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Inhibition of palmito (*Acanthophoenix rubra*) polyphenol oxidase by carboxylic acids

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The inhibition of palmito (*Acanthophoenix rubra*) polyphenol oxidase (PPO) is reported. Recently, two forms of palmito PPO were partially purified by hydrophobic chromatography. Inhibitory effects of various carboxylic acids on these two forms have been studied. Both forms showed identical behaviour towards the inhibitors studied. Cinnamic acid was found to have the greatest inhibitory effect ($K_i = 0{\cdot}06$ mM). When the inhibitory effects of acids from the benzoic acid family and from the cinnamic acid family were compared, it was found that acids which possess a double bond between the benzene ring and the carboxylic function showed the highest inhibitory effect. This inhibitory effect was decreased by substitutions on the benzene ring.

The influence of pH on the inhibitory effect of carboxylic acids on palmito PPO has also been investigated. K_i decreased with a decrease in pH. This effect is due to the fact that it is only the undissociated form (AH) of carboxylic acids that is responsible for inhibition of PPO. Inhibition constants (K_i) for the AH form have been recalculated and were found to remain constant in the pH range studied (for benzoic acid $K_i = 0{\cdot}14$ mM, for cinnamic acid $K_i = 0{\cdot}019$ mM, for sorbic acid $K_i = 0{\cdot}15$ mM).

INTRODUCTION

Polyphenol oxidases (PPO), in particular catecholases (EC 1.10.3.1), are the enzymes that cause enzymatic browning in the heart of palmito, *Acanthophoenix rubra* (Robert *et al.*, 1996*a*). These enzymes catalyse both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones, which polymerize to form brown or black pigments (Mayer & Harel, 1979; Vamos-Vigyazo, 1981; Burton, 1994; Griffith, 1994).

PPO are inhibited by various substances, among which are aromatic carboxylic acids. The inhibitory character of these compounds is linked to the presence of the benzene ring (Pifferi *et al.*, 1974). Inhibition by members of the benzoic and cinnamic acid series has previously been investigated (Walker, 1976; Gunata *et al.*, 1987; Janovitz-Klapp *et al.*, 1990; McEvily *et al.*, 1992; Kermasha *et al.*, 1993), and it was found that better inhibition was obtained when the –COOH group was directly attached to the benzene ring, while hydroxylation, methylation or esterification considerably decreased the inhibitory effects of these acids. However, the type of inhibition was found to depend on the substrate and the enzyme used, competitive, non-competitive or mixed-type inhibition having been obtained. Whatever the type of inhibition, the acid inhibitor attaches to a site different from the active site and hinders the binding of substrate to the enzyme through steric hindrance or by changing the protein conformation (Pifferi *et al.*, 1974).

We have previously studied the detailed inhibitory effects of cysteine (Robert *et al.*, 1996*b*) on palmito PPO. The aim of this paper is to establish a relationship between the structure of the molecule and its inhibitory effect and to study the effect of pH on this inhibition.

MATERIALS AND METHODS

Substrates and reagents were supplied by Sigma (St Louis, MO); phenyl-Sepharose CL4B was from Pharmacia.

