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Kinetic Study of Hydroxytyrosol Oxidation and Its Related Compounds by Red Globe Grape Polyphenol Oxidase

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ABSTRACT: Red Globe grape polyphenol oxidase, partially purified using phase partitioning with Triton-X114, was used to study the oxidation of hydroxytytosol (HT) and its related compounds tyrosol (TS), tyrosol acetate (TSA), and hydroxytyrosol acetate (HTA). The enzyme showed activity toward both monophenols (monophenolase activity) and *o*-diphenols (diphenolase activity) with a pH optimum (pH 6.5) that was independent of the phenol used. However, the optimal temperature for diphenolase activity was substrate-dependent, with a broad optimum of 25−65 °C for HT, compared with the maximum obtained for HTA (40 °C). Monophenolase activity showed the typical lag period, which was modulated by pH, substrate and enzyme concentrations, and the presence of catalytic amounts of *o*-diphenols. When the catalytic power (V_{max}/K_M) was determined for both activities, higher values were observed for *o*-diphenols than for monophenols: 9-fold higher for the HT/TS pair and 4-fold higher for HTA/TSA pair. Surprisingly, this ratio was equally higher for TSA (2.2-fold) compared with that of TS, whereas no such effect was observed for *o*-diphenols. This higher efficiency of TSA could be related to its greater hydrophobicity. Acetyl modification of these phenols not only changes the kinetic parameters of the enzyme but also affects their antioxidant activity (ORAC-FL assays), which is lower in HTA than in HT.

KEYWORDS: *hydroxytyrosol, tyrosol, polyphenol oxidase, grape, monophenolase activity, diphenolase activity*

INTRODUCTION

Phenolic compounds are important plant secondary metabolites found in a variety of foods, especially those used in the Mediterranean diet (olives, olive oil, grapes, wine). Their presence has been linked to the reduced incidence of cardiovascular disease, several types of cancer, and degenerative diseases.[1] Among them, hydroxytyrosol [2-(3,4-dihydroxyphenyl) ethanol; HT] and tyrosol [2-(4-hydroxyphenyl)ethanol; TS] and their corresponding acetate derivates (Figure 1) are considered multitargeted bioactive compounds,[2,3] acting as potent antioxidants,[4] cardioprotectives,[5,6] antimicrobials,[7] antimycotics,[8] antidiabetics,[9] neuroprotectives,[10] anti-inflammatories,[11] and antitumorals.[12] The biological activity of these phenols, along with their high degree of bioavailability, means that they are recommended for inclusion in the diet and for use as nutraceuticals. EFSA has given a positive opinion on the use of hydroxytyrosol (for example, olive polyphenol for protecting LDL particles from oxidative damage; http://efsa.europa.eu/en/efsajournal/doc/2033.pdf) and, in addition, it could be considered safe up to 20 mg/kg/day.[2]

The main sources of hydroxytyrosol and its related compounds in the diet are virgin olive oil[2,13,14] and wine, both white and red (1.85−3.89 mg/mL).[15−19] Their presence in wines is hypothesized to be due to the transformation of tyrosol into hydroxytyrosol during alcoholic fermentation.[15] This conversion could also be brought about by endogenous enzymes, especially the polyphenol oxidase (PPO) present in grapes. PPO (EC 1.14.18.1) is a bifunctional copper enzyme that catalyzes two different reactions in the presence of molecular oxygen, the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity).[20−22]

The present work represents the first detailed kinetic study of the oxidation of hydroxytyrosol and its related compounds (tyrosol, tyrosol acetate, and hydroxytyrosol acetate), which are assessed using partially purified Red Globe grape PPO. Interestingly, whereas its catalytic powers toward *o*-diphenols (HT and HTA) were similar, TSA was a better substrate than TS, probably due to its hydrophobicity. The presence of an additional acetyl group in HT and its related compounds also affected their FL-ORAC value, which was lower in *o*-diphenols than in monophenols (with HT > HTA and TSA > TS).

