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DIFFERENTIAL EFFECT OF PHENYLHYDRAZINE ON THE CATECHOLASE AND CRESOLASE ACTIVITIES OF *VITIS* CATECHOL OXIDASE

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Key Word Index—*Vitis* species; Vitoceae; grape; catechol oxidase; phenylhydrazine; cresolase activity.

Abstract—The simultaneous addition of phenylhydrazine and *p*-cresol to grape catechol oxidase resulted in enhanced oxidation of *p*-cresol. Carbonyl reagents such as hydrazine, borohydride and semicarbazide also enhanced cresolase activity but had no effect on catecholase activity. Pretreatment of the enzyme with periodate abolished cresolase activity. The effects of periodate and ascorbate or semicarbazide on cresolase activity were mutually reversible. The simultaneous addition of phenylhydrazine and 4-methylcatechol to the enzyme did not result in inhibition of the initial rate of oxidation of the phenolic substrate. It is concluded that phenylhydrazine does not react with a carbonyl group on the enzyme. The possible involvement of conformational changes in the enzyme, determining phenylhydrazine inhibition is discussed.

INTRODUCTION

WE HAVE previously shown that phenylhydrazine is an irreversible inhibitor of catechol oxidase.[1] Inhibition increased with the length of the incubation period, and was dependent on the oxygen concentration. However, the site of action of phenylhydrazine and the mechanism of inhibition were not determined.

Preliminary experiments indicated that *p*-cresol acts both as a substrate of the enzyme and as a reversible inhibitor of catecholase activity. Similar effects were reported by Kendall.[2] In the present work we report on the differential effect of phenylhydrazine on the oxidation of 4-methylcatechol and of *p*-cresol.[3,4]

RESULTS

Catecholase activity

Catecholase activity of grape catechol oxidase is inhibited 50% by 0·5 mM *p*-cresol and 10% by 0·05 mM of the compound. The catecholase activity of the enzyme from apricots was affected only by higher concentrations of the mono phenol (0·1 M for 50% inhibition). *p*-Cresol protected catecholase activity against inhibition by phenylhydrazine. Partially purified grape catechol oxidase was incubated in the presence of air with 1 mM phenylhydrazine and 1 mM *p*-cresol in 0·1 M citrate buffer pH 5 at room temperature. Samples were withdrawn at various time intervals, diluted 20-fold and assayed for catecholase activity. In the absence of *p*-cresol, the enzyme was rapidly inhibited (60% at 10 min, 95% at 25 mm), while in its presence the enzyme only lost 25% of its activity and

[1] LERNER, H. R., HAREL, E., LEHMAN, E. and MAYER, A. M. (1971) *Phytochemistry* **10**, 2637.
[2] KENDALL, L. P. (1949) *Biochem. J.* **44**, 442.
[3] NELSON, J. M. and DAWSON, C. R. (1944) *Advan. Enzymol.* **4**, 99.
[4] KIM, K. H. and TCHEN, T. T. (1962) *Biochim. Biophys. Acta* **59**, 569.

this was constant over 40 min. The protective effect of *p*-cresol was reversible. When *p*-cresol concentration was reduced, while maintaining the phenylhydrazine concentration, rapid inhibition of catecholase activity was observed (Fig. 1).

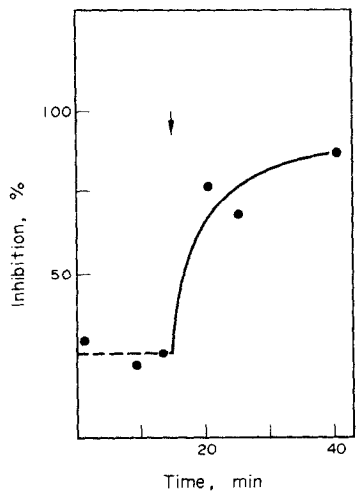

FIG. 1. REVERSIBILITY OF *p*-CRESOL PROTECTION OF CATECHOLASE ACTIVITY AGAINST INHIBITION BY PHENYLHYDRAZINE.
Grape catechol oxidase was preincubated with 1 mM *p*-cresol and 0·1 mM phenylhydrazine in 0·1 M citrate buffer pH 5·0. Samples were withdrawn, diluted 20-fold with buffer and assayed for catecholase activity in the presence of 5 mM 4-methylcatechol. At time indicated by arrow the incubation mixture was diluted 20-fold with 0·1 mM phenylhydrazine in buffer and samples withdrawn for determination of catecholase activity.