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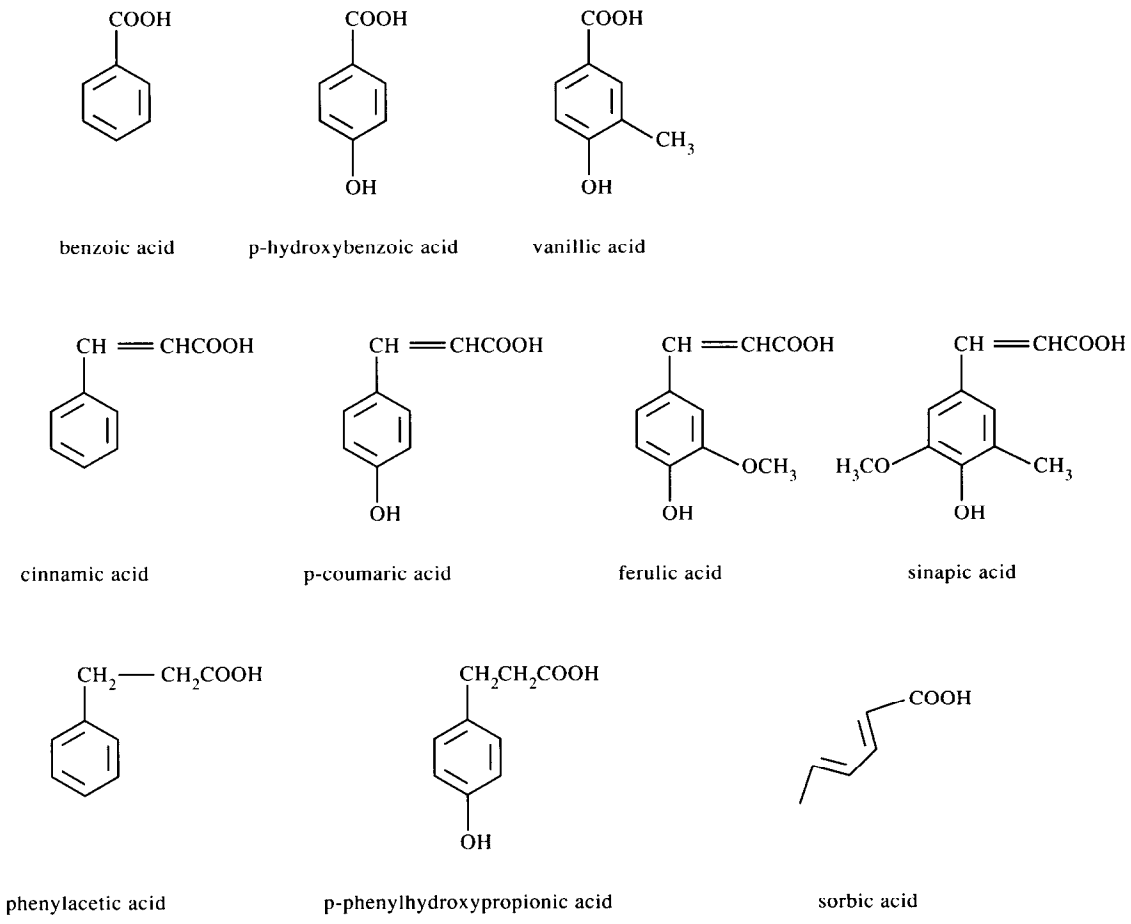

Fig. 1. Chemical structures of the carboxylic acid palmito PPO inhibitors used in the study.

PPO activity assay

For routine analysis, the substrate was 4-methylcatechol (20 mM) in 3 ml of a McIlvaine buffer solution at pH 5. PPO activity was assayed polarographically with a Clark oxygen electrode using an air-saturated substrate solution at 30°C. The rate of the reaction was calculated from the initial slope of the progress curve. Whenever there was a lag phase, the activity was calculated after this phase. The activity was expressed as nanomoles of oxygen consumed per second (nkat).

Inhibition studies were assayed at pH 5 with 4-methylcatechol concentration varying from 10 to 0·4 mM, without inhibitor and in the presence of three inhibitor concentrations. The effect of pH on inhibition was studied with benzoic acid, cinnamic acid and sorbic acid as inhibitors. Throughout the study, the same range of substrate concentrations was used and the inhibitor concentration was close to the K_i^{app} value at pH 5. All measurements were done in duplicate.

K_m and V_m values were determined using a non-linear regression data analysis program for IBM PC (Sigma Plot, Jandel Scientific).

Protein assay

Protein concentration was determined with the Bio-Rad reagent according to Bradford (1976). Absorbance at 280 nm was used to monitor protein in the column eluates.

