# Inhibition of Grape Polyphenol Oxidase by Several Natural Aliphatic Alcohols

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The inhibition of both cresolase and catecholase activities of grape polyphenol oxidase by several natural aliphatic alcohols formed during grape juice fermentation has been studied. Inhibition strongly increased as the hydrocarbonated chain of the alcohol increased, and a partial relation between the relative inhibitory potency and the octanol/water partition coefficient was found. Likewise, primary alcohols were more effective inhibitors than secondary, and these more than tertiary. Inhibition of 4-methylcatechol oxidation was linear-mixed and non pH dependent. Data obtained by incubation of the enzyme with different alcohols revealed an almost zero inactivation effect in the time range tested.

Polyphenol oxidase (PPO) (monophenol, dihydroxy-L-phenylalanine:oxygen oxidoreductase, E.C. 1.14.18.1) is a copper protein widely distributed in the phylogenetic scale, being responsible for browning in fruits and vegetables. It catalyzes both the ortho hydroxylation of monophenols to give *o*-diphenols (cresolase activity) and the further oxidation of *o*-diphenols to *o*-quinones (catecholase activity). Three different forms of binuclear copper in the active center involved in the reaction mechanism are known: met, oxy, and deoxy (Lerch, 1981; Robb, 1981).

This browning phenomenon is generally undesirable because of the unpleasant appearance and the concomitant development of off-flavor. So, their control is important in the processing of a high-quality product. Although a great number of inhibitors of PPD are known, the inhibition by aliphatic alcohols has only been cited by Montedoro and Cantarelli (1969) and Kidron et al. (1978) for ethanol, and few details were given. However, inhibition by monophenols and diphenols (Robb et al., 1966; Macrae and Duggleby, 1968; Hughes and Price, 1976; Cabanes et al., 1987a; Hider and Lerch, 1989) and by carboxylic acids has been thoroughly investigated (Krueger, 1955; Soler-Martínez et al., 1965; Pifferi et al., 1974; Walker and Wilson, 1975; Satô, 1980; Gunata et al., 1987). The aliphatic alcohols assume great technological importance in the case of grape, because they are biosynthesized during the course of fermentation and contribute to the aroma of the wine (Schreier, 1979).

In the present study, we have characterized the inhibition by several natural aliphatic alcohols of both cresolase and catecholase activities of partially purified grape PPO. The relationship between the hydrophobic character of these compounds and their relative potency to inhibit these two activities has also been investigated.

## MATERIALS AND METHODS

**Plant Material.** The grapes (*Vitis vinifera* L. cv Airen) used in this study were harvested at maturation stage at Villarrobledo (Albacete, Spain) and stored at −25 °C until use.

**Reagents.** *p*-Cresol and 4-methylcatechol were from Sigma Chemical Co. All other chemicals were of analytical grade and were used without further purification.

The octanol/water partition coefficient values for the different alcohols were taken from Rekker (1977).

**Methods.** Extraction of grape PPO and measurement of both cresolase and catecholase activities were performed as previously described (Valero et al., 1988).

The reaction media for catecholase activity contained in a final volume of 2.5 mL: 10 mM 4-methylcatechol (equivalent to $K_M$), 10 mM sodium acetate buffer (pH 4.75), the corresponding alcohol at the indicated concentration, and 0.35 μg/mL of grape PPO. The reaction media for cresolase activity contained a final volume of 1.5 mL: 0.35 mM *p*-cresol (equivalent to $K_M$), 10 mM phosphate buffer (pH 7.00), the corresponding alcohol at the indicated concentration, and 106 μg/mL of grape PPO.

One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of 4-methyl-*o*-benzoquinone/min.

$I_{50}$ was defined as the inhibitor concentration that yields 50% inhibition (Segel, 1975).

Protein concentration was determined by the method of Bradford (1976).

## RESULTS AND DISCUSSION

**Inhibition of Cresolase Activity.** The expression of cresolase activity is characterized by an initial lag period and the steady-state rate. The data in Figure 1 show the kinetics of *p*-cresol hydroxylation in the presence and absence (control) of different concentrations of 1-butanol. It can be seen that 1-butanol extends the lag period and also inhibits the rate of 4-methyl-*o*-benzoquinone formation (*A* at 400 nm) following the lag period. Dependence of steady-state rate and lag period with 1-butanol concentration is shown in Figure 1 (inset). Table I shows the effects of different alcohols on the lag period and steady-state rate, showing that inhibition is higher as the hydrocarbonated chain is longer and, furthermore, primary alcohols are more effective inhibitors than secondary and tertiary.

