Phytochemistry, Vol. 22, No. 10, pp. 2155–2159, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

MULTIPLE EFFECTS OF HYDROGEN PEROXIDE ON THE ACTIVITY OF AVOCADO POLYPHENOL OXIDASE*

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(*Revised received* 25 *April* 1983)

Key Word Index—*Persea americana*; Lauraceae; avocado fruit; polyphenol oxidase; hydrogen peroxide; multiple effect.

Abstract—Hydrogen peroxide (H_2O_2) affects polyphenol oxidase (PPO) of avocado mesocarp in several ways. In the absence of an exogenous hydrogen donor (AH_2), H_2O_2—at different concentrations (3.6–364 mM)—shortens the lag period of tyrosine hydroxylation and this effect was abolished when catalase was included in the reaction mixture. DOPA oxidation by avocado PPO is slightly increased by a relatively low concentration of H_2O_2 (3.3–30 mM), while higher concentrations of H_2O_2 decrease both the rate and the final level of dopachrome formed. Dopachrome and melanins were bleached in the presence of relatively high concentrations of H_2O_2 (50–500 mM). Preincubation of avocado PPO with H_2O_2 in the absence of a substrate resulted in the gradual loss of enzymatic activity, with the rate of loss of monophenolase activity being faster than that of *o*-dihydroxyphenolase activity. The possibility that relatively low *in situ* concentrations of H_2O_2 contribute indirectly to the low or high browning potential of avocado mesocarp is discussed.

INTRODUCTION

Browning in plant tissue is caused mainly by the oxidation of phenols present in the tissue by polyphenol oxidase (PPO) (EC 1.10.3.1) and their subsequent polymerization to dark-colored melanins. It was previously shown in our laboratory that mature fruits of two cultivars of avocado (Fuerte and Lerman) differ in their browning rates [1], their PPO activities [2], and their total phenol content [3]. It was found that the total and the specific activity of PPO [2] and the total phenol content [3] in these cultivars was directly correlated with their browning potential. However, changes in the browning potential of avocado fruit (Fuerte) during ripening of non-chilled and chilled fruits were not found to be directly correlated with PPO activity [4]. Rather, it was shown that the decrease in browning potential during ripening of Fuerte avocado as well as the decrease in fruit firmness paralleled more closely the decrease in the specific activity of catalase than of PPO [4].

Polyphenol oxidase of avocado, like that isolated from other sources, can carry out the following two different biochemical reactions:

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

(B) 2 *o*-dihydroxyphenol + O_2 → 2 *o*-quinone + H_2O

where AH_2 represents a reductant (co-substrate). Reactions A and B are catalysed by the monophenolase and the *o*-dihydroxyphenolase activities of PPO, respectively [5, 6].

In the absence of an exogenous reductant, the hydroxylation of monophenols (reaction A) is characterized by an initial lag period [7–11]. Certain reductants added exogenously can abolish the lag period [7–11]. *o*-Dihydroxyphenols are the most efficient reductants for the hydroxylation of monophenols by PPO [7]. The lag period can also be shortened by the addition of reductants that are not *o*-dihydroxyphenols [8–14]. For example, ascorbate, hydroquinone and ferrocyanide shortened the lag period of *p*-cresol hydroxylation by silkworm tyrosinase [12]. Sato [8] showed that ascorbate can serve as a reductant (AH_2) for the hydroxylation of *p*-coumaric acid to caffeic acid by mushroom tyrosinase. McIntyre and Vaughan [10] demonstrated that caffeic acid as well as ascorbate, NADH and dimethyltetrahydropteridine can each serve as a reductant during the hydroxylation of *p*-coumaric acid to caffeic acid carried out by spinach-beet phenolase. We have shown recently that exogenously added DOPA (3,4-dihydroxyphenylalanine) or ascorbate, but not DL-6-methyltetrahydropteridine or tetrahydrofolic acid, can effectively eliminate the lag period of tyrosine hydroxylation carried out by avocado PPO [15].