Extraction and purification of PPO

The palmito was harvested 1 day before extraction. Palmito stem was cut into pieces in liquid N_2 and then lyophilized and stored at −20°C until use.

A 10 g portion of lyophilized material was suspended in 100 ml of McIlvaine buffer at pH 6·8 containing 1·5% Triton X-100, 80 mM ascorbic acid and 10 g of wet polyvinylpolypyrrolidone for 30 s using an Ultra Turrax blender and then left for 15 min. The homogenate was filtered through eight layers of cheesecloth and centrifuged (40 000*g*, 40 min) at 4°C; the supernatant was used as the crude extract.

$(NH_4)_2SO_4$ was added to the supernatant to 20% saturation. After centrifugation (40 000*g*, 15 min, 4°C), $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation. The resulting pellet was resuspended in

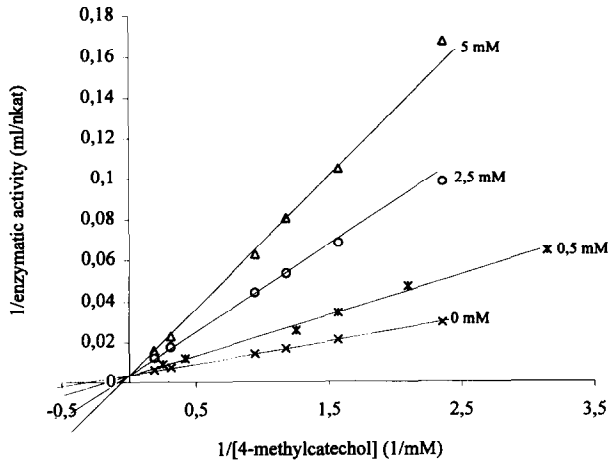

Fig. 2. Inhibition of palmito PPO (H1) by benzoic acid. Oxygen consumption was measured by an oxygen monitor at 30°C and at pH 5 (McIlvaine buffer) with 4-methylcatechol as substrate.

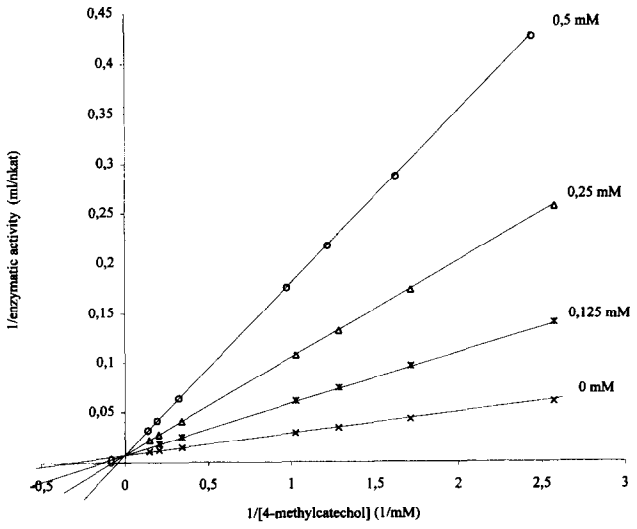

Fig. 3. Inhibition of palmito PPO (H1) by cinnamic acid. Oxygen consumption was measured by an oxygen monitor at 30°C and at pH 5 (McIlvaine buffer) with 4-methylcathecol as substrate.

50 ml of 50 mM sodium phosphate buffer at pH 6·5, containing 0·5 M $(NH_4)_2SO_4$ and 0·5 M KCl, and dialysed overnight against the same buffer. The dialysed enzyme was applied to a phenyl-Sepharose CL-4B column (5·3 $cm^2 \times 10$ cm), 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 activity was eluted using the same buffer containing $(NH_4)_2SO_4$ 0·1 M and KCl 0·1 M, and then with distilled water. The absorbance at 280 nm and the PPO activity were determined on each 6 ml fraction.

