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MONOPHENOLASE ACTIVITY OF AVOCADO POLYPHENOL OXIDASE*

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Key Word Index—*Persea americana*; Lauraceae; avocado fruit; polyphenoloxidase; monophenolase.

Abstract—Polyphenol oxidase of avocado mesocarp catalyses (a) the orthohydroxylation of monophenols like L-tyrosine, D-tyrosine, tyramine and *p*-cresol, and (b) the oxidation of the corresponding *o*-dihydroxyphenols to quinones. The rate of step b is much greater than that of step a. The hydroxylation of monophenols occurs after a lag period. DOPA or ascorbate effectively eliminate the lag but not DL-6-methyltetrahydropteridine or tetrahydrofolic acid. At 1.66×10^{-4} M, α,α-dipyridyl has no effect, while diethyldithiocarbamate at this concentration inhibits the hydroxylation reaction by 90%. The tyrosinase activity of avocado polyphenol oxidase is inactivated in the course of the reaction; this inactivation occurs faster and is more pronounced in the presence of exogenously added DOPA. This inactivation is partially prevented by a large excess of ascorbate. The K_m values indicate that tyramine, dopamine, *p*-cresol and 4-methyl catechol are better substrates for avocado polyphenol oxidase than tyrosine or DOPA.

INTRODUCTION

Polyphenol oxidases catalyse two distinct reactions:

(**A**) monophenol + O_2 + AH_2 → *o*-dihydroxyphenol + H_2O + A

(**B**) *o*-dihydroxyphenol + $\frac{1}{2}O_2$ → *o*-quinone + H_2O,

where AH_2 represents a reducing agent.

These reactions are catalysed by the monophenolase (cresolase, tyrosinase) and the dihydroxyphenolase activities of the PPO complex [1, 2]. Monophenolases of plant origin are characterized by broad substrate specificity compared with mammalian tyrosinase, which is relatively specific for tyrosine and DOPA [3]. PPOs‡ possessing both mono- and dihydroxyphenolase activities exist in a variety of plants [4–7], while other plants or different tissues of the same plant possess the latter activity and cannot act on monophenols [8–11].

PPO activity is conventionally assayed spectrophotometrically [10] or by measurement of oxygen uptake [2, 6], both methods being a reflection of the sum of reactions **A** and **B** outlined above. Kahn has previously reported that PPO of avocado, assayed by these methods, has dihydroxyphenolase but not monophenolase activity [12]. Monophenolase activity, in the absence of an appropriate hydrogen donor, is frequently characterized by a very long induction period [13–16]. The studies of Kahn with avocado PPO [12] were conducted under conditions where the reaction time used was too short to have overcome this expected induction period.

Pomerantz has developed a sensitive method for assaying tyrosinase activity [16]. The method allows the testing of potential hydrogen donors and consists of the determination of tritium released as water from L-[3,5-^{3}H]-tyrosine in the course of its hydroxylation (reaction **A**, outlined above). We have employed this assay in a search for monophenolase (tyrosinase) activity in avocado mesocarp, and in studies of inhibitors, reductants, and the causes of enzyme inactivation during the reaction.

RESULTS

Effect of enzyme concentration on time course of L-*tyrosine hydroxylation*

The rate of formation of ^{3}HOH from L-[3, 5-^{3}H]-tyrosine in the absence of an exogenous cofactor is demonstrated in Fig. 1. The hydroxylation is characterized by 3 consecutive phases: (a) an initial lag period (see inset graph), (b) a relatively constant rate of hydroxylation and (c) a slowing down of hydroxylation as the reaction ceases. The lag period was clearly demonstrated with small quantities of the enzyme and was found to be inversely proportional to enzyme concentration. The rate of hydroxylation during phase

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‡ Abbreviations used: PPO: polyphenol oxidase (*o*-diphenol: O_2 oxidoreductase, EC 1.10.3.1, also known as phenolase, phenoloxidase, catechol oxidase and tyrosinase); DOPA: 3,4-dihydroxyphenylalanine; dopamine: 3,4-dihydroxyphenyl ethylamine; DETC: diethyldithiocarbamate.