MATERIALS AND METHODS

Chemicals and Materials. Fully mature, bright red, thick waxy grapes of the var. Red Globe (Agrokasa, Valle de Ica, Peru) were obtained from a local supermarket. Hydroxytyrosol and tyrosolacetate esters were from TRC (Ontario, Canada). The rest of the reagents, including hydroxytyrosol and tyrosol, were from Sigma-Aldrich and were of the maximum purity available, except for Triton X-114, which was purified as described.[23]

Enzyme Purification. Red Goble grape PPO was extracted with Triton X-114 using the method described by Sánchez-Ferrer et al.[24] with some modifications. Grapes (150 g) were homogenized with 25 mL of cold 10 mM ascorbic acid in 10 mM phosphate buffer, pH 7.3. The resulting homogenate was filtered through eight layers of gauze

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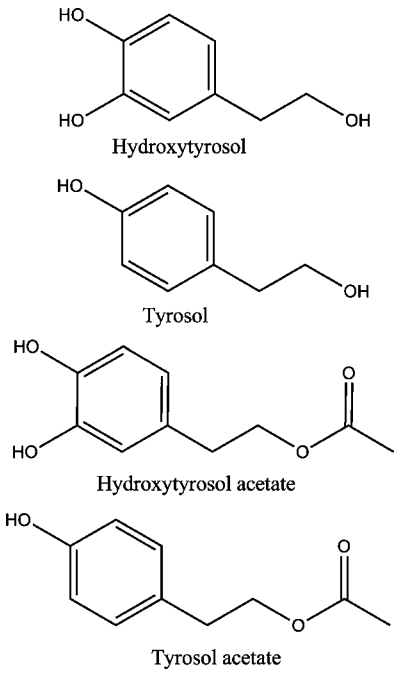

Figure 1. Chemical structures of hydroxytyrosol, tyrosol, and their corresponding acetate derivates.

and centrifuged at 4000*g* for 10 min at 4 °C. The supernatant was discarded, and the precipitate was extracted with 20 mL of 1.5% (w/v) Triton X-114 in 10 mM sodium phosphate buffer, pH 6.0. This extract was shaken gently overnight at 20 °C and centrifuged at 60000*g* for 15 min at 4 °C. The supernatant was mixed with 4% (w/v) Triton X-114. The mixture was subjected to temperature-induced phase partitioning by keeping it at 4 °C for 15 min and then warming it to 37 °C for 15 min. After this period, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins and phenolic compounds. This turbid solution was centrifuged at 5000*g* for 15 min at 30 °C. After centrifugation, the detergent-rich phase was discarded and the clear detergent-poor supernatant was used as enzyme source and stored at −20 °C.

Enzyme Activity. Diphenolase activity was determined spectrophotometrically following the conversion of HT and HTA into their corresponding *o*-quinones. Their molar absorption coefficients (ε) were obtained after instantaneous chemical oxidation of *o*-diphenols at different concentrations (0−0.5 mM) by sodium periodate in excess.[25] The values obtained for HT and HTA quinones at 400 nm were 950 and 1120 M^{-1} cm^{-1}, respectively.

The standard reaction medium at 25 °C contained 0.1 μg of a partially purified PPO, 10 mM sodium phosphate, pH 6.5, and 50 mM HT or HTA in a final volume of 1 mL. The standard monophenolase reaction medium at 25 °C contained 1.2 μg of a partially purified PPO, 10 mM sodium phosphate, pH 6.5, and 20 mM TS or TSA in a final volume of 1 mL. Monophenolase activity at the steady-state rate was calculated from the linear part of the product accumulation curve after the lag period.[26] One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of the corresponding *o*-quinone per minute. The effect of pH was measured in the corresponding standard medium at different pH values in 10 mM acetate (pH 4.0−5.5) or phosphate (pH 6.0−7.5) buffers. The effect of temperature was also measured in the standard reaction medium at different temperatures (5−75 °C). The protein content was measured by the bicinchoninic acid assay, using BSA as a standard.[27] Experiments were conducted with a minimum of three replicates per condition.

Determination of the Antioxidant Activity. Oxygen radical absorbance capacity with fluorescein as fluorescent probe (ORAC-FL) was used to determine the antioxidant activity in vitro using 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as free radical generator.[28] The latter is considered to produce the peroxyl radical as a result of heat, which damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are considered to protect the fluorescent molecule from oxidative degeneration. The reaction medium (200 μL) contained 30 nM fluorescein, 19 mM AAPH, and different concentrations of antioxidant in 75 mM sodium phosphate, pH 7.4. The reagents were added and measured in a microplate reader (Synergy-HT, Boptek, VT) as previously described.[28] Trolox C was used as a reference antioxidant.