Hydrogen peroxide (H_2O_2), at relatively low concentrations, has also been reported to shorten the lag period of monohydroxyphenol hydroxylation by mushroom tyrosinase [13, 14], *Neurospora* tyrosinase [16] and spinach-beet PPO [11]. Various oxygen radicals are known to be formed in the living tissue under aerobic conditions [17]. These radicals are unstable and are easily converted to H_2O_2, which is a relatively stable reduced form of oxygen. The possible role of H_2O_2 in the ageing of plant tissue and in the ripening of fruits has received considerable attention in recent years [18, 19]. In view of the above, it was of interest to study the nature of the effect of H_2O_2 on avocado PPO.

RESULTS

Figure 1 demonstrates that different levels of H_2O_2

*Contribution No. 611-E, 1982 series from the Agricultural Research Organization.

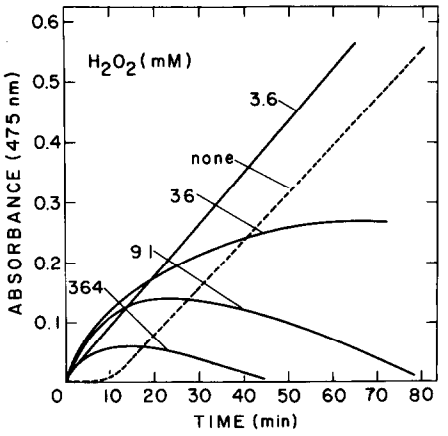

Fig. 1. The effect of different concentrations of H_2O_2 on avocado PPO activity using L-tyrosine as the substrate. The reaction mixture included, in a total volume of 11 ml, 2.3 mM L-tyrosine, 66 mM NaPi buffer (pH 6.5), 190 μg/ml avocado PPO (added last), and H_2O_2 as indicated.

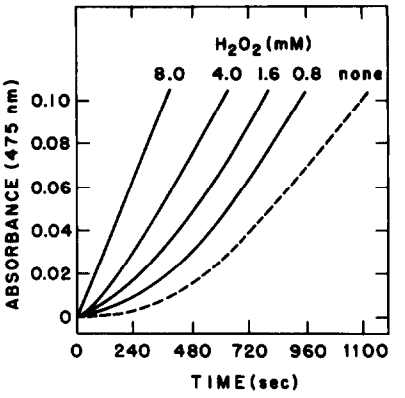

Fig. 2. The effect of H_2O_2 on the rate of tyrosine hydroxylation by avocado PPO. The reaction mixture included, in a total volume of 1.2 ml, 4.2 mM L-tyrosine, 33 mM NaPi buffer (pH 6.5), 160 μg/ml avocado PPO (added last), and H_2O_2 as indicated.

have an effect on (a) the lag period of tyrosine hydroxylation and (b) the rate of dopachrome formation (absorbance at 475 nm) by avocado PPO, as well as the stability of the melanin pigments thus formed. It can be seen that H_2O_2 in the range of 3.6–364 mM shortened the lag period of tyrosine hydroxylation by avocado PPO. Following the lag period, the kinetics of dopachrome formation was similar in the absence or presence of 3.6 mM H_2O_2. However, at higher H_2O_2 concentrations (36, 91, 364 mM), the dopachrome formation lasted for a limited time only; the higher the H_2O_2 concentration, the sooner was the slowdown in dopachrome formation. Prolonged incubation in the presence of relatively high concentrations of H_2O_2 (91, 364 mM) had an eventual "bleaching" effect (decrease in A_{475}) [20] on the dopachrome and melanin that had been formed earlier in the reaction (Fig. 1) (for further data, see Fig. 6 below).

Shortening of the lag period of tyrosine hydroxylation by avocado PPO in the presence of relatively low concentrations of H_2O_2 is described in Fig. 2 in more detail. The lag period in the absence of H_2O_2 was 360 sec, while in the presence of 0.8 mM H_2O_2 it was 240 sec. Increasing H_2O_2 concentrations shortened the lag period further, and at 8 mM H_2O_2 no lag period was detected at all. The rate of dopachrome formation seen, once the lag period was overcome, increased with increasing H_2O_2 levels (Fig. 2). Control experiments showed that in the absence of avocado PPO, incubation of tyrosine and NaPi buffer (pH 6.5) for 2 hr with low concentrations of H_2O_2 did not give any detectable increase in absorbance at 475 nm.

