ARTICLES

Studies on Inhibition of Mushroom Polyphenol Oxidase Using Chlorogenic Acid as Substrate

Selim Kermasha,* Mireille Goetghebeur, and Alan Monfette

Department of Food Science and Agricultural Chemistry, McGill University, Mcdonald Campus, 21111 Lakeshore, Ste Anne de Bellevue, Québec, Canada H9X 3V9

Maurice Metche and Bernard Rovel

Laboratoire de Biochimie Appliquée, ENSAIA, 2 Avenue de la Forêt de Haye, 54505 Vandoeuvre Cedex, France

The inhibitory effects of aromatic acids of the benzoic, cinnamic, and phenylalkanoic series on the enzymatic activity of a commercial purified mushroom polyphenol oxidase (PPO), using chlorogenic acid as a substrate, were determined by spectrophotometric and polarographic methods. The I_{50} values obtained were higher when determined by the polarographic method. Hydroxylation and methylation of the benzene ring as well as the addition of a second methylene group adjacent to the carboxylic acid decreased the inhibitory effect. Aromatic acids of the benzoic and cinnamic series appeared to have, respectively, an irreversible and most often a reversible inhibitory effect on PPO activity. The results also showed that the aromatic acids exhibited noncompetitive, mixed, and uncompetitive types of inhibition, with a range of K_i values varying from 0.26 to 56.7 mM. Spectrophotometric and polarographic methods failed to determine the type of inhibition for protocatechuic, caffeic, hydroxyphenylacetic, and hydroxyphenylpropionic acids. Benzoic, *o*-coumaric, *m*-coumaric, ferulic, phenylacetic, and phenylpropionic acids demonstrated different types of inhibition depending upon the method used. The results indicated that the type and degree of inhibitory effect of aromatic carboxylic acids are dependent upon both inhibitor and method used.

INTRODUCTION

Polyphenol oxidases (PPO) (EC 1.14.18.1) are enzymes belonging to the group of oxidoreductases which catalyze the hydroxylation of monophenols and the oxidation of *o*-diphenols to quinones (Mayer, 1987). Unfavorable browning of many food products has been of great concern to food technologists and processors. Although the darkening of food products is innocuous to consumers, it causes a decrease in market value and an economic loss (Walker, 1975; Chen et al., 1991).

The control of enzymatic browning has always been a challenge to the food-processing industry. The most widely used inhibitors are sulfites. However, the use of chemical agents as food additives is being reevaluated by the Food and Drug Administration (FDA) and, in some products, banned from use (Chen et al., 1991). Carboxylic acids of the cinnamic series were demonstrated to be inhibitors for the PPO activities of various origins such as potatoes (Macrae and Duggleby, 1968), cherries (Pifferi et al., 1974), and apples (Walker and Wilson, 1975). Walker (1969) suggested that *p*-coumaric and ferulic acids formed by the soft rot organism *Penicillium expansum* might account for the light color of the infected apple tissue. Benzoic acid and its derivatives showed an inhibitory effect on PPO activity in mushroom (Kuttner and Wagreich, 1953) and grape (Gunata et al., 1987). Kruegger (1955) and Janovitz-Klapp et al. (1990) reported various inhibitory effects of phenylalkanoic acids on PPO activity. The literature also indicates that the type and degree of inhibition of aromatic carboxylic acids on PPO activity are dependent on the structure of both substrate and inhibitor (Duckworth and Coleman, 1970; Jesus-Rivas et al., 1973; Walker and McCallion, 1980; Batitusti and Lourenço, 1985). Studies on the inhibitory effect of aromatic carboxylic acids on PPO activity were carried out by measuring either the absorbance of end-products (Pifferi et al., 1974; Batistuti and Lourenço, 1985) or the oxygen uptake (Gunata et al., 1987; Janovitz-Klapp et al., 1990). However, Golan-Goldhirsh and Whitaker (1984) reported that the nature of the effect of some reductants on mushroom PPO was dependent on the substrate and the assay method. Chlorogenic acid, which is one of the major phenolic compounds found in fruits and vegetables, was often used as substrate for the inhibition studies of PPO (Oszmianski and Lee, 1990).