RESULTS AND DISCUSSION

Enzyme Purification. Partially purified grape PPO var. Red Globe (PPO*rg*), obtained by using two-phase partitioning in Triton X-114,[28] displays activity toward the *o*-diphenol hydroxytyrosol (diphenolase activity) and shows a continuous increase in product accumulation (absorbance) with time (Figure 2, curve a). In addition, the enzyme was also active

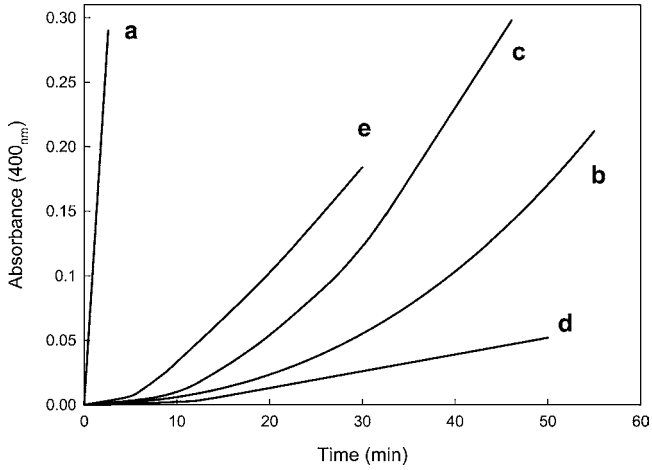

Figure 2. Enzymatic activities of Red Globe grape PPO: (a) diphenolase activity determined in the standard reaction medium; (b) monophenolase activity measured in the standard reaction medium; (c, d) same conditions as in (b) except for (c) different enzyme concentration (1.8 μg) or (d) different substrate concentration (0.1 mM); (e) presence of *o*-diphenol in the reaction medium (10 μM hydroxytyrosol).

toward the corresponding monophenol (tyrosol) (Figure 2, curves b−d). This monophenolase activity was characterized by a lag period (time required to reach the steady-state rate), which depends on several factors such as the enzyme source,[30] the concentration of monophenol used (compare curve b with curve d in Figure 2),[31] the enzyme concentration (Figure 2, curve c),[32] and the presence of catalytic amounts of *o*-diphenol in the reaction medium (Figure 2, curve e).[33]

Characterization of Diphenolase Activity. The diphenolase activity of PPO*rg* toward HT and HTA was greatly affected by pH (Figure 3a). The enzyme was active between pH 4 and 7.5, with an optimum at pH 6.5 (Figure 3a) for both substrates. This optimum pH was similar to that described for other grape PPOs, such as those from the varieties Koshu[34] and Dominga,[35] but contrasted with those recently found in other PPOs (pH 7.5−8.0) from *Lonicera japonica*, *Cleome gynandra*, and *Brassica oleracea*.[36−38]

PPO was affected by temperature over the range 5−75 °C (Figure 3b) and showed a differential behavior that depended on the diphenol used. Thus, the temperature profile toward HT showed a broad temperature optimum (25−65 °C), whereas the activity was halved at 75 °C. However, when HTA was used

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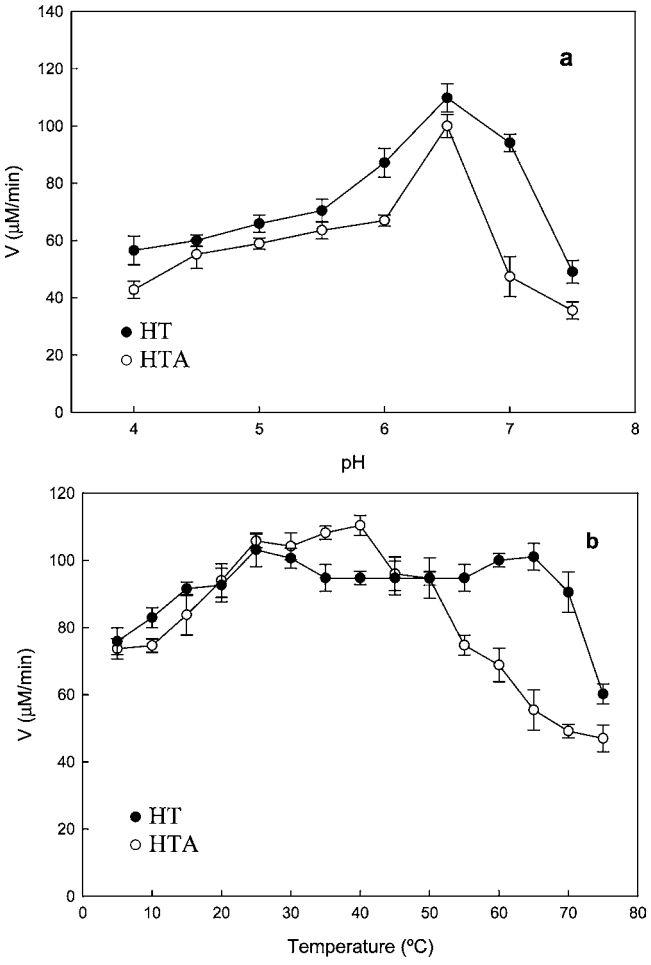