An inverse relationship between the lag period and enzyme concentration can be observed when PPO acts on *p*-cresol (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970; García-Carmona et al., 1979; García-

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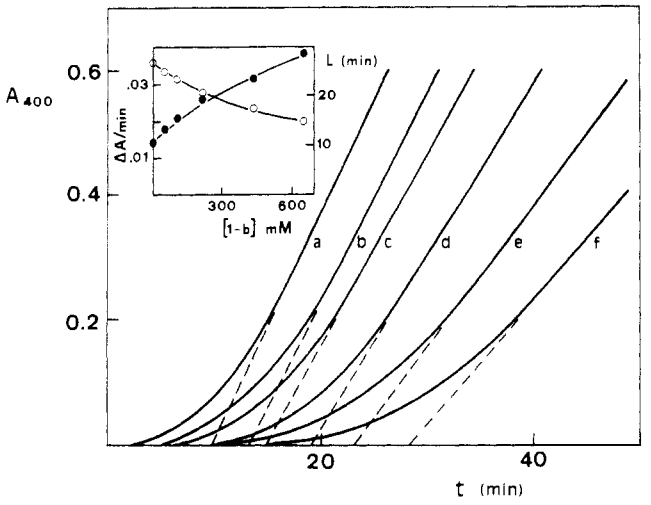


**Figure 1.** Effect of 1-butanol on the rate of *p*-cresol hydroxylation by grape PPO: (a) no inhibitor; (b) 54.64 mM, (c) 109.28 mM, (d) 218.56 mM, (e) 437.12 mM, and (f) 655.68 mM 1-butanol. The inset graph shows the dependence of the lag period (●) and the steady-state rate (○) with 1-butanol concentration (1-b).

**Table I. Inhibition of Cresolase Activity of Grape PPO by Several Aliphatic Alcohols**[a]

| alcohol | % inhibn | lag period, min |
|---|---|---|
| none | 0 | 9.9 |
| methanol | 1.5 | 12.4 |
| ethanol | 2.9 | 12.9 |
| 1-propanol | 16.6 | 14.8 |
| 1-butanol | 30.1 | 19.7 |
| 2-methylpropanol | 31.5 | 19.8 |
| 2-butanol | 25.8 | 18.5 |
| 2-methyl-2-propanol | 18.6 | 16.6 |
| 1-pentanol | 35.8 | 20.9 |
| 2-methylbutanol | 47.7 | 24.7 |

[a] In each case alcohol concentration was 220 mM.

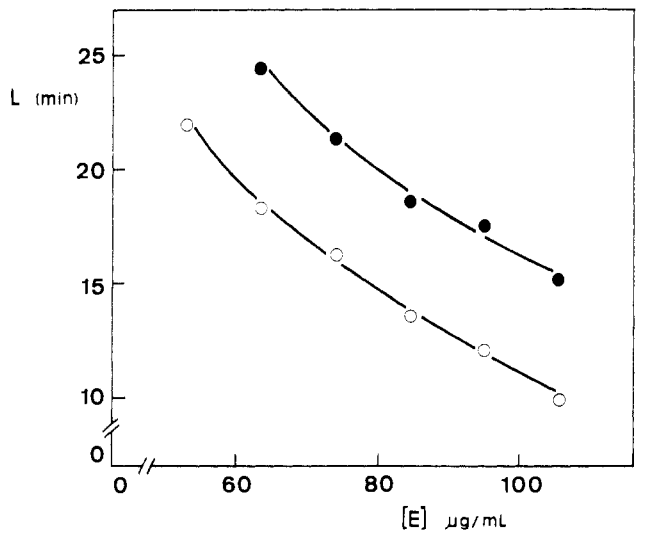


**Figure 2.** Effect of enzyme concentration on the lag period of cresolase activity of grape PPO in the absence of inhibitor (○) and in the presence of 109.28 mM 1-butanol (●).

Cánovas et al., 1981). As is shown in Figure 2, the same dependence of the lag period on enzyme concentration was obtained in the presence and absence of 1-butanol, indicating that alcohols inhibit PPO by binding to the active site of the enzyme in its oxy and met forms. However, they do not modify the complex kinetic relations established between these enzymatic forms and the chemical steps of the evolution of *o*-quinones generated by both cresolase and catecholase activities (Cabanes et al., 1987b).

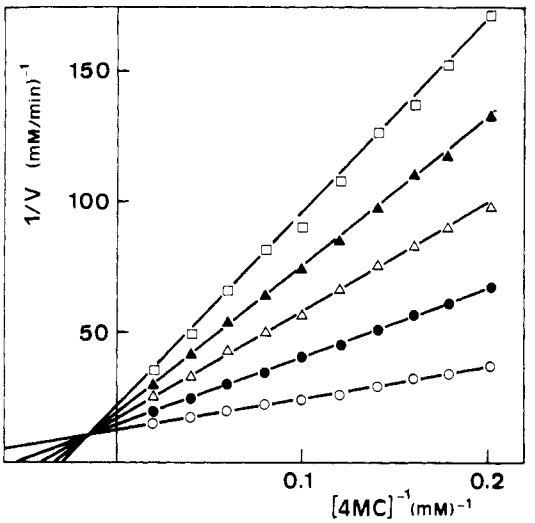


**Figure 3.** Lineweaver–Burk plot for 1-butanol inhibition with respect to 4-methylcatechol: (○) no inhibitor; (●) 109.28 mM, (△) 218.56 mM, (▲) 327.84 mM, and (□) 437.12 mM 1-butanol.

