# Resveratrol and a Novel Tyrosinase in Carignan Grape Juice

Regev Gilly,[†] Dekel Mara,[‡] Shoseyov Oded,[‡] and Kerem Zohar*[,†]

Institute of Biochemistry, Food Science and Nutrition, and Department of Horticulture, The Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

Polyphenol levels in wines are affected by the wine-making process. Resveratrol is one polyphenol which has been the subject of a commendable amount of recent research. In this work, we found that resveratrol is immediately degraded by tyrosinase. A novel tyrosinase was purified from Carignan grapes. The purification process included salting out and separation on a cation-exchange column, followed by gel filtration. Tyrosinase was purified in a homogeneous form by SDS−PAGE and was characterized: its specific activity toward 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) increased by a factor of 24 with an overall recovery of 3% of initial activity. The apparent molecular mass of the purified tyrosinase was 40 kDa as determined by SDS−PAGE, and 42 kDa as determined by gel filtration. Its activity was optimal at pH 6 and at 25 °C. The enzyme exhibited high activity toward phenylenediamine, epicatechin, pyrogallol, DOPA, and resveratrol. Tyrosinase activity was inhibited by KCN, thiourea, and $SO_2$. Resveratrol levels were stable following the removal of proteins from the juice, suggesting that early spraying of grapes with $SO_2$ is an important factor affecting the final amount of resveratrol in wine.

**Keywords:** *Resveratrol; tyrosinase; wine; grape; Vitis vinifera cv. Carignan*

## INTRODUCTION

Polyphenol oxidases (PPOs) occur widely in living organisms, particularly plants and animals, oxidizing a range of phenolic substrates to produce reactive quinones (*1*). The role of these enzymes in plant metabolism is not clear, although they may be involved in the plant's defense system (*1, 2*). In mammals, they are bifunctional enzymes catalyzing both the hydroxylation reaction that converts tyrosine to 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) and the oxidation reaction that converts DOPA to DOPA-quinone.

PPO enzymes extracted from different sources show varying degrees of utilization of phenolic substrates. Mushroom PPO has been shown to be active with both mono- and dihydroxyphenols, while PPO enzymes of plant origin, including grapes, generally utilize only dihydroxyphenols.

Originally, the term PPO referred to two enzymes − laccase or *p*-diphenol:oxygen oxidoreductase (EC 1.10.3.2), and catechol oxidase or *o*-diphenol:oxygen oxidoreductase (EC 1.10.3.1) (*3*). In general, they all have four copper atoms in their active site, use molecular oxygen as the final electron acceptor, and have high substrate specificities.

In the wine industry, enzymatic oxidation by PPO is an economically relevant problem. $SO_2$, which has long been known as an effective inhibitor of PPO (*4*), is added during the wine-making process. Although high levels of $SO_2$ may result in a lower quality wine, insufficient quantities during the wine-making process may result in enzymatic oxidation reactions that negatively affect the organoleptic and color properties of the wine.

Although PPOs, and particularly catecholase in grapes, have been studied widely (*5*−*10*), as have their effects on musts and wines (*4, 11, 12*), most of those studies were conducted on crude or only partially purified enzymes.

*Trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene), which has attracted a lot of attention because of its therapeutic value, is a phenolic compound present in grapes and wines. It has been widely reported in relation to its antifungal properties and its health benefits, including estrogenic and anticarcinogenic effects and protection against cardiovascular diseases (*13*−*20*). Resveratrol is found in the skins and the seeds of grape berries and is therefore usually found at higher levels in red wines than in white wines due to skin contact during fermentation. Resveratrol concentrations have been shown to increase during fermentation with the skins, but the amount extracted was dependent on the variety and enological conditions (*21*−*29*).

In this work, we purified a novel tyrosinase from red juice of Carignan grapes and demonstrated its activity toward resveratrol. Carignan (*Vitis vinifera* cv. Carignan) is one of the most abundant red grape varieties in the world that is used for the production of red wines. We suggest that a major contribution to the reduction of resveratrol levels in wines is due to the residual activity of grape tyrosinase in the juice, in addition to agrotechnical conditions.

