Role of Cinnamoyl Esterase Activities from Enzyme Preparations on the Formation of Volatile Phenols during Winemaking

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High levels of 4-vinylphenol and 4-vinylguaiacol were detected in wines made from grape juice initially treated with some enzyme preparations. Two enzyme activities, which operate successively, are responsible for this formation. First, the cinnamoyl esterase activity from enzyme preparation liberates cinnamic acids from their corresponding tartaric acid esters. Second, cinnamic acids are transformed into 4-vinylphenol and 4-vinylguaiacol by decarboxylase activity provided by the yeasts. This activity is quite stable throughout alcoholic fermentation. The high levels of volatile phenols in some enzymatically treated wines could be responsible for unpleasant phenolic off-flavors. During storage, these compounds decreased and corresponding ethoxyethylphenols increased.

INTRODUCTION

Volatile high-flavorant vinylphenols are known to be important contributors to wine flavor (Dubois et al., 1971; Boidron et al., 1988; Chatonnet et al., 1989; Etiévant, 1991). Their detection thresholds are generally low (Dubois, 1983; Etiévant and Bayonove, 1983; Etiévant, 1991). They have a characteristic aroma which, according to their amounts, contributes positively or negatively to wine flavor. In beer, it is known that an excessive formation of volatile phenols (>1 ppm) generates unpleasant phenolic off-flavors (Dadic et al., 1971; Thurston and Tubbs, 1981). In wines, the amounts of these compounds are generally low and usually limited by the concentrations of their precursors. Some phenolic acids are naturally present in grape juice. In particular, it was demonstrated (Steinke and Paulson, 1964; Albagnac, 1975; Versini, 1985; Dubourdieu et al., 1989) that 4-hydroxycinnamic acid (*p*-coumaric acid) and 3-methoxy-4-hydroxycinnamic acid (ferulic acid) could be changed into the corresponding vinylphenols (i.e., 4-vinylphenol and 4-vinylguaiacol) by *Saccharomyces cerevisiae* decarboxylase activity.

Recently, the possible use of glycosidases in enzyme preparations [pectinases and (hemi)cellulases] for wine flavor enhancement via release of glycosidically bound flavorants has attracted much attention (Gunata et al., 1990a,b; Dugelay, 1993). In some cases, we found evidence of very high levels of vinylphenols in wines made from juices initially treated with enzyme preparations (Dugelay, 1993). The pectinase preparations are commonly applied in wine technology to improve clarification (Pilnik, 1982) and color extraction (Canal-Llaubères, 1990).

Because of the importance of phenolic compounds in wine flavor, it was deemed worthwhile to investigate the origin of these volatile phenols. This is the purpose of the present work.

EXPERIMENTAL PROCEDURES

Grape Juice Winemaking. Grape juice (17 °Brix) from Muscat of Frontignan (INRA Experiment Station, Pech-Rouge, Narbonne, France, vintage 1990) was divided into three fractions. To one was added a commercial pectinase (P_1) and to another an experimental hemicellulase (P_2) enzyme preparation obtained from *Aspergillus niger*. The third juice fraction (control) was not treated with enzymes. Each fraction was fermented (20 °C) with two different *S. cerevisiae* yeast strains, widely used in winemaking (called in this work strains 1 and 2).

Wines (vintage 1990) were also prepared from grapes of Shiraz (red grape variety, INRA, Pech-Rouge), Gewürztraminer, Riesling, Muscat Ottonel (INRA, Colmar), and Sauvignon blanc (INRA, Angers) using enzyme preparation P_2 and yeast strain 2.

The extraction of volatile phenols (vinylphenols and ethoxyethylphenols) from wines (50 mL) after addition of 4-nonanol (10 μg) as internal standard was realized in triplicate using an Amberlite XAD-2 resin (Gunata et al., 1985). The volatile phenols were eluted with 50 mL of pentane/dichloromethane (2/1 v/v). The organic extract was concentrated by rectification at 30 °C and then analyzed by gas chromatography–mass spectrometry (Voirin et al., 1992; Dugelay, 1993). The wines were again extracted and analyzed in the same conditions after storage in an experimental cellar (16–18 °C) for 1 year.