**Inhibition of Catecholase Activity.** The effects of aliphatic alcohols on the catecholase activity of grape PPO were also tested.

Kinetic data obtained by using different concentrations of 4-methylcatechol (4MC) and different concentrations of 1-butanol were subjected to analysis by double-reciprocal plots. The results shown in Figure 3 indicated a mixed-type of inhibition (Dixon and Webb, 1979). To establish the type of inhibition and to determine $K_I$, replots of primary reciprocal data (slope vs [I] and $1/V$ axis intercept vs [I]) were made. These replots were linear (data not shown) showing that inhibition is linear-mixed. The system may be considered a mixture of partial competitive inhibition and pure noncompetitive inhibition, since the velocity decreased to zero when the alcohol concentration was increased (Segel, 1975). A similar result was obtained for the other alcohols, the $K_I$ values calculated for 1-butanol being 78 mM and for ethanol 845 mM.

When the inhibition of catecholase activity was studied as a function of pH, we found a total independence in the range of pH tested (3.5–7.5) (data not shown).

To determine the effect of alcohol concentration on PPO over time, similar levels of enzyme activity were exposed to increased levels of different alcohols and incubated. Aliquots of these preparations were assayed for catecholase activity at various times. Data obtained revealed that alcohols are inhibitors rather than inactivators of the enzyme: thus, for example, in the presence of 66% ethanol (11.432 M) and after 2-h preincubation, only 4% catecholase activity was lost. This result is in contrast to data obtained for carboxylic acids (Satô, 1980), where preincubation of the enzyme with oxalates could lead to complete loss of activity. This extremely low inactivation effect of alcohols is of great use because it allows one to study substrates and effectors of PPO insolubles in water and solubles in alcohol, since the degree of inhibition is known.

**Relationship between the Hydrophobic Character of Alcoholic Compounds and Their Relative Potency To Inhibit Grape PPO.** Table II shows the $I_{50}$ values calculated for the different alcohols tested for both cresolase and catecholase activities. The $I_{50}$ value for each alcohol is different for cresolase and catecholase activities, although both are catalyzed in the same

Table II. $I_{50}$ Values of Several Aliphatic Alcohols for both Cresolase and Catecholase Activities of Grape PPO[a]

| alcohol | $I_{50}$, M | |
|---|---|---|
| | cresolase | catecholase |
| ethanol | 10.4 | 1.8 |
| 1-propanol | 1.8 | 0.44 |
| 2-propanol | 4.1 | 0.70 |
| 1-butanol | 0.8 | 0.16 |
| 2-methylpropanol | 0.7 | 0.14 |
| 2-butanol | 1.1 | 0.26 |
| 2-methyl-2-propanol | 1.3 | 0.30 |
| 1-pentanol | 0.38 | 0.11 |
| 2-methylbutanol | 0.24 | 0.07 |
| 2-pentanol | 0.95 | 0.17 |

[a] Standard assay conditions were used except for the addition of 24 μM 4-methylcatechol for cresolase activity measurements, to avoid the lag period (Valero et al., 1988).

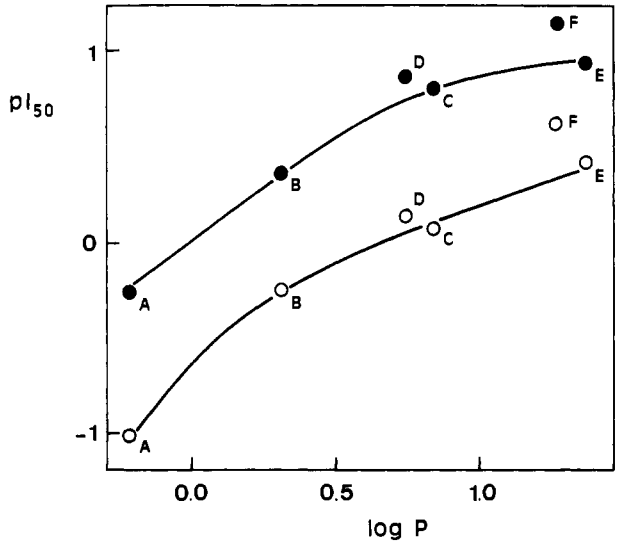


Figure 4. Logarithmic plot of $pI_{50}$ ($-\log I_{50}$) vs P for both cresolase (○) and catecholase (●) activities of grape PPO for primary aliphatic alcohols: A, ethanol; B, 1-propanol; C, 1-butanol; D, 2-methylpropanol; E, 1-pentanol; F, 2-methylbutanol.

active site (Lerch, 1981). This can be interpreted as being the result of different degrees of participation of the oxy and met forms of the enzyme in the reaction mechanism of both activities (García-Carmona et al., 1988) and also explain the nearly constant relation found between the $I_{50}$ values for cresolase and catecholase activities (4.6 ± 1.2).

