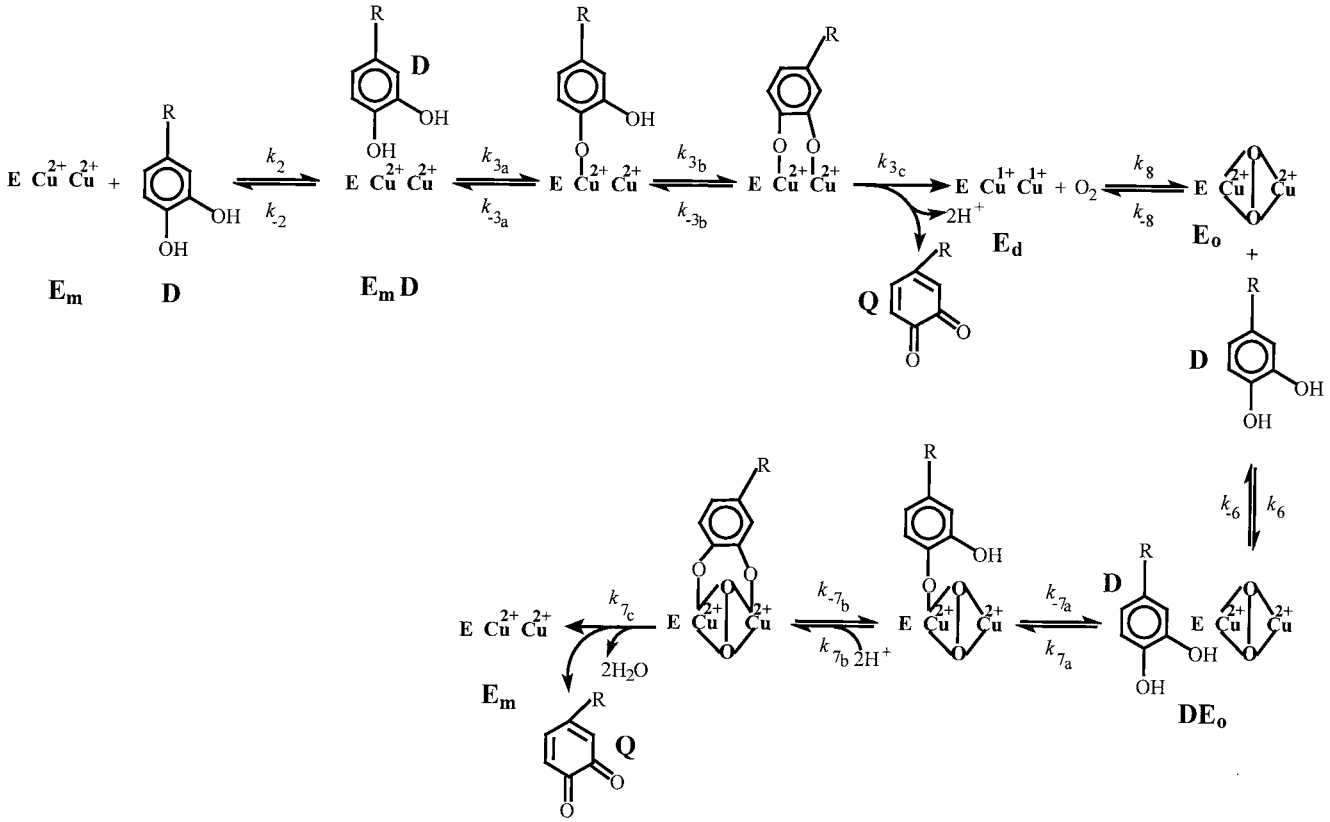
o-diphenol	δ_3	δ_4	*o*-diphenol	δ_3	δ_4
L-Dopa	146.920	146.011	DL-α-methyl-Dopa	146.720	146.183
DL-Dopa	146.921	146.014	L-isoproterenol	146.881	146.830
D-Dopa	146.920	146.012	DL-isoproterenol	146.890	146.829
L-α-methyl-Dopa	146.720	146.182			

[a] Conditions were saturating substrate concentration in D_2O at pH 4.3.

NMR Assays. Phenolic compounds with electron-withdrawing substituents are poorly oxidized (Solomon et al., 1996). Therefore, a side chain with high capacity to donate electrons will facilitate the hydroxylation and oxidation of monophenols and *o*-diphenols, respectively. To study the electron donor capacity of the side chain, the different isomers for *o*-diphenols were studied by means of NMR assays. The highest electron donor capacity will correspond to the highest electronic charge on the carbon atoms in *meta* (C3) and *para* (C4) positions of the aromatic ring and, therefore, to the lowest chemical displacement values (δ_3 and δ_4, respectively) (Table 3). It is of note that δ values were similar for the members (L-, DL-, and D-isomers) of each series of *o*-diphenols assayed.

On the basis of the NMR studies (Table 3), δ_3 and δ_4 values for each isomer of *o*-diphenol predicted the same nucleophilic power of the oxygen atom from the aromatic hydroxyl group to attack the copper atoms of the active

Scheme 1. Structural Reaction Mechanism for the Diphenolase Activity of PPO[a]



[a] $ECu^{2+}Cu^{2+}$, (E_m) metPPO; $ECu^{+}Cu^{+}$, (E_d) deoxyPPO; $ECu^{2+}Cu^{2+}-O_2$, (E_o) oxyPPO; D, *o*-diphenol; Q, *o*-quinone.

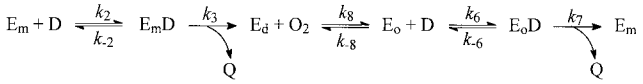
site of the enzyme, and therefore the isomers within each series of *o*-diphenol should show similar V^D_{max} values (Tables 1 and 2).

