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Theoretical Support for a Conformational Change of Polyphenol Oxidase Induced by Metabisulfite

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Polyphenol oxidases (PPO) are responsible for the oxidation of phenols into quinones that give brown or black pigments. The aim of the work presented in this paper was to study the mechanism of inhibition of palmito (*Acanthophoenix rubra*) PPO by metabisulfite. When monitored spectrophotometrically and with an oxymeter, a decrease in enzymatic activity was observed. When measured with the oxymeter, there was a decrease in oxygen comsumption, indicating enzyme inhibition. When monitored with the spectrophotometer, the existence of a lag phase was noted, indicating that the quinones formed either are reduced into phenols or react with metabisulfite to give a colorless complex. The oxidation of phenol took place during the lag phase, and when metabisulfite was depleted, the amount of oxygen was not enough to allow the oxidation of the substrate in the optimal conditions. The plot of inhibition versus metabisulfite showed an allosteric behavior of PPO with positive cooperativity. The incubation of PPO with metabisulfite resulted in inhibition of enzymatic activity. This inhibition was as strong as the concentration of metabisulfite was high. The plot of log(residual activity) versus incubation time showed a diphasic behavior. All of these results indicate that PPO inhibition by metabisulfite does not obey a first-order reaction and that the action of metabisulfite is accompanied by a conformational change of the enzyme. Accordingly, bisulfite would react with the enzyme and form an intermediate complex that is more stable than the native form. The decomposition of this complex would give an inactive enzymatic form (E_i) through a slower second step. Fitting experimental values to the equation describing a two-step denaturation model confirmed the structural changes observed by electrophoresis by other authors.

Keywords: *Acanthophoenix rubra; polyphenol oxidase; inhibition; metabisulfite*

INTRODUCTION

Polyphenol oxidases (EC 1.14.18.1, PPO) are oxidoreductases that catalyse two different reactions. First, the hydroxylation of monophenol into ortho-diphenols, which is called cresolase activity; and second, oxidation of ortho-diphenols into ortho-quinones, which is called catecholase activity (Mayer and Harel, 1979). The quinones thus formed are polymerized into brown or black pigments. These pigments are responsible for the enzymatic browning that causes commercial depreciation of fruits and vegetables (Sapers, 1993).

The first step of the reaction (formation of quinones) is reversible. Addition in the milieu of a reductor (for example, ascorbic acid) will limit accumulation of quinones (Varoquaux and Sarris, 1979; Labuza, 1992). Other reductors, like thiol compounds (L-cysteine) and sulfites, are used too. The action of ascorbic acid is restricted to the reduction of quinones into phenols, but different authors have shown a direct action of L-cysteine (Dudley *et al.*, 1989; Valero *et al.*, 1991; Robert *et al.*, 1995) and sulfites on the enzyme.

Metabisulfite exhibits a different mode of action on the enzymatic reaction; that is, it can reduce the quinone into the phenol (Walker, 1975). This compound can react with the reaction product and form a colorless compound that absorbs at 290 nm (Embs and Markakis, 1965). A direct action has also been shown by electrophoresis (Golan-Goldhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986). An hypothesis of the formation of a complex between bisulfite and enzyme resulting in an inactive enzymatic form has been established (Sayavedra-Soto and Montgomery, 1986). Metabisulfite irreversibly fixes the met and oxy forms of PPO (Valero *et al.*, 1992).

The aim of the work reported in this article was to study the inhibition mechanism of palmito (*Acanthophoenix rubra*) PPO by metabisulfite.

