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Kinetic Studies on Apple Polyphenol Oxidase

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The effect of oxygen concentration on the activity of purified apple polyphenol oxidase (PPO) was studied with 4-methylcatechol, chlorogenic acid, or (+)-catechin as phenolic substrate. For these three phenolic compounds, the kinetic pattern was consistent with a sequential mechanism for the binding of oxygen and phenol. Benzoic acid is a competitive inhibitor with respect to 4-methylcatechol and uncompetitive with respect to oxygen, which suggests that apple PPO combines with oxygen before it does with the phenolic substrate. Oxygen uptake of a two-phenolic compound mixture by apple PPO was studied in air-saturated solutions. An equation that contains only the kinetic parameters K_m and V_m of each phenol was developed. For the two mixtures tested, 4-methylcatechol/caffeic acid and (+)-catechin/chlorogenic acid, the experimental values of oxygen uptake were in excellent agreement with the calculated curves. The equation can be extended to a multicomponent solution and therefore can be used for the study of oxidability of mixtures of phenolic compounds in model solutions by PPO.

INTRODUCTION

Polyphenol oxidase (EC 1.14.18.1; PPO) is a copper-containing enzyme widely distributed in the plant kingdom (Mayer and Harel, 1979; Mayer, 1987). PPO is largely responsible for browning in fruits and vegetables, which is often commercially undesirable (Vamos-Vigyazo, 1981). Many works have been carried out by chemists and food technologists to prevent PPO action (Ponting, 1960). The enzyme catalyzes two different reactions involving molecular oxygen. The first type of reaction is the hydroxylation of monophenols leading to *o*-diphenols, often called monophenolase or cresolase. The second type of reaction is the oxidation of *o*-diphenols to *o*-quinones, often referred to as *o*-diphenolase or catecholase. If the latter activity is always present, enzyme preparations from several plants are often devoid of cresolase activity (Mayer and Harel, 1979; Whitaker, 1985). Many reports on plant catecholases were concerned with its specificity toward *o*-diphenols. However, in most cases, studies were carried out in air-saturated solutions. Therefore, K_m and V_m values determined in these conditions were only apparent, and the effect of oxygen concentration, the other substrate, on these parameters is unknown. The only few cases where such studies were performed with more or less purified extracts from French prunes (Ingraham, 1957), tea leaves (Gregory and Bendall, 1966), mushroom (Yamaguchi et al., 1969; Duckworth and Coleman, 1970), *Streptomyces glaucescens* (Lerch and Ettlinger, 1972), pear (Rivas and Whitaker, 1973), *Neurospora crassa* (Gutteridge and Robb, 1975), grape (Lerner and Mayer, 1976), potato (Matheis and Belitz, 1976), and spinach (Golbeck and Cammarata, 1981) gave contradictory results. Thus, using Lineweaver–Burk representations for the study of the concentration effect of oxygen and phenolic compounds, Gregory and Bendall (1966) obtained a series of parallel lines with all the phenolic substrates with the exception of chlorogenic acid. Such a pattern is consistent with a Ping-Pong mechanism. Duckworth and Coleman (1970) found that only pyrocatechol gave a series of parallel or very slightly convergent lines, whereas series of convergent lines were obtained with acetylcatechol and 4-methylcatechol. Whatever the phenolic substrate, all other authors found an intersecting pattern characteristic of a sequential mechanism. However, there were still some controversies since some workers indicated that it was an ordered mechanism where oxygen binds first (Ingraham,

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1957; Matheis and Belitz, 1977), whereas others postulated that it was either a random mechanism or an ordered one where oxygen does not bind first (Duckworth and Coleman, 1970; Gutteridge and Robb, 1975; Lerner and Mayer, 1976).

In another way, only few studies have been devoted to enzymatic oxidations of phenolic mixtures in model solutions (Cheynier et al., 1988; Cheynier and Van Hulst, 1988). Therefore, almost nothing is known on the mutual effect of different phenols on their oxidation kinetics by PPO. In enzymatic browning of fruit and vegetables, such knowledge is essential for a better understanding of the relationships among the degree of browning of bruised or cut tissues, their total and relative phenols contents, and their PPO activities.

