Phytochemistry, Vol. 26, No. 6, pp. 1573–1575, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

SUBSTRATES AND AROMATIC CARBOXYLIC ACID INHIBITORS OF GRAPE PHENOL OXIDASES

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(*Revised received* 10 *October* 1986)

Key Word Index—*Vitis vinifera*; Vitaceae; grape; catechol oxidase; fungal laccase; hydroxycinnamic derivatives; caffeoyl-tartrate; *p*-coumaroyl-tartrate; aromatic carboxylic acid inhibitors.

Abstract—The caffeoyl- and *p*-coumaroyl-tartrates which are the most abundant hydroxycinnamic derivatives in grapes, were found to be good substrates for grape phenol oxidases. Catecholase activity was inhibited by aromatic carboxylic acids, *p*-coumaric acid being the strongest inhibitor.

INTRODUCTION

Enzymic browning of grapes is mainly dependent on the oxidation of endogenous phenols catalysed by two phenol oxidases, grape catechol oxidase (EC 1.10.3.1) [1] and laccase (EC 1.10.3.2), produced in rotten grapes by the grey mould *Botrytis cinerea*. With the exception of flavonoids, hydroxycinnamic acid derivatives particularly caffeoyl- and *p*-coumaroyl-tartrates make up a large part of the phenolic components of grapes [2–6]. It has been shown recently that their level decreases markedly under the oxidative conditions which occur throughout the pre-fermentative steps of wine making [5–8]. However, no data are available about the affinity of grape catechol oxidase and *Botrytis* laccase towards these esters. This point was therefore investigated in the first part of this work. Previous work with fruits other than grapes showed an inhibition of catechol oxidase by hydroxycinnamic acids [9–13]. It thus appeared interesting to study this point in the second part of this work.

RESULTS AND DISCUSSION

Substrate affinity of grape catechol oxidase and Botrytis *laccase*

Table 1 gives K_m values for both enzymes for some phenolic substrates. As can be seen, *Botrytis* laccase displayed a higher affinity for all phenolics tested than grape catechol oxidase, in agreement with previous results [14]. Here, the highest affinity was found for caffeic acid.

Esterification of the carboxyl group of caffeic and *p*-coumaric acids with tartaric acid resulted in an increase in activity of the grape catechol oxidase. A similar effect was seen for caffeoyl-quinate (chlorogenic acid), although this ester does not occur in grapes [15, 16]. Caffeoyl- and *p*-coumaroyl-tartrates are the major phenolic components in free run grape juice with free caffeic and *p*-coumaric acids being present in only small quantities [7, 16, 17]. The large loss in these esters during grape juice preparation as a result of oxidation [5, 7] can be accounted for by this high affinity.

Table 1. K_m values of grape catechol oxidase and laccase from *B. cinerea* for some phenolic substrates

Substrates	Grape catechol oxidase K_m (mM)	Laccase K_m (mM)
4-Methylcatechol	7.5	0.39
(+)-Catechin	7.9	0.58
Caffeic acid	5.8	0.07
Caffeoyl-quinate (chlorogenic acid)	1.5	0.11
Caffeoyl-tartrate	2.1	0.11
p-Coumaric acid	2.0	0.25
p-Coumaroyl-tartrate	1.1	0.64

Inhibition of grape catechol oxidase by aromatic carboxylic acids

As shown in Table 2, the strongest inhibition of grape catechol oxidase was observed with *p*-coumaric, cinnamic and benzoic acids. Hydroxylation and methylation of the benzene rings of these acids decreased their inhibitory effects, as in other fruits [9, 13, 18]. This effect totally disappeared on mono- or di(*meta*) methylation of benzoic acid and di(*meta*) methylation of cinnamic acid. Substitution in an *ortho* position on the latter to give *o*-coumaric acid had no effect. Some authors, however, reported fairly high inhibition of catechol oxidase from other fruits by this acid, although not as great as that with cinnamic and *p*-coumaric acids [10, 13, 18]. The decrease or the loss of the inhibitory effects of substituted carboxylic acids may be due to steric hindrance [13].