Figure 3. Effect of (a) pH and (b) temperature on the diphenolase activity with HT and HTA.

as substrate, the profile showed a clear maximum at 40 °C, the activity decreasing gradually as the temperature increased, falling below 50% at 70 °C (Figure 3b, open circles). These values are higher than those described for other grape PPOs, such as those from Kosku (25−30 °C),[34] Airen (25−45 °C),[30] or Thompson Seedless (10−25 °C) varieties.[39] The PPO*rg* optimum temperature was also higher than those recorded for cauliflower PPO (55 °C),[38] soybean PPO (50 °C),[40] and mango PPO (55 °C).[41]

The kinetic parameters (V_{max} and K_M) were determined for both substrates (HT and HTA) using 0.1 μg/mL of partially purified PPO*rg* in 10 mM sodium phosphate, pH 6.5 (Table 1). The K_M value obtained for PPO*rg* with HT was higher than that described for mushroom PPO (21.6 vs 0.9 mM).[42] This K_M was also 3-fold higher than that obtained for HTA (Table 1). No previous data for HTA are available to make comparisons.

Table 1. Kinetic Parameters of Red Globe PPO[a]

	diphenolase activity		monophenolase activity	
	HT	HTA	TS	TSA
K_M (mM)	21.6	7	0.76	0.23
V_{max}* (μM/min/μg)	1711	590	6.7	4
V_{max}*/K_M (min^{-1})	0.079	0.084	0.008	0.017

[a]The reaction was assayed spectrophotometrically in the corresponding standard reaction medium (see Materials and Methods for details). V_{max} was normalized by enzyme concentration used in each activity.

Characterization of Monophenolase Activity. Monophenolase activity toward tyrosol (TS) and tyrosol acetate (TSA) slowly increased as the pH increased above pH 4.0, showing a broad maximum at around pH 6.5 (Figure 4a).

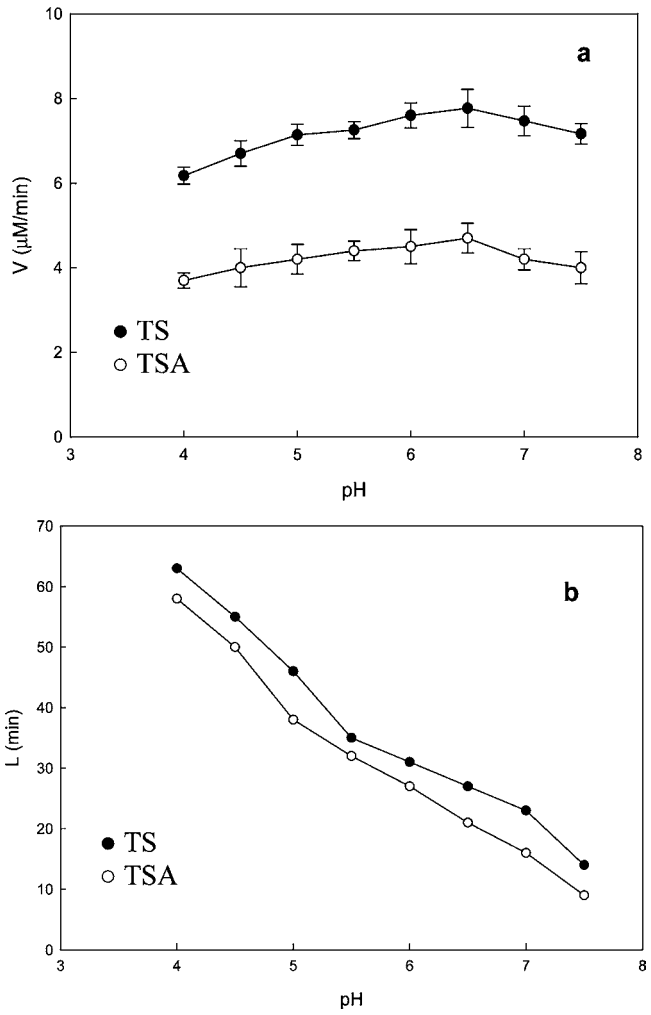

Figure 4. Effect of pH on the monophenolase activity (a) and on the lag period lag period (b) with TS and TSA.