The fractions corresponding to two peaks and containing PPO activity, H1 and H2 (Robert *et al.*, 1996*a*), were used as enzyme sources.

All steps were performed at 4°C.

RESULTS AND DISCUSSION

Inhibitor constants of the different carboxylic acids

Inhibition of palmito PPO by benzoic acid, cinnamic acid and their derivatives and by sorbic acid, phenylacetic acid and *p*-phenylhydroxypropionic acid (Fig. 1) has been investigated. 4-Methylcatechol was used as substrate. The inhibitory effect of benzoic acid, cinnamic acid and sorbic acid on the PPO form H1 were determined from Lineweaver–Burk double reciprocal plots, as illustrated in Figs 2–4. A competitive-type inhibition was obtained with these three acids. With the other carboxylic acids studied, except for *p*-coumaric and sinapic acids, the same competitive-type inhibition was obtained (Table 1). *p*-Coumaric (Fig. 5) and sinapic

Table 1. Inhibition type and inhibition constants for the carboxylic acid inhibitors studied

Inhibitor	Inhibition type	Enzyme form	
		Inhibition constant (K_i, mM)	
		H1	H2
Benzoic series			
Benzoic acid	Competitive	0·76	1·15
p-Hydroxybenzoic acid	Competitive	1·49	0·87
Vanillic acid	Competitive	7·87	7·95
Cinnamic series			
Cinnamic acid	Competitive	0·056	0·058
p-Coumaric acid	Non-competitive	0·067	0·074
Ferulic acid	Competitive	0·55	0·59
Sinapic acid	Non-competitive	4·05	5·63
Phenylacetic acid	Competitive	9·90	10·27
3-*p*-Hydroxyphenyl propionic acid	Competitive	3·71	3·07
Sorbic acid	Competitive	0·41	0·38

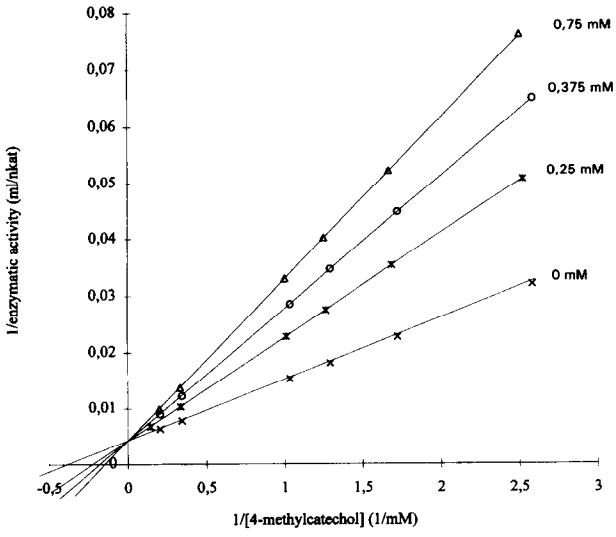

Fig. 4. Inhibition of palmito PPO (H1) by sorbic acid. Oxygen consumption was measured by an oxygen monitor at 30°C and at pH 5 (McIlvaine buffer) with 4-methylcatechol as substrate.

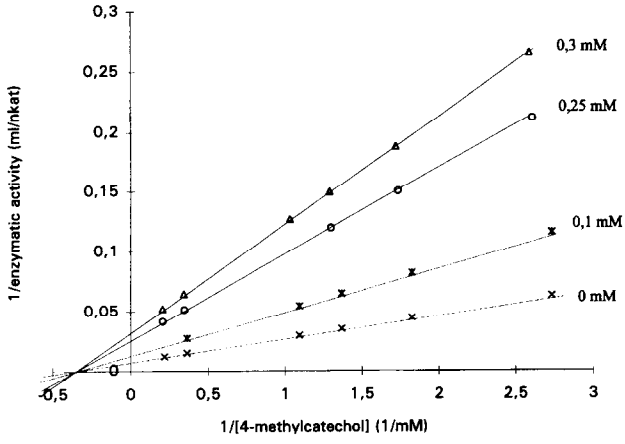

Fig. 5. Inhibition of palmito PPO (H1) by *p*-coumaric acid. Oxygen consumption was measured by an oxygen monitor at 30°C and at pH 5 (McIlvaine buffer) with 4-methylcatechol as substrate.

