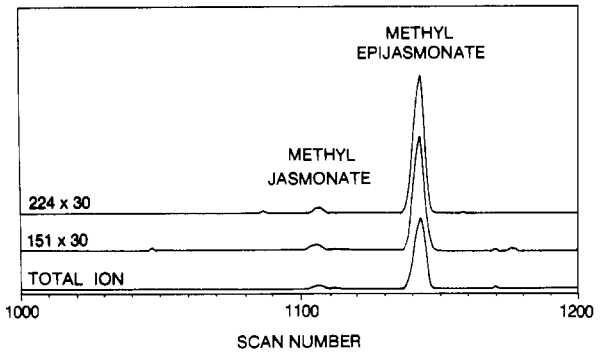

Figure 3. A mass fragmentogram of the methyl jasmonate epimers isolated from a lemon. The same gas chromatographic column described in Figure 2 was adapted to an Hitachi M-80 spectrometer and the 70-eV spectra were used to plot m/z 224 and 151.

thermodynamically less stable than the gauche arrangement of the methyl jasmonate epimer. However, methyl epijasmonate is the major form present in lemon peels and the only biologically active form present in the hairpencils of the oriental fruit moth (Baker et al., 1981; Nishida et al., 1982). Recent studies of the odor properties of synthetic methyl jasmonate stereoisomers (Acree et al., 1984) indicates that the (+)-epi isomer has the strongest odor for humans. Therefore, the original observation that an insect had a lemon-like odor is explained by the presence of methyl epijasmonate.

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Effect of Ascorbic Acid, Sodium Bisulfite, and Thiol Compounds on Mushroom Polyphenol Oxidase

Avi Golan-Goldhirsh and John R. Whitaker*

The effect of ascorbic acid, sodium bisulfite, glutathione (reduced), and dithiothreitol on the observed activity of mushroom polyphenol oxidase (PPO) was determined by spectrophotometry (color formation) and by polarography (O_2 uptake). By polarography, O_2 uptake began immediately on adding enzyme. Ascorbic acid and sodium bisulfite had little effect on the initial velocity while dithiothreitol and glutathione decreased the initial velocity by 35%, with no further decrease at higher concentrations. By spectrophotometry, there was an initial lag in the absorbance change followed by a slower increase in absorbance than for the control at zero time. By spectrophotometry, the I_{50} values were as follows: dithiothreitol, 0.06 mM; glutathione, 0.17 mM; sodium bisulfite, 0.20 mM; ascorbic acid, 0.24 mM. The direct effect of the reductants was determined by (1) incubation of the reductant with PPO and then measuring the remaining activity by polarography and (2) gel electrophoresis. At 0.1 mM, dithiothreitol caused a complete loss of activity after 70 min at 25 °C ($t_{1/2}$ = 8 min) while sodium bisulfite, glutathione, and ascorbic acid caused 50% inactivation after 28, 106, and 130 min, respectively, at 5 mM.

The effect of ascorbic acid, sodium bisulfite, and other reducing reagents on polyphenol oxidase (PPO) (EC 1.14.18.1) has been controversial over the years. The effects of ascorbic acid and sulfite have been most studied because of their extensive use in food processing. Early reports (Ingraham, 1956; Scharf and Dawson, 1958) indicated that ascorbic acid had no direct effect on the activity of PPO. More recently, Varoquaux and Sarris (1979) suggested that ascorbic acid neither inhibits nor activates the enzyme. However, several researchers (Baruah and Swain, 1953; Ponting, 1954; Mihály and Vámos-Vigyázó, 1976) reported inactivation of the enzyme by ascorbic acid. Duden and Siddiqui (1966) suggested a k_{cat} type of inactivation in the presence of ascorbic acid. Activation of PPO by ascorbic acid also has been reported (Krueger, 1950).

The effect of sulfite on PPO is also complex. It was shown to act as a reductant according to eq 1 (Ponting, 1960) and to react with quinones to form a colorless complex (LuValle, 1952; Schenck and Schmidt-Thomee, 1953). Direct inhibitory effect of sulfite on PPO was also shown (Embs and Markakis, 1965).

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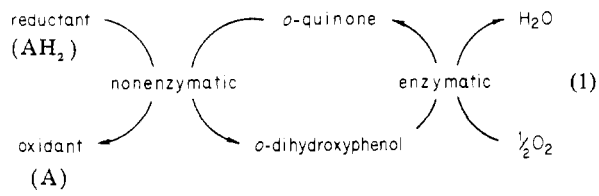

Most investigators have considered the effect of reducing agent on measured PPO activity to be due to a fast nonenzymatic reduction of the quinones formed enzymatically back to the o-dihydroxyphenolic substrates, according to eq 1 (Kubowitz, 1937, 1938; Sussman, 1961; Kertesz and Zito, 1962; Embs and Markakis, 1965). The observed time-related loss of PPO activity according to eq 1 (Kubowitz, 1937, 1938; Sussman, 1961; Kertesz and Zito, 1962; Embs and Markakis, 1965) has generally been associated with the effect of the product benzoquinone (or other product) on the active site of PPO (Wood and Ingraham, 1965; Dietler and Lerch, 1982), a k_{cat} type of inactivation.