MATERIALS AND METHODS

Enzymatic Extract. PPO was extracted from palmito according to the method of Robert *et al.* (1996a). Palmito stem was cut into pieces in liquid N_2 and then lyophilized and stored at −20 °C until use. Ten grams of lyophilized material was suspended in 100 mL of McIlvaine's buffer (pH 6.8; containing 1.5% Triton X-100, 80 mM ascorbic acid, and 10 g of wet insoluble PVPP) for 30 s with an Ultra Turrax blender and left for 15 min. The homogenate was centrifuged (40000*g*, 40 min) at 4 °C. A precipitate [20−60% saturation in $(NH_4)_2SO_4$] was prepared from the the supernatant. The pellet formed after centrifugation was resuspended in 50 mL of 0.5 M $(NH_4)_2SO_4$, 0.5 M KCl in 50 mM sodium phosphate buffer (pH 6.5) and dialyzed overnight against the same buffer. The dialysate was loaded on a phenyl-Sepharose CL-4B column (110 × 26 mm) equilibrated with the same buffer at a flow rate of 100 mL·h^{-1}. After elution of unbound proteins by the equilibration buffer, the PPO was eluted with using 0.05 M phosphate buffer (pH 6.8), 0.1 M KCl, and 0.1 M $(NH_4)_2SO_4$ following by distilled water. The fractions eluted with 0.05 M phosphate buffer (pH 6.8), 0.1 M KCl, and 0.1 M $(NH_4)_2SO_4$ were pooled and submitted to ion-exchange chromatography (Aquapore). This column was equilibrated with citrate buffer (pH 5). During

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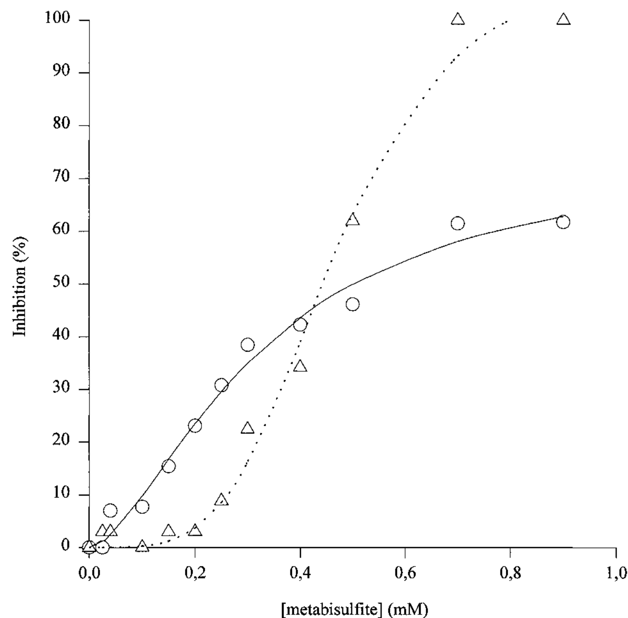


Figure 1. Inhibition of palmito PPO as a function of metabisulfite concentration. 4-Methylcatechol (20 mM, pH 5, 30 °C) was used as substrate. (○) Enzymatic activity followed with the oxymeter; (△) enzymatic activity followed with the spectrophotometer; (—) experimental values fitted to the Hill equation (oxymeter); (· · ·) experimental values fitted to the Hill equation (spectrophotometer).