The purpose of this work is to give further insight on the mechanism of PPO as influenced first by oxygen content and second by the presence of two phenolic substrates in model solutions using a purified PPO extract from apple (Janovitz-Klapp et al., 1989).

MATERIALS AND METHODS

Materials. Red Delicious apples picked at commercial maturity were used as an enzyme source. The PPO was 120-fold purified from cortex in three steps, extraction, fractional precipitations by ammonium sulfate, and hydrophobic chromatography with phenyl-Sepharose CL4B (Pharmacia), according to the method of Janovitz-Klapp et al. (1989). By isoelectrofocusing both in polyacrylamide gels and in liquid medium, two major bands (more than 95% of activity) were obtained at pH 4.5 and 4.8, respectively (Janovitz-Klapp et al., 1989) with the same properties (optimum pH and Michaelis constant) toward 4-methylcatechol and chlorogenic acid (unpublished results). Chlorogenic acid, caffeic acid, and (+)-catechin were from Extrasynthèse (Genay, France), and all other chemicals were of reagent grade from Sigma (St. Louis).

Assay Procedure. For routine PPO analysis, the substrate was 20 mM in 4-methylcatechol in 3 mL of a McIlvaine's buffer solution at pH 4.5. PPO was assayed by polarography with a Clark electrode using air-saturated substrate solution at 30 °C. For the study of oxygen concentration effect, gas mixtures of oxygen and nitrogen were prepared by means of two metering valves placed at the outlet of oxygen and nitrogen cylinders. Reaction mixtures were equilibrated with different oxygen levels by bubbling the appropriate gas mixture through the reaction solution at 30 °C for 10 min before the enzyme was added. Initial oxygen concentrations were determined with the Clark electrode. All the assays were performed in duplicate. Kinetics parameters K_m and V_m were determined by using a nonlinear regression data analysis program developed for IBM PC by Leatherbarrow (1987). PPO activity was expressed as nanomoles of oxygen consumed per second (nkat).

RESULTS

PPO is an enzyme that catalyzes a reaction between two substrates, a phenolic compound and oxygen. The study of varying each substrate concentration can give valuable information on its mechanism of action.

The effect of oxygen and 4-methylcatechol concentrations on the initial velocity is shown in Figure 1. The results given in double-reciprocal form lead to a series of lines which intersect to the left of the vertical axis and below the horizontal axis. Therefore, both apparent V_m and K_m for 4-methylcatechol increased when oxygen concentration increased and conversely for apparent V_m and K_m for oxygen with increasing 4-methylcatechol. Series of convergent lines to the left of the vertical axis were also obtained with two other phenolic substrates, namely, chlorogenic acid and (+)-catechin (data not shown). However, with chlorogenic acid, the intersection was on the horizontal axis, meaning that the apparent K_m for one substrate was

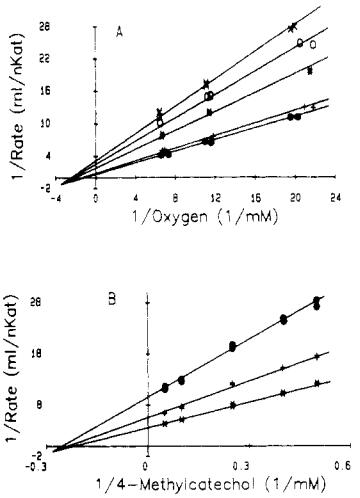

Figure 1. Effect of 4-methylcatechol and oxygen concentrations on the initial activity of apple PPO at pH 4.5 and 30 °C. (A) The different concentrations were (×) 2, (○) 2.5, (*) 4, (+) 10 and (●) 20 mM in 4-methylcatechol. (B) The different concentrations were (●) 0.05, (+) 0.08, and (*) 0.14 mM in oxygen.