On the basis of the mechanism of eq 1 and the known self inactivation of PPO (Asimov and Dawson, 1950; Vanneste and Zuberbühler, 1974; k_{cat} inactivation mechanism), we have recently suggested (Golan-Goldhirsh et al., 1984) that, in the presence of a substrate with fast k_{cat} inactivation and large enough excess of reductant, the enzymatic reaction would proceed with no color formation until the enzyme is completely and irreversibly inactivated. The reductant of first choice is ascorbic acid since it is permitted to be added to food products. During the course of our experiments on k_{cat} inactivation of mushroom PPO, it became clear that ascorbic acid decreases PPO activity on incubation with the enzyme in the absence of a polyphenolic substrate (Golan-Goldhirsh and Whitaker, 1984). Therefore, the direct effect of ascorbic acid and other reductants on PPO was further investigated.

We report here methodological considerations involved in studying the effect of reductants on mushroom PPO, as well as data on the kinetics and on the electrophoretic mobility of the enzyme as a result of treating PPO with reductants.

EXPERIMENTAL SECTION

Materials. Mushroom polyphenol oxidase (PPO; grade III, lot 91F-9650), bovine serum albumin (BSA), caffeic acid, dopamine, L-dopa (dihydroxyphenylalanine), DL-dopa, glutathione (reduced), and sodium bisulfite (as sodium metabisulfite, grade I) were from Sigma Chemical Co. L-Ascorbic acid and dithiothreitol were from Aldrich Chemical Co.; pyrocatechol was from Eastman Organic Chemicals. Human serum transferrin was from Behring Diagnostics, soybean trypsin inhibitor was from Nutritional Biochemical Corp., cytochrome c was from Boehringer, and ovalbumin was a gift (prepared by Dave Osuga of R. E. Feeney's laboratory). Other chemicals used for this work were of analytical grade. Deionized distilled water was used.

Methods. *Enzyme Assays.* A general assay is described. Specific modifications of the general assay are indicated when appropriate in the legends to the figures and tables.

The reaction, final volume of 1 or 3 mL, contained air-saturated 0.1 M sodium phosphate buffer, pH 6.5, substrate, and enzyme at 23 °C. Substrates were dissolved in 1×10^{-3} N H_2SO_4 to reduce autoxidation (Varoquaux and Sarris, 1979), and an appropriate aliquot was added to the reaction mixture. Reaction was started by addition of mushroom PPO (7 μg/mL of assay). The percent remaining activity was calculated as the activity of a reductant-treated sample compared to that of a reference sample treated identically but not including reductant.

The spectrophotometric assay was carried out on a Varian 635 spectrophotometer equipped with a thermostated cuvette holder and a 6051 recorder.

The polarographic assay was carried out on a YSI Model 53 biological oxygen monitor modified to permit the use of small reaction volumes (details are available upon request). Change in oxygen concentration was recorded continuously on a Honeywell recorder attached to the oxygraph.

Enzyme–Reductant Incubation. The enzyme and reductant were incubated together, in the absence of substrate, in a gyrotory water bath shaker, Model G76 (New Brunswick Scientific Co., Inc.), set at 25 °C.

The incubation mixture, final volume of 2.0 ml, contained air-saturated 0.108 M sodium phosphate buffer, pH 6.5, 0.96 mM disodium ethylenediaminetetraacetate (EDTA), 0.2 mg/mL mushroom PPO, and various concentrations of reductants. At time intervals, 50-μL aliquots were withdrawn and assayed polarographically in an assay containing 0.17 M sodium phosphate buffer, pH 6.5, and 2.4 mM DL-dopa in a final volume of 3.0 mL.

Polyacrylamide Gel Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE) were performed according to the "Hoefer Scientific Instrument Manual" (1980) with some modifications.

SDS–PAGE was carried out on 12 × 14 cm slab gels of 0.75-mm thickness [running gel, 12.5% acrylamide and 2.7% *N,N'*-methylenebis(acrylamide); stacking gel, 4% acrylamide and 2.7% *N,N'*-methylenebis(acrylamide); both gels contained 0.1% sodium dodecyl sulfate]. The electrode buffer contained 0.25 M Tris, 0.192 M glycine, and 0.1% SDS at pH 8.3. The sample buffer was 48.6 mM Tris, containing 1.5% SDS, 11.1% glycerol, 4 M urea, and 5% β-mercaptoethanol at pH 6.8. Samples dissolved in the sample buffer were heated in a boiling water bath for 5 min, cooled to room temperature, and applied to the gel. The electrophoretic run was carried out at room temperature (~23 °C) at 15 mA/slab gel. Staining was with 0.25% Coomassie Brilliant Blue R-250 in 45% methanol–9% acetic acid. The gel slab was destained first with 50% methanol–10% acetic acid for 0.5 h and then with 5% methanol–7% acetic acid.

Nondenaturing PAGE was carried out on 12 × 14 cm slab gels of 0.75-mm thickness [running gel, 10% acrylamide and 5% *N,N'*-methylenebis(acrylamide); stacking gel, 3.125% acrylamide and 20% *N,N'*-methylenebis(acrylamide)]. The electrophoretic run was at room temperature (~23 °C) at 15 mA/slab gel. Activity staining was done with DL-dopa as the substrate (Van Loon, 1971; Golan-Goldhirsh et al., 1984). Staining for protein was done as for SDS–PAGE, except that the gel was fixed for 0.5 h in 12.5% trichloroacetic acid before staining and was destained with 5% methanol–7% acetic acid.