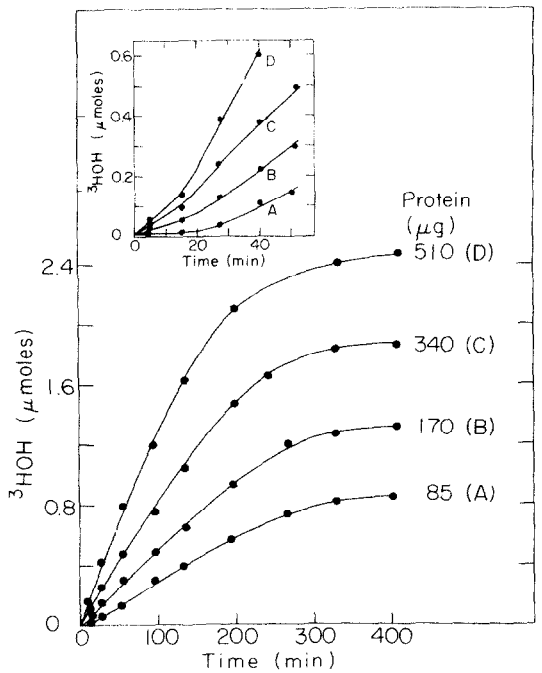

Fig. 1. The effect of enzyme concentration on the progress of tyrosine hydroxylation. The reaction mixture at 37° included, in a total volume of 5.3 ml: 9.75 μmol L-[3,5-^{3}H]-tyrosine (4.8×10^5 cpm/μmol), 195 μmol Na-Pi, pH 6.8, and various amounts of the partially purified PPO enzyme (85 to 510 μg protein).

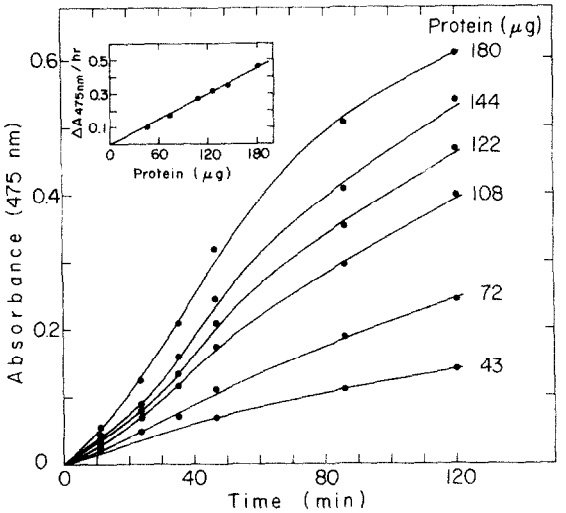

Fig. 2. The effect of enzyme concentration on the formation of dopachrome from L-tyrosine. The reaction mixture included, in a total volume of 4 ml: 5 μmol L-tyrosine, 100 μmol Na-Pi, pH 6.8, and various amounts of the partially purified PPO enzymes (43 to 180 μg protein). The reaction was measured at 37° using a Zeiss Spectrophotometer.

(b) is directly proportional to protein concentration up to at least 510 μg. We calculated that the partially purified avocado PPO hydroxylated 1.15 μmol tryosine per hr mg protein.

The kinetics of dopachrome formation from L-tyrosine determined spectrophotometrically (Fig. 2), is similar to that of L-tyrosine hydroxylation determined by the radioassay technique (Fig. 1).

As noted in Fig. 1, there is a slowing of the rate of tyrosine hydroxylation after *ca.* 200 min incubation. This phenomenon, often referred to as 'reaction-inactivation', was also observed when monohydroxyphenols like *p*-cresol and tyramine or when dihydroxyphenols like DOPA, dopamine and 4-methyl catechol were used as the substrate for K_m and V_{max} determinations (see Table 1). 'Reaction-inactivation' is known to occur with PPO of different origins [1, 17] and will be dealt with further below.

Inhibitors

DETC is an effective inhibitor of PPO but not of tyrosine hydroxylase, while the reverse is true for α,α-dipyridyl [18, 19]. The effect of these inhibitors on the hydroxylation of tyrosine by avocado PPO was tested in order to establish whether the hydroxylation reaction is carried out by a tyrosine hydroxylase or a tyrosinase. At 1.66×10^{-4} M, α,α-dipyridyl had no effect whereas DETC inhibited the reaction by 90%.