The aim of this study was to investigate the inhibitory effect of aromatic carboxylic acids on the oxidation of chlorogenic acid by a commercial purified mushroom tyrosinase activity, using comparative spectrophotometric and polarographic methods.

MATERIALS AND METHODS

Source of Enzyme. A commercial purified mushroom tyrosinase (Sigma Chemical Co., St. Louis, MO), with an activity of 2200 units/mg, was used throughout this study. One unit of enzyme activity is defined as an increase in absorbance, at 280 nm, of 0.001/min at pH 6.5 and at 25 °C in a reaction mixture containing L-tyrosine.

Enzyme Assays. The enzymatic assays were performed using two experimental methods, spectrophotometric and polaro-

* Author to whom correspondence should be addressed.

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graphic. The reaction mixtures had final volumes of 3 and 2 mL, respectively, for the spectrophotometric and polarographic assays containing tyrosinase (12 units/mL), chlorogenic acid (0.2–5.0 mM), and sodium phosphate buffer (pH 6.0, 0.1 mM).

The spectrophotometric assay was performed according to a modification of the method described by Oda et al. (1989). The spectrophotometric assay was carried out at 25 °C using a Beckman DU-65 spectrophotometer; the enzyme activity was followed by the increase in absorbance at 420 nm. The polarographic assay was carried out at 25 °C according to a modification of the procedure described previously by Okamoto et al. (1988) using a Gilson oxygraph equipped with a Clark electrode (Kermasha and Metche, 1986); the enzyme activity was followed by the oxygen uptake. The enzyme specificity was expressed as micromoles of product per minute per one enzyme unit.

Inhibition Studies. The inhibitory effects of a series of aromatic acids (benzoic acid, protocatechuic acid, gallic acid, *o*-coumaric acid, *m*-coumaric acid, caffeic acid, ferulic acid, phenylacetic acid, phenylpropionic acid, hydroxyphenylacetic acid, and hydroxyphenylpropionic acid) were investigated. Inhibitor concentrations varied from 1 to 10 mM. The enzyme assays were performed spectrophotometrically and by polarograph according to the procedures described above. The enzymatic reaction was initiated by the addition of the PPO to the temperature-equilibrated reaction mixture.

Inhibition reversibility or irreversibility was determined according to a mathematical method (Goetghebeur et al., 1992) which involves a plot of $v_0/(v_0 - v_i)$ vs $1/[I]$, where v_0 is the initial velocity without the presence of the inhibitor and v_i is the initial velocity but in the presence of the inhibitor. In such a graphic representation, a plot with a straight line that intercepts, by extrapolation, the y axis at a value of less than one or equal to and higher than one indicates, respectively, irreversible and reversible inhibitions. This mathematical determination should indicate the trend of the inhibition whether it is reversible or irreversible. Kinetic studies were determined according to Lineweaver and Burk (1934).

RESULTS AND DISCUSSION

To determine the absence or presence of contaminating oxidizing enzymatic activities, the commercially purified tyrosinase was examined using two specific substrates for laccase activity, 3,5-dimethoxy-4-hydroxybenzaldehyde azine (syringaldazine) (Dubourdieu et al., 1984) and 2,6-dimethoxyphenol. The results indicated that the commercial purified tyrosinase, used throughout this study, contains negligible laccase activity (0.03–0.4%).

The effects of aromatic acids of the benzoic, cinnamic, and phenylalkanoic series on PPO activity were determined spectrophotometrically and by the uptake of O_2.

Effect of Benzoic Acid and Its Derivatives on PPO Activity. The effect of different concentrations of each inhibitor varying from 1 to 10 mM are reported in Figure 1. Using both spectrophotometric and polarographic methods, the results indicate that benzoic acid was the strongest inhibitor (Figure 1A), whereas the hydroxylation of the benzene ring (Figure 1B,C) decreased its inhibitory effect. These results are in agreement with those reported by Pifferi et al. (1974) and Gunata et al. (1987), respectively, for sweet cherry and grape PPO. Pifferi et al. (1974) suggested that the decrease in the inhibitory effect of substituted benzoic acids on PPO activity may be due to steric hindrance.