RESULTS

Effect of Reductants on Observed PPO Activity in the Presence of Substrate. The effect of ascorbic acid, sodium bisulfite, glutathione (reduced), and dithiothreitol on the observed PPO activity was measured by two methods, a spectrophotometric determination of rate of increase in absorbance and a polarographic determination of uptake of O_2. The reactions were initiated by the addition of PPO to the temperature-equilibrated buffer–substrate–reductant-containing solution.

The two methods of analysis gave initial velocities that were proportional to enzyme concentration and had a

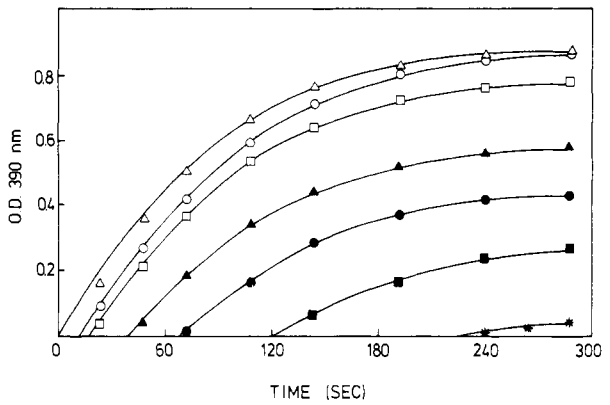

Figure 1. Spectrophotometrically observed reaction of mushroom PPO with pyrocatechol at 23 °C in the presence and absence of ascorbic acid. The 1-mL reaction mixture contained 0.1 M sodium phosphate buffer, pH 7.1, 5 mM pyrocatechol, 14 μg/mL mushroom PPO, and the following ascorbic acid concentrations (mM): (△) 0; (○) 0.1; (□) 0.2; (▲) 0.3; (●) 0.5; (■) 0.7; (*) 0.8.

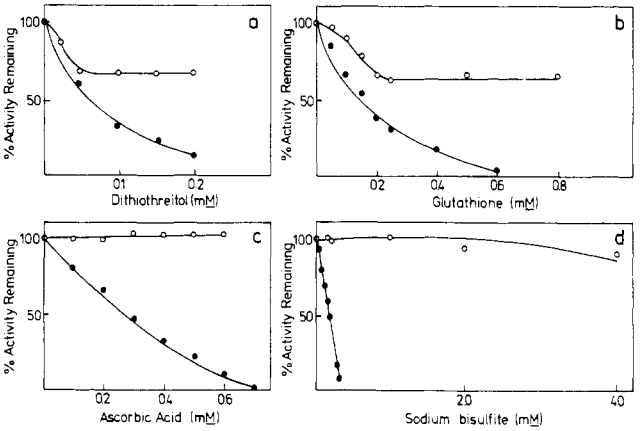

Figure 2. Effect of reductants concentration on mushroom PPO activity, measured spectrophotometrically (●) and by oxygraph (○) at 23 °C. The spectrophotometric and oxygraph assays contained the same concentration of reactants: 0.1 M sodium phosphate buffer, pH 6.5, 10 mM pyrocatechol, 7 μg/mL mushroom PPO, and the indicated concentrations of dithiothreitol (a), glutathione (reduced) (b), ascorbic acid (c), and sodium bisulfite (d). The final volumes of the spectrophotometric and oxygraph assays were 1.0 and 3.0 mL, respectively. The reactions were started by enzyme addition.

constant relative ratio of measured change with pyrocatechol as the substrate in the absence of added reductants. However, in the presence of reductants, the results were quite different. The polarographic technique showed an immediate increase in O_2 consumption on adding the enzyme, whereas there was a lag period before any change in absorbance was measurable. This difference permitted a separation between the effect of reductant on measurement of product formation and its direct effect on PPO.

As shown by the kinetic data of Figure 1, obtained in the presence of various concentrations of ascorbic acid, there was a lag period before any change in absorbance at 390 nm was measured. This lag period was longer the higher the ascorbic acid concentration. Also, the rate of increase in absorbance at 390 nm following the end of the lag period was decreased as the ascorbic acid concentration increased. In contrast, there was no effect of ascorbic acid on O_2 uptake measured polarographically (Figure 2).

When the initial rates of absorbance change at 390 nm following the lag period were plotted vs. the time of the lag period, extrapolation to zero lag period gave the same initial rate as in the absence of added reductant (Golan-

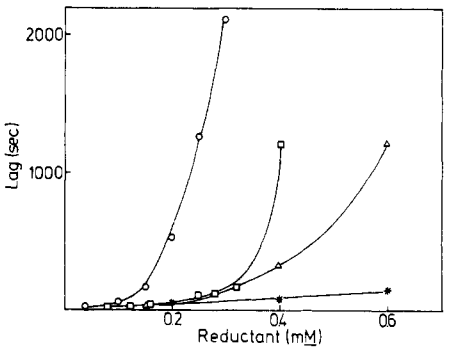

Figure 3. Effect of reductants concentration on the length of the lag period as measured spectrophotometrically at 23 °C. The lag period was estimated from the interception of the extrapolated linear portion of the progress curve with the base line, after absorbance at 390 nm started to increase in the reaction mixture (data not shown). The reaction conditions were as described in Figure 2. (○) Dithiothreitol; (□) sodium bisulfite; (△) glutathione (reduced); (*) ascorbic acid.