Effect of different reductants on progress of L-*tyrosine hydroxylation*

Exogenous reductants shorten the lag period of tyrosine hydroxylation by tyrosinase [13, 20]. Fig. 3 demonstrates that L-DOPA is an effective hydrogen donor for the hydroxylation of tyrosine by avocado PPO since a concentration of 2×10^{-5} M eliminates the lag period seen in its absence. The nature of inhibition exerted after 10–20 min by higher DOPA concentrations will be dealt with below.

Figure 4 demonstrates that ascorbate also shortens the lag in tyrosine hydroxylation. The effectiveness of ascorbate is proportional to its concentration in the range of 2.2 to 11.2×10^{-4} M (Fig. 4). At *ca.* 2×10^{-4} M, both ascorbate and L-DOPA eliminate the lag (Figs. 3 and 4). However, the inhibition seen after long incubation with elevated concentrations of L-DOPA (Fig. 3) was not observed with ascorbate (Fig. 4).

The addition of tetrahydrofolic acid or of DL-6-methyltetrahydropteridine (in the range of $1.8–3.6\times10^{-4}$ M and $0.43–8.6\times10^{-4}$ M, respectively) did not shorten the lag period seen in the absence of these reductants.

Reaction inactivation

The data in Fig. 1 show that in the absence of a hydrogen donor tyrosine hydroxylation slows after *ca* 200 min and then ceases, and the data in Fig. 3 demonstrate that high concentrations of L-DOPA slow the reaction earlier. Thus, the reaction slows after 40 min with 2×10^{-4} M L-DOPA and after 10 min with 9.5×10^{-4} M. After 180 min incubation, the extent of hydroxylation at 9.5×10^{-4} M L-DOPA was only 35% of that in the absence of L-DOPA (Fig. 3).

The possibility that the observed inhibition is a result of accumulation of DOPA oxidation products was tested in several experiments. The data in Fig. 5 show that tyrosine hydroxylation was inhibited in the case of an enzyme that had been exposed to elevated levels of DOPA oxidation products for 90 min (part **B**) compared with an enzyme exposed to low levels for the same period (part **A**). The possibility was further

Table 1. Comparison between specific activity and apparent K_m of mono- and dihydroxyphenols

Compound No.	Substrate	μmol quinone/hr/mg protein (V_{max})	Apparent K_m (mM)
1	L-Tyrosine	7.3	2.0
2	D-Tyrosine	7.9	2.0
3	L-DOPA	1977.0	5.2
4	D-DOPA	1511.2	6.0
5	Tyramine	12.0	0.55
6	Dopamine	4692.0	0.25
7	*p*-Cresol	91.0	0.65
8	4-Methyl catechol	2010.0	0.90

Assays were conducted in a total volume of 20 ml consisting of 0.1 M Na-Pi, pH 6.8, substrate, and the partially purified avocado PPO. Different substrate concentrations were used for apparent K_m determination. A single substrate concentration (where the enzyme exhibited maximum activity) was used for sp. act. determination. Samples were kept at 24° with continuous gentle shaking. The activities of compounds 1 to 4 were assayed at 480 nm, of compounds 5 and 6 at 465 nm and of compounds 7 and 8 at 410 nm. Absorbance values were converted to μmol quinones using appropriate E_m values [37]. For K_m determinations, the substrate concentration was plotted against the initial reaction velocity during the first minute in the case of dihydroxyphenols, and against the constant velocity seen after the lag period in the case of monohydroxyphenols. From a plot of the rate of reaction vs substrate concentration, the concentration at which the rate of the reaction was *ca* 50% of the estimated maximum rate (V_{max}) was taken to represent the apparent K_m.

tested by minimizing the formation of DOPA oxidation products by the addition of a large excess of ascorbate, since ascorbate rapidly reduced DOPA-quinone to DOPA non-enzymatically. The rate and extent of tyrosine hydroxylation were much greater in the presence of 90 μmol of ascorbate than in its absence (Fig. 6). However, even with the ascorbate the reaction slowed after *ca* 180–120 min of incubation, although under these conditions only 15% of the tyrosine was hydroxylated. The addition of 1 μmol