independent of the concentration of the other substrate. With (+)-catechin, the lines intersect above the horizontal axis, indicating that the apparent K_m for one substrate decreased with increasing concentration of the other substrate. Therefore, for the three phenolic substrates tested, the apparent V_m was always increased by increasing amounts of oxygen, whereas the apparent K_m was either increased (4-methylcatechol), unchanged (chlorogenic acid), or decreased [(+)-catechin]. These experimental results suggest a sequential mechanism for the binding of phenolic compound and oxygen to apple PPO. In such a mechanism, the enzyme can bind the substrates either in a compulsory order or randomly. However, in the latter case (random mechanism), straight lines are obtained only if the rapid equilibrium assumptions are satisfied, i.e., all binding and dissociation steps are very rapid compared to the catalytic step; otherwise, the double-reciprocal plots are hyperbolic (Segel, 1975). According to this author, the rapid equilibrium approach is not valid for most enzymes that catalyze ordered reaction sequences. Thus, one can think that apple PPO catalyzes an ordered mechanism where one of the substrates is required to bind to the enzyme before the second substrate can bind. Since the velocity equation of this mechanism is symmetrical for the two substrates, no conclusions about the order of addition of oxygen and phenolic compound to apple PPO can be made from the above experiments, and additional data are required.

Therefore, the inhibition mode of benzoic acid was studied by considering first 4-methylcatechol (Figure 2A) and then oxygen (Figure 2B) as the variable substrate. Benzoic acid is competitive with 4-methylcatechol since it does not affect the apparent V_m and increased the apparent K_m (Figure A). The apparent K_i value was 0.64 mM. This is in agreement with our previous results concerning the effect of aromatic carboxylic acids on the apple PPO (Janovitz-Klapp et al., 1990). Thus, benzoic acid can bind only with enzymatic forms of apple PPO which are free of the phenolic compound Ph, i.e., E or E–O_2 (if the latter exists). The parallel pattern (Figure 2B) obtained when oxygen was the variable substrate indicates that benzoic acid is uncompetitive with oxygen and the apparent inhibition constant K_i was found close to 0.62 mM. In that case, the inhibitor can bind only enzymatic forms which contain oxygen, i.e., E–O_2 and E–O_2–Ph. Therefore, these two propositions are only realized if the E–O_2 form is present,

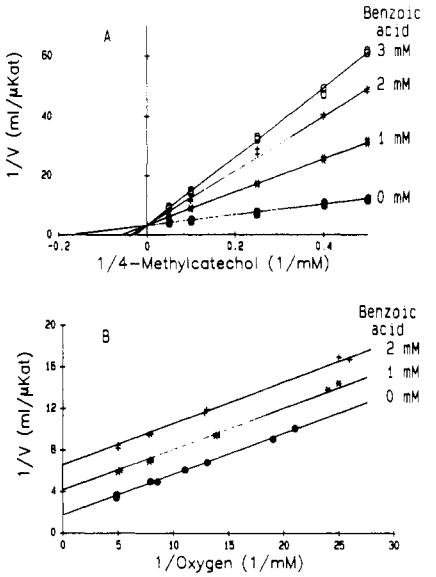

Figure 2. Inhibition of apple PPO by benzoic acid at pH 4.5 and 30 °C (A) with respect to 4-methylcatechol (in air-saturated solutions) and (B) with respect to oxygen (10 mM in 4-methylcatechol). (●) 0, (*) 1, (+) 2, and (○) 3 mM in benzoic acid.

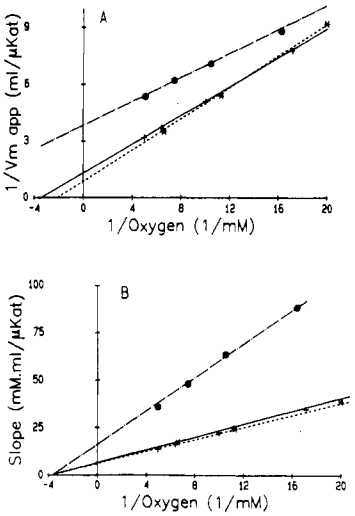

Figure 3. Replots of the effect of oxygen concentrations on the activity of apple PPO for three phenolic substrates. (A) Replots of intercepts (apparent V_m) vs the reciprocal of oxygen concentration. (B) Replots of slopes vs the reciprocal of oxygen concentration. (*) 4-Methylcatechol, (+) chlorogenic acid, and (●) (+)-catechin.

which implies that oxygen is the first substrate to be bound by apple PPO.

