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Influence of maturity on grape tyrosinase activity

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Abstract. Enzymatic browning of grape must remains a major issue in winemaking, especially when grapes are affected by grey rot. This browning, caused mainly by the enzyme tyrosinase, leads to the formation of melanins that affects seriously the colour of white and red wines. Tyrosinase is known to oxidize ortho-diphenols into colorless ortho-diquinones that later polymerize into brown pigments. However, little recent research has been done on tyrosinase in grape must, and notably, no studies have yet investigated the effect of grape ripeness on tyrosinase activity. This study aims to fill that gap by analyzing how grape maturity influences the enzyme's activity.

1. Introduction

The enzymatic browning of grape must is still a major problem in oenology today [1] being particularly serious when the grapes have been infected by grey rot [2]. Browning is an oxidation process that causes certain foods to turn brown, often resulting in consumer rejection [3]. This is particularly critical in the case of grape must, as grape must is highly susceptible to enzymatic browning [4].

Tyrosinase (EC 1.14.18.1) is the main enzyme responsible for the browning of grape must derived from healthy grapes. The mechanism of oxidation provoked by this enzyme results in the conversion of ortho-diphenols into ortho-diquinones, which subsequently polymerize to create melanins. These diquinones formed at first are colourless, but the melanins produced later through chemical polymerization exhibit a yellow-brown hue. These melanins contribute to the deepening of the yellow colour in white wines (a phenomenon known as browning) and lead to colour degradation in red wines [4]. During last years very few information about tyrosinase from grape must has been reported [5,6,7,8,9] and to the best of our knowledge, none of these studies have explored the impact of ripeness on tyrosinase activity. Therefore, the aim of this research was to examine how grape maturity influences tyrosinase activity.

2. Materials and methods

2.1. Chemical reagents and equipment

Polyvinylpolypyrrolidone (PVPP), was purchased from Sigma-Aldrich (Madrid, Spain). Caftaric acid (purity ≥ 99.9 %) was purchased from Biosynth S. R. O. (Bratislava, Slovakia). Ethanol (96 % vol.) and hydrochloric acid (purity ≥ 36.5 %) were supplied by Fisher Scientific (Madrid, Spain).

The equipment used was as follows: a spectrophotometer UV-Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a thermostatic bath (6,000,137 Selecta, Abrera, Barcelona, Spain); and a CB Standard Balance (Cobos, Barcelona, Spain).

2.2. Preparation of grape must and removal of phenolic compounds

Healthy grapes from the cultivars Macabeo and Muscat of Alexandria variety (Variety number VIVC, n.d.: 8241) and Macabeo (Variety number VIVC, n.d.: 13127) were picked at different maturity levels from the experimental vineyard of the Rovira i Virgili University (Mas dels Frares, Constantí, Tarragona: 41° 08′ 44.1″ N; 1° 11′ 51.0″

E) during the 2023 vintage harvest. The bunches were frozen in plastic bags at -20 ºC until the moment of the analysis.

Bunches were defrosted for 24 hours at room temperature and berries were manually destemmed and ground with a blender (Silvercrest SSM550C1, Kompernass GMBH, Bochum, Germany). Then, 20 mg/Kg of pectolytic enzyme (Lallzyme C-Max™, Lallemand, Inc., Montreal, Canada) was added and the sample was macerated at 4 ºC for 24 h to favour the extraction of the tyrosinase enzyme from the grape skin fragments, with the method applied in our previous work [9]. After maceration the sample was sieved, manually pressed and the solid parts were separated by centrifugation, obtaining a clean must. The must was not supplemented with sulphur dioxide to avoid tyrosinase inhibition.

To prevent interference from other polyphenols and ensure that tyrosinase activity was measured exclusively with a single substrate in the reaction medium, all phenolic compounds were entirely removed from the grape must. The phenolics were removed using polyvinylpolypyrrolidone (PVPP). A suspension of 200 g/L in distilled water was prepared and 10 mL was placed in separation columns, before water removal with a vacuum pump. Using the vacuum pump, 100 mL of grape must was percolated through these columns, and the process was then repeated until a Total Polyphenol Index (TPI) lower than 2 was obtained. The TPI was determined by measuring the absorbance at 280 nm of the must in a quartz cuvette [10].

