pH-Dependent Effect of Sodium Chloride on Latent Grape Polyphenol Oxidase

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This paper presents a kinetic study of the effect of sodium chloride on the catecholase activity of latent grape polyphenol oxidase. The modifier showed a strongly pH dependent inhibitory effect at pH values <5 but acted as an activator at higher values. Other salts such as potassium nitrate and sulfate also activated the enzyme, indicating an unspecific activation effect by ionic strength. Furthermore, at pH values <5, at which the enzyme followed Michaelis−Menten behavior, the presence of sodium chloride gave rise to a lag period in the product accumulation curves, generating positive kinetic cooperativity in the steady-state kinetics. In contrast, at higher pH values, at which the enzyme showed a lag phase and exhibited negative cooperativity, the presence of a salt decreased cooperativity. The inhibition data are consistent with a mechanism by which chloride ions bind to both protonated forms of the enzyme, free enzyme and the enzyme−substrate complex, the latter species undergoing a conformational change. This mechanism was simulated by computer, and good agreement was obtained with the experimental results.

Keywords: *Polyphenol oxidase; chloride; inhibition; activation; slow transition*

INTRODUCTION

Polyphenol oxidase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1; PPO) is a bifunctional, copper-containing enzyme widely distributed in the phylogenetic scale that, in the presence of molecular oxygen, catalyzes both the *o*-hydroxylation of monophenols to give *o*-diphenols (cresolase activity) and the further oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The *o*-quinones thus generated are very unstable and rapidly react with themselves and with amino acids or proteins, polymerizing to the brown or black pigments (Mason and Peterson, 1965; Matheis and Whitaker, 1984; García-Carmona et al., 1988) that are responsible for melanization in animals and browning in plants. This browning phenomenon is generally undesirable in food technology because of the unpleasant appearance and the concomitant development of off-flavor. Owing to its technological importance therefore, numerous studies have been devoted to the inhibition of the enzyme from different sources by different substances (Ferrar and Walker, 1996).

In vegetables, this enzyme is located in the chloroplast thylakoid membranes (Mayer, 1987), in some species in a latent form. The latent enzyme can be activated in vitro by different treatments such as trypsin (Tolbert, 1973), fatty acids (Golbeck and Cammarata, 1981), aging (Lieberei and Biehl, 1978), acid and base shock (Kenten, 1957; Lerner et al., 1972), detergents (Moore and Flurkey, 1990), and cations (Söderhall et al., 1985). When the enzyme is activated by pH changes in the medium, the process is accompanied by a change in the Stoke's radius of the protein, which suggests the involvement of conformational changes during activation (Lerner et al., 1972; Lerner and Mayer, 1975). More recently, evidence has emerged that slow pH-induced conformational changes probably occur during the catalytic cycle of latent grape PPO (Valero and García-Carmona, 1992a,b).

The fact that halides can inhibit was demonstrated a long time ago from PPO of many sources (Krueger, 1955; Robb et al., 1966; Wong et al., 1971), although its effect on the latent enzyme has not been studied. The inhibition is strongly pH dependent and increases with decreasing pH, a fact that has been explained by the possible involvement of histidine residues in complexing the metal at the active site (Krueger, 1955; Peñafiel et al., 1984). Furthermore, techniques such as electron paramagnetic resonance (EPR) and Raman resonance have pointed to an interaction between halides and copper at the active site of PPO (Himmelwright et al., 1980) and other copper-containing enzymes such as hemocyanin (Himmelwright et al., 1978, 1979) and laccase (Winkler et al., 1982). Such inhibition has been claimed to be both competitive and noncompetitive in nature (Robb et al., 1966; Peñafiel et al., 1984).

It is the purpose of the present paper to describe a kinetic study of the effect of sodium chloride on the catecholase activity of PPO extracted in its latent state from chloroplast thylakoid membranes of grapes (*Vitis vinifera* cv. Airen).

MATERIALS AND METHODS

4-*tert*-Butylcatechol was purchased from Aldrich (Madrid, Spain) and used without further purification. Catechol, 4-methylcatechol, 3,4-dihydroxybenzoic acid, and L-3,4-dihydroxyphenylalanine (L-Dopa) were from Sigma (Madrid, Spain). Triton X-114 was obtained from Fluka AG (Madrid, Spain) and

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condensed as described by Bordier (1981), except that 100 mM sodium phosphate buffer (pH 7.3) was used. The detergent phase of the third condensation had a concentration of 30% (w/v) Triton X-114 and was used as the stock detergent solution for all of the experiments.