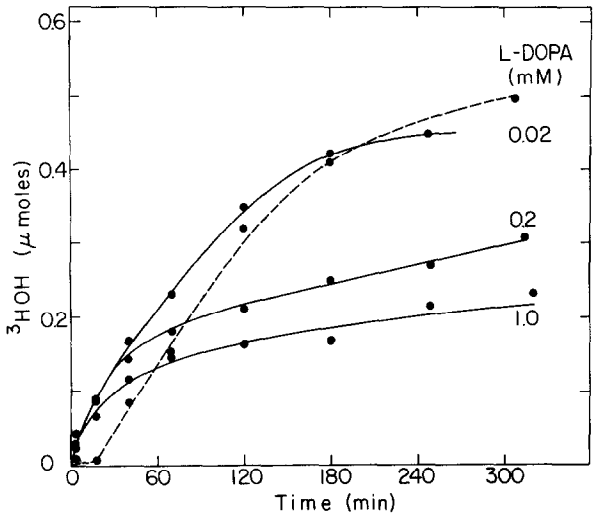

Fig. 3. L-DOPA as a reductant for tyrosine hydroxylation. The reaction mixture at 37° included, in a total volume of 5.3 ml: 6.5 μmol L-[3,5-^{3}H]-tyrosine (9×10^5 cpm/μmol), 200 μmol Na-Pi, pH 6.8, 0.35 ml of the partially purified avocado PPO (76 μg protein), and L-DOPA as indicated.

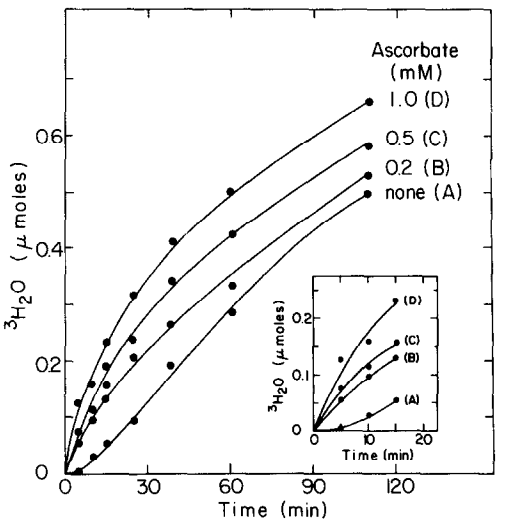

Fig. 4. Ascorbic acid as a reductant for tyrosine hydroxylation. The reaction mixture at 37° included, in a total volume of 4.5 ml: 6.5 μmol L-[3,5-^{3}H]-tyrosine (7.2×10^5 cpm/μmol), 180 μmol Na-Pi, pH 6.8, the partially purified avocado PPO (90 μg protein), and ascorbate as indicated.

L-DOPA alone eliminated the lag in tyrosine hydroxylation, but the initial rate of hydroxylation was much higher when DOPA and ascorbate were both added. Furthermore, the total amount of hydroxylation was higher than when L-DOPA was added alone. The data in Fig. 6 thus suggest that the elevated level of DOPA-quinone and other oxidation products derived from L-DOPA is responsible for the inhibition of

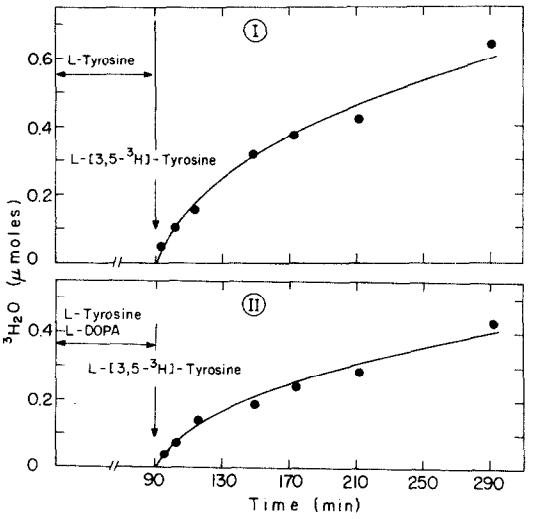

Fig. 5. The effect of preincubation of PPO with L-DOPA. The reaction mixture at 37° included, in a total volume of 3.8 ml: 6.5 μmol L-tyrosine, 200 μmol Na-Pi buffer, pH 6.8, and 1 ml of partially purified avocado PPO (180 μg protein) without (I) or with (II) 1 μmol L-DOPA. At the end of the 90-min incubation period, 0.2 ml of L-[3,5-^{3}H]-tyrosine was added to each flask (indicated by the arrow). (The final sp. act. of tyrosine was 12×10^5 cpm/μmol.)

