Phytochemistry, Vol. 27, No. 6, pp. 1589–1592, 1988.
Printed in Great Britain.

0031-9422/88 $3.00+0.00
Pergamon Press plc.

EFFECTS OF SOME REDUCTANTS ON THE ACTIVITY OF CHERIMOYA POLYPHENOL OXIDASE

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(*Revised received* 30 *September* 1987)

Key Word Index—*Annona cherimolia*; Annonaceae; cherimoya; polyphenol oxidase; ascorbic acid; cysteine; mercaptoethanol; hydrogen peroxide.

Abstract—Ascorbate, cysteine, mercaptoethanol and hydrogen peroxide (H_2O_2) affect the polyphenol oxidase (PPO) activity of cherimoya epicarp in different ways. At relatively low concentrations, 0.1 mM to 0.2 mM, ascorbate, cysteine and mercaptoethanol decrease melanin formation from catechol without modifying the rates of the reaction whereas at higher concentrations, they decrease both the rates of the reaction and the final product levels. One to 10 μM cysteine or mercaptoethanol has no affect on the rate of hydroxylation of tyramine, but 20 μM cysteine or mercaptoethanol inhibit almost completely monophenol hydroxylation; these compounds increase the lag phase. 0.1–0.3 mM ascorbate reduces the lag period in the hydroxylation of tyramine and increases the rate of oxidation but higher levels of reductant produce a rapid drop in the rate of oxidation. One to 20 mM H_2O_2 lowers the final melanin levels from cathecol, but increases the concentrations of these products from DOPA and dopamine. High levels of H_2O_2 (40–320 mM) decrease the final amount of melanins formed from each substrate due to a bleaching effect. Pre-incubation of partially purified PPO in the presence of H_2O_2 in the absence of a substrate results in the total inactivation of the enzyme with the monophenol oxidase activity being lost at a faster rate than the *o*-diphenol oxidase activity.

INTRODUCTION

Enzymatic browning in fruits has been defined as an oxidative process catalysed by polyphenoloxidases (PPO) (EC 1.10.3.1) in which phenolic compounds are oxidized and subsequently polymerized giving dark-coloured melanins [1].

PPO from cherimoya epicarp shows two distinct catalytic activities (the oxidation of *o*-diphenols to *o*-quinones and the hydroxylation of monophenols), as has been observed for PPOs from other sources [2, 3]. In the absence of an exogenous reductant, the hydroxylation of monophenols is characterized by an initial lag period [4, 5]. Some reductants added exogenously can either reduce or abolish the lag period. *ortho*-Diphenols have been described as the most efficient reductants for the hydroxylation of monophenols by PPO [6] but there are many other reductants as effective as *o*-dihydroxyphenols, namely ascorbate, thiol derivatives and hydrogen peroxide [3, 7–9].

Vaughan and Butt [9] have shown that the lag period for hydroxylation of *p*-coumaric acid catalysed by PPO from spinach leaves is shortened in the presence of some diphenols, NADH and dimethyltetrahydropterine. Neumann *et al.* [7] have observed that the lag period for potato tyrosinase activity can be eliminated in the presence of 3 μM ascorbic acid and Kahn and Pomerantz [3] also showed the same effect for avocado PPO. However, some controversy exists with regard to the effect of ascorbate on PPO activity since there are reports that ascorbic acid inhibits the enzyme [10, 11].

Glutathione and dithiothreitol have been shown to decrease the initial rate in the oxidation of catechol catalysed by mushroom PPO [12].

Hydrogen peroxide (H_2O_2) formed in plant tissues from oxygen radicals may contribute to the browning of fruits. H_2O_2 at low concentrations in the absence of an exogenous hydrogen donor, has been reported to shorten the lag period of tyrosinase hydroxylation by mushroom tyrosinase [13, 14], *Neurospora* tyrosinase [15], spinach-beet PPO [5] and avocado mesocarp PPO [16]. These enzymes are inactivated by high concentrations of H_2O_2 [5, 13–16]. *ortho*-Diphenol oxidation by avocado PPO has been shown to increase at low concentrations of H_2O_2 (3.3–30 mM) and to decrease at high concentrations [15].

This study was designed to evaluate the influences of ascorbate, cysteine, mercaptoethanol and hydrogen peroxide on the monophenol hydroxylase and *o*-diphenol oxidase activities of PPO cherimoya epicarp.

RESULTS AND DISCUSSION

Effects of ascorbate, cysteine, and mercaptoethanol on monophenol hydroxylase and o-diphenol oxidase activities of PPO cherimoya epicarp

The effects of different concentrations of ascorbate on catechol oxidation by PPO cherimoya epicarp are shown in Table 1. Low concentrations of ascorbate scarcely modified the rate of catechol oxidation while higher concentrations decreased the rate of reaction. Figure 1 shows the effects of several concentrations of ascorbate

Table 1. Effects of different concentrations of ascorbic acid on the *o*-diphenol oxidase activity of PPO cherimoya epicarp

Ascorbic acid concentrations (mM)	Initial rates (ΔOD/min)	Lag period (min)
0.0	0.252	0.0
0.1	0.250	0.5
0.2	0.250	1.0
0.5	0.018	3.5
1.0	0.000	—

The reaction mixture included, in a total volume of 3 ml, 1.66 mM catechol, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (1 μg/ml) and ascorbic acid in the above concentrations.