Sodium chloride and all other chemicals were of standard analytical grade and purchased from Merck (Darmstadt, Germany). Halide-buffered stock solutions were prepared by adjusting the pH in the presence of the salt (Rouet-Mayer and Philippon, 1986).

The Airen grape berries used in this study were harvested and processed as previously described (Valero et al., 1988a). PPO was extracted from grape berries in a latent form by using temperature-induced phase separation in Triton X-114 at pH 7.3 (Sánchez-Ferrer et al., 1989). The clear solution thus obtained, after dialysis against 1 mM sodium phosphate buffer (pH 7.3), was used as enzyme source. To avoid any possible activation of the enzyme by endogenous proteases, phenylmethanesulfonyl fluoride (PMSF) and *N*-ethylmaleimide were added to give final concentrations of 0.5 and 5 mM, respectively, in all purification steps and in the dialysis buffer. This did not significantly affect PPO activity.

The catecholase activity of the enzyme was assayed by monitoring at 25 °C the increase in absorbance at 400 nm due to the formation of the 4-*tert*-butyl-*o*-quinone ($\epsilon = 1150\ M^{-1}\ cm^{-1}$) (Waite, 1976). The steady-state rate (V_s) was defined as the slope of the linear zone of the product accumulation curve. The lag period was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa axis. Unless otherwise stated, the reaction medium contained 4.5 mM 4-*tert*-butylcatechol and sodium chloride at the indicated concentration in 50 mM sodium acetate buffer (pH 3.5−5.5) or 50 mM sodium phosphate buffer (pH 5.5−6.5) and 0.073 unit of enzymatic activity, in a final volume of 1 mL. The reaction was started by addition of the enzyme. One unit of enzyme activity was taken as the amount of enzyme that produces 1 μmol of 4-*tert*-butyl-*o*-benzoquinone/min as measured at pH 4.5 under the above experimental conditions in the absence of the salt.

The steady-state rate data versus substrate concentration were initially analyzed graphically by double-reciprocal plots (Neet, 1980). Those showing linearity were fitted to the Michaelis−Menten equation. In the case of double-reciprocal plots with concave lines, the steady-state rates were fitted to the Hill equation. Statistical analysis was carried out by fitting the data by nonlinear regression using the SigmaPlot Scientific Graphing System.

Repetitive spectra of the oxidation of 4-*tert*-butylcatechol by PPO or sodium periodate in the presence of sodium chloride were obtained with a Uvikon 940 spectrophotometer from Kontron Instruments (Zürich, Switzerland). Oxygen consumption throughout the time course of the reaction was monitored with an oxygen electrode (Rank Brothers Co., Norfolk, England).

The reaction mechanism was simulated by means of a homemade computer program elaborated in QBASIC, which is available to interested readers on request. This program calculates initial and steady-state rates, the product concentration curve, and lag period, as well as parameters related to cooperativity. Values for the kinetic constants were arbitrarily chosen.

RESULTS AND DISCUSSION

Figure 1A shows the pH dependence obtained for the catecholase activity of latent grape PPO in the absence and in the presence of sodium chloride. Two well-differentiated zones may be seen in the plot: below pH values of ∼5 a strongly pH dependent inhibitory effect was observed, the degree of inhibition increasing with the acidity of the reaction medium, which is in agreement with the literature concerning PPO from other biological sources (Krueger, 1955; Robb et al., 1966; Wong et al., 1971; Peñafiel et al., 1984). However, above