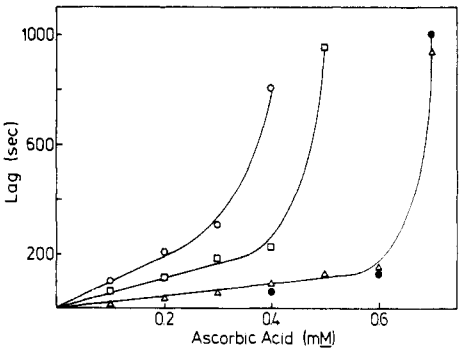

Figure 4. Effect of ascorbic acid concentration on the lag period in reaction of mushroom PPO with different substrates, measured spectrophotometrically at 23 °C. The reaction, in 3 mL, contained 0.08 M sodium phosphate buffer, pH 6.5, 0.9 mM EDTA, 7 μg/mL mushroom PPO, different concentrations of ascorbic acid (as shown), and substrate. The following substrates, their concentrations, and wavelength at which reaction was measured are (○) 2.4 mM L-dopa (475 nm), (□) 3.3 mM caffeic acid (420 nm), (●) 15 mM pyrocatechol (390 nm), and (△) 12 mM dopamine (470 nm). The lag period was determined as described in Figure 3.

Goldhirsh et al., 1984). Based on this analysis, it is reasonable to conclude that the rate of initial increase in absorbance following the end of the lag period reflects primarily the effect of reductant concentration on inactivation of PPO, while the length of the lag period, affected by reductant concentration, is the sum of effect of the reduction of the initial product, benzoquinone, back to pyrocatechol and the rate of inactivation of PPO.

The effect of concentration of ascorbic acid, sodium bisulfite, reduced glutathione, and dithiothreitol on the length of the lag period in a reaction mixture of mushroom PPO and pyrocatechol is shown in Figure 3. The effectiveness of the reductants in inhibiting onset of color (A_{390nm}) formation in the reaction mixture was in the order DTT > sodium bisulfite > glutathione (reduced) ≫ ascorbic acid. The relative effect of the reductants on the onset of color formation by mushroom PPO with other substrates (L-dopa, caffeic acid, and dopamine) was similar to that of pyrocatechol (data not shown).

The effect of ascorbic acid concentration on the lag period when DL-dopa, caffeic acid, pyrocatechol, and dopamine were used as substrates is shown in Figure 4. Table I shows the relationship between the lag period with the four substrates in the presence of 0.30 mM ascorbic acid (from Figure 4) and V_{max} and K_m for these substrates

Table I. Comparison of the Lag Period for Different Substrates in the Presence of 0.30 mM Ascorbic Acid with V_{max}, K_m, and v_0 in the Absence of Ascorbic Acid for PPO-Catalyzed Oxidation of These Substrates

substrate	lag period,[a] s	V_{max},[b] $\Delta\%O_2$ min^{-1} mg^{-1}	K_m,[b] mM	v_0, $\Delta\%O_2$ min^{-1} mg^{-1}
L-dopa	310	9.9	0.24	9.0
caffeic acid	180	10.5	0.80	8.4
dopamine	55	34.0	1.20	30.9
pyrocatechol	<55	62.6	1.49	56.9

[a] From the data of Figure 4. [b] Determined polarographically.

Table II. Effect of Polyphenol Oxidase Concentration on the Length of the Ascorbic Acid Caused Lag Period[a]

mushroom PPO, $\mu g/mL$ of assay	lag period, s
2.5	65
4.2	40
5.8	31
7.5	24

[a] Enzymatic activity was measured spectrophotometrically as described in the legend of Figure 4, except that several enzyme concentrations were used (as indicated) at fixed ascorbic acid (50 μM) and L-dopa (2.4 mM) concentrations.

in the absence of reductants. There appears to be a correlation between the lag period and V_{max} and K_m of the substrate. This relationship is best seen for v_0, calculated by using the Michaelis–Menten equation, the initial substrate concentration, V_{max}, and K_m. The higher the v_0, the faster ascorbic acid is oxidized, and the higher the concentration of ascorbic acid required to prevent onset of color (A_{390nm}) formation. As might be expected, the lag period is inversely proportional to the enzyme concentration (Table II) since more product is formed per unit time to react with the reductant (ascorbic acid in this case).

The data in Figure 2 compare the observed effects of reductant concentration as measured by the spectrophotometric method and by the polarographic method. The activity measured polarographically was the initial rate measured immediately on addition of PPO. The activity measured spectrophotometrically was the "initial rate" immediately following the end of the lag period (Figure 1).

