Changes in pH-Dependent Grape Polyphenoloxidase Activity during Maturation

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Catecholase and cresolase activity of grape polyphenoloxidase was investigated during development and maturation of the Monastrell and Airen grape berries. Catecholase activity of Monastrell grape polyphenoloxidase increased or decreased depending on the pH used to measure it, while in Airen grape it always increased independently of the pH used. Two significant days of higher catecholase activity were found for the former variety, one after heavy rainfall and the other just at véraison. The apparent optimum pH of Monastrell grape catecholase, a red grape, shifted from pH 4.0 to 5.5 at véraison and then back to pH 4.0 but that of Airen, a white grape, was pH 4.0 throughout. Cresolase activity measured at pH 7.0 increased slightly in both cultivars during the seven weeks of the study.

Enzymatic browning of musts and wines is mainly dependent on the oxidation of endogenous phenols (Sapis et al., 1983b) by grape polyphenoloxidase (EC 1.14.18.1) (PPO). This enzyme catalyzes both the ortho-hydroxylation of monophenols to give *o*-diphenols (cresolase activity) and the further oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The quinones thus formed lead by polymerization to the creation of brown pigments, which cause a change in the color and flavor of the juice, changing the quality of the final processed product.

The importance of this enzyme in wine-making has attracted the attention of many investigators since the 1960s as described in a recient revision (Mayer, 1987) to discover its kinetic parameters (Lerner and Mayer, 1976; Sánchez-Ferrer et al., 1988), its location (Ivanov, 1967), and the best moment for grape harvest to produce a high-quality wine without or at least with reduced enzymatic browning. The variation of PPO activity during grape maturation has been studied in different red and white varieties from different countries (United States, Israel, France); however, the literature reveals no systematic evolution of grape PPO activity during maturation in spite of the fact that the evolution of other grape parameters (pH, degrees Brix, phenols, etc.) change in a similar way (Kidron et al., 1978; Wissemann and Lee, 1980; Sapis et al., 1983a).

The present study follows catecholase activity extracted from a red and white grape cultivar commonly grown in Spain and assayed at several pHs from a short time after fruit set at 1-week intervals until harvest. Cresolase activity was also measured at pH 7.0 in these extracts.

MATERIALS AND METHODS

Plant Material. Grapes (*Vitis vinifera* L.) were picked weekly from mid-July (after fruit set) until harvest (mid-September), during the 1987 growning season in the southeast of Spain and analyzed the same day for cresolase and catecholase activities. Two varieties of grape were chosen for their differing browning capacities: one red (cv. Monastrell, picked at Jumilla, Spain) and the other white (cv. Airen, picked at Villarrobledo, Spain).

Extraction of Cresolase and Catecholase. Grape cresolase and catecholase were extracted according to the method described by Harel and Mayer (1971). The grapes were cut from the pedicel and flushed with the skin, and the flesh was placed in 100 mM phosphate buffer (pH 7.3) containing 10 mM sodium ascorbate. They were then homogenized in a blender for 15 s, filtered through eight layers of gauze, and centrifuged at 4000*g* for 15 min. The precipitate was extracted for 30 min with 1.5% of Triton X-100 in 100 mM phoshate buffer (pH 7.3) and then centrifuged at 60000*g* for 15 min. The supernatant was subjected to ammonium sulfate fractionation between 45% and 95% saturation at 4 °C. The precipitate was resuspended and after dialysis was used as enzyme source.

Table I. Changes in the Maturation Parameters

variety	weight, g		pH		deg Brix		phenol, mg/mL	
	AFS[a]	H[b]	AFS	H	AFS	H	AFS	H
Monastrell	0.5	1.7	2.5	3.7	6	23	9.7	1.3
Airen	0.8	2.1	2.5	3.3	5	18	6.5	2.1

[a] AFS = value obtained after fruit set (mid-July). [b] H = value obtained at harvest (mid-September).