Detection of Esterase Activity in Enzyme Preparations. Several samples from the same Muscat of Frontignan grape juice (treated with 100 ppm of SO_2 to avoid the oxidation of phenolic substrates), clarified by centrifugation (6000*g*, 4 °C, 15 min), were subsequently treated with 12 different enzyme preparations (basically pectinases, cellulases, and hemicellulase) at the amount of 5 g/hL. Each sample was incubated at 20 °C for 20 h, filtered (0.45 μm), and immediately analyzed by high-performance liquid chromatography (HPLC) (column RP 18, detection 313 nm) (Romeyer et al., 1982; Gunata et al., 1986; Boursiquot, 1987) to evaluate changes in cinnamoyltartaric esters. Identification of compounds was made using standard compounds, ferulic and *p*-coumaric acids (Fluka), caffeic and *o*-coumaric acids (Merck), and *p*-coumaroyl- and caffeoyltartaric acid esters [extracted and purified from grape according to the methods of Singleton et al. (1978, 1985) and Gunata et al. (1986)]. *o*-Coumaric acid served as internal standard (1.1 ppm in sample).

Detection of Yeast Strains Containing Decarboxylase Activity. *S. cerevisiae* strains were maintained at 4 °C on malt wickerham agar slants. Culture media were prepared according to the method of Albagnac (1975), to which was added *p*-coumaric acid (1 ppm). The media were filtered (0.45 μm) and inoculated with 10^6 yeast cells/mL *S. cerevisiae* strain 1 or 2.

After the end of alcoholic fermentation (28 °C), samples were withdrawn. A part of each sample was filtered and directly analyzed by UV spectrometry to follow the change in the spectrum of *p*-coumaric acid (Albagnac, 1975). Another part was extracted on Amberlite XAD-2 resin, and after elution with pentane/dichloromethane (2/1 v/v), 4-vinylphenol was analyzed by GC–MS (Gunata et al., 1985; Voirin et al., 1992; Dugelay, 1993).

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Table I. Vinylphenol Contents in Muscat of Frontignan Wines Originating from Juices Treated with Enzyme Preparations (P_1 and P_2) and Fermented with Two Different Yeast Strains (1 and 2)

yeast strain	enzyme preparation	vinylphenols, ppb	
		4-vinylguaiacol	4-vinylphenol
strain 1	none	–[a]	<1
strain 2	none	–	<1
strain 1	P1	–	<1
strain 1	P2	–	<1
strain 2	P1	25	137
strain 2	P2	160	1396

[a] –, not detected.

Table II. Effect of Enzyme Preparation (P_2) in the Formation of Vinylphenols in Wines Fermented by Yeast Strain 2

wine	vinylphenols, ppb			
	4-vinylguaiacol		4-vinylphenol	
	nontreated	enzyme treated	nontreated	enzyme treated
Gewürztraminer	38	127	48	286
Riesling	275	687	804	1354
Muscat Ottonel	–[a]	–	–	–
Sauvignon	–	45	–	–
Shiraz[b]	–	25	–	391

[a] –, not detected. [b] Red wine.

Stability of Yeast Decarboxylase Activity during the Alcoholic Fermentation. The aim was to evaluate yeast decarboxylase activity at different stages of fermentation. First, the native cinnamate derivates in grape juice were oxidized by air bubbling at 20 °C to test yeast decarboxylase activity against the high added levels. The oxidation was followed by HPLC analysis (column RP 18, in the same conditions previously described); the major fraction of phenolic substrates had disappeared after 1 h of air bubbling. The oxidized juice was clarified by filtration (0.45 μm), SO_2 (100 ppm) was added, and the juice was divided into different batches. These batches were then inoculated with *S. cerevisiae* yeast strain 2 (10^6 cells/mL) possessing decarboxylase activity. The *p*-coumaric and ferulic acids (respectively, 61 and 55 μmol/L) were added to juice at different stages (0, 57, 63, 144, 428 h) of the fermentation. The fermentation was followed by the analysis of sugars (glucose and fructose) according to Boehringer enzymatic assay (Bergmeyer et al., 1974). One batch (control) was fermented without addition of cinnamic acids. After the end of the fermentation, samples were extracted on Amberlite XAD-2 resin and volatile phenols were analyzed by GC–MS.