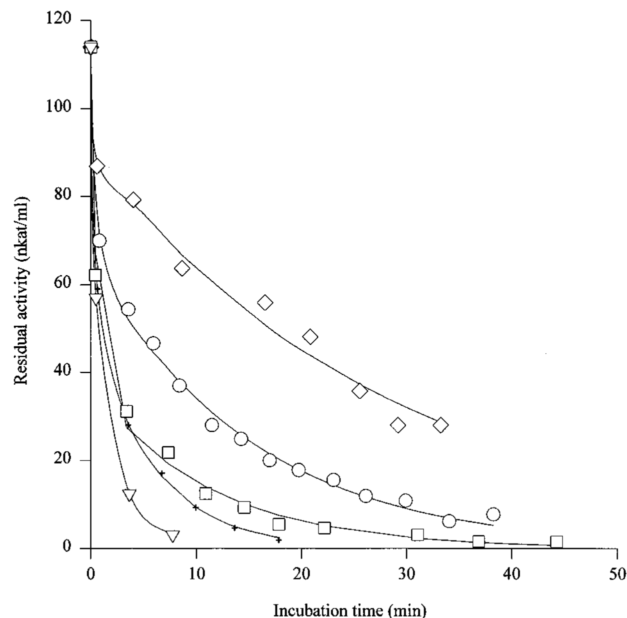


Figure 2. Residual activity versus incubation time in presence of different concentrations of metabisulfite: (◇) 0.1 mM; (○) 0.25 mM; (□) 0.3 mM; (+) 0.4 mM; (▽) 0.75 mM . 4-Methylcatechol (20 mM, pH 5, 30 °C) was used as substrate, and oxygen consumption was measured with an oxymeter.

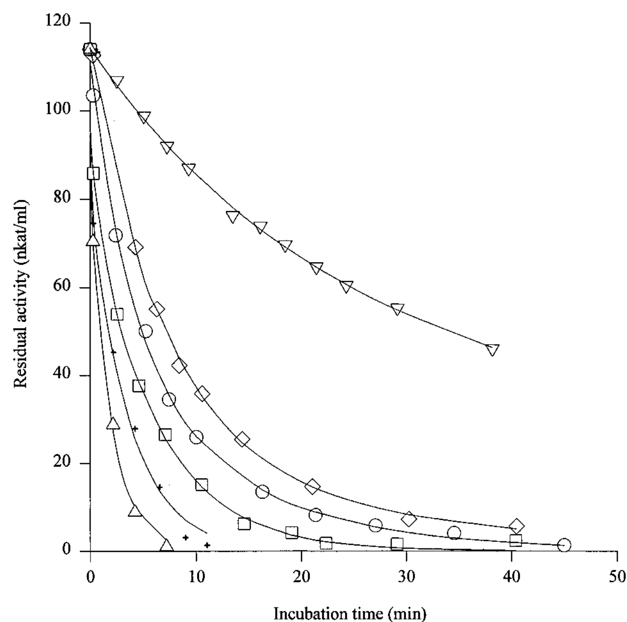


Figure 3. Residual activity versus incubation time in presence of different concentrations of metabisulfite: (▽) 0.05 mM; (◇) 0.1 mM; (○) 0.25 mM; (□) 0.3 mM; (+) 0.4 mM; (△) 0.75 mM. 4-Methylcatechol (20 mM, pH 5, 30 °C) was used as substrate, and oxygen consumption was measured with a spectrophotometer.

elution with a linear gradient (0 to 0.5 M) of $(NH_4)_2SO_4$, PPO eluted at 0.3 M $(NH_4)_2SO_4$. For all the kinetic studies, enzyme extracted after ion-exchange chromatography (25-fold purification) was used. A molecular sieving and a specific coloration after electrophoresis of the extract indicated the existence of only one PPO isoform.

Reagents. 4-Methylcatechol and potassium metabisulfite were purchased from Sigma Aldrich.

Methods. Enzymatic activity was measured spectrophotometrically at 395 nm (where the appearance of quinones is measured) and with an oxymeter (with which oxygen consumption is measured) with different concentrations of metabisulfite (0−0.9 mM). The substrate was 4-methylcatechol (20 mM) prepared in a McIlvaine's buffer at pH 5 and at 30 °C, and was saturated with air.

The enzyme was incubated with different concentrations of metabisulfite. Aliquots were taken at different incubation times and enzymatic activity was tested spectrophotometrically and with an oxymeter. The enzymatic test was the same as just described.