## MATERIALS AND METHODS

Tyrosinase, resveratrol, pyrogallol, phenylenediamine, DL-3,4-dihydroxyphenyl-alanine (DL-DOPA), chlorogenic acid, caffeic acid, epicatechin, catechol, guaiacol, KCN, sodium azide, thiourea, Bradford reagent, and tyrosine were purchased from Sigma (St Louis, MO). Sodium acetate and acetic acid were purchased from Baker J. T. (Phillipsburg, NJ).

**Grapes.** Carignan grapes (Vitis *vinifera* cv. Carignan) were

* Corresponding author. Tel: 972-8-9489278. Fax: 972-8-9476189. E-mail: kerem@agri.huji.ac.il.

[†] Institute of Biochemistry, Food Science and Nutrition.

[‡] Department of Horticulture.

10.1021/jf0011079 CCC: $20.00 
Published on Web 02/14/2001

harvested at commercial maturity and were pressed to produce juice at the Carmel Mizrahi winery, Zichron Yaacov, Israel.

**HPLC Analysis.** HPLC analysis was performed to detect resveratrol.The HPLC system (Thermo Separation Products, Riviera Beach, FL) consisted of an auto-sampler (AS3000), injector (100 $\mu$L), column oven (30 °C), pump (P3000), and diode array detector (UV6000). A reverse-phase hexyl-phenyl column (250 × 4.6 mm, "Luna" Phenomenex, Torrance, CA) was employed. Elution was performed using water and methanol, both acidified with 0.01% formic acid, at a flow rate of 1 mL/min. A linear gradient program was developed:

| time (min) | water (%) | MeOH (%) |
|---|---|---|
| 0 | 70 | 30 |
| 3 | 70 | 30 |
| 18 | 10 | 90 |
| 21 | 10 | 90 |
| 22 | 70 | 30 |

*Trans*-resveratrol was monitored at 306 nm.

**Ultrafiltration.** The juice was de-proteinated by ultrafiltration through a C-10 membrane filter (Spectrum Medical Industries Inc., Los Angeles, CA).

**PPO Assay.** PPO activity was measured using DOPA as a substrate. The reaction was carried out at 25 °C with 20 mM acetate buffer, pH 6.5, and 6.7 mM DOPA in a total volume of 700 $\mu$L. The increase in absorbance at 475 nm was monitored.

**Purification of Tyrosinase.** The experiment was carried out at 0 to 4 °C. Protein concentration was determined by Bradford reagent, as described previously (*9*), using bovine serum albumin (BSA) as the standard. Ammonium sulfate was added to 4.0 L of grape juice at a saturation level of 60%. The juice was then centrifuged for 10 min at 10 000*g*, and the resultant precipitate was mixed with 80 mL of 20 mM acetate buffer, pH 3.5, containing 0.04 mM copper to maintain enzyme activity. The precipitate was dialyzed against the same buffer.

**Ion-Exchange Chromatography.** The crude protein preparation was loaded onto a cation-exchange column (1 mL HiTrap SP, Pharmacia, Uppsala, Sweden) that had been equilibrated with 20 mM acetate buffer, pH 6.5. After washing the column with the buffer, bound proteins were eluted by a stepwise linear gradient of 0 N to 1 N NaCl at a flow rate of 1 mL/min:

| time (min) | 0 N NaCl (%) | 1 N NaCl (%) |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 100 | 0 |
| 5 | 85 | 15 |
| 8 | 85 | 15 |
| 14 | 70 | 30 |
| 17 | 70 | 30 |
| 17 | 60 | 40 |
| 20 | 60 | 40 |
| 20 | 50 | 50 |
| 23 | 50 | 50 |
| 23 | 20 | 80 |

Fractions (1 mL) were collected and the protein concentration and enzyme activity were determined. Active fractions were centrifuged and concentrated by ultrafiltration as described above.

**Gel Filtration.** HPLC−gel filtration chromatography on a Superdex 75 HR 10/30 column was performed at a flow rate of 0.8 mL/min. Aliquots (1 mL) were collected and the protein concentration and enzyme activity were determined. Fractions exhibiting tyrosinase activity were pooled.

The apparent molecular mass of the enzyme was estimated using BSA, egg albumin, extracellular domain from bovine growth hormone, and lysozyme as marker proteins. Five consecutive injections of the markers indicated less than 3% variability in retention times.