RESULTS AND DISCUSSION

1. Vinylphenols in Wines. As shown in Table I only a trace level of 4-vinylphenol was detected in Muscat of Frontignan wines fermented with two yeast strains (1 or 2). The same data have been observed when the wine was obtained from the juice initially treated with enzyme preparations P_1 or P_2 and fermented with yeast strain 1. However, high levels of 4-vinylguaiacol and 4-vinylphenol were detected when juice was fermented with yeast strain 2 after treatment of the juice with enzyme preparations. Formation of vinylphenol was particularly great (around 1.4 ppm) when enzyme preparation P_2 was used. Ten staff members from the Institute who participated in the sensory evaluation described an unpleasant phenolic odor (solvent, medicinal) in this wine. Relatively high amounts of vinylphenols were also detected when white wines from Gewürztraminer and Riesling were prepared in the presence of enzyme preparation P_2 and yeast strain 2 (Table II). The same occurred when a red wine from Shiraz was prepared. Thus, yeast decarboxylase activity appears not to be inhibited by phenolic compounds of red wine, contrary to the previous observations (Dubourdieu et al., 1990).

The amounts of vinylphenols in wines made from different grape cultivars are quite different (Table II). Vinylphenols are not detected in enzyme-treated or nontreated wines of Muscat Ottonel grape, while they are already present at high levels in nontreated Gewürztraminer and Riesling wines. This can be explained by the presence of cinnamic acids in Gewürztraminer and Riesling juices which could be transformed into volatile phenols by yeast strain 2.

It should be emphasized that the grape juice contains cinnamic acid precursors of vinylphenols (Boursiquot, 1987) which are good substrates for grape polyphenol oxidase (Gunata et al., 1987). The level of these compounds depends greatly on the type of grape juice processing before fermentation (Gunata et al., 1986; Cheynier et al., 1989), which can explain the difference of vinylphenols observed in this study.

On the basis of these observations, the presence of cinnamoyl esterase activity in the enzyme preparations was studied, since *S. cerevisiae* uses cinnamic acids as substrates but not cinnamate tartaric esters, which are the main native forms of cinnamate derivatives in grapes (Ribéreau-Gayon, 1964; Nagel et al., 1979; Romeyer et al., 1983; Singleton et al., 1985).

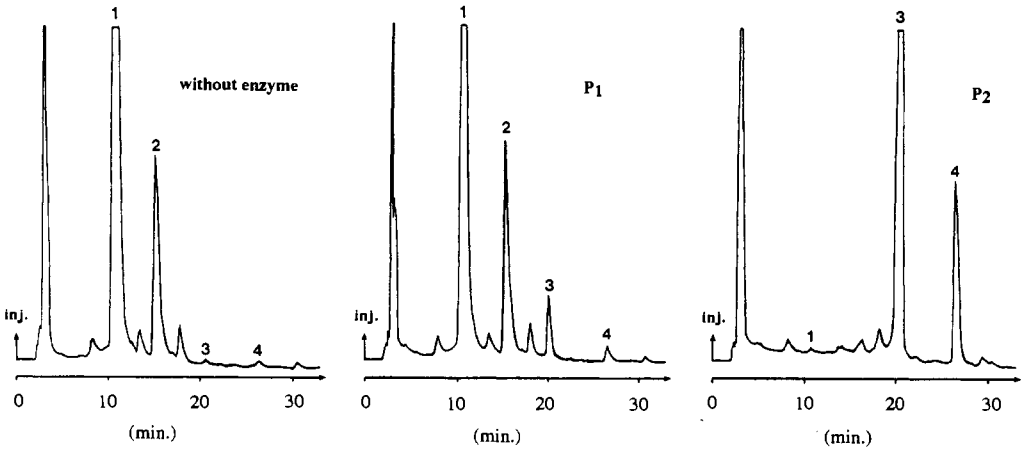

Figure 1. HPLC chromatograms (RP 18, detection 313 nm) of cinnamic acid derivates of grape juice from Muscat of Frontignan incubated (20 °C, 20 h) without enzymes and with enzyme preparations P_1 and P_2. 1, caffeoyltartaric acid ester; 2, *p*-coumaroyltartaric acid ester; 3, caffeic acid; 4, *p*-coumaric acid.

Table III. Changes in Cinnamoyl Tartrates and Free Cinnamic Acid Contents of a Muscat of Frontignan Grape Juice after Incubation (20 °C, 20 h) with Various Commercial and Experimental Enzyme Preparations