It can also be seen that an increase in the number of carbon atoms results in a strong increase in the degree of inhibition. Likewise, primary alcohols were more effective inhibitors than secondary, and these more than tertiary, probably due to the primary alcohols having better accessibility to the active site. These results suggested to us that the inhibition is caused more by the hydrophobic chain than by the alcohol function. To examine this effect, we correlated the hydrophobic character of these compounds with their respective octanol/water partition coefficient ($P$), since the literature reveals best correlations with use of this parameter (Laane et al., 1986, Pérez-Guillermo et al., 1987), and we made a logarithmic plot of $P$ vs $pI_{50}$ ($-\log I_{50}$) for the different primary alcohols (Figure 4). Data obtained indicated that hydrophobic forces are involved in the alcohol binding to the enzyme, although other types of forces must also be involved (the competition between the alcohol function and the substrate, for example) since the relation is nonlinear, which would explain the mixed-type inhibition obtained. Another fact to be kept in mind is that the degree of inhibition caused by the structural isomers is different, although the $P$ parameter practically does not distinguish between them. Thus, for example, in the case of five-carbon atoms, the $I_{50}$ value calculated for 1-pentanol is higher than the one for 2-methylbutanol. The same occurs for 1-butanol and 2-methylpropanol. These results indicated that the hydrophobic site at which these compounds bind into the enzyme must be small, and the lower degree of inhibition of the linear chain compounds may be explained by a smaller accessibility to the hydrophobic pocket of the enzyme.

The results shown here contribute to the study of the relation between PPO and the alcohols biosynthesized during the fermentation process, since these compounds contribute to the flavor of the wine, and it is well-known that PPO also influences it (Kidron et al., 1978; Sapis et al., 1983).

ACKNOWLEDGMENT

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**Registry No.** PPO, 9002-10-2; ethanol, 64-17-5; 1-propanol, 71-23-8; 2-propanol, 67-63-0; 1-butanol, 71-36-3; 2-methylpropanol, 78-83-1; 2-butanol, 78-92-2; 2-methyl-2-propanol, 75-65-0; 1-pentanol, 71-41-0; 2-methylbutanol, 137-32-6; 2-pentanol, 6032-29-7.

# Involvement of Microorganisms in Accelerated Degradation of EPTC in Soil

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Accelerated EPTC (*S*-ethyl dipropylcarbamothioate) degradation was confirmed in a mixed culture of microorganisms derived from a soil with enhanced degradation (history soil) by using $^{14}$C-labeled EPTC. The antibacterial agent chloramphenicol (D-(−)-*threo*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]acetamide) markedly suppressed $^{14}CO_2$ evolution while the antifungal agent cycloheximide (4-[(2*R*)-2-((1*S*,3*S*,5*S*)-3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide) did not, suggesting that soil bacteria play a significant role in enhanced EPTC degradation. A fast EPTC bacterial degrader (FD1) strain and a slower one (SD1), which were isolated by a soil enrichment technique from a history soil, were capable of utilizing EPTC as a sole carbon source. Vernolate (*S*-propyl dipropylcarbamothioate), butylate (*S*-ethyl bis(2-methylpropyl)carbamothioate), or cycloate (*S*-ethyl cyclohexylethylcarbamothioate) were also degraded by these bacteria in a pattern similar to that in a soil with enhanced degradation. Inoculation of nonhistory soil with FD1 strain induced accelerated degradation of the herbicide in the soil at rates similar to those in field soils exhibiting EPTC accelerated degradation.

Accelerated biodegradation of pesticides in soil following repeated application and consequent loss of their efficacy against the target pest has been demonstrated with carbamothioate herbicides, as well as with many other pesticides (Kaufman et al., 1985; Roeth, 1986; Katan and Aharonson, 1989). This phenomenon has been shown to be linked with previous application of the same pesticide (history soils) or a structurally similar one and accompanied by an adaptation and/or enrichment of specific pesticide-degrading soil microorganisms. Although it is well accepted that soil microorganisms play a significant role in the degradation of soil-incorporated carbamothio-

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