**SDS−PAGE.** Protein samples were run under partially denaturing conditions: samples were not heated and were diluted with Laemmli buffer (*30*), without reducing agent. The gel was treated with 2-propanol to remove the SDS. Then, activity staining was carried out by immersing the gel for 15 min in 20 mM acetate buffer, pH 6.5, containing 20 mM DOPA at 25 °C. Following activity staining, the gel was stained with Coomassie Brilliant Blue.

Proteins were also run under fully denaturating conditions: samples were boiled for 3 min in Laemmli buffer containing $\beta$-mercaptoethanol as a reductant and 2% SDS. The gel was stained in a silver-nitrate solution.

**Characterization of Tyrosinase.** The effect of pH on enzyme activity was determined at 25 °C, using DOPA as the substrate. The following buffers (100 mM) served to achieve the indicated pH: acetate, from pH 3.0 to 6.0; potassium phosphate, from pH 6.0 to 8.0; glycine−NaOH from pH 8.0 to 11.

The effect of temperature on enzyme activity was also determined using DOPA as the substrate. The enzyme was incubated in 20 mM acetate buffer (pH 6.5) at various temperatures (0−80 °C) for 10 min prior to addition of the substrate.

The Michaelis−Menten values using either DOPA or resveratrol as substrates were determined by the method of Cash et al. (*31*). The activity was measured at various concentrations of DOPA (0.2−10 mM) and resveratrol (0.1−1 mM) at pH 6.5 and 25 °C. The $K_m$ and $V_{max}$ values were obtained from a Lineweaver−Burk plot.

Substrate specificity toward ten phenolic compounds was measured according to the methods of Benjamin and Montgomery (*32*) and Zhou et al. (*33*) using various phenolic substrates. To overcome the nonenzymatic oxidation effect, similar solutions which had no enzyme added served as blanks. The reaction was carried out at 25 °C with acetate buffer (pH 6.5).

The inhibitory effect of various compounds on enzyme activity was determined by preincubating the enzyme with a 20-$\mu$L solution of each inhibitor at 25 °C for 2 min, and initiating the reaction by adding 6.7 mM DOPA (980 $\mu$L). The concentrations of each inhibitor were 0.5, 1.25, 2.5, and 5 mM. The concentrations of $SO_2$ were 0.094, 0.468, 0.936, and 1.872 $\mu$M.

**Tyrosinase Content in Skins and Juice.** Whole grapes (6.5 gr) were peeled by hand. The flesh weighed 4.3 g and the skins weighed 2.2 g. The skins were ground in liquid nitrogen using a mortar and pestle. Flesh and skin juice (20 $\mu$L each) were assayed for tyrosinase.

## RESULTS

**Degradation of Resveratrol by Fungal Tyrosinase.** *Trans*-resveratrol eluted at an Rt of 14.5 min in the HPLC system. When resveratrol was added externally to Carignan grape juice, to a final concentration of 0.2 mM, it was completely depleted within 5 min. However, when resveratrol was added to deproteinated juice, the levels of the added resveratrol did not change for 24 h. To assess the possible enzymatic degradation of resveratrol, we added mushroom tyrosinase (15 U/mL) to the solution, which again resulted in the complete depletion of resveratrol.

**Purification of Tyrosinase from Carignan Grapes.** Assaying for PPO activity in Carignan grapes revealed high activity using DOPA as the substrate: 17 U/mL was measured in the skins, 51 U/mL was measured in the flesh. Carignan juice, prepared in a commercial winery, was used for further purification of grape tyrosinase, as summarized in Table 1. The first step consisted of precipitation by ammonium sulfate (60% saturation) followed by dialysis, which resulted in the loss of 50% of the original tyrosinase activity. (Ammonium sulfate cuts of 30% and 50% proved to decrease the total recovery at the precipitation step). Chromatography on a cation-exchange column resulted in the additional loss of 50% of the remaining activity and an 8.4-fold purification. A chromatogram of the cation-exchange column eluate shows that most of the proteins eluted with the

**Table 1. Purification of Tyrosinase from Carignan Grapes**

| | total protein (mg) | total tyrosinase activity (U)*$10^{-8}$ | specific activity (U/mg) | fold (%) | yield (%) |
|---|---|---|---|---|---|
| juice | 520 | 32692 | 63 | 1 | 100 |
| amonium sulfate | 71 | 15466 | 218 | 3.4 | 47 |
| cation-exchange | 4.54 | 8322 | 1833 | 29 | 25 |
| gel filtration−HPLC | 0.0076 | 1050 | 138158 | 2193 | 3 |