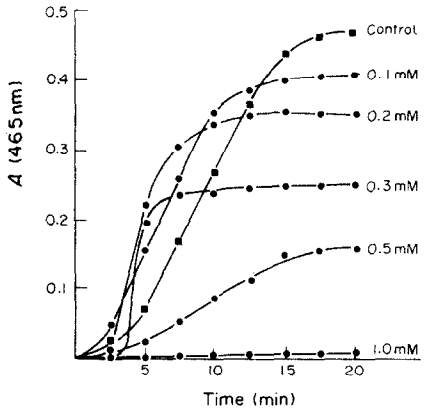

Fig. 1. Effects of different concentrations of ascorbic acid on the monophenol hydroxylase activity of PPO cherimoya epicarp. The reaction mixture included, in a total volume of 3 ml, 1.66 mM tyramine, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (11.7 μg/ml) and ascorbic acid in the above concentrations.

on the hydroxylation of tyramine catalysed by PPO cherimoya epicarp. 0.1–0.3 mM ascorbate shortened the lag period in tyramine hydroxylation from 5 min, in the control extract, to 2.5 min, in the extracts incubated with 0.1–0.3 mM ascorbate; higher concentrations decreased the rate of tyramine hydroxylation from 0.040 ΔOD/min, in the control extract, to 0.013 and 0.001 ΔOD/min when 0.5 or 1.0 mM ascorbate was used. When either catechol or tyramine was used as substrate, all concentrations of ascorbate assayed decreased the final amount of coloured compounds formed.

The effectivity of ascorbic acid as a cofactor in the hydroxylation of tyramine by PPO of cherimoya epicarp is probably due to not only its capacity to act as a reductant but also to its ability to reduce dopaminochrome, formed by the *o*-diphenol activity of the enzyme, to dopamine, thus increasing the pool of diphenols in the reaction.

Cysteine and mercaptoethanol showed a similar behaviour to ascorbate with regard to their effect on the reaction of catechol oxidation. Rates of tyramine hydroxylation catalysed by PPO cherimoya epicarp were unchanged below 10 μM cysteine (0.040 ΔOD/min), but 20 μM cysteine inhibited almost completely monophenol hydroxylation; on the other hand the lag period for this reaction was increased to 3, 6, 10 or 15 min respectively when 1, 2, 5 or 10 μM cysteine was used (Table 2). The effect of mercaptoethanol on monophenol hydroxylase activity of PPO cherimoya epicarp was similar to cysteine.

Thiol compounds appear to be inadequate hydrogen donors in the monophenol hydroxylation of monophenols by PPO cherimoya. The increase in the apparent lag period may be due to the reduction of *o*-quinones formed in the oxidative reaction from *o*-diphenols thus preventing the formation of coloured compounds. A condensation reaction between *o*-quinones and thiol compounds could be responsible for the increase in the lag period during monophenol hydroxylation and colour inhibition during *o*-diphenol oxidation catalysed by PPO cherimoya epicarp.

Inactivation of PPO cherimoya epicarp by hydrogen peroxide

Hydrogen peroxide has been reported to shorten the lag period in monophenol hydroxylation catalysed by some PPO enzymes [13–16], however this compound did not modify the lag period in the catalysis of tyramine by cherimoya PPO (Table 3). Relatively low concentrations of H_2O_2 increased the levels of the final coloured compounds formed from tyramine and DOPA, respectively, and decreased the levels of these melanins formed from catechol. Higher concentrations of H_2O_2 decreased the rate of tyramine hydroxylation and DOPA and catechol oxidation (Tables 3, 4 and Fig. 2). High levels of H_2O_2 probably attack the melanins formed during the reaction leading to a bleaching effect [17].

PPO from several plant sources are inactivated by high concentration of H_2O_2 [13, 15]. PPO from cherimoya epicarp was also inactivated when the enzyme was preincubated at different H_2O_2 concentrations. The rate of catechol oxidation in the control extract after two hr of

Table 2. Effects of different concentrations of cysteine on the monophenol hydroxylase activity of PPO cherimoya epicarp

Cysteine concentrations (mM)	Initial rates (ΔOD/min)	Lag period (min)
0	0.040	3
1	0.040	6
2	0.040	9
5	0.040	13
10	0.040	18
20	0.000	—

The reaction mixture included, in a total volume of 3 ml, 1.66 mM tyramine, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (11.7 μg/ml) and cysteine in the above concentrations.