The values of I_{50} (Table I) obtained for carboxylic acids of the benzoic series were higher when determined by the oxygen uptake method compared to those measured by the increase in absorbance; these findings are especially true for gallic acid and to a lesser extent for protocatechuic acid. A possible explanation is that consumption of oxygen would be inhibited in a different way from product formation, depending both on the location of the inhibitor site and on its structure. Pifferi et al. (1974) suggested

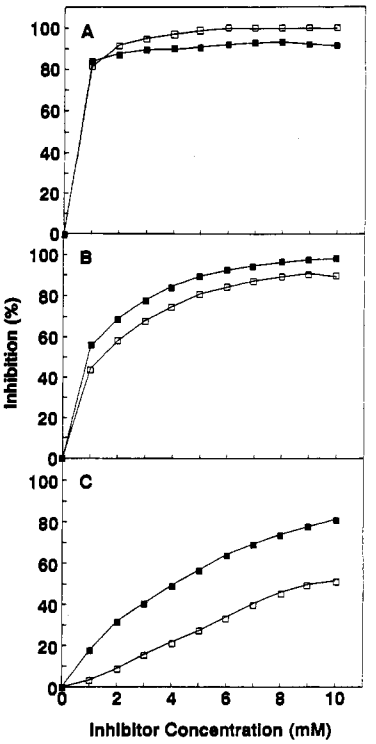

Figure 1. Inhibitory effect of concentrations of benzoic acid and its derivatives on the oxidation of chlorogenic acid by mushroom PPO, measured spectrophotometrically (■) and by oxygraph (□): (A) benzoic acid, (B) protocatechuic acid, and (C) gallic acid.

the existence of various interactions between the inhibitory sites and the active site.

The mathematical determination (Goetghebeur et al., 1992) of the reversibility or the irreversibility of the inhibitory effects of carboxylic acids indicates (Table I) that, using both spectrophotometric and polarographic methods, benzoic acid and its derivatives appeared to have an irreversible inhibitory effect on mushroom PPO activity.

Table I also shows that, using the polarographic method, benzoic acid was a noncompetitive inhibitor for PPO activity. These results suggest that benzoic acid binds at a different site than chlorogenic acid and that the substrate–inhibitor–enzyme complex is inactive; this inhibitor may also prevent the proper positioning of the catalytic center (Segel, 1976). However, using the spectrophotometric method, benzoic acid showed an uncompetitive inhibitory effect, which suggests that the inhibitor binds only to the enzyme–substrate complex which results in the formation of an inactive complex (Segel, 1976). Pifferi et al. (1974) showed that benzoic acid was determined to be a noncompetitive inhibitor of the mixed type when the enzymatic assay was performed by a spectrophotometric method. However, benzoic acid showed a competitive inhibitory effect on the oxidation of catechol by PPO when the enzyme activity was measured spectrophotometrically (Duckworth and Coleman, 1970) and with an oxygen electrode (Jesus-Rivas and Whitaker, 1973). The results (Table I) show that the K_i values for benzoic acid were significantly higher (4.1 times) when the enzymatic assay was performed spectrophotometrically compared to those determined by the polarographic method. These findings indicate that K_i values are dependent on whether the enzyme assays were performed as a determination of the end products (spectrophotometer) or the oxygen uptake (polarograph). Using catechol as substrate, Duckworth and Coleman (1970) reported a K_i value of 1 μM for benzoic acid with the spectropho-

Table I. Inhibitory Effect of Benzoic Acid and Its Derivatives on Mushroom Polyphenol Oxidase Using Chlorogenic Acid as Substrate

kinetic parameter	method[a]	inhibitor		
		benzoic acid	protocatechuic acid	gallic acid
I_{50},[b] mM	spectrophotometer	0.60	1.12	4.27
	polarograph	0.61	1.53	9.30
reversibility[c]	spectrophotometer	irreversible	irreversible	irreversible
	polarograph	irreversible	irreversible	irreversible
K_i,[d] mM	spectrophotometer	0.26	*e*	14.8
	polarograph	1.16	*e*	56.7
type of inhibition	spectrophotometer	uncompetitive	*f*	mixed
	polarograph	noncompetitive	*f*	mixed

[a] Two methods of determination of enzymatic activity were used. [b] Inhibitor concentration that yields 50% inhibition. [c] Mathematical determination of the reversibility or irreversibility of the inhibitory effect (Goetghebeur et al., 1992). [d] Inhibition dissociation constant. [e] Uncalculable. [f] Undefined type of inhibition.