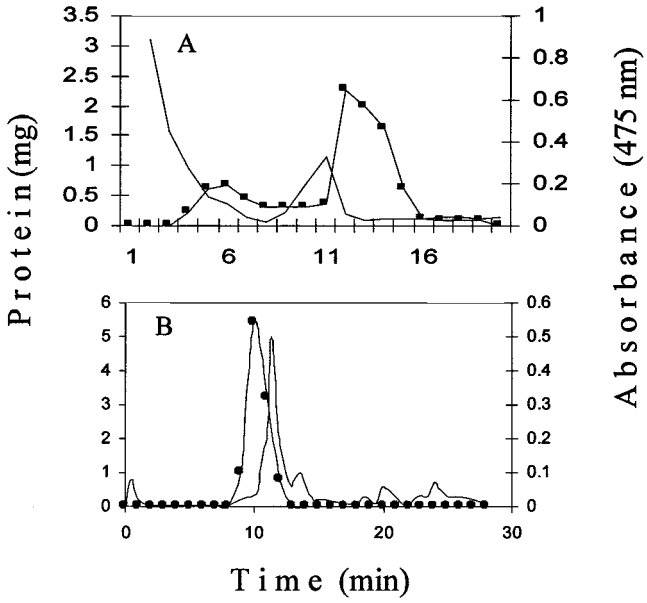


**Figure 1.** Elution profiles of grape PPO. (A) Protein fraction obtained by salting-out with ammonium sulfate from salt-soluble proteins of Carignan juice eluted by cation-exchange and (B) protein fraction obtained from cation-exchange chromatography (fractions 11−16), eluted by HPLC−Superdex 75. (●) Tyrosinase activity; (−) protein concentration.

front (Figure 1A). Two active peaks were detected: a smaller one at 0.15 M NaCl and a large one at 0.25 M NaCl.

To further purify the enzyme, an HPLC−gel filtration column was used (Figure 1B). The specific activity increased by a factor of 75, with an overall recovery of around 3% of the initial activity. A few proteins were observed, but only one active peak was detected, at 10.6 min.

Three active fractions at different stages of purification were analyzed as shown in Figure 2A, B, C. Staining for tyrosinase activity using DOPA (Figure 2A) revealed an active protein band with an apparent mass of 40 kDa. Coomassie Blue and silver staining of the different fractions (Figure 2B, C) indicated the progressive purification of the enzyme preparation to near homogeneity. The purified enzyme was used for further characterization.

**Apparent Molecular Mass.** The apparent molecular mass of the Carignan tyrosinase was estimated by HPLC−gel filtration (Figure 3) using molecular weight markers. It was estimated to be 42 kDa, which was in agreement with the SDS−PAGE analysis (Figure 2B).

**Effect of pH and Temperature on Carignan Tyrosinase Activity.** The enzyme was active from pH 4 to 11, showing optimum activity at pH 6 (Figure 4). Optimum activity was found at 25 °C (Figure 5). The activity at 4 °C was approximately 50% of that at the optimum temperature.