RESULTS AND DISCUSSION

Inhibition of Enzymatic Activity as a Function of Metabisulfite Concentration. For this experiment, the enzymatic extract was prepared in the presence of metabisulfite and the inhibition of enzymatic activity was tested with the mixture. The results obtained with the spectrophotometer and the oxymeter are presented in Figure 1. Regardless of the instrumental method used, an inhibition of the enzymatic activity was observed. The results obtained with the oxymeter showed a decrease in the oxygen consumption, indicating that the enzymatic reaction was inhibited by metabisulfite. The inhibition observed with the spectrophotometer was equivalent to that observed with the oxymeter for the concentrations of metabisulfite ranging from 0 to 0.4 mM. For example, when 0.2 mM metabisulfite was used, an enzymatic decreases of 12 and 23% were observed spectrophotometrically and with the oxymeter, respectively. For a metabisulfite concentration >0.4 mM, the inhibition observed spectrophotometrically was higher than that with the oxymeter. For example, for 0.5 mM, the inhibition obtained with the spectrophotometer was near 65% and that with the oxymeter was only 45%.

With metabisulfite, the recordings obtained with the spectrophotometer showed a lag phase; there was no increase of absorbance at 395 nm (data not shown). During this lag phase, the quinones formed are reduced to phenols (Walker, 1975) or react with metabisulfite and give a colorless compound (Embs and Markakis,

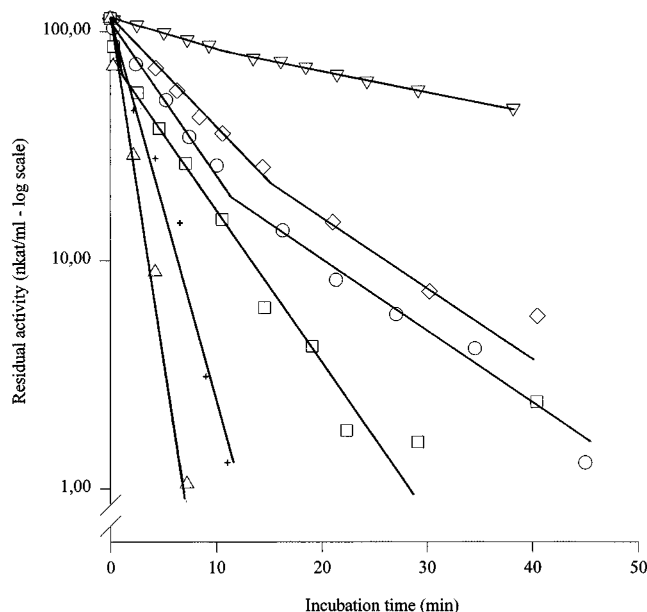


Figure 4. Residual activity (log scale) versus incubation time with different concentrations of metabisulfite: (◇) 0.1 mM; (○) 0.25 mM; (□) 0.3 mM; (+) 0.4 mM; (▽) 0.75 mM. Activity was followed spectrophotometrically.

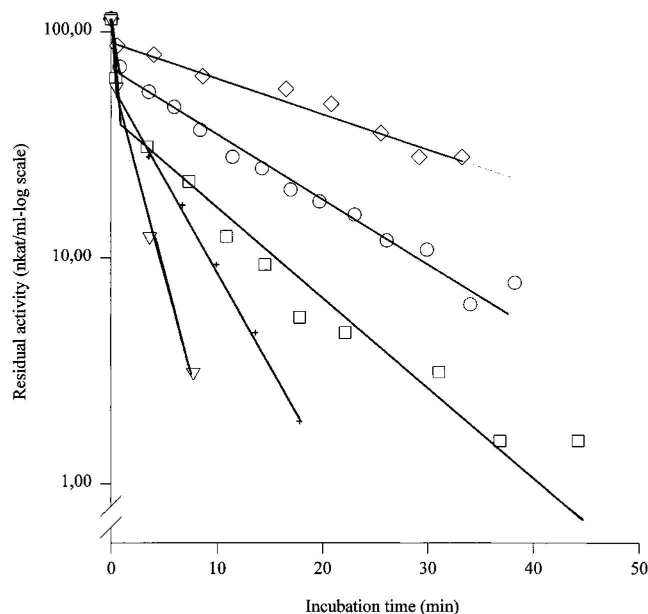


Figure 5. Residual activity (log scale) versus incubation time with different concentrations of metabisulfite: (▽) 0.05 mM; (◇) 0.1 mM; (○) 0.25 mM; (□) 0.3 mM; (+) 0.4 mM; (△) 0.75 mM. Activity was followed with an oxymeter.