2.3. Stock solutions of caftaric acid, sulphur dioxide, ascorbic acid and glutathione.

Stock solution of caftaric acid (30 mM) was prepared with oxygen-free distilled water acidified to pH = 3.5 with hydrochloric acid. The oxygen-free acidified water was prepared by purging it with nitrogen for 10 minutes.

2.4. Measurement of the apparent tyrosinase activity

The apparent tyrosinase activity was determined by the methodology previously developed by our group [9]. We refer to tyrosinase activity as "apparent" because it was assessed solely by measuring the browning of the reaction medium. This browning results not only from the enzymatic oxidation caused by tyrosinase but also from subsequent polymerization reactions of quinones, which produce the melanins responsible for the observed browning. Briefly, aliquots of 1.8 mL of the grape must, from which all phenolic compounds had been removed, were introduced in spectrophotometer microcuvettes with a 10 mm optical path length. Afterwards, caftaric acid was added at concentrations of 0, 0.25, 0.50, 1.0 and 1.5 mM. The total volume was adjusted to 2 mL using distilled water. The microcuvettes were manually shaken to homogenize and saturate the must with oxygen, and periodic measurements of absorbance at 420 nm were made to monitor the formation of brown pigments, until asymptomatic behaviour was observed. Experiments were carried out in triplicate at 25 ºC. Since pH has a strong influence on tyrosinase activity [9], all measurements were carried out at pH = 3.50 and also at the natural pH of each sample, in order to allow for normalized comparison of activities. The tyrosinase activity was calculated based on the maximum reaction velocity, as explained in the next section.

2.5. Determination of kinetic constants

The kinetic constants were calculated by the Michaelis-Menten [11](Michaelis & Menten, 1913) and Lineweaver-Burk [12] (Lineweaver & Burk, 1934) models, which make it possible to determine the Michaelis constant (KM) and maximum reaction velocity (Vmax). Experiments were considered to follow the Michaelian model if the linear adjustment coefficients (r2) of the Lineweaver-Burk plot were greater than 0.95 and the point of intersection with the Y axis was on the positive side.

2.6. Statistical analysis

Results are expressed as mean values ± standard deviation of three replicates. Treatments were compared with one-factor analysis of variance (ANOVA) using the XL-STAT 2024.3.0 software (Addinsoft, Paris, France).

3. Results and discussion

Figure 1 shows the changes in absorbance at 420 nm according to incubation time for Muscat of Alexandria at three different levels of maturity.

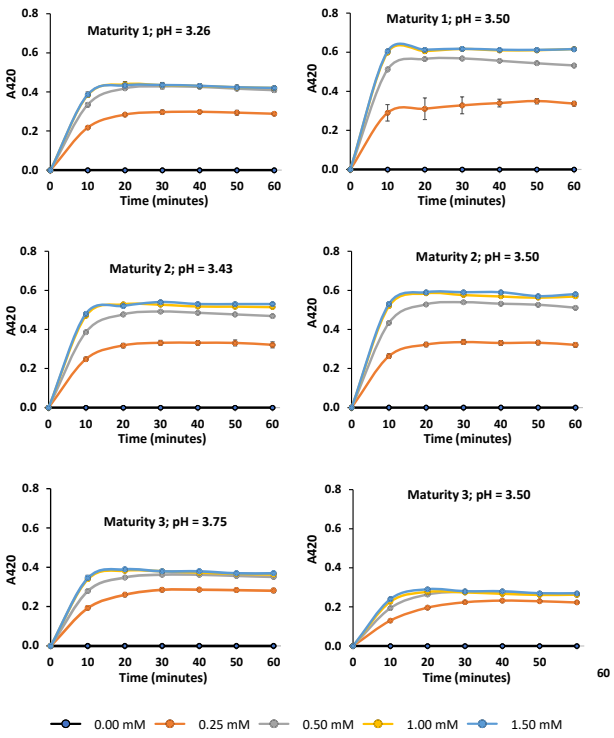

Figure 1. Tyrosinase browning kinetics for Muscat of Alexandria in function of maturity.