Measured polarographically, ascorbic acid, up to 0.6 mM, had no effect on initial rate of O_2 uptake. Sodium bisulfite had a small effect (~10%) only at 4 mM. On the other hand, 0.1 mM dithiothreitol and 0.2 mM reduced glutathione decreased the initial rate by about 35% compared to the control. No additional effect was seen at higher concentrations of these thiol compounds. Whether this effect is due to reduction of a disulfide bond important for full enzymatic activity (Jolley et al., 1969; Golan-Goldhirsh et al., 1984) remains to be determined.

Quite different effects of concentration of the four reductants on the initial rate following the end of the lag period are seen when the activity is determined spectrophotometrically at 390 nm (Figure 2). All four reductants had a marked effect on the observed initial rate. The data indicate that all reductants would cause complete inactivation of PPO at sufficiently high concentrations. The I_{50} values (concentration of reductant required to reduce the observed activity by 50%) from the data of Figure 2 are as follows: dithiothreitol, 0.06 mM; glutathione (reduced), 0.17 mM; sodium bisulfite, 0.20 mM; ascorbic acid, 0.24 mM.

The difference between the results determined polarographically and spectrophotometrically, as reported in Figure 2, provides data on the inactivation of PPO.

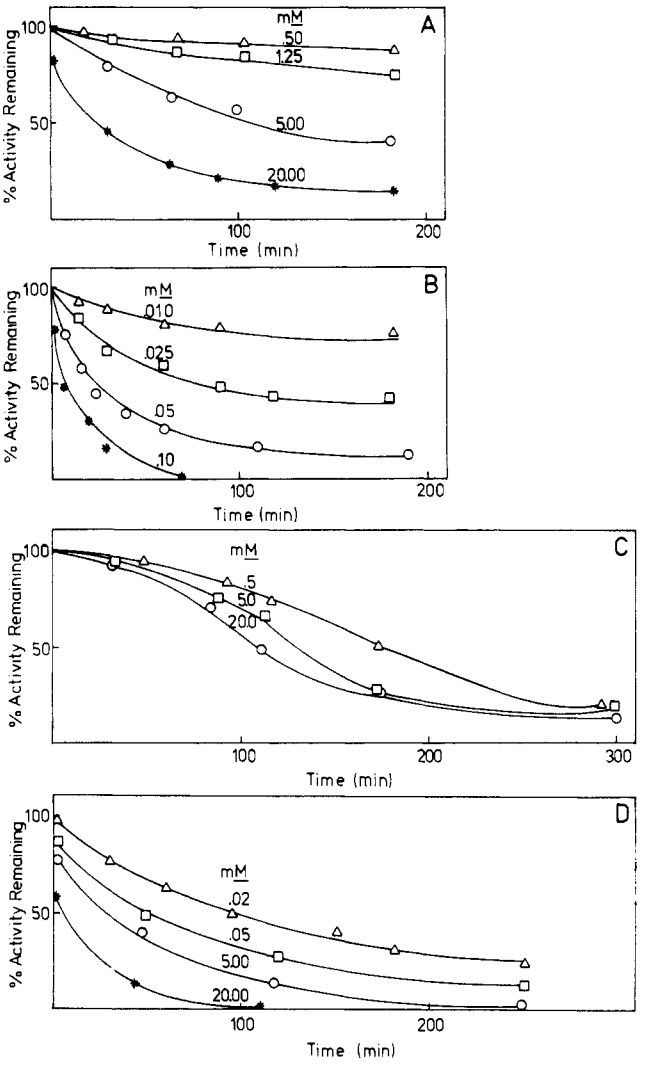

Figure 5. Rate of inactivation of mushroom PPO when incubated alone with reductants. Mushroom PPO was incubated with different concentrations of reductants (as indicated) in a 2-mL incubation mixture containing 0.108 M sodium phosphate buffer, pH 6.5, 0.96 mM EDTA, and 0.2 mg/mL mushroom PPO, in a shaking water bath at 25 °C. At the time intervals shown, a 50-μL aliquot was withdrawn and assayed by oxygen consumption (see Experimental Section) for the remaining activity. (A) Glutathione (reduced); (B) dithiothreitol; (C) ascorbic acid; (D) sodium bisulfite.

Whether the observed inactivation of PPO is due to the k_{cat} mechanism (inactivation of PPO by products of the reaction) or direct effect of the reductant will be addressed below.

Direct Effect of Reductants on PPO. The direct effect of reductants on inactivation of PPO was tested by incubating the enzyme, in the absence of substrate, with dithiothreitol, glutathione (reduced), ascorbic acid, and sodium bisulfite separately, and the activity remaining was assayed polarographically on aliquots withdrawn during the incubation.