1965). In these two cases, the reaction takes place and oxygen is consumed. After the lag phase, when all the metabisulfite has been used, the quinones formed can accumulate again in the milieu, but the activity measured has decreased.

The reaction stoichiometry shows that a half mole of oxygen is consumed when one mole of quinone is formed. In the hypothesis of a reaction between quinone and phenol, this reaction is equimolar (one mole of metabisulfite will react with one mole of quinone and give either the colorless complex or the original phenol; Labuza et al., 1992). The substrate used has been saturated with air at 30 °C, giving an oxygen concentration of 0.22 mM (Wise and Taylor, 1985). When 0.5 mM metabisulfite is added in the reactional milieu, 0.25 mM oxygen will be consumed during the lag phase. After the lag phase, the amount of oxygen will not be enough to allow the oxidation of the phenol in optimal conditions. That is why for metabisulfite concentrations >0.6 mM the enzymatic activity measured was nearly zero.

The plot of inhibition versus metabisulfite concentration is sigmoidal. The experimental values were fitted according to the following equation adapted from the Hill equation (Segel, 1975):

$$\% \text{ inhibition} = a[\mathrm{I}]^n/(b^n + [\mathrm{I}]^n) \quad (1)$$

In eq 1, a and b are constants, n is the Hill coefficient, and [I] is the inhibitor concentration, which in this case is metabisulfite. This kind of plot indicates that PPO with metabisulfite presents an allosteric response. The plot of inhibition shows positive cooperativity; that is, the binding of one molecule of metabisulfite induces conformational changes of the enzyme and facilitates the fixation of other molecules of inhibitor. On the other hand, this conformational change does not facilitate the fixation of the substrate.

PPO Incubation in the Presence of Metabisulfite. The effect of metabisulfite on activity was studied by incubating enzyme in the presence of metabisulfite. The enzymatic activities were measured with the oxymeter (Figure 2) and with the spectrophotometer (Figure 3). Both instrumental methods indicated an inhibition of the enzymatic activity as a function of incubation time. This inhibition was as strong as the metabisulfite concentration was high.

Table 1. Calculated Values of the Kinetic Parameters for the Metabisulfite Two-Step Inhibition Model[a]

metabisulfite, mM	R_1 (nkat/mL)	R_2 (nkat/mL)	k_1 (s^{-1})	k_2 (s^{-1})
0.1	114 ± 4.08	89.42 ± 8.39		0.0355 ± 0.00326
0.25	114 ± 1.76	65.80 ± 2.32	2.51 ± 0.50	0.068 ± 0.0030
0.3	114 ± 1.19	43.45 ± 2.25	2.98 ± 0.23	0.11 ± 0.006
0.4	114 ± 0.59	48.66 ± 1.97	2.57 ± 0.18	0.17 ± 0.007
0.75	114 ± 0	36.75 ± 0	2.44 ± 0	0.38 ± 0

[a] R_1, R_2, k_1, and k_2 were obtained by nonlinear fitting of the experimental values from oxymeter readings to eq 2.

Table 2. Calculated Values of the Kinetics Parameters for the Metabisulfite Two-Step Inhibition Model[a]

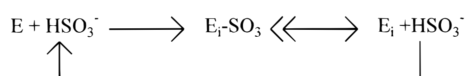
metabisulfite, mM	R_1 (nkat/mL)	R_2 (nkat/mL)	k_1 (s^{-1})	k_2 (s^{-1})
0.05	114.3 ± 0.59	50.17 ± 32.6	0.057 ± 0.031	0.012 ± 0.012
0.1	115.1 ± 0.84	17.42 ± 9.50	0.15 ± 0.019	0.039 ± 0.019
0.25	111.6 ± 1.39	35.07 ± 15.49	0.27 ± 0.074	0.082 ± 0.020
0.3	114.00 ± 1.21	78.31 ± 2.90	4.52 ± 0.83	0.16 ± 0.007
0.4	114 ± 2.37	78.34 ± 10.09		0.27 ± 0.027
0.75	114 ± 2.38	74.21 ± 28.07		0.48 ± 0.10

[a] R_1, R_2, k_1, and k_2 were obtained by nonlinear fitting of the experimental values from spectrophotometer readings to eq 2.