Enzyme Assay. Both cresolase activity toward *p*-cresol and catecholase activity toward 4-methylcatechol were measured spectrophotometrically by the appearance of 4-methyl-*o*-benzoquinone at 400 nm, as described previously (Sánchez-Ferrer et al., 1988). Unless otherwise stated, the reaction media for catecholase activity at 30 °C contained 25 mM of 4-methylcatechol at different pHs in 10 mM acetate (pH 3.5–5.5) or phosphate (pH 6.0–7.0) buffers in a final volume of 1 mL. The reaction media for cresolase activity at 30 °C contained 0.5 mM *p*-cresol in 10 mM phosphate buffer (pH 7.0) in a final volume of 1 mL.

The specific activity was defined as the amount of enzyme that produces 1 μmol of 4-methyl-*o*-benzoquinone/min per mg of protein.

Analytical Methods. Phenolic compounds were extracted from the grapes with use of 80% ethanol (Kidron et al., 1978) and determined spectrophotometrically (Singleton and Rossi, 1965). The color intensity of the grape juice was determined at 420 and 520 nm (Wissemann and Lee, 1980). The soluble solids (degrees Brix), used in agriculture as an index of maturity for grapes, was measured using a hand refractometer. Protein content of the samples was determined by the dye-binding method (Bradford, 1976), using BSA as standard.

Electrophoresis. SDS-PAGE was carried out as described (Angleton and Flurkey, 1984) with the method of Laemmli (1970). Samples were mixed with glycerol and bromophenol blue before application onto 7.5% polyacrylamide gels. Electrophoresis was carred out for 6 h at room temperature. Gels were stained for PPO activiy in 100 mL of 10 mM acetate buffer (pH 5.0) containing 5 mM L-β-3,4-dihydroxyphenylalanine (L-Dopa).

RESULTS

Grape Development. The content of phenolic compounds, sugar content (degrees Brix), pH, and weight were measured. As shown in Table I, both varieties presented the same trends: The phenolic compounds (which were greater in the red variety) decreased during maturation,

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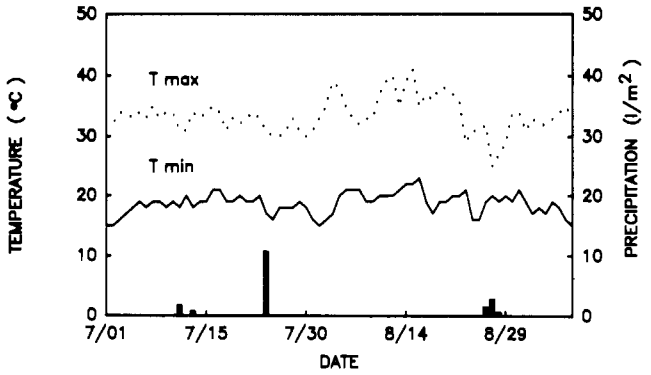

Figure 1. Metereological data of the red variety (cv. Monastell) vineyard during the 1987 growing season.

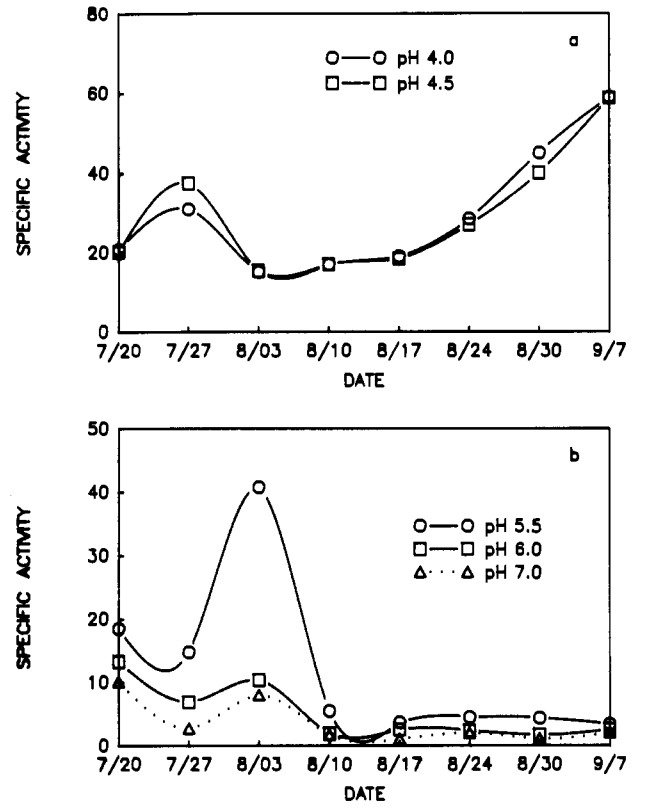

Figure 2. Changes in catecholase activity of Monastrell grape polyphenoloxidase during maturation with the pH used to measure it: (a) below pH 5.0; (b) above pH 5.0.

while weight, pH, and degrees Brix increased.