Table II. Inhibitory Effect of Aromatic Carboxylic Acids of the Cinnamic Series on Mushroom Polyphenol Oxidase Using Chlorogenic Acid as Substrate

kinetic parameter	method[a]	inhibitor			
		o-coumaric acid	*m*-coumaric acid	caffeic acid	ferulic acid
I_{50},[b] mM	spectrophotometer	0.73	*c*	0.98	0.71
	polarograph	1.12	0.66	5.00	0.85
reversibility[d]	spectrophotometer	reversible	*c*	reversible	reversible
	polarograph	reversible	reversible	reversible	irreversible
K_i,[e] mM	spectrophotometer	*f*	0.29	*f*	*f*
	polarograph	2.70	0.68	*f*	1.69
type of inhibition	spectrophotometer	*g*	uncompetitive	*g*	*g*
	polarograph	mixed	uncompetitive	*g*	noncompetitive

[a] Two methods of determination of enzymatic activity were used. [b] Inhibitor concentration that yields 50% inhibition. [c] Uncalculable because of a weak inhibitory effect. [d] Mathematical determination of the reversibility or irreversibility of the inhibitory effect (Goetghebeur et al., 1992). [e] Inhibition dissociation constant. [f] Uncalculable. [g] Undefined type of inhibition.

tometric method, whereas Janovitz-Klapp et al. (1990) indicated a K_i value of 640 μM with the polarographic method.

The results (Table I) indicate that both methods failed to demonstrate the nature of inhibition of PPO activity by protocatechuic acid when chlorogenic acid was used as substrate. These findings may suggest the presence of a complex inhibition phenomenon that may involve various types of interactions and interference. On the other hand, Batistuti and Lourenço (1985) showed that protocatechuic acid demonstrated, spectrophotometrically, a noncompetitive inhibitory effect on potato PPO activity when chlorogenic acid was used as substrate; these authors suggested that the substrate and the inhibitor may bind at different sites on the enzyme molecule.

Gallic acid demonstrated (Table I) a noncompetitive inhibition of the mixed type for the PPO activity, using both polarographic and spectrophotometric methods. These results suggest that gallic acid reduces the affinity of the substrate for the enzyme, yet it does not bind at the active site. These findings may also indicate that the dissociation of the enzyme–substrate complex was prevented (Segel, 1976).

Overall, these results and those reported in the literature suggest the presence of different inhibitory effects of aromatic carboxylic acids of the benzoic series on PPO activity. The differences in type and degree of inhibition between benzoic acid and its derivatives may be related to various interactions of these compounds with the enzyme.

Effect of Aromatic Carboxylic Acids of the Cinnamic Series on PPO Activity. The results (Table II; Figure 2) show that the degree of inhibition of PPO activity by aromatic acids of the cinnamic series decreased in the order *m*-coumaric acid > ferulic acid > *o*-coumaric acid > caffeic acid. However, I_{50} values were higher when the enzymatic assays were determined by oxygen uptake compared to those performed spectrophotometrically. The different inhibitory effects obtained for *m*-coumaric (Figure 2A) and *o*-coumaric acid (Figure 2B) may be related to a steric hindrance, due to the ortho substitution (Pifferi et al., 1974). The results (Figure 2B–D) indicate that the addition of a methoxy group to the benzene ring decreased the inhibitory effect of the substituted cinnamic acid but to a lesser extent than that resulting from the addition of a hydroxy group; this suggests that the inhibitory effect of the substituted cinnamic acids could be dependent on