However, the pH clearly affected the lag period, which decreased as pH increased (Figure 4b). These results are similar to those described for Monastrell grape PPO using *p*-cresol as substrate[43] and for banana pulp PPO using tyramine as substrate.[44]

Increasing the concentration of both monophenols (TS and TSA) produced an increase in the lag period of partially purified PPO*rg* (data not shown), an effect similar to that described for other plant PPOs,[26,44−46] although the exact profile depends on the enzyme source used.[32] The kinetic constants obtained (K_M and V_{max}) toward TS and TSA (Table 1) were 0.76 mM and 8.1 μM min^{-1} and 0.23 mM and 4.99 μM min^{-1}, respectively. These values were in a range similar to those reported for the monophenolase activity of Monastrell grape, potato, and eggplant PPOs using *p*-cresol as substrate.[26,29,43] However, the K_M for TS was 5-fold higher than that described for mushroom PPO (0.72 vs 0.15 mM).[42] The V_{max}/K_M ratio (catalytic power) was used to compare the

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action of PPO*rg* toward monophenols and diphenols, using a normalized enzyme concentration (Table 1). This ratio was higher for *o*-diphenols than for monophenols, 9-fold higher for the HT/TS pair, and 4-fold higher for the HTA/TSA pair. Of note was the fact that the catalytic power was 2-fold higher for TSA than for TS, whereas this effect was not evident in a comparison with diphenols. This higher efficiency for TSA compared with TS could be related with its higher hydrophobicity, which would improve binding at the active site. In fact, the active site in the case of Grenache grape PPO contains six conserved tryptophans and eight conserved phenylalanines.[47]

The lag period was also affected by the enzyme concentration in the reaction medium, decreasing hyperbolically as the concentration increased (Figure 5a, solid circles),

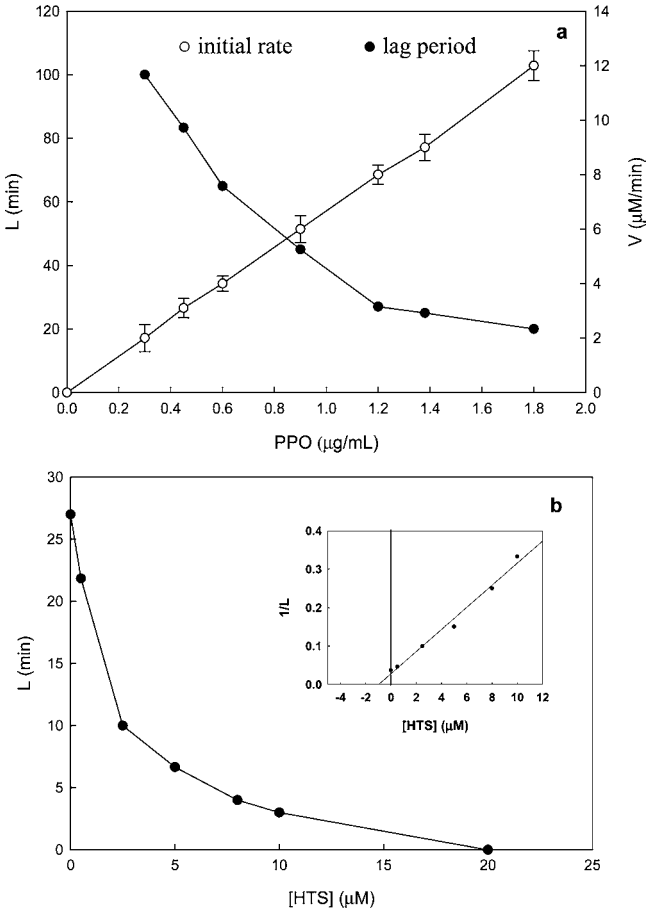

Figure 5. (a) Effect of enzyme concentration on the initial rate and on the lag period of the monophenolase activity. (b) Effect of HT concentration on the shortening lag period. In the standard reaction medium, the concentration of the HT was modified from 0 to 20 μM. (Inset) Evaluation of the activation constant, K_{act}, using the Pomerantz equation (see text for details).