acids were shown to be non-competitive inhibitors. The two forms of PPO isolated by hydrophobic chromatography had the same behaviour towards the inhibitors studied. The type of inhibition observed depended on the substrate used. Hence, no general rule can easily be established with regard to the type of inhibition observed with carboxylic acids. Gunata *et al.* (1987) observed a competitive-type inhibition with cinnamic and benzoic acids as inhibitors with 4-methylcatechol as substrate, while a non-competitive-type inhibition was observed with the same acids with caffeic acid as substrate. Walker and Wilson (1975) suggested the existence of two distinct sites on the enzyme: one for the binding of the substrate and another, adjacent, site for binding the inhibitor. The type of inhibition also depends on the origin of the PPO studied. With cinnamic acid, a mixed-type inhibition was observed for potato PPO (Macrae & Duggleby, 1968).

K_i values obtained with each inhibitor are given in Table 1. The two forms H1 and H2 have close K_i values. Inhibitors from the cinnamic series have a greater inhibitory effect than those from the benzoic series. With cinnamic acid a K_i of 0·06 mM is obtained which is ten times less than the value obtained with benzoic acid ($K_i = 0{\cdot}8$ mM). The same inhibitory pattern was observed by Martinez-Cayuela *et al.* (1988) on

Table 2. Effect of pH on values of inhibition constants for benzoic, cinnamic and sorbic acids

	pH			
	3·6	4	4·6	5
Benzoic acid, 1 mM, pK_a 4·2				
K_i^{app} (mM)	0·191	0·267	0·507	1·15
β	0·80	0·61	0·20	0·14
$K_{i(AH)}$ (mM)	0·15	0·16	0·10	0·16
Cinnamic acid, 0·1 mM, pK_a 4·46				
K_i^{app} (mM)	—	0·0254	0·0574	0·058
β	—	0·74	0·42	0·22
$K_{i(AH)}$ (mM)	—	0·019	0·024	0·013
Sorbic acid, 0·05 mM, pK_a 4·76				
K_i^{app} (mM)	—	0·191	0·346	0·38
β	—	0·85	0·42	0·36
$K_{i(AH)}$ (mM)	—	0·16	0·14	0·14

Oxygen consumption was measured by an oxygen monitor at 30°C.

$$\beta = \frac{[AH]}{[A^-] + [AH]}$$

where [AH] and [A^-] are the concentrations of the undissociated and dissociated forms of the corresponding acid, respectively. Only the AH form inhibits PPO.

cherimoya PPO, where cinnamic acid and *p*-coumaric acid exhibited the strongest inhibitory effect. These observations lead us to suppose that the double bond found between the benzene ring and the carboxylic acid function improves the inhibitory character of the molecule. However, the presence of the benzene ring does not seem to play an essential part in the inhibition. In fact, we obtained an important inhibition for sorbic acid (an aliphatic carboxylic acid) (K_i = 0·38 mM on the H2 form). It appears that a conjugated system involving the COOH group is required.

Substitution by a –OH group in the *para*-position (*p*-hydroxybenzoic acid and *p*-coumaric acid) does not appear to influence the inhibitory effect of the corresponding carboxylic acids. However, a single *meta*- or a dimethoxy substitution considerably decreased the inhibitory power of the carboxylic acid. Indeed, the K_i increased 10-fold in the presence of vanillic and ferulic acids and increased 72-fold with sinapic acid. Moreover, no inhibition was observed with syringic acid (results not shown). Compared to benzoic acid, phenylacetic acid was a very weak inhibitor. Here, the –COOH group and the benzene ring are separated by a $-CH_2$ group. Thus it appears that the further away the –COOH group is from the benzene ring the more the inhibitory effect decreases. As regards *p*-hydroxyphenylpropionic acid, elongation of the lateral chain and *p*-hydroxy substitution appeared to improve the inhibitory character of the carboxylic acid. This compound is as good an inhibitor as sinapic acid.