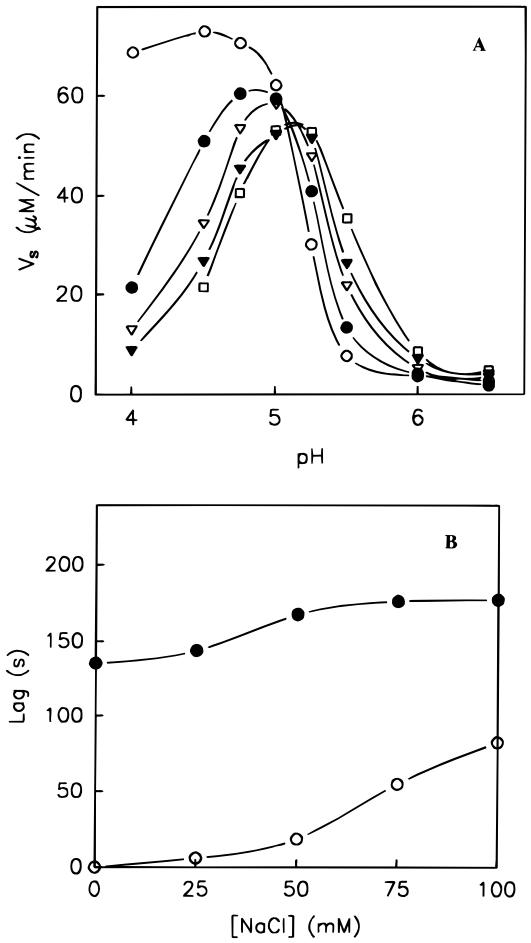


Figure 1. (A) Variation of catecholase activity of latent grape PPO in the presence of NaCl as a function of pH. Conditions were as indicated under Materials and Methods, at the following NaCl concentrations: (○) 0, (●) 25, (▽) 50, (▼) 75 and (□) 100 mM. (B) Variation of lag period against chloride concentration at pH (○) 4.5 and (●) 5.5. Conditions were as in (A).

this pH sodium chloride was found to have an activation effect on PPO. Such an effect has been described hitherto only by Söderhäll et al. (1985), who observed in vitro that millimolar concentrations of $CaCl_2$ activated prophenoloxidase from carrot, although the activation effect was attributed to the presence of Ca^{2+} ions rather than Cl^-.

On the other hand, it has been demonstrated that latent grape PPO shows a lag period in the expression of its catecholase activity above pH 5, whereas under more acid pH conditions the steady state is reached immediately (Valero and García-Carmona, 1992a). However, in the activity assays performed at these pH values in the presence of NaCl, a lag phase was observed in the product accumulation curves, the duration of which increased with chloride concentration, as can be seen in Figure 1B. This lag period in the expression of the catecholase activity of PPO in the presence of sodium chloride has never been previously described.

It was confirmed that these two phenomena (activation above pH 5 and appearance of a lag period) were not untrue results by following the time course of the reaction with other *o*-diphenolic substrates (15 mM catechol, 10 mM 4-methylcatechol, 15 mM 3,4-dihydroxybenzoic acid, and 1.5 mM L-Dopa) and also by monitoring oxygen consumption during catalysis (data not shown). Furthermore, using rapid scan spectros-

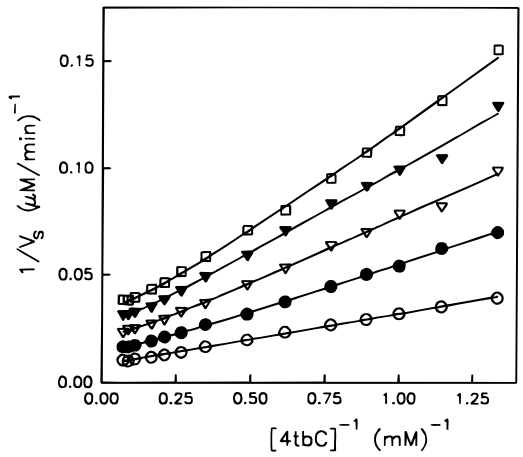


Figure 2. Double-reciprocal plot of inhibition of PPO by NaCl at pH 4.5. Conditions were as in Figure 1A. The points represent the experimental data. The lines represent the fit of these experimental data to the Hill equation.

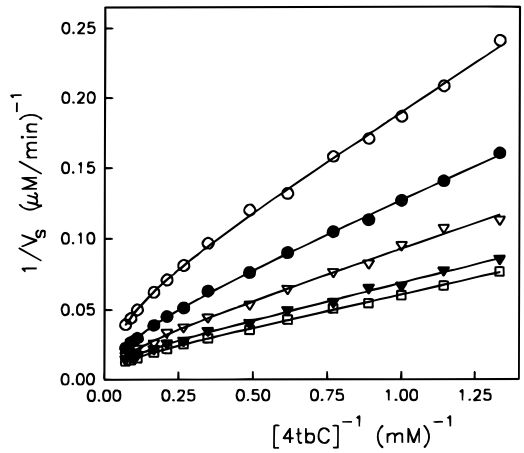


Figure 3. Double-reciprocal plot of activation of PPO by NaCl at pH 5.5. Conditions were as in Figure 1A, except the enzyme concentration used was 0.13 unit. The points represent the experimental data. The lines represent the fit of these experimental data to the Hill equation.

copy with PPO and sodium periodate (Valero et al., 1988b), it was demostrated that the stability of the quinone product of the oxidation reaction was the same in the presence and in the absence of sodium chloride (data not shown).