It is well established that dopachrome (peak at 475 nm) is the main pigmented intermediate formed in the course of conversion of tyrosine to melanin by tyrosinase [21]. The data in Fig. 3 show the changes in absorption spectra observed during incubation of tyrosine plus avocado PPO in the absence or presence of H_2O_2 (parts A and B, respectively). It is clear that added H_2O_2 enhances the peak at 475 nm, indicating that more dopachrome is being formed in the presence of H_2O_2 than in its absence. The close similarity of the spectra of part A and part B (Fig. 3),

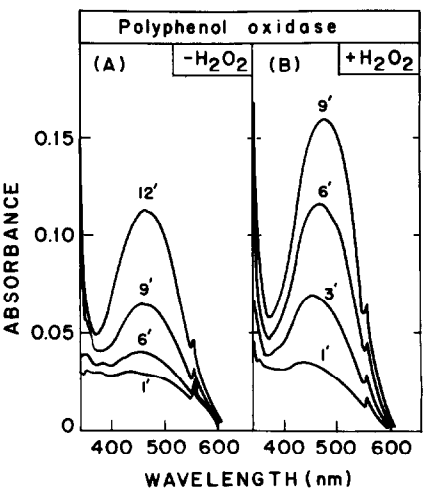

Fig. 3. Absorption spectra obtained by the action of avocado PPO on tyrosine in the absence and presence of H_2O_2 (parts A vs B). Reaction mixture A included, in a total volume of 3 ml, 5 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5) and 7 μg/ml avocado PPO (added last). Reaction mixture B was the same as that of A, except that it contained 6.6 mM H_2O_2. The absorption spectrum of each reaction was scanned in a Varian 635 spectrophotometer at the indicated times (min) after the addition of the enzyme (time zero).

establishes that the observed increase in absorbance at 475 nm presented earlier (Figs 1 and 2) is not due to an artifact resulting from a non-enzymatic interaction of H_2O_2 with dopachrome or related intermediates, but is the result of increased dopachrome formation by avocado PPO in the presence of H_2O_2 compared with in its absence.

The effect of H_2O_2 in shortening the lag of tyrosine hydroxylation by avocado PPO was counteracted when enough catalase was included in the reaction mixture. As

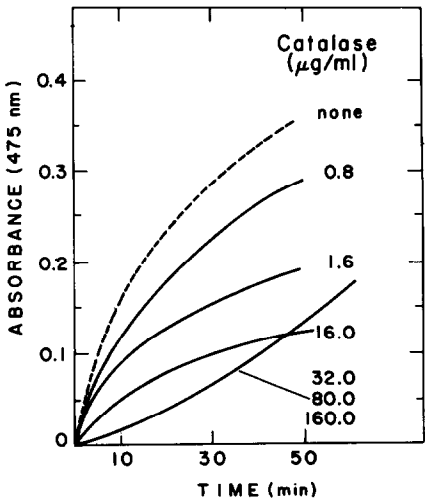

Fig. 4. The effect of H_2O_2 on the rate of tryrosine hydroxylation by avocado PPO in the absence and presence of catalase. The reaction mixture included, in a total volume of 12.5 ml, 4 mM L-tyrosine, 40 mM NaPi buffer (pH 6.5), 4 mM H_2O_2, 200 μg/ml avocado PPO (added last), and catalase as indicated.

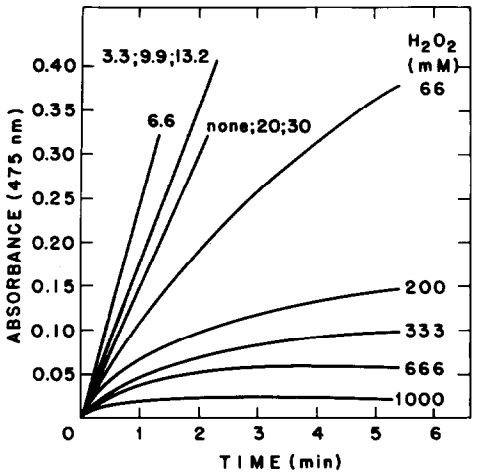

Fig. 5. The effect of high concentrations of H_2O_2 on *o*-dihydroxyphenolase activity of avocado PPO. The reaction mixture included, in a total volume of 3 ml, 6.7 mM DL-DOPA, 50 mM NaPi buffer (pH 6.5), 17 μg/ml avocado PPO (added last), and H_2O_2 as indicated.