Figure 2 shows the changes in absorbance at 420 nm according to incubation time for Macabeo at three different levels of maturity.

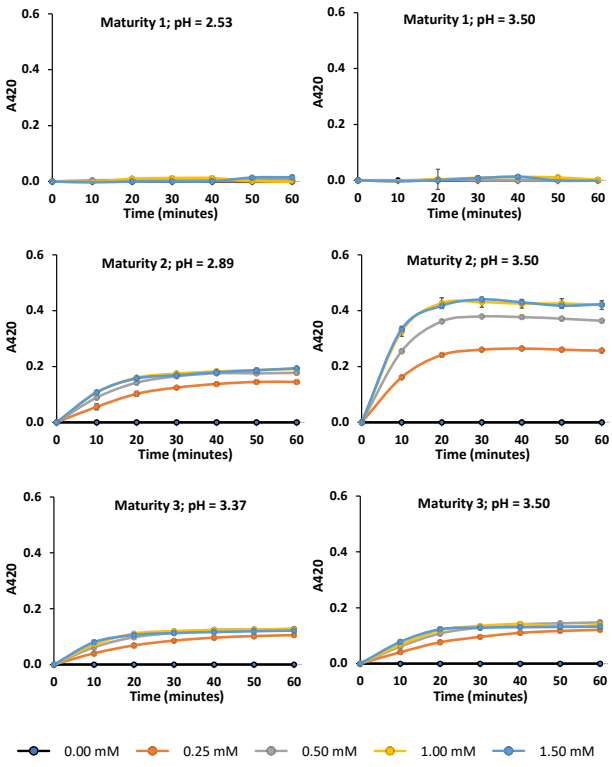

Figure 2. Tyrosinase browning kinetics for Macabeo in function of maturity.

As expected, the absorbance at 420 nm for both varieties increased over time in all cases, and this browning effect was more pronounced when the concentration of caftaric acid was higher. These tyrosinase browning kinetics were measured at the corresponding pH of each sample and also at pH = 3.50 because pH exerts a great influence on tyrosinase activity [9] and consequently it is needed in order to allow for normalized comparison of activities.

In order to better compare the tyrosinase activity of Muscat of Alexandria and Macabeo as a function of ripeness, Michaelis-Menten and Lineweaver-Burk plots were created (Figures 3 and 4).

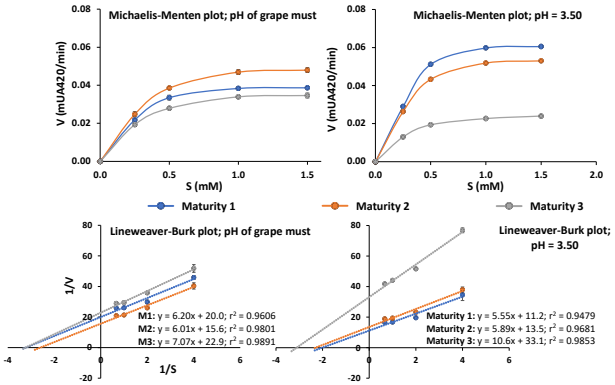

Figure 3. Tyrosinase browning kinetics for Muscat of Alexandria in function of maturity.

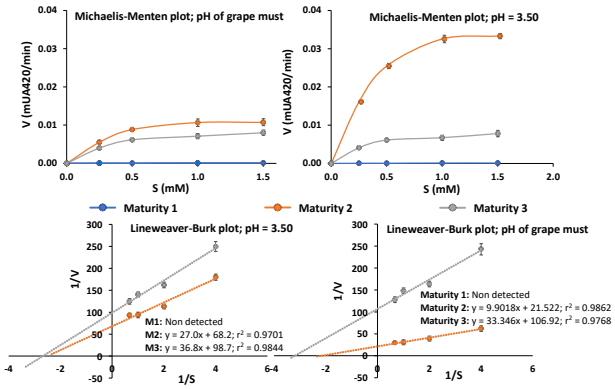

Figure 4. Tyrosinase browning kinetics for Macabeo in function of maturity.