The trend of these parameters during maturation followed the same pattern as previously described for other grape varieties' phenolic content (Crippen and Morrison, 1986; Romeyer et al., 1983), pH (Traverso-Rueda and Singleton, 1973), degrees Brix, and weight (Sapis et al., 1983a).

Temperature and rainfall are two important factors influencing the physiological and biochemical changes during the development and maturation of the grapes, and because these factors are outside human control, metereologial data during the growning and maturation period of the grapes must be taken into account to understand their relationship with the trend of PPO. The metereological data of the 1987 maturation period from the vineyard of the red variety are presented in Figure 1, showing that the berries had been subjected to heat stress due to the high temperatures (32–40 °C) and very little rainfall.

Evolution of Catecholase Activity. The trend of catecholase activity during maturation was followed at different pHs in 10 mM acetate (pH <5.5) or phosphate

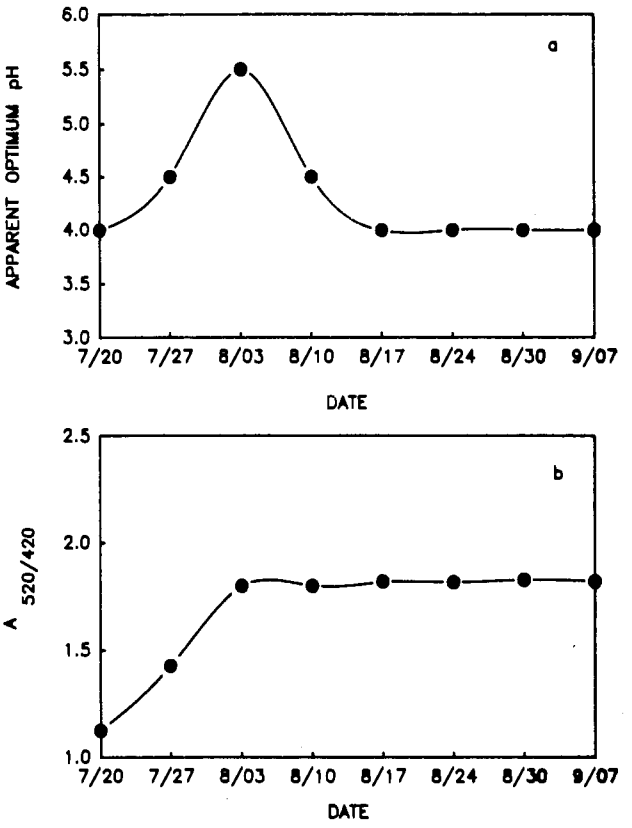

Figure 3. (a) Changes in the apparent optimum pH of Monastrell grape polyphenoloxidase. (b) Ratio between absorbance at 520 and 420 nm of the red variety during maturation.

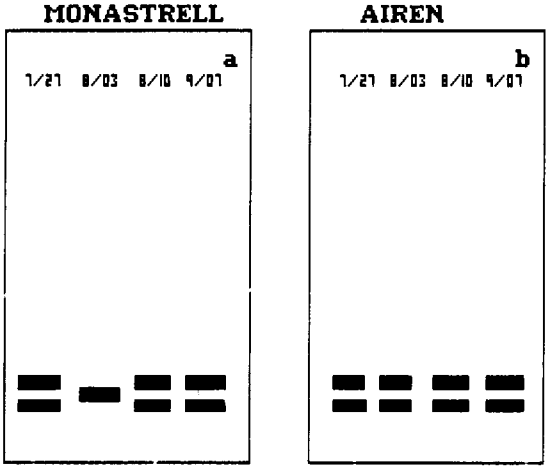

Figure 4. SDS gel electrophoresis of grape polyphenoloxidase during maturation: (a) red grape (cv. Monastrell); (b) white grape (cv. Airen). All lanes contained 50 μg of protein.