seen in Fig. 4, the lag period was abolished in the presence of 4 mM H_2O_2. However, when catalase at a concentration of 32 μg/ml or higher was included, dopachrome formation was delayed appreciably, showing a lag period of 15 min. A catalase concentration of 16 μg/ml or lower had an effect only on the rate and total concentration of dopachrome formed, but apparently was not high enough to degrade immediately all the H_2O_2 present in the reaction. In view of this, it can be concluded that H_2O_2, at relatively low concentrations, shortens the lag period of tyrosine hydroxylation to DOPA by avocado PPO.

The mechanism by which H_2O_2 shortens the lag period of hydroxylation of monohydroxyphenols by tyrosinase is not yet understood [11, 13, 14, 16, 22, 23] but it has been suggested as being due to the ability of H_2O_2 to reduce *o*-quinones back to *o*-dihydroxyphenols [9] or to the ability of H_2O_2 to form oxytyrosinase [13, 14, 22, 23]. Oxytyrosinase is postulated by some investigators [13, 14, 22, 23] but questioned by others [16, 24] to be an active intermediate form of tyrosinase isolated from mushroom, *Neurospora* and plants.

The effect of H_2O_2 *on o-dihydroxyphenolase activity of avocado PPO*

The effect of various concentrations of H_2O_2 on the *o*-dihydroxyphenolase activity of avocado PPO was also tested. It was found that relatively low concentrations of H_2O_2 (2–13.2 mM) slightly increased the rate of DOPA conversion to dopachrome with a maximum of 20% increase in the presence of 6.6 mM H_2O_2 but only 10% increase in the presence of 3.3 or 13.2 mM H_2O_2. Intermediate and high concentrations of H_2O_2 (66–1000 mM) decreased the rate and the final level of dopachrome formation (Fig. 5).

Melanins are known to be attacked by excess H_2O_2 [20]. First, a clear brown solution is formed, which eventually becomes pale yellow ("bleached"). The "bleaching" of the dopachrome–melanin was formed during a 3 min incubation of avocado PPO with tyrosine yielding an A_{475} of 0.4. At that time (indicated by the arrow) aliquots were withdrawn and exposed to various concentrations of H_2O_2. It can be seen that the dopachrome–melanin pigments were destroyed (bleached) following a 3 min exposure to 50 mM H_2O_2 or immediately upon exposure to 500 mM H_2O_2 (Fig. 6).

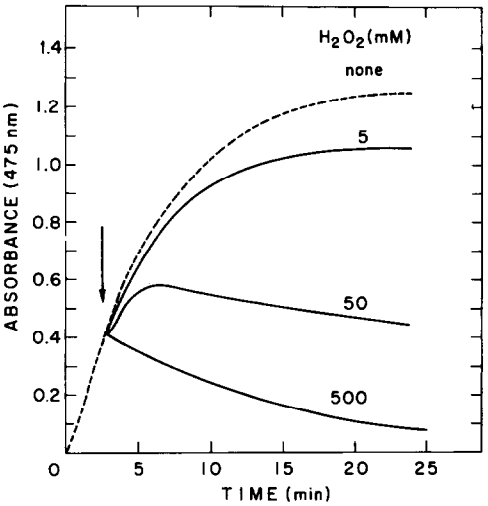

Fig. 6. The effect of H_2O_2 on dopachrome and related products formed by the oxidation of DOPA by avocado PPO. A large reaction mixture (185 ml) containing 6.7 mM DL-DOPA, 50 mM NaPi buffer (pH 6.5) and 135 μg/ml avocado PPO (added last) was preincubated for 3 min. Aliquots of this preincubated mixture were then transferred (at the time indicated by the arrow) to either control flasks (no H_2O_2) (– – –) or to flasks containing H_2O_2 (———) as indicated.