Figures 2 and 3 show Lineweaver−Burk double-reciprocal plots of the steady-state rate against 4-*tert*-butylcatechol concentration at pH 4.5 and 5.5, respectively. It can be seen that the control activity plot was lineal at pH 4.5, with the system following hyperbolic kinetics. However, the presence of sodium chloride in the reaction medium induced the appearance of concave upward plots, which indicates positive kinetic cooperativity (Neet, 1980). This became more pronounced as the inhibition increased, as can be seen by the greater curvature of the plots. As a reflection of the inhibitory effect observed, the curves became steeper and pivoted anticlockwise as the sodium chloride concentration increased. At pH 5.5, on the other hand (Figure 3), the latent enzyme displayed negative cooperativity in the absence of NaCl (Valero and García-Carmona, 1992b), an effect that gradually decreased in the presence of the salt. Furthermore, reflecting the activation effect observed at this pH value, the slopes of the curves decreased clockwise with increasing sodium chloride

Table 1. Variation of Hill Coefficient as a Function of NaCl Concentration

[NaCl] (mM)	pH 4.5	pH 5.5	pH 5.5[a]
0	1.0	0.53	0.56
25	1.16	0.60	0.61
50	1.26	0.66	0.66
75	1.35	0.70	0.78
100	1.38	0.72	0.82

[a] These data were obtained in the presence of KNO_3 at the same concentration.

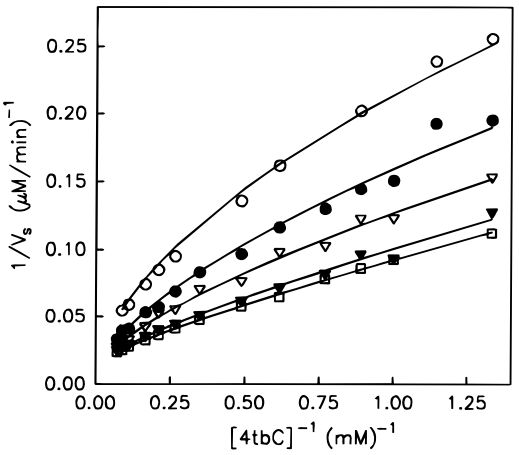


Figure 4. Double-reciprocal plot of activation of PPO by KNO_3 at pH 5.5. Conditions were as in Figure 1B. The points represent the experimental data. The lines represent the fit of these experimental data to the Hill equation.

concentrations. To estimate the degree of cooperativity, we fitted these data using nonlinear regression to the Hill equation

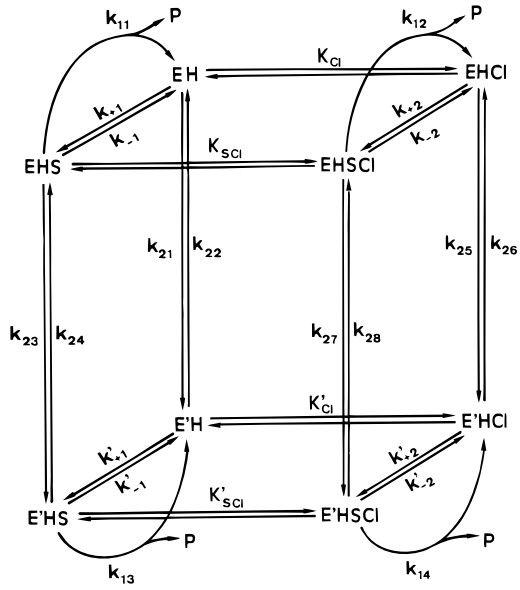
$$v = V_m[S]^h/(K_H{}^h + [S]^h) \quad (1)$$

where h is the Hill coefficient, widely used as an index of cooperativity, and K_H is known as the constant of the Hill equation. The results obtained are shown in Table 1, positive kinetic cooperativity ($h > 1$) being obtained when chloride acted as inhibitor (pH <5) and negative cooperativity of the latent enzyme disappearing ($h \rightarrow 1$) at higher pH values, when it acted as an activator.

To confirm that the salt activated PPO at pH values >5, similar experiments were performed in the presence of other salts such as potassium nitrate and sulfate. Figure 4 shows the results obtained in the presence of KNO_3 plotted in double-reciprocal form. It is again clear that PPO activity was enhanced by the presence of the salt, with the progressive decrease of the curvature of the plots (concave downward) indicating a progressive decrease of kinetic negative cooperativity as potassium nitrate concentrations rose. The corresponding Hill coefficients are also shown in Table 1. These results indicate that PPO is unspecifically activated by ionic strength.