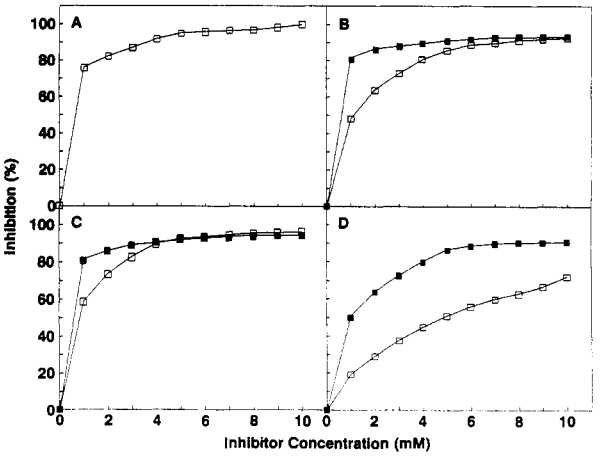

Figure 2. Inhibitory effect of concentrations of aromatic acids of the cinnamic series on the oxidation of chlorogenic acid by mushroom PPO, measured spectrophotometrically (■) and by oxygraph (□): (A) *m*-coumaric acid, (B) *o*-coumaric acid, (C) ferulic acid, and (D) caffeic acid.

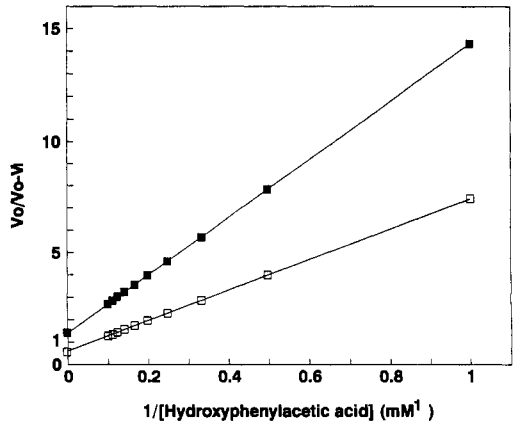

Figure 3. Determination of the reversibility or the irreversibility of the inhibitory effect of ferulic acid on the oxidation of chlorogenic acid by mushroom PPO. Shown is a plot of $v_0/(v_0 - v_i)$ vs 1/[I] (where v_0 is the initial velocity without inhibitor and v_i is the initial velocity but in the presence of the inhibitor): irreversible inhibition with polarographic method (□) and reversible inhibition with spectrophotometric method (■).

the number and the nature of the added groups on the benzene ring. Pifferi et al. (1974) reported that the addition of electron-attracting or electron-donating groups on the benzene ring of carboxylic acids, respectively, increased or decreased the inhibitory effect on cherry PPO activity.

The inhibitory effect of caffeic acid (Figure 2D), measured by the oxygen uptake method, was found to be much lower than that determined by the spectrophotometric method. The high uptake of oxygen could be explained by an autoxidation phenomenon of caffeic acid (Cilliers and Singleton, 1990).

Using both spectrophotometric and polarographic methods, the mathematical determination (Goetghebeur et al., 1992) suggests (Table II) that *o*-coumaric, *m*-coumaric, and caffeic acids have a reversible inhibitory effect on PPO activity. However, the results (Table II; Figure 3) suggest that ferulic acid has reversible and irreversible inhibitory effects on PPO activity, determined, respectively, by the spectrophotometer and by the polarograph.

The results (Table II) show that *o*-coumaric acid exhibited a noncompetitive inhibition of the mixed type with the polarographic method, whereas the spectrophotometric method failed to determine the type of inhibition. Walker (1969) reported that *o*-coumaric acid demonstrated a noncompetitive inhibitory effect with a K_i value of 2.70 mM. Table II also shows that, using both spectrophotometric and polarographic methods, *m*-coumaric acid was an uncompetitive inhibitor for the enzymatic oxidation of chlorogenic acid. The K_i values for *m*-coumaric acid (Table II) were found to be 0.29 and 0.68 mM, respectively, for the spectrophotometric and polarographic methods. However, Walker and Wilson (1975) reported that *m*-coumaric acid demonstrated a noncompetitive inhibitory effect on the oxidation of chlorogenic acid by apple PPO, with a K_i value of 0.2 mM.