Inactivation of avocado PPO by relatively high concentrations of H_2O_2

Jolley *et al.* [13] and Guttridge and Robb [16] stated briefly that mushroom tyrosinase [13] and *Neurospora* tyrosinase [16] are inactivated by high concentrations of H_2O_2. However, the rate of inactivation of tyrosinase from either source by H_2O_2 was not reported by these groups.

The data in Fig. 7 illustrate that avocado PPO is inactivated by relatively high concentrations of H_2O_2. Thus, for example, 50% monohydroxyphenolase of avocado PPO was lost after 15 min preincubation in the presence of 1.7 mM H_2O_2, and 50% *o*-dihydroxyphenolase of avocado PPO was lost after 14 min preincubation in the presence of 8.5 mM H_2O_2 (Fig. 7, parts A and B, respectively). Comparison of parts A and B in Fig. 7, indicates that the ability of avocado PPO to hydroxylate tyrosine (part A) was lost faster than its ability to oxidize DOPA (part B) as a result of exposure of the enzyme to relatively high concentrations of H_2O_2. For example, after exposure to 8.5 mM of H_2O_2, 50% monohydroxyphenolase activity was lost within 6 min, while 50% *o*-dihydroxyphenolase activity was lost after 14 min.

The inactivation of an enzyme by high concentrations of H_2O_2 is often attributed to modification of aromatic amino acids at the active site of the particular enzyme under study [25–30]. Different mechanisms have been proposed to account for the way by which H_2O_2 causes such amino acid modification [28, 31]. Other possible explanations for H_2O_2 inactivation have been proposed. In the case of propyl-4-hydroxylase, inactivation by high concentrations of H_2O_2 was attributed to the dissociation of the active form of the enzyme into two dimers [32].

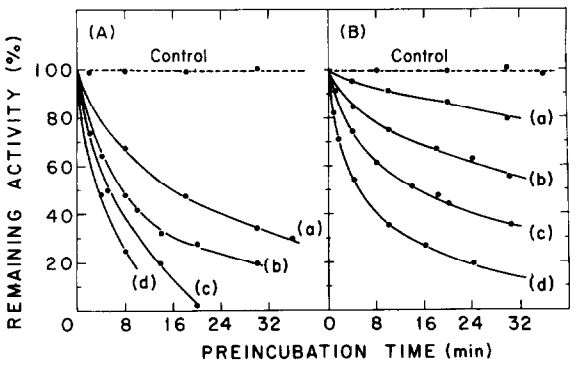

Fig. 7. Loss of monohydroxy- and *o*-dihydroxyphenolase activities of avocado PPO as a result of preincubating the enzyme with different concentrations of H_2O_2. In a total volume of 70 ml, avocado PPO (170 μg/ml) in the presence of 55 mM NaPi buffer (pH 6.5) was preincubated without (- - - -) (control) or in the presence of (———) H_2O_2. Concentrations (mM) as follows: a = 1.7; b = 3.4; c = 8.5; d = 17.0. At time zero, and at known times thereafter, 5-ml samples of each preincubated mixture were withdrawn, exhaustively dialysed against 0.05 M NaPi buffer (pH 6.5), and the activity on either L-tyrosine (part A) or on DL-DOPA (part B) was tested by adding the samples to flasks containing 5 ml of either 20 mM L-tyrosine in 80 mM NaPi buffer (pH 6.5) (part A) or 20 mM DL-DOPA in 80 mM NaPi buffer (pH 6.5). The final volume of each reaction mixture during the assay was 10 ml. Part A: Monohydroxyphenolase activity (ΔA_{475} hr^{-1}) was computed from the linear portion of each curve obtained after the lag period (data not shown). At time zero, activity of the control as well as of a, b, c and d was 0.19. Part B: *o*-Dihydroxyphenolase activities (ΔA_{475} min^{-1}) were computed from the linear rates of the curves during the first 2 min (data not shown). Activity at time zero was 0.24 for the control as well as for a, b, c and d.