The process of inhibition can be divided into two parts. In the first part, the inhibition seemed to be fast, and the second part, the inhibition was slower. For example, when 0.25 mM metabisulfite was followed spectrophotometrically (Figure 3), 70% inhibition was observed during the first 10 first min, whereas only 26% inhibition was noted from 10 to 30 min of incubation. The same observation was made when the activity was followed with the oxymeter. Thus, for the same concentration of metabisulfite, there was 60% inhibition in the first 6 min and only 32% inhibition between 6 and 30 min of incubation. The inhibition observed when followed with the spectrophotometer is stronger than the inhibition obtained with the oxymeter. The explanation just given relative to the existence of the lag phase is valid again here, and this phenomenon in addition to the direct inhibition (inactivation after covalent modification with sulfite) of the enzyme by metabisulfite resulted in more inhibition.

When log(residual activity) was plotted versus incubation time (Figures 4 and 5), it was impossible to draw a staight line through the experimental points. Therefore, the inhibition kinetics do not obey a first-order reaction.

The inhibition of enzymatic activity by metabisulfite and incubation of the enzyme in the presence of metabisulfite indicate that PPO inhibition doesn't obey a first-order reaction and that the action of metabisulfite is accompanied by a structural modification of the enzyme. The following mechanism of inhibition has been proposed by Sayavedra-Soto and Montgomery (1986):



In this mechanism, bisulfite would form a complex with the enzyme. The decomposition of this complex would give an inactive enzymatic form (E_i) through a slow second step, which would regenerate metabisulfite.

Studies published for thermal denaturation (Nury *et al.*, 1989; Violet et Meunier, 1989; Robert *et al.*, 1995) and L-cysteine inhibition (Robert *et al.*, 1996b) used eq 1; we have used this equation in the case of inhibition of PPO by metabisulfite. Equation 2 describes the following two-step denaturation model in which N, X, and D are the native, intermediate, and denatured forms of the enzyme, respectively, and k_1 and k_2 are deactivation constants:

$$\mathrm{N} \xrightarrow{k_1} \mathrm{X} \xrightarrow{k_2} \mathrm{D}$$

$$R = \left(R_1 - \frac{R_2 k_1}{k_1 - k_2}\right) e^{-k_1 t} + \left(R_2 \frac{k_1}{k_2 - k_1}\right) e^{-k_2 t} \qquad (2)$$

The fitted values obtained with eq 2 are near the experimental values (Figures 2 and 3). The fitted values are presented in Tables 1 and 2. The value of R_1, which is the specific activity of the native enyzmatic form, is dependent on the concentration of metabisulfite. On the other hand, R_2 varied with inhibitor concentration, suggesting the existence of more than one intermediate form, the structure of which is dependent on the experimental conditions.

Previous studies of PPO inhibition by metabisulfite have shown a modifiction of the ionization state and of the structure after incubation (Sayavedra-Soto and Montgomery, 1986). In this study, we demonstrated by calculation the existence of an intermediate compound that appeared during the inhibition process. Our theorical study of palmito PPO inhibition by metabisulfite confirm the changes observed by electrophoresis by Sayavedra-Soto and Montgomery (1986) and Golan-Goldhirsh and Whitaker (1984). The intermediate form could be the complex formed between enzyme and metabisulfite according to the model proposed by Sayavedra-Soto and Montgomery (1986).

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