All four reductants caused loss of enzymatic activity (Figure 5). With dithiothreitol, glutathione (reduced), and sodium bisulfite, the rate of inactivation decreased with time, following roughly a first-order rate process. With ascorbic acid there was an initial slow rate of inactivation followed by a faster rate of inactivation that decreased with time (Figure 5). This peculiar effect of ascorbic acid on mushroom PPO has been explored in more detail (Golan-Goldhirsh and Whitaker, 1984). The results suggest that ascorbic acid undergoes a change to a more reactive

Table III. Inactivation of Mushroom Polyphenol Oxidase with Reductants[a]

reductant	% activity remaining after treatment time of				
	1 min	6 h	10 h	23 h	after 24-h dialysis
none	100	92	96	86[b]	69
ascorbic acid	100	–[c]	–	1[b]	2
sodium bisulfite	90	7	1[b]	–	0
dithiothreitol	1[b]	–	–	–	35
glutathione (reduced)	112	37	19	10[b]	9

[a] Treatment with each reductant was done in 2 mL consisting of 0.14 M sodium phosphate buffer, pH 6.5, 0.5 mM EDTA, 5 mM of reductant, and 2 mg of mushroom PPO, except in the ascorbic acid treatment where 0.4 mg of enzyme was used. Incubation was in a shaking water bath at 25 °C. Remaining activity was measured polarographically as described under Experimental Section. [b] Remaining activity, at which time the reaction was dialyzed against distilled water at 4 °C. [c] Not measured.

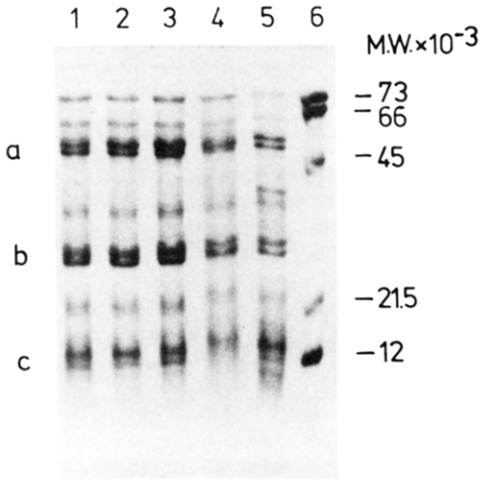

Figure 6. Electrophoretogram of SDS–polyacrylamide gel electrophoresis of reductant-treated mushroom PPO. Mushroom PPO was treated with different reductants as described in Table III. Treatments according to lanes: 1, sodium bisulfite; 2, glutathione; 3, dithiothreitol; 4, ascorbic acid; 5, control (no reductant); 6, standard proteins as shown (from top to bottom, human transferrin, bovine serum albumin, ovalbumin, soybean trypsin inhibitor, and cytochrome c). Approximately 130 μg of protein/lane was applied to the gel.

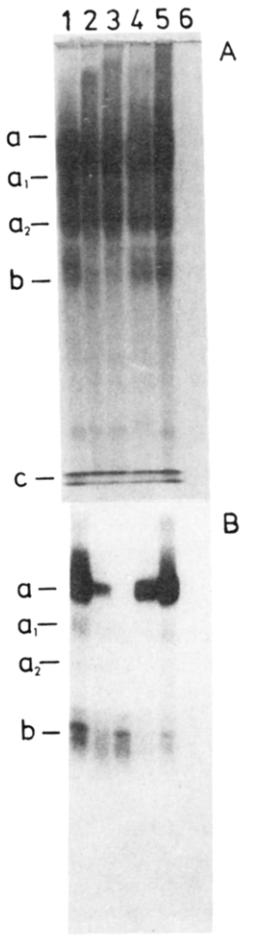

Figure 7. Electrophoretogram of nondenaturing polyacrylamide gel electrophoresis of reductant-treated mushroom PPO. Mushroom PPO was treated with different reductants as described in Table III. After electrophoresis, one part of the gel (A) was stained for protein with Coomassie Brilliant Blue dye and the other part (B) for activity (see Experimental Section). Treatments according to lanes: 1, no reductant; 2, ascorbic acid; 3, sodium bisulfite; 4, glutathione (reduced); 5, dithiothreitol; 6, blank. Approximately 150 and 300 μg of protein/lane were applied to the gel in part A and part B, respectively.

species during the early stage of incubation.

Dithiothreitol was the most effective in inactivating PPO. At 0.1 mM, inactivation was complete after 70 min ($t_{1/2}$ = 8 min at 0.1 mM) while 0.5 mM sodium bisulfite, glutathione (reduced), and ascorbic acid caused 50% inactivation after 28, 106, and 130 min, respectively. Dithiothreitol was much more effective in inactivating PPO when incubated alone with PPO than in the presence of substrate (compare the data of Figures 2 and 5).

The effect of incubation of PPO with the reductants for longer times is shown in Table III. The activity left was 1% of the control in the presence of 5 mM dithiothreitol within 1 min, 5 mM sodium bisulfite in 10 h, and 5 mM ascorbic acid in 23 h. With 5 mM gluthathione, the activity left was 10% of the control activity after 23 h of incubation. No recovery of activity was observed after dialysis of the reductant-treated samples in the case of sodium bisulfite, glutathione, and ascorbic acid (2% vs. 1% within experimental error), indicating irreversible inactivation. There was a 35% increase in activity following dialysis of the dithiothreitol-treated sample, suggesting that at least part of the inactivation may be due to reduction of one or more disulfide bonds needed for maximum activity. Note that a similar 35% effect was observed on the initial rate as measured polarographically (Figure 2).

Electrophoresis was performed in the absence (PAGE) and presence of sodium dodecyl sulfate (SDS–PAGE) on the dialyzed samples of reductant-treated PPO as described in Table III, in order to detect any electrophoretic mobility changes or loss of any of the isozymes of PPO caused by incubation with the reductants.