The appearance of both a lag period and positive kinetic cooperativity in the steady state of the expression of the catecholase activity of PPO in the presence of sodium chloride at pH values >5 is a phenomenon that has never been previously described. This phenomenon can be interpreted according to the concept of "hysteresis" proposed by Frieden (1970), who defined hysteretic enzymes as those that respond slowly (in terms of kinetic characteristics) to a rapid change in ligand (substrate or modifier) concentration. The change

Scheme 1. Mechanism Proposed for the Action of NaCl (Cl) on Latent PPO[a]



[a] EH and E′H represent different conformations of the enzyme that are slowly interconvertible compared with the other steps in the mechanism, and both of which can bind substrate with different affinities.

in ligand concentration presumably induces a conversion of one enzyme form to another form that has different kinetic properties. Thus, the observed lag period may be caused by a slow conformational change in the protein induced by the presence of sodium chloride to a catalytically more active form. Chloride would bind to protonated forms of the enzyme, in accordance with the observation that inhibition increases as pH decreases. Cooperativity in the steady-state kinetics is mechanistically related to the existence of the slow transition and is dependent on the relative turnover rates of the two conformational forms of the enzyme. These two conformations must be similar enough to catalyze the same reaction but dissimilar enough to have different catalytic or binding rate constants (Ainslie et al., 1972). Therefore, the protonated enzyme−chloride complex must undergo a slow conformational change, and these two conformations (EHCl and E′HCl) must also comply with these conditions if the increase in the duration of the lag period and the appearance of positive kinetic cooperativity are to be explained. A mechanism in which chloride ions bind only to protonated free enzyme species (EH and E′H), such as a competitive-type inhibitor, could explain the appearance and the increase in the lag period in the progress curves; however, it could not generate the positive cooperativity observed experimentally at pH <5 (Table 1). This reasoning was confirmed by computer simulation of the corresponding mechanism, leading us to propose the mechanism depicted in Scheme 1 to explain the action of chloride on PPO, where it also binds to protonated enzyme−substrate complexes (EHS and E′HS), the complexes being enzyme−substrate−chloride productive. This scheme is also supported by the dependence of maximal velocity on substrate concentration, since curves from Figure 2 do not intersect the ordinate axis at the same point as would be predicted for a competitive-type mechanism.

The kinetic behavior of this mechanism was studied by computer simulation, with values being assigned to the different kinetic constants. The results obtained for a determined set of kinetic constants and initial conditions are shown in Table 2. It can be seen that the kinetic behavior of the system obtained in these simulation conditions accurately reflects experimental results, indicating that the model adequately describes the inhibition of latent PPO by sodium chloride and explaining some of the different results published in the literature with respect to the type of inhibition.

Table 2. Data Obtained by Computer Simulation of the Mechanism Shown in Scheme 1

$[Cl]_0$ (mM)	lag (s)	V_s (M s^{-1})	h
0	2.9	7.86×10^{-6}	1.00
25	14.3	1.92×10^{-6}	1.13
50	24.5	1.28×10^{-6}	1.21
75	33.2	1.03×10^{-6}	1.25
100	40.7	9.08×10^{-7}	1.28

[a] The kinetic constants used were as follows: $K_{Cl} = 5 \times 10^{-3}$ M; $K'_{Cl} = 0.1$ M; $k_{+1} = 10^6$ $M^{-1}s^{-1}$; $k_{-1} = 10$ s^{-1}; $k_{+2} = 5 \times 10^4$ $M^{-1}s^{-1}$; $k_{-2} = 10^3$ s^{-1}; $k'_{+1} = 10^7$ $M^{-1}s^{-1}$; $k'_{-1} = 10$ s^{-1}; $k'_{+2} = 10^7$ $M^{-1}s^{-1}$; $k'_{-2} = 1$ s^{-1}; $k_{11} = 10^4$ s^{-1}; $k_{12} = 2.5 \times 10^2$ s^{-1}; $k_{13} = 10^4$ s^{-1}; $k_{14} = 5 \times 10^2$ s^{-1}; $k_{21} = 4 \times 10^{-2}$ s^{-1}; $k_{22} = 10^{-2}$ s^{-1}; $k_{24} = 10^{-4}$ s^{-1}; $k_{25} = 10^{-5}$ s^{-1}; $k_{27} = 10^{-2}$ s^{-1}. The initial conditions were $S_0 = 4.5 \times 10^{-3}$ M and $E_0 = 10^{-9}$ M.

Supporting Information Available: Derivation of analytical expressions for the time dependence of the species involved in Scheme 1; the equations thus obtained were implemented in a computer program elaborated in QBASIC to simulate the kinetic behavior of mechanism described by Scheme 1 (7 pages). Ordering information is given on any masthead page.

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