Table 3. Effects of different concentrations of H_2O_2 on the monophenol hydroxylase activity of PPO cherimoya epicarp

H_2O_2 concentrations (mM)	Initial rates (ΔOD/min)	Lag period (min)
0.0	0.040	3
0.5	0.038	3
1.0	0.038	3
3.0	0.036	3
8.0	0.034	3
20.0	0.028	3
40.0	0.018	3
160.0	0.017	3
320.0	0.013	3

The reaction mixture included, in a total volume of 3 ml, 1.66 mM tyramine, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (11.7 μg/ml) and H_2O_2 in the above concentrations.

incubation without H_2O_2 was 0.128 ΔOD/min while in extracts incubated with 4.4, 35.2, 176 or 352 mM H_2O_2 the rates were 0.100, 0.062, 0.008 and 0.002 ΔOD/min (Table 5). Tyramine hydroxylation is also inhibited in the presence of H_2O_2 although the effect is more pronounced than in the case of catechol oxidation. Thus, in the control extract, the rate of reaction was 0.034 ΔOD/min while incubation with 4.4, 35.2, 176 or 352 mM H_2O_2 gave rates of 0.016, 0.006, 0.000 and 0.000 ΔOD/min, respectively.

The inactivation by H_2O_2 has been attributed either to a modification of active site aromatic amino acids [18, 19] or to the dissociation of active enzyme into two dimers [20]. This last hypothesis may be applied to cherimoya PPO since extraction of the enzyme using active surface agents causes its dissociation leading to a measurable loss of activity, especially for monophenol hydroxylase [21]. Cells continuously produce H_2O_2 as a

Table 4. Effects of different concentrations of H_2O_2 on the *o*-diphenol oxidase activity of PPO cherimoya epicarp

H_2O_2 concentrations (mM)	Initial rates (ΔOD/min)	Lag period (min)
0	0.264	0
1	0.252	0
8	0.240	0
20	0.232	0
40	0.230	0
80	0.228	0
160	0.192	0
320	0.180	0

The reaction mixture included, in a total volume of 3 ml, 1.66 mM DOPA, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (1 μg/ml) and H_2O_2 in the above concentrations.

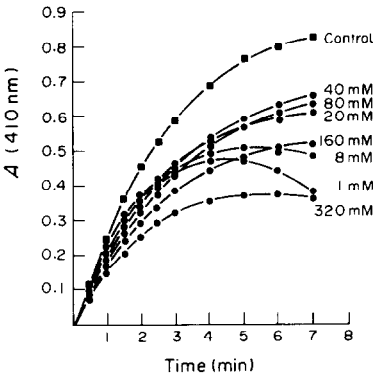

Fig. 2. Effects of different concentrations of H_2O_2 on the *o*-diphenol oxidase activity of PPO cherimoya epicarp. The reaction mixture included, in a total volume of 3 ml, 1.66 mM catechol, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (1 μg/ml) and H_2O_2 in the above concentrations.

Table 5. Effects of preincubation of PPO cherimoya epicarp with different concentrations of H_2O_2 on its *o*-diphenol oxidase activity

	Activity (% control)			
H_2O_2 (mM)	10 min	30 min	60 min	120 min
0.0	100	100	100	100
4.4	85	81	80	80
35.2	62	58	55	54
176.0	25	18	14	13
352.0	15	8	3	2

2.5 ml of enzyme extract (140 μg/ml) were incubated with 2.5 ml of H_2O_2 in 50 mM sodium phosphate buffer (pH 6.5). Samples were taken after 10, 30, 60 and 120 min of the incubation period and dialysed against 25 mM sodium phosphate buffer (pH 6.5) for 15 hr at 4°. *ortho*-Diphenol oxidase activity was determined as in Fig. 2, without including H_2O_2 in the assay medium, and was computed during the first two min of reaction as ΔOD_{410}/min.

consequence of oxygen free radicals formed in plant tissues [16]; thus H_2O_2 could have a regulatory effect on the oxidation of phenolic compounds or could have a role on the regulation of active PPO enzyme contributing towards the development or inhibition of browning products.

EXPERIMENTAL

PPO purification. Partially purified PPO of cherimoya epicarp was prepared using 20 g plant tissue and 200 ml cold Me_2CO (−20°). The tissue was homogenized in a Sorvall Omni-Mixer, filtered onto a filter paper and washed with 200 ml cold Me_2CO and 200 ml cold Et_2O. The residue obtained was dried and stored at 4°. 25 g Me_2CO- dried powder were resuspended in a 0.1% cysteine–0.1 M Pi buffer (pH 6.5), homogenized in a Sorvall Omni-Mixer for 5 min. at medium speed and centrifuged at 10000 *g* for 15 min. The supernatant was fractionated with $(NH_4)_2SO_4$ and the 40–75% fraction was resuspended into 9–15 ml of 50 mM Pi buffer (pH 6.5); this extract was dialysed for 24 hr at 4° against 25 mM Pi buffer (pH 6.5) and further fractionated through a Sephadex G-200, 3.8 × 100 cm glass column giving a peak with a high PPO activity which remained stable at 2–4° for at least 10 days. Partially purified PPO was 70 times more active compared to the crude extract.

Protein was determined by the method of ref. [22].

Monohydroxyphenolase and o-dihydroxyphenolase activities were assayed spectrophotometrically as described in ref. [23].

Acknowledgement—We would like to thank Dr González-Pacanowska for her assistance in the translation of the text.

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