The electrophoretic mobility of the several proteins in commercially available PPO was little affected by treatment with dithiothreitol, glutathione (reduced), ascorbic acid, or sodium bisulfite, as determined by either SDS–PAGE (Figure 6) or nondenaturing PAGE (Figure 7A). However, following ascorbic acid and sodium bisulfite treatment, there was a decrease in intensity or disappearance of the protein bands in zone b (lanes 2 and 3 of Figure 7A) and in zones a, a_1, and a_2. The activity staining showed there were major effects on the activity of the isozymes (Figure 7B). Ascorbic acid treatment caused marked reduction in the intensity of activity staining of all bands (a, a_1, a_2, and b of lane 2, Figure 7B); sodium bisulfite treatment completely inactivated the isozymes of bands a, a_1, and a_2 (lane 3, Figure 7B), with perhaps some decrease in activity in zone b. Note that no meas-

urable activity was detected following sodium bisulfite treatment after dialysis (Table III). The isozyme of zone b appears to be somewhat more resistant to inactivation by sodium bisulfite than those of zones a, a_1, and a_2. Differential sensitivity of banana PPO isoenzymes to sodium bisulfite was reported by Montgomery and Sgarbieri (1975). When a lower concentration of PPO (0.2 vs. 1.0 mg/mL as shown in Table III) was treated with sodium bisulfite (0.5 mM), complete inactivation of all bands on PAGE was obtained (data not shown).

Glutathione treatment partially reduced the activity left in band a (lane 4, Figure 7B); the activity of bands a_1, a_2, and b were substantially decreased. The partial reversibility of the effect of dithiothreitol on PPO (Table III) is also shown on PAGE (lane 5, Figure 7B). Except for slight reduction in activity in bands a_2 and b, the intensity of activity staining bands in dithiothreitol-treated and reference samples (lanes 5 and 1, respectively; Figure 7B) was virtually the same.

DISCUSSION

The data from this work clearly indicate that the *observed* effect of reductants on mushroom polyphenol oxidase (PPO) is dependent on the assay method. Failure to appreciate this has caused much confusion in the past as to the effect of reductants on the activity of PPO. Mayer et al. (1966) have suggested that polarography is the preferred method to assay polyphenol oxidase even in the absence of reductants.

Under similar experimental conditions, except for the time at which the rate of reaction was measured, the polarographic method indicated less decrease in enzyme activity in the presence of reductants than did the spectrophotometric method (Figure 2). During the lag period before a change in A_{390nm} can be measured, several reactions may take place. The lack of color (A_{390nm}) development during the lag period is generally attributed to the nonenzymatic reduction of the benzoquinone formed by the enzymatic oxidative reaction back to the colorless *o*-dihydroxyphenol (eq 1). In addition, complexing of quinones with bisulfite (LuValle, 1952) and addition reactions between quinones and thiols (glutathione and dithiothreitol) can also lead to colorless products (Mason, 1955). These additional reactions of glutathione, dithiothreitol, and sodium bisulfite might explain their higher effectiveness, compared to ascorbic acid, in preventing color formation. Much additional work is needed on the prevention of browning by food-compatible thiol compounds (e.g., glutathione and L-cysteine) to replace sodium bisulfite because of its adverse side effects (Schwimmer, 1981).

In addition to the nonenzymatic reactions that prevent color formation, the enzyme is being inactivated by a k_{cat} mechanism (Golan-Goldhirsh et al., 1984; Golan-Goldhirsh and Whitaker, 1984) as well as by the direct effect of the reductants on the enzyme as shown by this work.

Incubation of PPO with dithiothreitol, glutathione (reduced), sodium bisulfite, or ascorbic acid rapidly inactivated the enzyme in the absence of substrate (Figure 5). Ascorbic acid was more effective in inactivating PPO after incubation with the enzyme for approximately 1 h, under the conditions tested (Figure 5). This unusual effect of ascorbic acid on mushroom PPO will be described in more detail elsewhere (Golan-Goldhirsh and Whitaker, 1984).

The spectrophotometric assay cannot be used alone to study the details of the observed effect of reductants on PPO. However, in conjunction with the polarographic method, much information about the effect of reductants on PPO can be obtained. From a practical point of view, the spectrophotometric method is useful to determine the optimum reductant concentration required to inhibit browning (Figures 3 and 4). One must still test these results on the biological system (fruit or vegetable). For example, from our experience ascorbic acid inhibits browning in avocado extracts as measured spectrophotometrically, but ascorbic acid addition enhanced discoloration in avocado pulp. Glutathione (reduced) and L-cysteine were effective in preventing the browning of avocado pulp (Golan-Goldhirsh and Whitaker, 1984).

The data reported here indicate some differences between the effect of dithiothreitol and the effect of glutathione (reduced), sodium bisulfite, and ascorbic acid on mushroom PPO in the absence of substrate. Inactivation by glutathione (reduced), sodium bisulfite, and ascorbic acid was irreversible as judged by the polarographic assay of the dialyzed solution (Table III), although after electrophoresis some isoenzymes regained activity (Figure 7B). On the other hand, there was a 35% regain of original activity on dialysis of the dithiothreitol-treated PPO measured polarographically and probably considerably more following electrophoresis (Figure 7B). The different observed effects of the reductants on different isoenzymes of mushroom PPO (Figure 7B) require additional work.