Using the polarographic method, the ferulic acid demonstrated (Table II) a noncompetitive inhibitory effect on PPO activity, whereas the spectrophotometric method failed to determine the type of inhibition. These results are partially in agreement with those reported by Walker and Wilson (1975) and Batistuti and Lourenço (1985), who showed that ferulic acid exhibited, respectively, noncompetitive and mixed-type inhibitory effects on potato and apple PPO. However, Macrae and Duggleby (1968) and Gunata et al. (1987) reported that ferulic acid has a competitive inhibitory effect, respectively, on potato and grape PPO activities.

The results (Table II) indicate that both methods failed to determine the type of inhibition of PPO by caffeic acid. However, caffeic acid was considered as a substrate rather than an inhibitor for the PPO activity (Hyodo and Huritani, 1965; Okamoto et al., 1988; Sachde et al., 1989). The double nature of caffeic acid, i.e., as a substrate and as an inhibitor, could explain the failure to determine the type of inhibition.

Effect of Aromatic Carboxylic Acids of the Phenylalkanoic Series on PPO Activity. The results (Table III; Figure 4A) show that phenylacetic acid has a strong inhibitory effect on chlorogenic acid oxidation by mushroom PPO. The I_{50} values obtained with phenylacetic acid are close to those obtained with benzoic acid (Table I). Krueger (1955) reported a strong inhibitory effect of phenylacetic acid on the oxidation of *p*-cresol by mushroom PPO; however, Janovitz-Klapp et al. (1990) showed that phenylacetic acid had a weak inhibitory effect on the oxidation of methylcatechol by apple PPO. The results (Table III; Figure 4A,B) also show that the presence of a second methylene group between the benzene ring and the carboxylic group of phenylacetic acid (e.g., phenylpropionic acid) decreased the degree of inhibition of PPO activity. Moreover, the results (Figure 4A,C) show that the hydroxylation of phenylacetic acid (e.g., hydroxyphenylacetic acid) also decreased the degree of inhibition of PPO. The results (Figure 4D) demonstrate that the combined presence of a second methylene group between the benzene ring and the carboxylic group and the hydroxylation of the ring (e.g., hydroxyphenylpropionic acid) resulted in the lowest inhibitory effect on mushroom PPO activity.

Using both spectrophotometric and polarographic methods, phenylacetic and phenylpropionic acids (Table III) appear to have, respectively, reversible and irreversible inhibitory effects on PPO activity. However, by using the polarographic method both acids show (Table III) a noncompetitive mixed-type inhibitory effect on PPO activity. Although the spectrophotometric assay showed that phenylpropionic acid exhibited (Table III) an uncompetitive inhibition of PPO activity, it failed to demonstrate the nature of inhibition by phenylacetic acid. On the other hand, both methods failed to demonstrate the nature of the inhibitory effects of hydroxyphenylacetic acid hydroxyphenylpropionic acids on PPO activity. Macrae and Duggleby (1968) reported that hydroxyphenylpropionic acid was used as a substrate for the potato PPO activity, whereas Janovitz-Klapp et al. (1990) reported that phenylacetic and hydroxyphenylpropionic acids demonstrated a competitive inhibitory effect on grape PPO activity. The double nature of hydroxyphenylpropionic acid as substrate and as inhibitor may be related to a complex kinetic phenomenon.

The various results obtained in our laboratory suggest that the decrease in the inhibitory effects resulted from the addition of a second methylene group adjacent to the carboxylic group and from the methylation and the hydroxylation of aromatic carboxylic acids may be related to steric interferences in the interaction between the benzene ring and the enzyme. Pifferi et al. (1974) reported the presence of a very low inhibitory effect of aliphatic acids on PPO activity and suggested that this may be related to the absence of the benzene ring. These authors concluded then that the benzene ring of the aromatic carboxylic acids may have an important role in the inhibition mechanism.