DISCUSSION

Browning in most plant tissues is caused mainly by the enzyme polyphenol oxidase (PPO). PPO catalyses the hydroxylation of various monohydroxyphenols and the oxidation of various *o*-dihydroxyphenols present in the tissue to *o*-quinones. The *o*-quinones thus formed are subsequently polymerized (non-enzymatically) to dark-colored melanins. The activity of PPO and the total content of polyphenols are usually considered as the main factors contributing to the browning potential of the tissue. The data presented above indicate that H_2O_2 is an additional factor that may contribute to the potential of the tissue to darken. In the living tissue H_2O_2 is continually being produced enzymatically (by enzymes such as ascorbic acid oxidase, glucose oxidase, uricase, β-hydroxy acid oxidase, flavoprotein oxidases), by auto-oxidation of ascorbic acid and transition metals, by dismutation of superoxide anion ($O_2^{\cdot-}$) (spontaneously or by superoxide dismutase), or by interaction of $O_2^{\cdot-}$ with ascorbate, thiols or catechols [17 and references therein]. High concentrations of H_2O_2 are toxic to the cell and it is thought that reactions involving degradation of H_2O_2 developed in the cell to protect it against such potential toxicity [17]. Hydrogen peroxide can be decomposed enzymatically (by the action of catalase, peroxidase or glutathione peroxidase), by an interaction with transition metal ions (Fe^{2+}, Cu^{+}) (Fenton reaction) [33], or by an interaction with $O_2^{\cdot-}$ (Haber–Weiss reaction) [34].

Due to the above, H_2O_2 is usually maintained in the cell at a relatively low concentration. Determination of H_2O_2 by a very sensitive method based on the chemiluminescence of luminol with H_2O_2 [19] showed that potato tubers, germinating castor beans and tomatoes contain relatively low concentrations of H_2O_2 (*ca* 0.22, 0.14 and 0.1 mM H_2O_2, respectively). Ripening of fruits has recently been shown to be associated not only with an increase in the rate of respiration and ethylene production, but also with an increase in H_2O_2 [35].

It was previously shown in our laboratory [36] that PPO of avocado mesocarp exists in a supernatant and a particulate fraction (mostly in the microbodies) and, as the fruit ripens, more PPO is associated with the particulate fraction relative to the supernatant fraction. Using sucrose density gradients, we have seen differences in the densities of organelles isolated from unripe vs ripe avocado fruits (cv Fuerte) [36]. Platt-Aloia and Thomson [37], using scanning electron microscopy, detected definite breakdown in the structure of the cell wall but no changes in the ultrastructure of plastids and vacuoles during ripening of avocado (cv Hass). The ultrastructural changes of cell wall and of some subcellular organelles in the avocado fruit undoubtedly lead to subsequent decompartmentation of the enzymes, substrates and 'cofactors' [38, 39].

Dopamine, caffeic acid, epicatechin, catechin, chlorogenic acid, leucoanthocyanidins, *p*-coumaric acid, tyramine and serotonin are among the phenols known to be

present in avocado mesocarp [3, 40–42]. Additional phenols are present, but their identification is not certain [3, 40]. The low concentration of H_2O_2 in the cell, regulated by the various enzymatic and non-enzymatic reactions mentioned above, can have an important effect on the rate of hydroxylation of monohydroxyphenols and a small effect on the rate of oxidation of *o*-dihydroxyphenols present in avocado mesocarp. H_2O_2 can thereby contribute indirectly to the low or high browning potential of the tissue, particularly during ripening of the avocado fruits.

EXPERIMENTAL

Partially purified PPO (40–75% ammonium sulfate fraction) of commercially mature avocado (*Persea americana* Mill.) fruits (cv Fuerte) was prepared as described previously [43]. Monohydroxyphenolase and *o*-dihydroxyphenolase were assayed spectrophotometrically as described elsewhere [43]. H_2O_2 concn was determined spectrophotometrically at 240 nm using Em (240 nm) of 43.6 $M^{-1}cm^{-1}$. Protein was determined by the method of Lowry *et al.* [44].

Acknowledgement—This research was supported by grant no. I-223-80 from BARD, the United States–Israel Binational Agricultural Research and Development Fund.

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