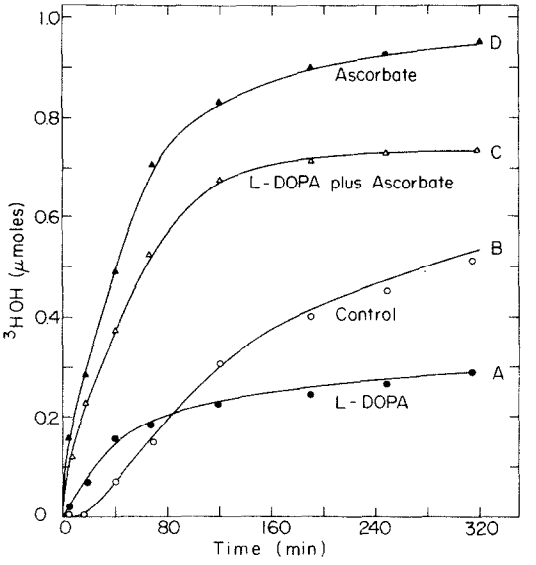

Fig. 6. The effect of ascorbate in minimizing inhibition caused by oxidation products of DOPA. The reaction mixture at 37° included, in a total volume of 5.3 ml: 6.5 μmol L-[3,5-^{3}H]-tyrosine (9×10^5 cpm/μmol), 200 μmol Na-Pi, pH 6.8, and 0.35 ml of the partially purified avocado PPO (76 μg protein). Where indicated, L-DOPA (1 μmol) and/or ascorbic acid (90 μmol) were included.

tyrosine hydroxylation. The fact that tyrosine hydroxylation in the presence of L-DOPA and ascorbate (Fig. 6,C) was lower than that seen with ascorbate alone (Fig. 6,D) indicates that ascorbate has a limited effect on the extent of relief of inhibition exerted by DOPA-quinone, in agreement with the findings of other investigators [1].

The ability of excess ascorbate to overcome part of the inhibitory effect of L-DOPA on tyrosine hydroxylation was seen also in another type of experiment, presented in Fig. 7. The enzyme was preincubated alone (A) (control) or in the presence of 5 μmol L-DOPA (B), 90 μmol ascorbate (C), or 5 μmol L-DOPA plus 90 μmol ascorbate (D). After 45 min of preincubation, the reaction mixtures were supplemented so that each contained 5 μmol L-DOPA, 90 μmol ascorbate and labelled tyrosine (designed by the arrow in Fig. 7). Samples were withdrawn at intervals to measure the ^{3}HOH released. At the end of the 45 min preincubation, reaction mixture B was dark red whereas A, C and D were colorless and remained so throughout the experiment, indicating that there was sufficient ascorbate in the incubation to reduce continuously the DOPA-quinone to DOPA. The data in Fig. 7 show that as a result of preincubating the enzyme with L-DOPA (B), the subsequent rate of tyrosine hydroxylation was appreciably inhibited over the control (A) despite the presence of ascorbate after the preincubation period. This again indicates that reaction of the enzyme with DOPA oxidation products is the likely cause of the subsequent inhibition of hydroxylation.

The data in Fig. 7 also show that preincubating the enzyme in the presence of L-DOPA and ascorbate together (D) partially relieved the inhibition exerted by preincubation with L-DOPA alone (B). However, the rate of tyrosine hydroxylation in a reaction preincubated with ascorbate alone (C) was somewhat higher than that seen with an enzyme that was preincubated with L-DOPA and ascorbate together. This was so despite the fact that, as noted above, reaction mixtures A, C and D were colorless, which indicates the presence of little or no quinones.