(pH >5.5) for the two varieties. The red (cv. Monastrell) presented a different pattern of catecholase activity evolution, depending on the pH used to measure it (Figure 2).

The catecholase activity measured in buffers below pH 5.0 increased during maturation while the catecholase activity measured in buffers above pH 5.0 decreased during maturation. This indicates the presence of at least two different catecholases in Monastrell grapes. One catecholase has an apparent optimum at pH 5.5 and the other has an apparent optimum at pH 4.0. Figure 3a shows that the apparent optimum pH for catecholase activity temporarily shifted from pH 4.0 to 5.5. This shift coincided with the time of véraison or grape anthocyanin formation as shown by a change in the ratio of absorbance at 520 to 420 nm on 8/03 (Figure 3b). The presence of at least three

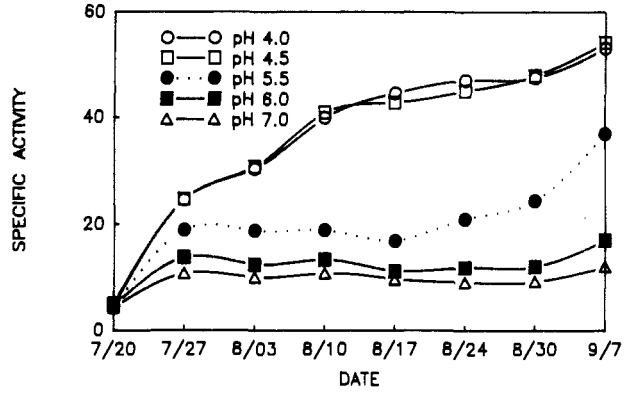

Figure 5. Airen grape polyphenoloxidase during maturation. Trends in catecholase activity with the pH used to measure it.

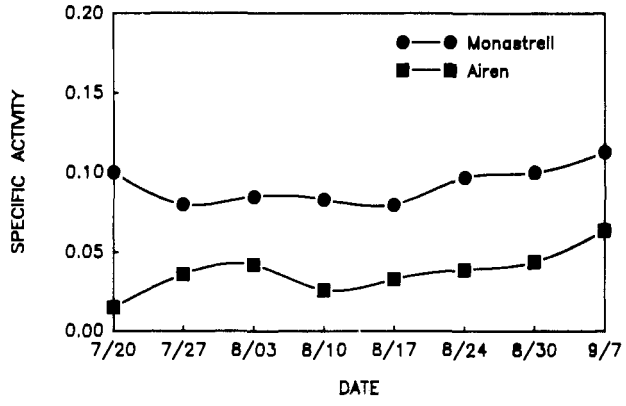

Figure 6. Trend of cresolase activity during grape maturation.

catecholase enzymes in Monastrell grapes was also indicated by the polyphenoloxidases separated by gel electrophoresis (Figure 4a). The relative activities of two polyphenoloxidases visible in the gel disappeared during the week of 8/03 revealing the presence of a third. This coincided with a shift in apparent optimum pH and the week of véraison.

This result could be correlated with the changes in proteins and in anthocyanins that take place during the véraison (Kluba et al., 1978). This change in electrophoretic band pattern has been described for other plant enzymes such as $NADP^+$–malic enzyme from tomato, where only one of the four activity bands was visible at the mature green stage (close to the beginning of the climacteric respiration rise), while it was not visible at the small green, red, or overripe (postclimacteric) stages (Hobson, 1974).

That these changes in catecholase activity are related to the formation of anthocyanins is supported by the observation that there was no shift in the optimum pH of the catecholase activity measured in the white grape Airen (Figure 5), nor was there any change in the polyphenoloxidases separated by gel electrophoresis during the 7 weeks of the study (Figure 4b). The catecholase activity of the extracts from both cultivars measured at pH 4.0 increased continously during the 7 weeks of the study with one exception. On 7/27 the catecholase activity of the Monastrell grape was double that of the week before and the week after (Figure 2a). This coincided with the only heavy rainfall observed during the study. In contrast, the cresolase activity extracted from both cultivars increased much less than did the catecholase activity (Figure 6).