These results indicated that the reactivity of the different isomers within each series of *o*-diphenols was the same. This NMR study supported the sequence of V^D_{max} values spectrophotometrically obtained. If the electronic density (reactivity) is exactly the same (Table 3), then the nucleophilic power of the oxygen from the hydroxyl group to attack the copper atoms of the active site of the enzyme is the same. Therefore, the transformation rate (V^D_{max}) of the enzyme on several isomers of each series of substrates is the same (Tables 1 and 2).

In the different *o*-diphenols assayed, it should be noted that $\delta_3 > \delta_4$ (Table 3). In other words, the capacity of nucleophilic power in C4 is higher than that in C3, and therefore the attack of the oxygen from the hydroxyl group, which is supported by C3, is the limiting step in the reaction mechanism. However, the binding of the substrate to the copper atoms of active site could cause a distortion, which could provoke electronic, steric, or hydrophobic effects of the side substituent on the different amino acids of the active site (Scheme 1). L-α-Methyl-Dopa had a lower δ_3 value and a bigger side-chain substituent than those for L-Dopa; the overall effect made L-Dopa a better substrate than L-α-methyl-Dopa for pear and strawberry PPOs. These enzymes poorly oxidized L-isoproterenol because of its high δ_3 value and bulky side-chain substituent, and therefore it is the worst PPO substrate assayed.

Regarding K^D_m values, it should be noted that the spatial orientation of the side-chain substituent provoked stereospecificity in pear and strawberry PPOs.

Scheme 2. Kinetic Reaction Mechanism for the Diphenolase Activity of PPO[a]



[a] E_m, *met*PPO or oxidized form of PPO with $Cu^{2+}-Cu^{2+}$ in the active site; E_d, reduced form of PPO with $Cu^{+}-Cu^{+}$ in the active site; E_o, *oxy*PPO (E_dO_2 or $E_mO_2^{2-}$); D, diphenol; Q, *o*-quinone; N, nucleophile (MBTH); NQH, MBTH-quinone adduct.

Therefore, PPO showed more affinity for L-isomers than for D-isomers. Moreover, when the side-chain substituent was bigger, the enzyme showed less affinity toward the substrate (higher K^D_m value) (Tables 1 and 2).

The diphenolase reaction mechanism of PPO (Scheme 2) involves several rate constants for the analytical expressions V^D_{max} and K^D_m (Rodríguez-López et al., 1992; Ros et al., 1994):

$$V^D_{max} = k_3k_7[E]_0/(k_3 + k_7) \quad (1)$$

$$K^D_m = k_2k_3(k_{-6} + k_7) + k_6k_7(k_{-2} + k_3)/[k_2k_6(k_3 + k_7)] \quad (2)$$

The V^D_{max} expression involves rate constants that only rule transformation steps in the reaction mechanism. Previous studies reported that the rate-limiting step in the diphenolase activity of PPO could be the step ruled by k_3 (García-Cánovas et al., 1985; García-Moreno et al., 1994). The K^D_m expression involves rate constants, which rule transformation and binding steps in the reaction mechanism. Considering eq 1, the V^D_{max}

expression is directly dependent on the kinetic constant k_3. However, K_m^D is not directly dependent on this kinetic constant (eq 2).

The sequence of K_m^D values obtained for the different enantiomers (L- < DL- < D) could be explained by the spatial orientation of the side chain, which could affect one or several rate constants in the K_m^D expression for the diphenolase activity of PPO (Rodríguez-López et al., 1992; Ros et al., 1994).

To sum up, previous studies reported that electron-withdrawing side substituents in the aromatic ring of phenolic compounds caused their poor oxidation by PPO (Solomon et al., 1996). A substituent with high capacity to donate electrons will increase this PPO-catalyzed reaction. If the electron donor capacity is the same (similar δ_3 and δ_4 values for *o*-diphenols) for several substrates (enantiomers of *o*-diphenols), the oxidation rate will be the same. On the basis of the kinetic (Tables 1 and 2) and NMR (Table 3) assays, pear and strawberry PPOs did not show stereospecificity in the transformation reaction rate (V_{max}^D) of several isomers of *o*-diphenols. From the sequence of K_m^D values obtained, pear and strawberry PPOs showed stereospecificity in their affinity toward these chiral substrates (Tables 1 and 2). Moreover, the decrease of the size of the side substituent in the aromatic ring increased the affinity ($1/K_m^D$), velocity (V_{max}^D), and catalytic power (V_{max}^D/K_m^D) properties of PPO toward *o*-diphenolic substrates.

ABBREVIATIONS USED

AB, sodium acetate buffer; C3, carbon atom in position 3 (*meta*) of the *o*-diphenolic ring; C4, carbon atom in position 4 (*para*) of the *o*-diphenolic ring; D_2O, deuterium oxide (heavy water); δ_3, chemical displacement of the carbon atom in the *m*-position of *o*-diphenols; δ_4, chemical displacement of the carbon atom in the *p*-position of *o*-diphenols; DMF, *N*,*N*′-dimethylformamide; K_m, Michaelis constant; K_m^D, Michaelis constants of PPO toward *o*-diphenols; MBTH, 3-methyl-2-benzothiazolinone hydrazone; NMR, nuclear magnetic resonance; PB, sodium phosphate buffer; PPO, polyphenol oxidase; $[S]_0$, initial substrate concentration; TX-114, Triton X-114; V_{max}, maximum steady-state rate; V_{max}^D, maximum steady-state rate for diphenolase activity; V_{ss}, steady-state rate.

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