	caffeoyltartaric acid ester, μmol/L	*p*-coumaroyltartaric acid ester (*trans*), μmol/L	*p*-coumaroyltartaric acid ester (*cis*), μmol/L	caffeic acid, μmol/L	*p*-coumaric acid (*trans* + *cis*),[a] μmol/L
initial amount in juice	19.8	7.6	7.2	0.5	–[b]
commercial preparations					
pectinase 1	19.2	7.5	6.3	4.3	1.0
pectinase 2	16.3	4.3	6.7	0.3	0.2
pectinase 3	14.6	4.0	2.5	0.4	–
pectinase 4 (=P_2)[c]	13.2	3.6	2.5	5.8	1.7
pectinase 5	10.1	2.1	4.7	9.8	4.1
cellulase 1	15.5	4.5	4.4	0.9	–
cellulase 2	13.0	7.9	7.3	1.3	–
experimental preparations					
pectinase 1	5.6	2.4	3.6	15.1	6.5
pectinase 2	4.9	2.0	3.2	18.0	10.0
pectinase 3	1.7	1.4	1.7	16.5	11.1
cellulase 1	6.6	4.1	4.7	15.4	7.2
hemicellulase (=P_1)[c]	18.0	6.5	6.3	1.2	–

[a] Coelution in our HPLC analysis conditions. [b] –, not detected. [c] P_1 and P_2, see Experimental Procedures.

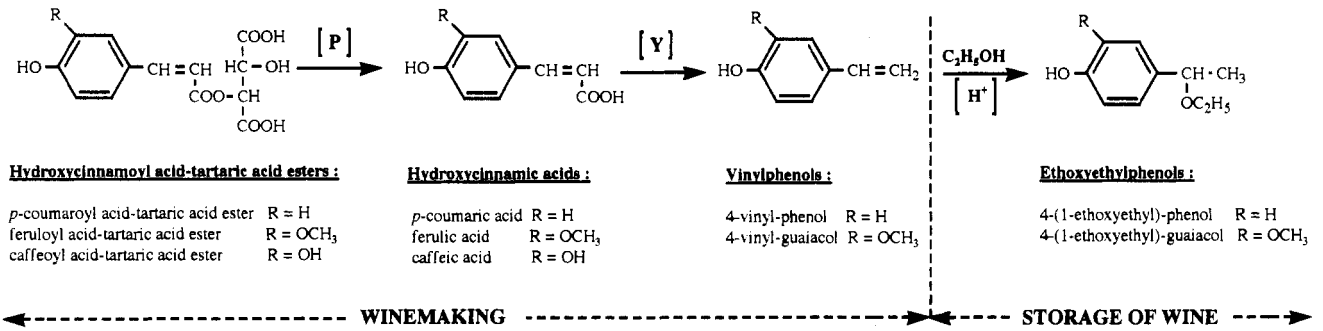

Figure 2. Scheme of vinylphenol formation during winemaking and formation of ethoxyethylphenols during wine storage. [P], enzyme preparation; [Y], yeast.

Table IV. Effect of Addition of Ferulic (55 μmol/L) and *p*-Coumaric (61 μmol/L) Acids at Various Stages of Fermentation on the Formation of Vinylphenol and Vinylguaiacol

	vinylphenols,[a] μmol/L	
	4-vinyl-guaiacol	4-vinyl-phenol
without addition of cinnamic acids	3.8	2.5
with addition of cinnamic acids during fermentation		
at t = 0 h (183 g/L sugar)	40	54
at t = 57 h (100 g/L sugar)	42	57
at t = 63 h (63 g/L sugar)	46	64
at t = 144 h (<2 g/L sugar)	41	67
at t = 428 h (<1 g/L sugar)	50	70

[a] Vinylphenols were analyzed after the end of the fermentation.

2. Cinnamoyl Esterase Activity in Enzyme Preparations. Caffeoyl- and *p*-coumaroyltartaric acid esters are the most abundant cinnamate esters in grape juices (Ong et al., 1978; Nagel et al., 1979; Singleton et al., 1985; Boursiquot, 1987). Hydrolysis of these compounds was studied after incubation (20 h, 20 °C) of grape juice with P_1 and P_2 enzyme preparations (Figure 1).

The levels of caffeoyl- and *p*-coumaroyltartaric acid esters declined dramatically with a concomitant increase in the corresponding free phenolic acids in the presence of enzyme preparation P_2. However, enzyme preparation P_1 had little effect on caffeoyltartaric acid esters and only trace levels of *p*-coumaric acid were detected after 48 h of incubation.

Feruloyltartaric acid ester, present at low concentration in grape juice (Romeyer et al., 1983; Boursiquot, 1987), was not detected in our HPLC analysis conditions, nor were its subsequent hydrolysis products. It has been reported that pectinase preparations were able to hydrolyze this ester in grape juice and wine (Baranowski and Nagel, 1981; Gunata et al., 1986; Spanos and Wrolstad, 1990).