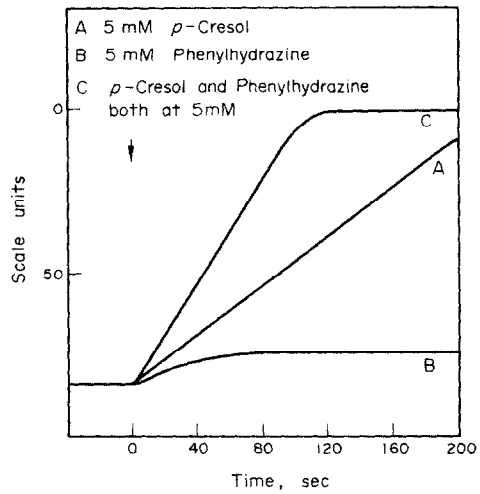

FIG. 2. OXIDATION OF PHENYLHYDRAZINE AND *p*-CRESOL BY SOLUBILIZED *Vitis* CATECHOL OXIDASE.
Electrode tracings of oxygen consumption. Enzyme in 0·1 M citrate buffer pH 5·0. Arrows indicate addition of substrates.

4-Methylcatechol did not protect the catecholase activity against inhibition by phenylhydrazine. Initial rates of the reaction were unaffected at several concentrations of 4-methylcatechol, when phenylhydrazine and substrate were added together. It appears, therefore, that the site of binding of the enzyme by phenylhydrazine and the enzyme site for 4-methylcatechol are different.

Cresolase activity

Since phenylhydrazine apparently links to the enzyme at the *p*-cresol binding site, it seemed possibile that it inhibits cresolase activity. At high concentrations, grape catechol oxidase oxidised phenylhydrazine, the reaction proceeding for a very short time. The initial rate of oxidation of phenylhydrazine was about 40% of that for *p*-cresol (Fig. 2). When oxygen consumption was followed in the presence of both *p*-cresol and phenylhydrazine, the rate of oxidation was 50% higher than the combined rates of oxidation of either substrate alone (Fig. 2). Therefore, phenylhydrazine does not inhibit cresolase activity; but enhanced it.

Since phenylhydrazine reacts with carbonyl groups, the effect of other carbonyl reagents on cresolase activity was studied. As a reference compound, known to activate cresolase activity, ascorbic acid was used. Cresolase activity was enhanced by 50% by 2×10^{-5} M 1,1-methylphenylhydrazine, 10^{-4} M semicarbazide, 5×10^{-5} M hydrazine

and 2×10^{-3} M aminoguanidine. Sodium borohydride at 5×10^{-4} M caused 100% enhancement. None of these compounds enhanced catecholase activity. 2-Mercaptoethanol, hydroxylamine and ethanolamine at corresponding concentrations inhibited cresolase activity. Amino acids have also been reported to activate cresolase.[6] We also found that glycine and proline at 30–40 mM activated cresolase although this activation was somewhat lower than the one produced by semicarbazide. Biuret and glutamate had no effect on cresolase activity.

The activation of cresolase we observed with carbonyl reagents is similar to the activation reported for ascorbate[2] or hydroxyphenols.[5] Carbonyl reagents thus seem to act in the same way as ascorbate in enhancing cresolase activity. The action of ascorbate and the dihydroxyphenols has usually been ascribed to their activity as reducing agents. Bordner and Nelson[6] have shown that an oxidizing agent such as ferricyanide induces a lag in the rate of oxygen consumption and inhibits cresolase activity. They observed that hydrogen peroxide activated mushroom cresolase and considered it a reducing agent. In our system, hydrogen peroxide, inhibited catechol oxidase irreversibly. Sodium periodate, however, did not inhibit catecholase activity, but reversibly inhibited the increased cresolase activity given by semicarbazide or ascorbate (Table 1). On the other hand, acetone 10 mM or acetophenone 10 mM did not reverse the increase in cresolase activity induced by 1 mM semicarbazide. The reversal by periodate and the lack of reversal by acetone or acetophenone indicates that the activation of cresolase by carbonyl reagents is probably not due to a reaction with an enzyme carbonyl group. The possible involvement of carbonyl groups in the inhibitory action of phenylhydrazine on catechol oxidase was further studied using sodium borohydride. Since carbonyl groups are readily reduced by borohydride, the effect of pretreatment of the enzyme with borohydride on inhibition by phenylhydrazine was studied. Reduction of the enzyme by borohydride did not affect the inhibitory action of phenylhydrazine, nor the time required to obtain 50% inhibition of enzyme activity.

TABLE 1. REVERSIBLE INHIBITION BY PERIODATE ON CRESOLASE ACTIVITY OF *Vitis* CATECHOL OXIDASE

Treatment	Relative activity %
None	100
Semicarbazide 1 mM	515
Semicarbazide 1 mM followed by $NaIO_4$ 2 mM	0
As above followed by 25 mM semicarbazide	415
1 mM Ascorbate, no substrate added	150
Ascorbate + *p*-cresol	770
Ascorbate + 2 mM $NaIO_4$, then *p*-cresol	0
As above followed by 5 mM ascorbate	600

Grape catechol oxidase was treated with either ascorbate or semicarbazide. Activity was measured in 0·1 M acetate buffer at pH 4·6, 5 mM *p*-cresol as substrate.