Replots of $1/v$ axis intercepts (Figure 3A) permit K_{mO2}, K_{mPh}, and V_m to be determined, whereas K_{iO2} [the nomenclature of Segel (1975) is used for the constant K denomination; in the nomenclature of Cleland (1963) K_{mO2}, K_{mPh}, and K_{iO2} are indicated as K_{O2}, K_{Ph} and K_{iO2}, respectively] can be obtained from the slope replots (Figure 3B). The kinetic parameters for the different substrates are given in Table I. It is interesting to note that 4-methylcatechol is the best substrate for apple PPO since theoretical V_m values for chlorogenic acid and (+)-catechin are slightly higher than 60% and 20%, respectively, of the value obtained for that substrate. In other words, K_{iO2}, which represents the equilibrium constant between apple PPO and oxygen, is independent of the phenolic compound at 0.29 mM. This value is close to the oxygen concentration of air-saturated aqueous solution at 30 °C (Wise and Naylor, 1985). Therefore,

Table I. Kinetic Parameters of Apple PPO for Several Phenolic Substrates at Varied Oxygen Concentrations and at pH 4.5

substrate	V_m, % found for 4-methylcatechol	K_{mPh}, mM	K_{mO2}, mM	K_{iO2}, mM
4-methylcatechol	100	7.1	0.54	0.28
chlorogenic acid	63	4.2	0.28	0.29
(+)-catechin	22	4.3	0.08	0.29

Table II. Apparent V_m and K_m of Apple PPO for Four Phenolic Substrates in Air-Saturated Solutions at 30 °C and pH 4.5

substrate	V_m, % found for 4-methylcatechol	K_m, mM
4-methylcatechol	100	5.2
chlorogenic acid	93	4.2
(+)-catechin	54	6.2
caffeic acid	7.5	0.14

during routine activity assay, PPO is approximately half-saturated by oxygen. Lastly, K_{mO2} values, which represent the ratio of the rates of quinone formation to the $E–O_2$ complex dissociation, are very different from one phenol to another. Thus, compared to the rate of $E–O_2$ complex formation, the rate of quinone formation is twice slower with chlorogenic acid and 7 times slower for (+)-catechin than with 4-methylcatechol.

Kinetic data concerning PPO in the literature are scarce and somewhat contradictory. Thus, the PPO mechanism was considered a Ping-Pong mechanism (Gregory and Bendall, 1966) or a sequential one with a random or a compulsory order of binding (Duckworth and Coleman, 1970; Gutteridge and Robb, 1975; Lerner and Mayer, 1976). Moreover, the latter authors considered that if it is an ordered mechanism, oxygen does not bind first, whereas Ingraham (1957) and Matheis and Belitz (1977) indicated that oxygen is the first substrate. Many reasons can be raised to explain such discrepancies. First, in numerous enzymatic extracts such as those from fungi (Duckworth and Coleman, 1970; Gutteridge and Robb, 1975), grape (Lerner and Mayer, 1976), and potato (Matheis and Belitz, 1977) both mono- and diphenolase activities are present. In this case, the mechanism study is further complicated since *o*-diphenol has to be considered both a product of and a substrate for the enzyme. Second, some authors (Duckworth and Coleman, 1970) have used a spectrophotometric assay for the measurement of PPO activity, and the part played by secondary products in the absorption, which is very difficult to determine, was not taken in account. Third, progress curves of change in oxygen concentration obtained during polarography assay have been used by different authors to determine the instantaneous velocity at different oxygen concentrations (Ingraham, 1957; Lerner and Mayer, 1976; Matheis and Belitz, 1977). This last method can lead to erroneous results since the initial velocity was not measured for each oxygen concentration. It is well-known, on one hand, that secondary products can play an important role in the oxygen consumption by a nonenzymatic process and, on the other hand, that PPO undergoes inactivation during its catalysis (Golan-Goldhirsch and Whitaker, 1985).