Cinnamate esterase activity was then evaluated in a large number of commercial or experimental enzyme preparations (eight pectinases, two cellulases, and one hemicellulase) (Table III). The activity varied largely according to the preparation. Three pectinase, two cellulase, and the hemicellulase preparations showed weak activities. Four pectinase preparations and one cellulase preparation exhibited high esterase activities. Consequently, the low initial concentration of free cinnamic acids

Table V. Stability of Vinylphenols during Wine Storage (16–18 °C) and Occurrence of Ethoxyethylphenols

	vinylphenols, ppb				ethoxyethylphenols, ppb			
	4-vinylguaiacol		4-vinylphenol		4-(1-ethoxyethyl)guaiacol		4-(1-ethoxyethyl)phenol	
wine[a]	1 month	1 year	1 month	1 year	1 month	1 year	1 month	1 year
Muscat of Frontignan	160	156	1396	360	–[b]	39	23	411
Gewürztraminer	127	74	286	12	–	14	3	10
Riesling	687	191	1354	244	64	182	185	435
Muscat Ottonel	–	–	–	–	–	–	–	–
Shiraz	25	–	391	59	–	–	32	41

[a] Wines obtained using enzyme preparation P2 and yeast strain 2. [b] –, not detected.

(0.5 μmol/L) in the juice increased to 14–30 μmol/L. *p*-Coumaric acid was not detected in the initial grape juice, and its concentration reached 11 μmol/L in enzyme-treated juice. Previous works demonstrated that caffeic acid was not a substrate of yeast decarboxylase as were *p*-coumaric and ferulic acids (Albagnac, 1975; Dubourdieu et al., 1990). We reported here the values obtained for caffeic acid. First, its formation was readily detectable due to the abundance of caffeoyltartaric acid ester in grape juice (Singleton et al., 1985; Boursiquot, 1987) and, second, the enzyme hydrolysis of this ester was consistent with the hydrolysis of coumaroyltartaric acid ester.

3. Detection of Yeast Cinnamic Acid Decarboxylase Activity. On the basis of the previous data, decarboxylase activity against *p*-coumaric acid was investigated in two yeast strains used in this work. Decarboxylase activity was detected in yeast strain 2, on the basis of a hypsochromic shift in the UV absorption spectrum of medium with this strain, contrary to strain 1 (Albagnac, 1975). Formation of vinylphenol was confirmed by GC–MS analysis of the media after extraction on Amberlite XAD-2 resin. Only the medium fermented with yeast strain 2 contained vinylphenol. A great number of enological *S. cerevisiae* strains possess cinnamic acid decarboxylase activity (Albagnac, 1975; Dubourdieu et al., 1990).

4. Stability of Yeast Decarboxylase Activity during Fermentation. According to present regulations, the addition of exogenous enzyme preparations to juices must be made before the end of fermentation. It was important to know if yeast decarboxylase activity was stable throughout fermentation to determine the best moment to add enzymes, thus avoiding the increase in the amounts of yeast cinnamic acid substrates.

Table IV indicates that yeast decarboxylase is quite stable, since *p*-coumaric and ferulic acids added at different stages of fermentation were quite totally transformed into 4-vinylphenol and 4-vinylguaiacol, respectively. It also appears that due to the good stability of yeast decarboxylase the moment of addition of enzyme preparation to the juice during fermentation does not influence the level of vinylphenol produced.

5. Stability of Vinylphenols during Wine Storage. The high levels of vinylphenol and vinylguaiacol decreased notably after 1 year of storage at 16–18 °C (Table V). The concentration of vinylphenol seems to decline more than that of vinylguaiacol. These compounds are partly transformed into corresponding ethoxyethylphenols, and unpleasant off-flavor decreased. More results concerning ethoxyethylphenols will be published in a forthcoming paper.

6. Conclusion. This study gave evidence of the origin of the high amounts of vinylphenols encountered in wines from juices initially treated with some enzyme preparations. The cinnamoyl esterase activity from added enzyme preparations increased the concentration of cinnamic acids, which in turn are decarboxylated to vinylphenols by decarboxylase produced by *S. cerevisiae* during fermentation (Figure 2). Five pectinase preparations of the eight studied here contained noticeable cinnamoyl esterase activity. Moreover, the majority of enological yeast strains possess decarboxylase activity. Consequently, particular attention should be directed to the presence of undesirable enzymatic activity in enzyme preparations to avoid the formation of unpleasant phenolic off-flavors during winemaking.

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