Attempts to remove possible carbohydrate moieties from the enzyme which might react with phenylhydrazine were made. Pretreatment with α- or β-glucosidase, or treatment of the enzyme with periodate did not affect inhibition of enzyme activity by phenylhydrazine. Pretreatments of the enzyme with semicarbazide had no effect on the catecholase activity

[5] LERNER, A. B., FITZPATRICK, T. B., CALKINS, E. and SUMMERSON, W. H. (1949) *J. Biol. Chem.* **178,** 185.
[6] BORDNER, C. A. and NELSON, J. M. (1939) *J. Am. Chem. Soc.* **61,** 1507.

of the enzyme at pH 5, or on the kinetics of enzyme inhibition by phenylhydrazine. 1,1-Methylphenylhydrazine acted in the same way as phenylhydrazine. These results indicate that a carbonyl group is not involved in phenylhydrazine inhibition.

DISCUSSION

As a working hypothesis we assumed that phenylhydrazine was oxidized at the cresolase site of catechol oxidase, thus causing irreversible inhibition of the enzyme. Catecholase activity of grape catechol oxidase was protected against phenylhydrazine by *p*-cresol. (Fig. 1). However, apricot catechol oxidase which is much less sensitive to *p*-cresol inhibition was not protected in this fashion at the *p*-cresol concentration used. Phenylhydrazine did not inhibit the initial rates of catecholase or cresolase activity of the grape enzyme. These results make it extremely unlikely that phenylhydrazine reacts at the cresolase site of catechol oxidase. It is also clear from our results that phenylhydrazine does not bind to a carbonyl group on the enzyme.

In addition to inhibiting catecholase activity, phenylhydrazine also enhanced cresolase activity. The enhancement of cresolase activity by reducing agents and its inhibition by oxidizing reagents has long been known.[6] Generally speaking, enhancement of activity correlates well with reducing properties of the promoters and vice versa, inhibition correlates with oxidizing properties. Our results are consistent with the view that phenylhydrazine, semicarbazide and borohydride are acting as reducing agents. The various effects of different agents on catechol oxidase activity can be reconciled, assuming that they cause conformational changes in the enzyme. We have previously shown that O_2 can induce a conformational change in grape catechol oxidase.[7] Application of the induced fit hypothesis[8] would require that *p*-cresol also induces a conformational change in the enzyme. The requirement for O_2 in order to obtain phenylhydrazine inhibition might be ascribed to a conformational change, induced by O_2, which exposed a site to phenylhydrazine attack. *p*-Cresol might induce a change masking this site, thereby protecting the enzyme. The effects of amino acids reported here and in the literature[6,9,10] may also be due to changes in enzyme conformation. Since almost all effectors of cresolase either inhibit catecholase activity or do not alter it, it seems possible that conformational changes favourable to cresolase activity tend to reduce catecholase activity. From these results it also appears the active site of grape catechol oxidase for cresolase activity differs from that for catecholase activity. However, the nature of the binding of phenylhydrazine to the enzyme is still unclear. We hope to resolve this question using ^{14}C-labelled phenylhydrazine.

EXPERIMENTAL

Enzymes. Grape catechol oxidase was prepared as previously described[1] except that the pH at all stages of the procedure was kept at pH 7.[7] Catechol oxidase from apricots was prepared as described by Lerner *et al.*[1]

Enzyme activity was determined by measuring oxygen consumption using a Clark polarographic electrode.[11] Catecholase activity was determined using 4-methylcatechol 5 mM as substrate in 0·1 M citrate buffer pH 5. Cresolase activity was determined using *p*-cresol 5 mM as substrate in 0·1 M acetate buffer pH 4·6.

[7] LERNER, H. R., MAYER, A. M. and HAREL, E. (1972) *Phytochemistry* **11**, 2415.
[8] KOSHLAND, D. E., JR. (1960) *Advan. Enzymol.* **22**, 45.
[9] MILLER, W. H. and DAWSON, C. R. (1942) *J. Am. Chem. Soc.* **64**, 2344.
[10] KARKHANIS, Y. and FRIEDEN, E. (1961) *J. Biol. Chem.* **236**, PC1.
[11] MAYER, A. M., HAREL, E. and BEN-SHAUL, R. (1966) *Phytochemistry* **5**, 783.

Chemicals. 4-Methylcatechol was recrystallized from *n*-hexane. *p*-Cresol was redistilled. Phenylhydrazine was purified by decolorization of its aqueous solution with active charcoal, followed by precipitation of the hydrochloride with HCl.

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