PPO is an enzyme able to act on a wide variety of phenolic compounds with large differences among their kinetic parameters (Vamos-Vigyazo, 1981). In this respect, we have determined the values of apparent V_m and K_m at pH 4.5 and 30 °C for four phenolic compounds (Table II). Among 4-methylcatechol, chlorogenic acid, (+)-catechin, and caffeic acid, the first one is the best substrate for apple PPO, the two following are the most abundant

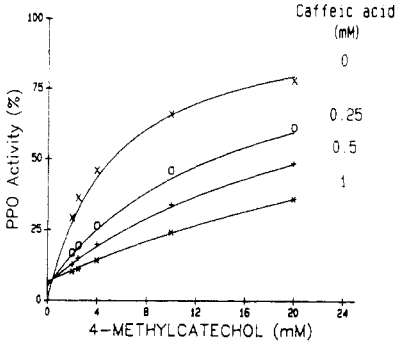

Figure 4. Effect of caffeic acid addition on initial oxygen uptake at pH 4.5 and 30 °C by apple PPO at different concentrations of 4-methylcatechol. (×) 0, (○) 0.25, (+) 0.5, and (*) 1 mM in caffeic acid. Full lines are curves calculated by using eq A with $V_{m1} = 100$, $K_{m1} = 5.2$ mM, $V_{m2} = 7.5$, and $K_{m2} = 0.14$ mM (Table II). Results are expressed in percent of V_m with respect to 4-methylcatechol.

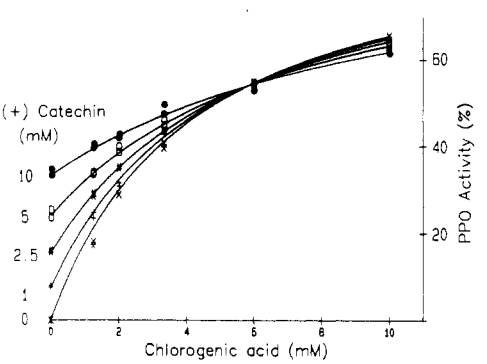

Figure 5. Effect of (+)-catechin addition on initial oxygen uptake at pH 4.5 and 30 °C by apple PPO at different concentrations of chlorogenic acid. (×) 0, (+) 1, (*) 2.5, (○) 5, and (●) 10 mM in (+)-catechin. Full lines are curves calculated by using eq A with $V_{m1} = 93$, $K_{m1} = 4.2$ mM, $V_{m2} = 54$, and $K_{m2} = 6.2$ mM (Table II). Results are expressed in percent of V_m with respect to 4-methylcatechol.

natural substrates in apple fruit, and the last one has both very low apparent V_m and K_m compared to those obtained for the three other substrates. To our knowledge, kinetic studies of the PPO activity on model solutions of two phenolic compounds have never been carried out. The results obtained during incubation of 4-methylcatechol between 2 and 20 mM with apple PPO in the presence of caffeic acid between 0 and 1 mM are illustrated on Figure 4. Whatever the 4-methylcatechol concentration used, increasing amounts of caffeic acid decrease the oxygen uptake. In this respect, caffeic acid can be considered an inhibitor of the 4-methylcatechol oxidation by apple PPO. A too rapid analysis after Lineweaver–Burk transformation (not shown) leads to the conclusion that it acts as a competitive inhibitor. However, due to the very low values of both apparent K_m and V_m for caffeic acid, one can assume that when this compound is present, a great part of apple PPO is in the form of a caffeoyl–PPO complex which is very slowly transformed in quinone, leaving a small part of enzyme available for the 4-methylcatechol oxidation.