In the measurements carried out at the natural pH of each ripeness level, it is important to consider that pH has a significant effect, causing an apparent increase in activity as the grapes ripen—greater than what would be observed at a constant pH. In the case of Muscat of Alexandria, the Michaelis-Menten plot suggests that tyrosinase activity increases between the first and second stages of ripeness, to decrease thereafter. For Macabeo, no tyrosinase activity was detected at the first ripeness stage, probably because the grapes were too unripe, and a decrease in activity is observed between the second and third stages, following a pattern similar to that of Muscat of Alexandria.

When tyrosinase activity was normalized to a constant pH (pH = 3.50), it was observed that the activity tended to decrease with increasing ripeness in the case of Muscat of Alexandria. A similar trend was observed for Macabeo, although no activity was detected at the first ripeness stage

Lineweaver-Burk plots were used to determine the kinetic constants of tyrosinase for each of the two grape varieties as a function of ripeness, considering both the natural pH of the grapes and the standardized pH of 3.50. Tables 1 (Vmax) and 2 (K_M) show these kinetic constants.

Table 1. Maximum velocity (Vmax)

Vmax (mUA420/min)	Muscat of Alexandria		Macabeo	
	pH of grape must	pH = 3.50	pH of grape must	pH = 3.50
Maturity 1	0.502 ± 0.013 B	8.96 ± 0.41 C	Non detected	Non detected
Maturity 2	0.644 ± 0.021 C	7.41 ± 0.17 B	0.015 ± 0.001 B	0.047 ± 0.002 B
Maturity 3	0.437 ± 0.016 A	3.02 ± 0.05 A	0.010 ± 0.001 A	0.009 ± 0.002 A

Table 2. Maximum velocity (Vmax)

KM (mM)	Muscat of Alexandria		Macabeo	
	pH of grape must	pH = 3.50	pH of grape must	pH = 3.50
Maturity 1	0.311 ± 0.008 A	0.497 ± 0.024 C	Non detected	Non detected
Maturity 2	0.387 ± 0.012 B	0.436 ± 0.010 B	0.476 ± 0.014 A	0.553 ± 0.022 B
Maturity 3	0.309 ± 0.01 A	0.321 ± 0.005 A	0.447 ± 0.017 A	0.374 ± 0.030 A

The Vmax data clearly indicate that tyrosinase activity levels are significantly higher in Muscat of Alexandria than in Macabeo, suggesting a greater susceptibility to browning in Muscat of Alexandria than in macabeo grapes. These results also confirm that, at the natural pH of the must, tyrosinase activity increases from the first to the second ripeness stage and then decreases. However, in the case of Macabeo, no activity was detected in the least ripe grapes, likely because the grapes were excessively unripe. When tyrosinase activity was measured at a standardized pH (pH = 3.50), the results were more consistent and indicated that tyrosinase activity decreases with increasing ripeness, although this decrease is partially offset by the rise in grape must pH.

Regarding the Michaelis constant (K_M), only slight variations were observed when measurements were taken at the natural pH of each ripening stage. In contrast, when measurements were performed at a standardized pH (pH = 3.50), K_M was found to decrease as the grape matured. These results suggest that the affinity of tyrosinase for their substrates increases with ripeness.

4. Conclusions

These results indicate that tyrosinase activity levels vary significantly among wine grape varieties, which undoubtedly influences the susceptibility of the musts from different cultivars to enzymatic browning. In adition, it is clear that pH exert a significant influence on tyrosinase activity, with activity being significantly lower at lower pH levels. On the other hand, in both varieties, it is observed that tyrosinase activity increases between the first two maturity points and then decreases thereafter when the measurements were performed at the natural pH of the grape must. In contrast, when the measurements were conducted at a standardized pH (pH = 3.50), tyrosinase activity appeared to decrease with increasing maturity. Further studies are needed to determine the effect of tyrosinase activity at the time of harvest on the oxidability of the must.

5. Acknowledges

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