Substrate specificity

Since our present studies with L-tyrosine revealed that avocado PPO does possess tyrosinase-type activity, we tested the ability of avocado PPO to act on some other monophenols. It was found that avocado PPO acts on D-tyrosine, tyramine, and *p*-cresol (Table 1). The general patterns of quinone formation from these monophenols as a function of time, enzyme concentration and substrate concentration were similar to that seen when dopachrome was formed from L-tyrosine (Fig. 2).

K_m and specific activity (V_{max}) values for these monophenols and their corresponding dihydroxyphenols are shown in Table 1. As can be seen in Table 1, the V_{max} for DOPA and 4-methyl catechol is *ca* 200 to 250-fold, and for dopamine *ca* 40-fold, higher than for the corresponding monohydroxy phenols.

K_m values obtained for the D- and L-isomers of tyrosine were identical while those of D- and L-isomers of DOPA were nearly equal. However, the reaction rates are much higher when L- and D-DOPA, rather than L- or D-tyrosine, are substrates. This means that the hydroxylation of tyrosine, rather than the oxidation of DOPA, is rate-limiting.

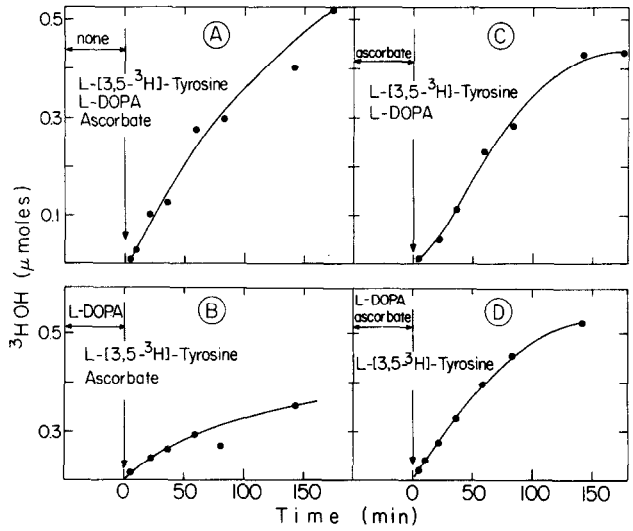

Fig. 7. The effect of preincubation of PPO with ascorbate and L-DOPA. Partially purified avocado PPO (1 ml) in 0.2 M Na-Pi, pH 6.8, (180 μg protein) was preincubated in a total volume of 3.2 ml that included the following additions: (A) none; (B) 5 μmol L-DOPA; (C) 90 μmol ascorbate; (D) 90 μmol ascorbate and 5 μmol L-DOPA. After 48 min preincubation, each reaction mixture was supplemented (where needed), so as to contain, in a total volume of 6.7 ml: 5 μmol L-DOPA, 90 μmol ascorbate and 6.5 μmol L-[3,5-^{3}H]-tyrosine (4.7×10^5 cpm/μmol). The experiment was conducted at 37°.

DISCUSSION

Two distinct enzymes hydroxylate tyrosine to DOPA: tyrosine-3-hydroxylase (L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (EC 1.14.3.0) and tyrosinase. They can be distinguished on the basis of substrate specificity, response to inhibitors, efficiency of different hydrogen donors, and the presence or absence of pigments formed as end products of the reaction [18, 19]. Based on these criteria, our findings prove that the hydroxylation of tyrosine by the partially purified avocado extract is due to PPO-tyrosinase and not to tyrosine hydroxylase.

The enzymatic oxidation of L-tyrosine [20, 21] and other monophenols [13, 15] is accompanied by a typical lag or induction phase before a constant reaction velocity is reached. Our data show that the kinetics of hydroxylation of tyrosine assayed by the rate of release of ^{3}HOH from L-[3,5-^{3}H]-tyrosine or by the rate of dopachrome formation from L-tyrosine was similar. Both reactions proceeded after a lag period. The higher the enzyme concentration, the shorter the lag observed (Figs. 1, 2) probably because of the increased concentration of endogenous reductants at a higher concentration of enzyme.