This can be calculated by assuming that enzyme acts independently on both phenolic substrates S_1 and S_2, on one hand, and that the amount of oxygen consumed per mole of oxidized phenol is the same for S_1 and S_2, on the other hand. Then

$$E + S_1 \rightleftharpoons ES_1 \xrightarrow{k_{p1}} E + P_1 \quad \text{with } K_{m1} = [E][S_1]/[ES_1]$$

$$E + S_2 \rightleftharpoons ES_2 \xrightarrow{k_{p2}} E + P_2 \quad \text{with } K_{m2} = [E][S_2]/[ES_2]$$

Total oxygen uptake v_t is

$$v_t = v_1 + v_2 = k_{p1}[ES_1] + k_{p2}[ES_2]$$

If both sides of the equation are divided by E_t (the sum of all enzyme species), the results are

$$\frac{v_t}{E_t} = \frac{k_{p1}[ES_1] + k_{p2}[ES_2]}{[E] + [ES_1] + [ES_2]}$$

$$\frac{v_t}{E_t} = \frac{(k_{p1}[ES_1]/[E]) + (k_{p2}[ES_2]/[E])}{1 + ([ES_1]/[E]) + ([ES_2]/[E])}$$

$$v_t = E_t \frac{(k_{p1}S_1/K_{m1}) + (k_{p2}S_2/K_{m2})}{1 + (S_1/K_{m1}) + (S_2/K_{m2})}$$

Since $V_{m1} = k_{p1}E_t$ and $V_{m2} = k_{p2}E_t$

$$v_t = \frac{(V_{m1}S_1/K_{m1}) + (V_{m2}S_2/K_{m2})}{1 + (S_1/K_{m1}) + (S_2/K_{m2})} \qquad \text{(A)}$$

One can see in Figure 4 that experimental values were in good agreement with the curves calculated by using eq A and the kinetic parameters determined for each phenol (Table II).

The same study was carried out with two other phenols, chlorogenic acid and (+)-catechin, the apparent K_m and V_m of which were not very different compared to those of the previous experiment (Table II). Both experimental values and calculated curves of oxygen uptake are given in Figure 5, with again an excellent agreement between them. In other words, all curves intersect in one point close to 6 mM in chlorogenic acid. For this value, oxygen uptake is independent of the amount of (+)-catechin present in the solution. Coordinates S_{1c} and v_c of this point can be calculated since it corresponds to the S_1 value for which the derivative of v_t with respect to S_2 is zero. It comes $S_{1c} = K_{m1}V_{m2}/(V_{m1} - V_{m2})$ and $v_c = V_{m2}$. For chlorogenic acid and (+)-catechin, the theoretical value is $S_{1c} = 5.8$ mM. This particular point was also present for 4-methylcatechol with caffeic acid, but the S_{1c} value is very low due to the low value of V_{m2} and therefore is far below the experimental S_1 values that we have used for this experiment.

In conclusion, it seems that eq A allows the calculation of initial oxygen uptake by PPO in the presence of two phenolic substrates. However, when the enzymatic reaction was followed by spectrophotometry, the kinetics obtained did not agree with eq A. Different reasons can explain this fact. First, the absorption coefficients of the different quinones are unknown and a fortiori their respective contributions to the final absorption. Second, the quinones and peculiarly those from catechin were unstable and very rapidly underwent nonenzymatic polymerization (Mathew and Parpia, 1971). Lastly, some quinones enzymatically produced are likely to react nonenzymatically with phenols (Monties, 1967; Singleton, 1987; Cheynier et al., 1988). According to Cheynier et al. (1988), such coupled oxidations can be very rapid and are dependent of the respective redox potential of the different quinone/phenol couples present. Nevertheless, eq A can be extended to a more complex solution containing more than two phenolic compounds without any special difficulty. It allows the prediction of initial oxygen uptake provided the relative concentrations and the kinetic parameters of each individual phenol present in the solution are known. It can be used in the study of the oxidability of phenolic compounds in model solutions and therefore could be of interest in the understanding of enzymatic browning extent in relation to the phenolic content of fruits and vegetables.

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LITERATURE CITED

Cheynier, V.; Van Hulst, M. W. J. Oxidation of trans-caftaric acid and 2-S-Glutathionylcaftaric acid in model solutions. *J. Agric. Food Chem.* **1988**, *36*, 10–15.

Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.* **1988**, *53*, 1729–1732.

Cleland, W. W. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. 1. Nomenclature and rate equations. *Biochim. Biophys. Acta* **1963**, *67*, 104–137.

Duckworth, H. W.; Coleman, J. E. Physicochemical and kinetic properties of mushroom tyrosinase. *J. Biol. Chem.* **1970**, *245*, 1613–1625.

Golan-Goldhirsch, A.; Whitaker, J. R. Kcat inactivation of mushroom polyphenol oxidase. *J. Mol. Catal.* **1985**, *32*, 141–147.

Golbeck, J. H.; Cammarata, K. V. Spinach thylakoid polyphenol oxidase. Isolation, activation and properties of the native chloroplast enzyme. *Plant Physiol.* **1981**, *67*, 977–984.

Gregory, R. P. F.; Bendall, D. S. The purification and some properties of the polyphenol oxidase from tea (*Camellia sinensis* L.). *Biochem. J.* **1966**, *101*, 569–581.

Gutteridge, S.; Robb, D. The catecholase activity of *Neurospora* tyrosinase. *Eur. J. Biochem.* **1975**, *54*, 107–116.

Ingraham, L. L. Variation of the Michaelis constant in polyphenol oxidase catalyzed oxidations: substrate structure and concentration. *J. Am. Chem. Soc.* **1957**, *79*, 666–669.

Janovitz-Klapp, A.; Richard, F.; Nicolas, J. Polyphenoloxidase from apple. Partial purification and some properties. *Phytochemistry* **1989**, *28*, 2903–2907.

Janovitz-Klapp, A.; Richard, F.; Goupy, P.; Nicolas, J. Inhibition studies on apple polyphenoloxidase. *J. Agric. Food Chem.* **1990**, *38*, 926–931.

Leatherbarrow, R. J. In *Enzfitter, a non linear Regression Data Analysis Program for the IBM PC*; Elsevier: Amsterdam, 1987.

Lerch, K.; Ettlinger, L. Purification and characterization of a tyrosinase from *Streptomyces glaucescens*. *Eur. J. Biochem.* **1972**, *31*, 427–437.

Lerner, H. R.; Mayer, A. M. Reaction mechanism of grape catechol oxidase. A kinetic study. *Phytochemistry* **1976**, *15*, 57–60.

Matheis, G.; Belitz, H. D. Studies on browning of potatoes. 3. Kinetic properties of potato phenoloxidase. *Z. Lebensm. Unters. Forsch.* **1976**, *163*, 191–195.

Mayer, A. M. Polyphenol oxidases in plants. Recent progress. *Phytochemistry* **1987**, *26*, 11–20.

Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* **1979**, *18*, 193–215.

Monties, B. Potentiel d'oxydoréduction des polyphénols. Caractérisation potentiométrique des constituants phénoliques du jus de pomme et des boissons. *Ann. Technol. Agric.* **1967**, *16*, 251–268.

Ponting, J. D. The control of enzymatic browning of fruits. In *Food enzymes*; Schultz, H. W., Ed.; Avi: New York, 1960.

Rivas, N. J.; Whitaker, J. R. Purification and some properties of two polyphenol oxidases from Bartlett pears. *Plant Physiol.* **1973**, *52*, 501–507.

Segel, I. H. In *Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme system*; Wiley-Interscience: New York, 1975.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines, and model systems: Observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69–77.

Vamos-Vigyazo, L. Polyphenoloxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49–127.

Whitaker, J. R. Mechanisms of oxidoreductases important in food component modification. In *Chemical Changes in Food during Processing*; Richardson, T., Finley, J. W., Eds.; Avi: New York, 1985.

Wise, R. R.; Naylor, A. W. Calibration and use of a Clark-type oxygen electrode from 5 to 45 °C. *Anal. Biochem.* **1985**, *146*, 260–264.

Yamaguchi, M.; Henderson, H. M.; Hwang, P. M.; Campbell, J. D. Effect of oxygen concentration on o-diphenol oxidase activity. *Anal. Biochem.* **1969**, *32*, 178–182.

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