These results show that aromatic acids are good inhibitors of palmito PPO when the carboxyl group is separated from the benzene ring by a conjugated double bond. The presence of a –OH group in the *para*-position does not appear to influence the inhibitory effects of aromatic carboxylic acids. The presence of a double bond in the α-position of the –COOH group seemed to be relatively important. Aliphatic carboxylic acids are known to be very weak inhibitors of PPO (Pifferi *et al.*, 1974; Martinez-Cayuela *et al.*, 1988), while sorbic acid has a good inhibitory effect. It is the presence of two conjugated double bonds that confers sorbic acid with an inhibitory power equivalent to that of ferulic acid.

Effect of pH on inhibition of palmito PPO by benzoic, cinnamic and sorbic acids

The influence of pH on inhibition of palmito PPO by sorbic, benzoic and cinnamic acids was studied in the pH range 3·6–5 with a 4-methylcatechol concentration range of 0·4–5 mM.

The same type of inhibition as that observed at pH 5 is observed for the three inhibitors at different pH. The effects of pH on the inhibitory pattern of benzoic, cinnamic and sorbic acids are shown in Figs 6–8. These double reciprocal plots show that the more acidic the pH the greater the inhibitory effect. This was confirmed by calculating the K_i values (Table 2).

In aqueous solution, an equilibrium exists between the undissociated form (AH) of the enzyme and its dissociated form (A^-). At pH values below the pK_a of the corresponding acid, AH is the predominant form, while at pH values above the pK_a the dissociated form predominates. For each of the three acids studied, the inhibitory effect improved the more acidic the pH.

In a preceding paper (Robert *et al.*, 1995), we showed the existence of a protonated form of the enzyme at acidic pH. There was a 20% difference between the enzyme activity at pH 5 and that at pH 3·6. In the experiment reported in this paper, there was a difference

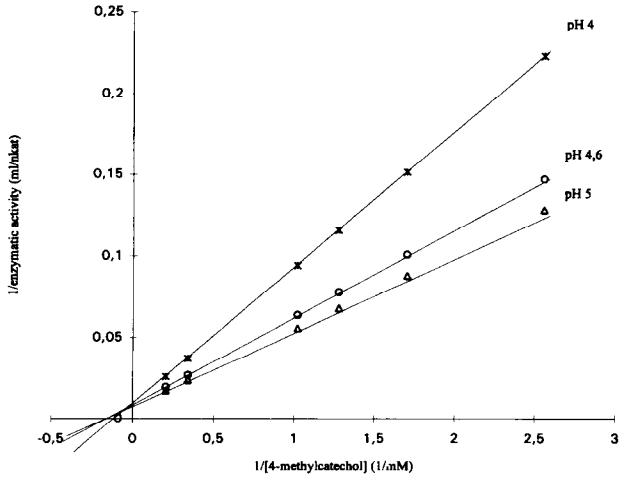

Fig. 6. Lineweaver–Burk double reciprocal plots showing inhibition of palmito PPO by sorbic acid (0·5 mM) at different pH (McIlvaine buffer). Oxygen consumption was measured by an oxygen monitor at 30°C with 4-methylcatechol as substrate.