Table III. Inhibitory Effect of Aromatic Carboxylic Acids of the Phenylalkanoic Series on Mushroom Polyphenol Oxidase Using Chlorogenic Acid as Substrate

		inhibitor			
kinetic parameter	method	phenylacetic acid	phenylpropionic acid	hydroxyphenylacetic acid	hydroxyphenylpropionic acid
I_{50},[b] mM	spectrophotometer	0.65	1.94	3.66	7.63
	polarograph	0.81	2.20	*c*	*c*
reversibility	spectrophotometer	reversible	irreversible	irreversible	*c*
	polarograph	reversible	irreversible	*c*	*c*
K_i,[e] mM	spectrophotometer	*f*	2.33	*f*	*f*
	polarograph	1.38	5.22	*f*	*f*
type of inhibition	spectrophotometer	*g*	uncompetitive	*g*	*g*
	polarograph	mixed	mixed	*g*	*g*

[a] Two methods of determination of enzymatic activity were used. [b] Inhibitor concentration that yields 50% inhibition. [c] Uncalculable because of a weak inhibitory effect. [d] Mathematical determination of the reversibility or irreversibility of the inhibitory effect (Goetghebeur et al., 1992). [e] Inhibition dissociation constant. [f] Uncalculable. [g] Undefined type of inhibition.

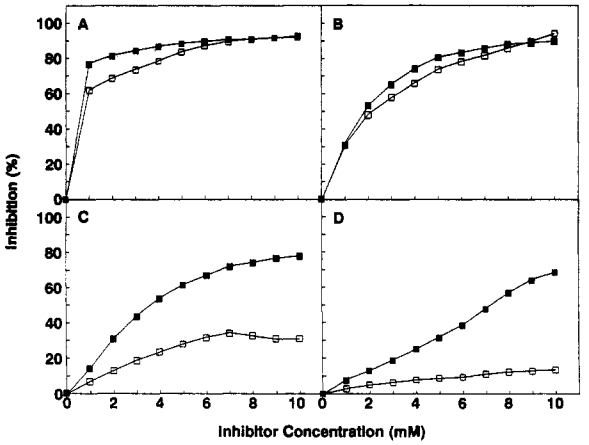

Figure 4. Inhibitory effect of concentrations of aromatic acids of the cinnamic series on the oxidation of chlorogenic acid by mushroom PPO measured spectrophotometrically (■) and by oxygraph (□): (A) phenylacetic acid, (B) phenylpropionic acid, (C) hydroxyphenylacetic acid, and (D) hydroxyphenylpropionic acid.

The differences in type and degree of inhibition of PPO activity, demonstrated by the change in the method used, may be related to the complex structure of the PPO molecule. Lerch (1987) reported that PPO enzymes contain a coupled copper pair which is the active site of interaction with both molecular oxygen and organic substrates. Pifferi et al. (1974) indicated that PPO enzymes may contain inhibitory sites which are distinct from active sites; these authors suggested the presence of various interactions between the different sites.

The spectrophotometric and polarographic methods have basically different principles in measuring the enzyme activity, since the first is a determination of the absorbance of end products, whereas the second is a measurement of the oxygen uptake. The differences in results obtained by the two methods suggest the presence of a complex kinetic phenomenon which may involve various interactions between active site and inhibitory sites.

The discrepancies between our results and those reported in the literature, as well as the conflicting results among different authors, may be related to differences in the conditions of the assays. Another possible explanation may also be the differences in PPO molecular structure (Lerch, 1987), which could lead to various interactions between active site and inhibitory sites.

Conclusion. The results gathered in this study indicate that the type and degree of inhibitory effects of aromatic carboxylic acids on mushroom PPO activity are dependent upon both inhibitor and method used, spectrophotometer or polarograph. The presence of an additional methylene group adjacent to the carboxylic group as well as the hydroxylation or the methylation of the benzene ring decreased the inhibitory effect of aromatic carboxylic acids. The aromatic carboxylic acids showed various trends with regard to the reversibility or the irreversibility of inhibition. Moreover, these inhibitors exhibited noncompetitive, mixed, and uncompetitive types of inhibition. The results gathered in this study indicate that the inhibition of PPO by aromatic carboxylic acids is a complex phenomenon.

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