Esterification of the carboxyl group of benzoic and cinnamic acids leads to a considerable decrease of their inhibitory strength on grape catechol oxidase. The same occurs in apricots [9] and cherries [13] in which catechol oxidase inhibition with benzoic acids esters was lower than with the corresponding free acid. It has been

Table 2. Inhibition of grape catecholase activity by benzoic, cinnamic acids and their derivatives

Inhibitors (benzoic acid and its derivatives)	4-Methylcatechol inhibition (%)	Inhibitors (cinnamic acid and its derivatives)	4-Methylcatechol inhibition (%)
Benzoic acid	43	Cinnamic acid	46
p-Hydroxybenzoic acid	36	*p*-Coumaric acid	71
Gallic acid (3,4,5-trihydroxybenzoic)	22	*o*-Coumaric acid	0
Vanillic acid (4-hydroxy-3-methoxybenzoic)	0	Ferulic acid (4-hydroxy-3-methoxycinnamic)	25
Syringic acid (4-hydroxy-3,5-dimethoxybenzoic)	0	Sinapic acid (4-hydroxy-3,5-dimethoxycinnamic)	0
Ethyl-*p*-hydroxybenzoate	10	*p*-Coumaroyl-tartrate	5
Methyl-*p*-hydroxybenzoate	20	—	—
p-Hydroxybenzaldehyde	5	—	—

Activity determined in 0.1 M phosphate–citrate buffer, pH 5.0, with 2.5 mM 4-methylcatechol as a substrate. In each case inhibitor concentration was 2.5 mM.

suggested that for strong inhibition aromatic acid inhibitors require a free carboxylic group substituted directly on to the benzene ring [19].

The type of inhibition of grape catechol oxidase by aromatic carboxylic acids and K_i values depended on the structures both of the substrate and of the inhibitors (Table 3). This is in agreement with other results obtained for other fruit catechol oxidases [13, 18].

K_i values reported here for cinnamic and benzoic acid inhibitors of grape catechol oxidase are somewhat higher than those found for apple catechol oxidase [18], but lower than values given for potato tuber oxidase [20].

Our K_i values show that of the aromatic carboxylic acids studied here, *p*-coumaric acid was the most powerful inhibitor of grape catechol oxidase.

EXPERIMENTAL

Preparation of extract with catechol oxidase activity. Sound grapes (cv Merlot) were harvested at maturity. They were immediately stored in a freezer at − 20° until analysis. At the time of testing, the deseeded grapes (10 g) were immersed in liquid N_2 and then ground in the presence of liquid N_2. The resultant powder was suspended in 100 ml 0.1 M phosphate–citrate buffer, pH 7.0, at 2°, containing 3 ml 1.5% tween 80 (for solubilization of the enzyme), 200 mg ascorbic acid and 2.5 g Polyclar AT. The slurry was gently stirred at 2° for 30 min and then centrifuged (25 000 *g*, 2°, for 15 min). The resultant supernatant was used immediately as the enzyme source.

No laccase activity was present in the enzyme extract as indicated by the absence of activity towards hydroquinone [14] or benzidine [21].

Laccase from B. cinerea. *B. cinerea* was grown on a liquid medium consisting of a mixture of grape juice and malt extract. Crude laccase extract was prepared according to the procedures described elsewhere [22].

Substrates. *trans*-Caffeoyl and *trans*-*p*-coumaroyl tartrates were isolated from grapes and purified by HPLC [7]. 4-Methylcatechol was recrystallized from *n*-hexane before use. All other substrates were of reagent grade and were used without further purification.

Enzyme assay. Enzyme activities were determined polarographically by measuring the rate of O_2 consumption with a Clark-type oxygen electrode (Yellow Springs Instruments, Co, U.S.A.). The reaction mixture (15 ml) consisted of 0.1 M phosphate–citrate buffer, pH 5.0 (for catechol oxidase) or pH 4.75 (for laccase), containing substrate, and inhibitor when used, equilibrated with air at 30°. The reactions were initiated by adding enzymic extracts.

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Table 3. Inhibitory effect of aromatic carboxylic acids upon grape catechol oxidase

	Substrates					
	4-Methylcatechol		Caffeic acid		(+)-Catechin	
Inhibitor	Type of Inhibition	Inhibition constant K_i (mM)	Type of Inhibition	Inhibition constant K_i (mM)	Type of Inhibition	Inhibition constant K_i (mM)
p-Coumaric acid	Competitive	0.95	Non-competitive	1.33	Non-competitive	0.32
Cinnamic acid	Competitive	1.2	Non-competitive	2.06	Non-competitive	0.55
Benzoic acid	Competitive	2.2	Non-competitive	3.7	Competitive	0.88
Ferulic acid	Competitive	3.0	Non-competitive	7.0	Competitive	0.96

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