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Registry No. L-Ascorbic acid, 50-81-7; glutathione (reduced), 70-18-8; dithiothreitol, 3483-12-3; polyphenol oxidase, 9002-10-2; L-DOPA, 59-92-7; caffeic acid, 331-39-5; dopamine, 51-61-6; pyrocatechol, 120-80-9; sodium bisulfite, 7631-90-5.

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Volatile Sulfides of the Amazonian Garlic Bush

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The essential oil from *Adenocalymma alliaceum* leaves (ca. 0.04%) was investigated with GC and GC–MS. Of the 13 compounds tentatively identified, 9 are likely to be part of a homologous series. The most abundant components were diallyl disulfide and diallyl trisulfide, which comprise 62% of the oil.

The garlic bush (Portuguese, cipô d'alho) is a widespread plant in Brazil, particularly in the north where the principal species is *Adenocalymma alliaceum* Miers. Upon the sample handling of the large leaves, a strong odor exudes, similar to that of garlic (*Allium sativum*). Although infusions of the leaves are used in treating colds and fevers (Pio Correa, 1931; Figueiredo, 1979), its principal use is in the substitution of garlic, especially in the interior regions of difficult access.

The ethereal extracts of steam distillates from fresh leaves of the garlic bush were first studied by Apparao et al. (1978), using packed column gas chromatography–mass spectrometry (GC–MS). The principal components of the extracts to be the diallyl di-, tri-, and tetrasulfides.

Not surprisingly, these are also among the major constituents in garlic (Oaks et al., 1964; Brodnitz et al., 1971). As part of an ongoing study of the essential oil of amazonian plants, we have characterized the volatiles of garlic bush, using capillary column gas chromatography–mass spectrometry, and we now report the presence of additional alkenyl sulfides.

EXPERIMENTAL SECTION

Plant material of *A. alliaceum* was collected in Aurá, near Belém, state of Pará, and an exsiccate (no. 69734) has been placed in the herbarium at the Emilio Goeldi Museum, in Belém. The air-dried leaves were subjected to steam distillation, and the volatile oil obtained was dried in the presence of sodium sulfate. The yield was 0.04%.

The volatile oil was analyzed with a Carlo Erba 4160 gas chromatography (FID), with a 30 m × 0.25 mm fused silica capillary column containing a 0.25 μm film of SE-54. Hydrogen was used as the carrier gas, adjusted to a linear velocity of 33 cm/s (measured at 150 °C); the split flow was adjusted to give a 20:1 ratio, and the septum sweep was a constant 10 cm^3/min. Splitless injection of 2 μL, on a 1:1000 *n*-hexane solution, was followed by a delay of 30 s before beginning the purge. Injection was done with the oven at 50 °C. After a 3-min initial wait, the temperature was programmed at 6 °C/min to 230 °C.

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Table I. Constituents of *A. alliaceum*

peak no.	compound[a]	Kovats indices	% of oil[b]
1	diallyl sulfide	850	trace
2	allyl methyl disulfide	910	trace
3	dithiacyclopentene	945	2.18
4	allyl propyl disulfide	1048	trace
5	diallyl disulfide	1065	31.38
6	allyl methyl trisulfide	1123	0.96
7	3-vinyl-1,2-dithi-5-ene	1160	2.60
8	trithiacyclohexene (tent.)	1168	6.67
9	3-vinyl-1,2-dithi-4-ene	1205	4.71
10	allyl propyl trisulfide	1262	0.75
11	diallyl trisulfide	1296	30.55
12	allyl methyl tetrasulfide	1535	2.51
13	diallyl tetrasulfide	1690	1.23

[a] All of the compounds were identified by comparison of their mass spectra with those in the data system library or from the literature and are considered tentative. [b] Relative to the quantitation report of the Incos data system.

Gas chromatography–mass spectrometry was performed on a Finnigan 4021 GC–MS system, which includes on Incos data system. An identical 30-m SE-54 silica capillary column was installed in the GC, in a Grob-type injector, and directly coupled to the ion source of the MS. Helium was used as carrier gas, and injection and oven-programming temperatures were the same as above except a 4 °C/min gradient was used. The mass spectrometer was in EI mode at 70 eV. The quadrupole filter was scanned from 34 to 434 daltons once every second, and resulting spectra were stored on computer discs for later recall.

Identifications were based on mass spectra by comparison with those in the data system library and confirmed by retention data (Kovats, 1958).

RESULTS AND DISCUSSION

A gas chromatogram of the essential oil of the garlic bush is presented in Figure 1. From this GC trace one can see the simplicity of the mixture and the prominence of two components, peaks 6 and 13. These were tentatively confirmed to be the diallyl di- and trisulfides, as reported by Apparao et al. (1978). Diallyl tetrasulfide (peak 15) was also found to be present, although not as abundant as reported. For each of these sulfides, the MS matched almost perfectly with that given by Apparao et al. (1978). Several other major peaks were present, most of which

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