DISCUSSION

The results presented in this paper reveal a complex process of evolution of PPO activities during development and maturation of the grape. Several factors are involved in this process, such as the weather (temperature, rainfall), the content of phenolics and anthocyanins, and the changes in protein synthesis during grape development.

The weather not only acts on shortening or lengthening the time of development and maturation of the berries but also contributes to rapid physiological or biochemical changes (Hrazdina et al., 1984). The content of phenolics and anthocyanins is very closely related with the weather, since high temperatures have been correlated with a decrease of phenols and sudden heat stress with a large decrease in anthocyanin content of the grape berries (Kliewer, 1977). High rainfall also decreases the phenolic concentration of Trebbiano grapes (Piretti et al., 1980). We found in this study that all these changes in berry metabolism induced by the weather also affect the trend of PPO, increasing, for example, after a high rainfall.

The pH used in the study of the evolution of PPO activity during maturation is an important experimental parameter to be borne in mind since, as is shown in this paper, the same PPO has an opposite trend (increasing or decreasing) depending on the pH used to follow it. This behavior was not dependent on the kind of buffer used, since the enzyme showed the same activity in acetate or phosphate buffer at a given pH (pH 5.5). The different trends could be correlated with the balance between the enzymatic forms found in the electrophoresis during maturation for Monastrell grape PPO. This balance could be critical at véraison, the moment when major changes in general metabolism of grape take place (Hrazdina et al., 1983), giving as a result the prevalence of only one enzymatic form with a well-defined bell-shaped optimum pH.

The dependence of PPO on the pH is a varietal characteristic, since the above conclusion is only true for the red variety, while the white variety did not show this dependence. This implies that the changes in the catecholase activity during maturation are related to the formation of the anthocyanins.

The activity of the white variety increased during maturation in agreement with the results found for other white varieties, such as Dutchess and Pinot Blanc (Wissemann and Lee, 1980) and Ugni Blanc and Clairette (Sapis et al., 1983a). The only discrepancy with these results was found by Kidron et al. (1978), since the solubilized Clairette PPO decreased regularly with the grape maturation, with a slight increment at the end of maturation.

This discrepancy found in the bibliography for the trends of white grape PPO could be explained by the different pH used to measure it by the different authors: pH 4.8 (Kidron et al., 1978) and pH 6.5 (Wissemann and Lee, 1980), even with the same extraction method. On the other hand, it could be explained by the different methods of extraction used: For example, in the Clairette variety, PPO was extracted from the chloroplast fraction with 1% Triton X-100 (Kidron et al., 1978), and in other experiments with the same variety, PPO was extracted from the chloroplast fraction after being shaken for 30 min at 0 °C with Polycar AT to set the phenolic compounds without any detergent (Sapis et al., 1983a). The increase in the activity during maturation found by the latter authors did not reflect the true quantity of enzyme present in the grape but only measured the release of increasing amounts of enzyme produced by disruption of chloroplast membrane during ageing (Lieberei and Biehl, 1978). The former authors (Kidron et al., 1978), although taking into consideration the influence of aging by the use of a method of extraction with detergent, did not remove the phenolic compounds, which are well-known as inactivator agents

of PPO (Loomis, 1974; Garcia-Cánovas et al., 1987), and because of their presence the evolution pattern could be affected.

The cresolase activity, characterized by a lag period (defined as the intercept on the abscissa obtained by extrapolation of the linear part of the product accumulation curve), has never been followed during maturation perhaps because it is considerably less than the catecholase activity in grape PPO and, in addition, because the optimal conditions for measuring this activity (pH, temperature, etc.) were not sought. Attempts have been made to measure it in the same way as catecholase activity, but the assays might not even have been extended during the necessary time for cresolase activity to leave its lag period, which can last more than 1 h, if the same enzyme concentration as in the catecholase activity measurement is used (Cabanes et al., 1987; Garcia-Carmona et al., 1987; Sánchez-Ferrer et al., 1988).

CONCLUSIONS

From our investigation into changes in grape polyphenoloxidase from a red and a white variety with different browning capacities during maturation, we conclude that the catecholase enzymes in red grapes change at the moment of véraison, coinciding with the formation of anthocyanin.

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