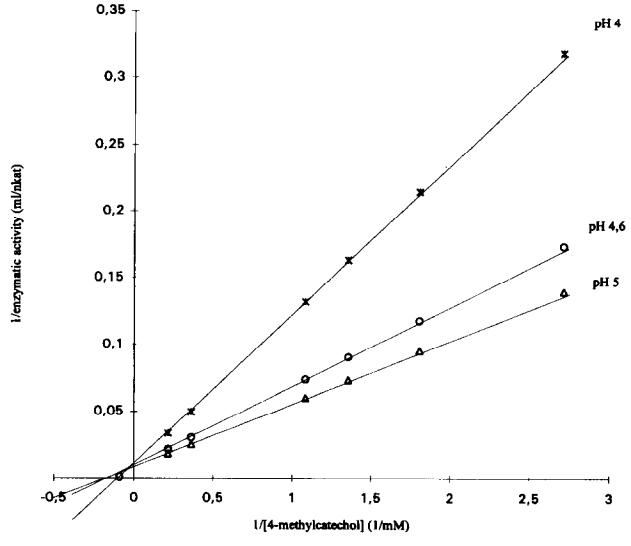

Fig. 7. Lineweaver–Burk double reciprocal plots showing inhibition of palmito PPO by benzoic acid (1 mM) at different pH (McIlvaine buffer). Oxygen consumption was measured by an oxygen monitor at 30°C with 4-methylcatechol as substrate.

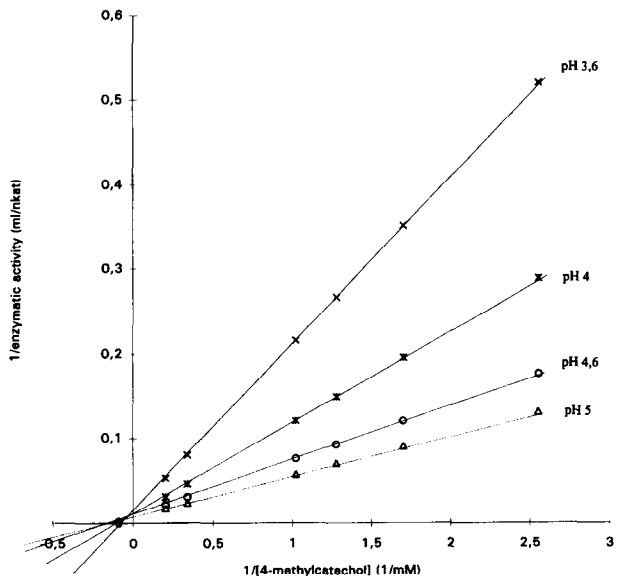

Fig. 8. Lineweaver–Burk double reciprocal plots showing inhibition of palmito PPO by cinnamic acid (0·1 mM) at different pH (McIlvaine buffer). Oxygen consumption was measured by an oxygen monitor at 30°C with 4-methylcathechol as substrate.

of 80% between the activity at pH 5 and the activity at pH 3·6 with benzoic acid, for example. This higher difference can not be explained solely by the existence of a protonated form of the enzyme sensitive to the action of the inhibitors. Hence we suggest that there is an effect of pH on the inhibitor, and that the undissociated form of the carboxylic acid is responsible for the enzyme inhibition.

For each acid, the relative importance (β) of AH over A^- can be estimated knowing the corresponding pK_a values.

$K_{i(AH)}$ values have been recalculated (Table 2) assuming that only the form AH is the inhibitor. In fact, a proportional increase in the undissociated form is observed when the pH decreases. The calculated $K_{i(AH)}$ values vary only slightly with pH. The observed pH-dependent changes in K_i values can be explained by the fact that less of the undissociated form of the inhibitor is present when the pH increases. Therefore, at a given concentration, the inhibitory effectiveness of the acid decreases with an increase in pH.

The same results were obtained by Janovitz-Klapp *et al.* (1990) for inhibition of apple PPO: the inhibitor constant values were independent of pH when the undissociated form AH was considered. According to Robb *et al.*, 1966, the non-ionized form of the inhibitor would react with the copper atom located in the active site of the PPO enzyme to form a complex.

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