The lag period in phenol hydroxylation by monophenolase can be shortened by the addition of reducing agents which act as co-substrates [13–16, 21]. Our data show that both L-DOPA and ascorbate were effective in eliminating the lag, whereas neither DL-6-methyl tetrahydropteridine nor tetrahydrofolic acid were able to shorten the lag. Theoretically the hydroxylation of tyrosine should require the presence of a substrate amount of reducing agent. However, since DOPA is generated during the hydroxylation of tyrosine, even small amounts of endogenous or exogenous reducing agent apparently bring about the accumulation of enough DOPA to cause the reaction to be autocatalytic. Thus the rate of hydroxylation of tyrosine in the absence of an added reducing agent eventually reaches the rate attained after the addition of 0.1 or 1.0 μmol of DOPA.

Our data show that ascorbate enhances the rate of the monophenolase reaction better than L-DOPA (cf. Fig. 4 vs 3, cf. Fig. 6 vs 7). However, when DOPA and ascorbate are compared as to their ability to shorten the lag, DOPA at 2×10^{-5} M is equally effective to ascorbate at the higher concentration of 2×10^{-4} M. This is different from the situation reported by Neumann *et al.* [22], who showed that the lag period of potato tyrosinase was eliminated with pyrocatechol at 3.0×10^{-6} M while it still existed with a concentration of ascorbate 1000 times higher. The effectiveness of ascorbate as a cofactor in the hydroxylation of tyrosine by avocado PPO is probably due in part to its ability to reduce DOPA-quinone to DOPA non-enzymatically and thus increase the pool of DOPA in the reaction mixture.

Vaughan and Butt [13] have shown with phenolase of spinach leaves that the lag period of *p*-coumaric acid hydroxylation can be shortened with NADH, dimethyltetrahydropterine, protocatechuic acid, catechol, 4-methyl catechol or DOPA. The initial lag period in the hydroxylation of *p*-cresol and of *p*-coumaric acid by mushroom phenolase can be eliminated by the presence of the corresponding dihydroxyphenols 4-methyl catechol [14] and caffeic acid [15], respectively. Trace amounts of catechol decreased the lag phase of hydroquinone hydroxylation by mushroom phenolase [2].

L-DOPA was the best electron donor for hamster melanoma tyrosinase, while catecholamines, NADPH ascorbate (even at high concentrations) or 6,7-dimethyl-5,6,7,8-tetrahydropteridine substituted poorly for DOPA [2]. Ascorbate reduces the induction period of silkworm tyrosinase acting on *p*-cresol or on

tyrosine [23]. Similarly, high concentrations of ascorbate eliminated the induction period of DOPA formation with crude mushroom tyrosinase [24]. Mushroom phenolase [15] and spinach beet phenolase [13] react similarly to ascorbate during hydroxylation of *p*-coumaric acid to caffeic acid.

A gradual decrease in the rate of monophenol hydroxylation by phenolases from some sources is observed in the course of the reaction [1, 17, 25]. This reaction–inactivation might be the result of the action of the plant tyrosinase on tyrosyl residues within the enzyme to form DOPA residues and to the oxidation of DOPA residues to quinones [26].

Mason [27] and Pierpoint [28] enumerated different non-enzymatic reactions that may occur between *o*-quinones and amino and thiol groups present in proteins. From studies with ^{14}C-phenol and mushroom tyrosinase, Wood and Ingraham [29] obtained evidence showing the formation of a compound between the enzyme protein and the labelled product of phenol oxidation. These investigators proposed that reaction–inactivation is the result of the reaction of a nucleophilic group in the vicinity of the active site with *o*-quinone molecules [29]. The quinone complex formed is thought to be capable of breaking up to generate an active enzyme and quinone or to form an inactive enzyme–quinone adduct [1, 17].

The monophenolase of avocado PPO is probably inactivated to a large extent by the oxidation products formed in the reaction. This is supported by the fact that ascorbate can relieve part of the high inhibition on avocado PPO exerted by DOPA and the small inhibition observed in a reaction mixture consisting of tyrosine alone (Fig. 6). However, even a large excess of ascorbate could not eliminate the inhibition (Fig. 7), perhaps because ascorbate reacts only with free quinones and has no effect on the protein–quinone complex formed during the reaction [1], or because DOPA itself might cause inactivation.