whereas the steady-state rate increased linearly (Figure 5a, open circles). These results were similar to those obtained for PPO from other sources using *p*-coumaric and *p*-cresol as substrates, respectively.[26,48] In no case could the lag period be abolished by increasing the enzyme concentration, because, as shown the Figure 5a (solid circles), this curve is asymptotic and the value is not zero. The lag can only be completely removed by catalytic amounts of the corresponding *o*-diphenols,[31,33,49] the effect of which can be studied, according to Pomerantz and Warner,[33] using the empirical formula

$$\frac{1}{l} = \frac{1}{L} + \frac{1}{L} \times \frac{[o\text{-diphenol}]}{K_{act}}$$

where l represents the lag period in the presence of *o*-diphenol, L, the lag period in the absence of *o*-diphenol, and K_{act}, the diphenol activation constant.

Increasing amounts of HT reduced the lag period of the monophenolase activity until it was abolished at 20 μM (Figure 5b). The activation constant K_{act} calculated from the reciprocal of the lag period in the presence of HT (Figure 5b, inset) was 1 μM. This value was lower than those obtained for potato (4.5 μM)[29] and eggplant (7.9 μM) PPO[26] using 4-methylcatechol as activator. In addition, it was 134-fold lower than that of HTA (K_{act} = 134 μM), perhaps due to the higher hydrophobicity of the TSA/HTA pair.

To test the effect of this higher hydrophobicity of HTA on the antioxidant activity, fluorescein (FL) fluorescence decay was induced by AAPH (ORAC-FL method) in the presence of this compound (Figure 6), comparing the curves with those of

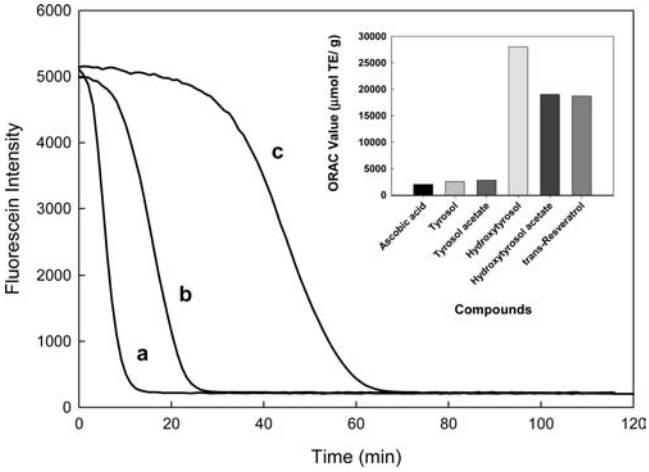

Figure 6. ORAC-FL assay. Fluorencesce decay curve was induced by APPH in the presence of HTA. Only three concentration curves (a, 0 μM; b, 0.75 μM; c, 3.75 μM) are shown for clarity. (Inset) Values obtained with different antioxidants. The values are expressed as μmol of Trolox equivalent (TE) per gram.

TS, TSA, HT, ascorbic acid, and resveratrol. The values obtained (Figure 6, inset) showed that HTA had lower antioxidant capacity than HT, but activity similar to that of resveratrol (19000 vs 18690 μmol TE/g). The antioxidant activity of both monophenols (TS and TSA) was similar to that of ascorbic acid, but far from that of their corresponding *o*-diphenols.

In conclusion, this paper provides for the first time a detailed kinetic characterization of the monophenolase and diphenolase activity of Red Globe grape PPO toward the TS/HT pair and the corresponding acetates (TSA/HTA). Acetyl modification, which changes the hydrophobicity of the latter monophenol compound, could be related with its higher catalytic power compared with that of the TS. In addition, this modification lowers the antioxidant activity of HTA compared with HT. Further research is required to determine the physiological role of grape PPO in the presence of HT in wine, if any.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

HT, hydroxytyrosol; HTA, hydroxytyrosol acetate; TS, tyrosol; TSA, tyrosol acetate; PPO, polyphenol oxidase; FL, fluorescein; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride

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