Tyrosinase preparations from a variety of sources demonstrate activity not only towards a specific *o*-monophenol but also towards the corresponding *o*-dihydroxyphenol [4–6, 9, 10, 18, 20, 30]. Of the phenols tested in this work, dopamine, tyramine, *p*-cresol and 4-methyl catechol have the highest affinity for the enzyme; L- and D-tyrosine, intermediate; and L- and D-DOPA the lowest. L- and D-tyrosine have the same affinity towards avocado PPO (Table 1) as is the case for *V. tyrosinaticus* tyrosinase [31]. This is different from the situation with mammalian [32] and *Pseudomonas* [18] tyrosinases, which have, respectively, ten times and twice as high an affinity towards L-tyrosine compared with D-tyrosine. The K_m values of avocado PPO varied among the substrates tested by a factor of 30, whereas V_{max} values (specific activities) showed a 1000-fold range of variation (Table 1).

Golan *et al.* have recently shown that the browning potential of freshly cut mature ripe fruits of Fuerte avocado is much higher than that of Lerman [33]. The same authors showed later that the total and specific activities of PPO of Fuerte were higher than those of Lerman and that the level of *o*-dihydroxyphenols was the same in the fruits of the two cultivars [34]. In view of the present study, it will be interesting to determine the level of monophenols in the mesocarp of the two avocado cultivars, since they are potential substrates for the monophenolase activity of the avocado PPO complex and may account in part for the greater browning potential of Fuerte.

EXPERIMENTAL

Partially purified PPO (40–75% ammonium sulfate fraction) of commercially mature avocado (*Persea americana* Mill.) fruits (cr Fuerte) was prepared as described previously [35]. Protein was determined by the method of ref. [36]. Monophenolase activity was assayed by a radioassay method (a) or spectrophotometrically (b). Dihydroxyphenolase activity was assayed spectrophotometrically (b).

(a) *Radioassay.* The hydroxylation of tyrosine to DOPA was measured by determining the amout of ^{3}HOH released from L-[3,5-^{3}H]-tyrosine (New England Nuclear) by a slight modification of the procedure of ref. [16]. The reaction mixture contained L-[3,5-^{3}H]-tyrosine, Na-Pi buffer, pH 6.8, a hydrogen donor where appropriate, and partially purified avocado PPO in the total vol. and final concns indicated in the figure and table legends. Since the [^{3}H]-tyrosine is subject to radiation decomposition, which leads to the release of ^{3}HOH, the sample of labelled tyrosine was evaporated to dryness in each expt shortly before dilution with unlabelled tyrosine. The reaction was initiated by addition of enzyme and the mixture was gently shaken at 37 ± 1° in a 25 ml flask. At time zero, and at set intervals thereafter, 0.5–0.8 ml samples were withdrawn from the reaction mixture, added to tubes containing 0.5 ml 20% *meta*-phosphoric acid, and then passed through a column (2.5 × 8 cm) packed with mixed bed ion exchange resin (Crystalab Inc., Hartford, Conn). The column was washed with H_2O to make an effluent vol. of 30 ml, a vol. sufficient to recover essentially all the ^{3}HOH. A sample (0.5 ml) of the effluent was mixed with 5 ml of scintillation fluid and counted. In reactions without a hydrogen donor or with a poor one, the reaction was characterized by a lag period followed by a relatively constant rate of hydroxylation. Activity was estimated from the portion of the curve where the rate of ^{3}HOH released was constant with time. Activity is expressed in μmol of ^{3}HOH formed per unit time, since the amount of tyrosine hydroxylated is equivalent to the amount of ^{3}HOH formed [16]. The rates are corrected for reactions observed with no enzyme or boiled enzyme controls.

(b) *Spectrophotometric assay.* Unless otherwise specified, the standard reaction mixture consisted of 5–10 ml of 0.1 M Na-Pi buffer, pH 6.5, and 5–10 ml of freshly prepared 0.02-M mono- or dihydroxyphenol. The assay was initiated by the addition of the enzyme, and was conducted at either 24° or 37° with continuous shaking. The formation of phenol oxidation products was followed at the appropriate wavelength [37] using either a Zeiss Model PMQ II spectrophotometer equipped with an automatic recorder, or a Gilford Model #N-3000 spectrophotometer. Activity was estimated from the linear portions of curves. Where indicated, change in *A* (ΔA) values were converted to μmol substrate oxidized using molar extinction coefficients of the corresponding *o*-quinones [37].

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