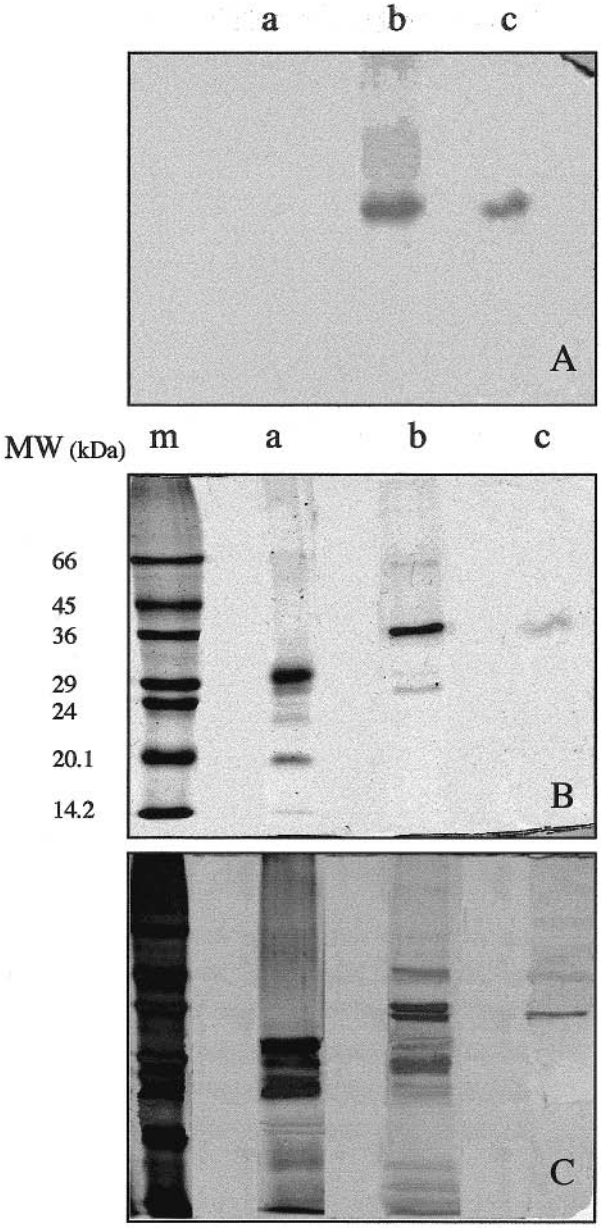


**Figure 2.** SDS−PAGE of (a) whole Carignan juice; (b) protein fraction obtained from cation-exchange chromatography (fractions 11−16); (c) protein fraction obtained from HPLC−gel filtration (fractions 9−12); and markers (m). A, activity staining; B, Coomassie Brilliant Blue staining; C, silver nitrate staining.

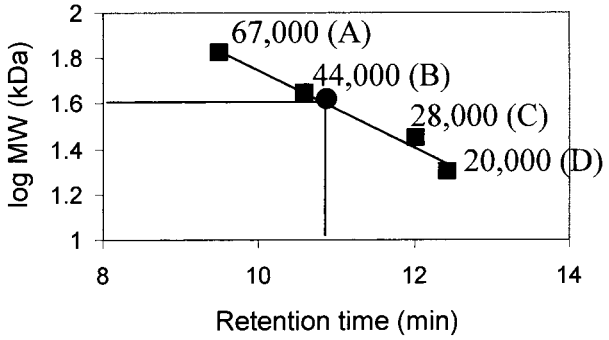


**Figure 3.** Determination of the apparent molecular mass of tyrosinase from Carignan grapes by gel filtration−HPLC.

**Kinetic Properties.** The values of $K_m$ and $V_{max}$ of fungal tyrosinase, after 15 min with DOPA, were 0.92

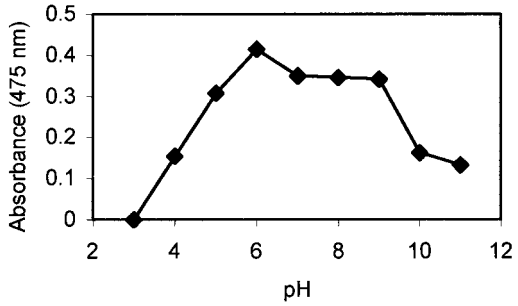


**Figure 4.** Effect of pH on Carignan tyrosinase activity.

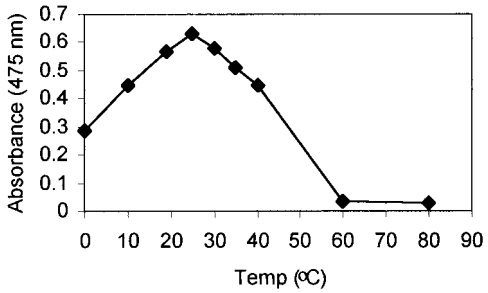


**Figure 5.** Effect of temperature on Carignan tyrosinase activity.

mM and $4 \times 10^{-6}$ mol/min, respectively. The respective values for purified tyrosinase after 60 min were 2.43 mM and $0.91 \times 10^{-6}$ mol/min (Figure 6A, C). The values of $K_m$ and $V_{max}$ for fungal tyrosinase with resveratrol were 0.35 mM and $0.8 \times 10^{-6}$ mol/min, and for the purified tyrosinase were 0.14 mM and $0.24 \times 10^{-6}$ mol/min (Figure 6B, D).

**Substrate Specificity.** Carignan tyrosinase activity toward various substrates is shown in Table 2. The enzyme was able to oxidize mono-, di-, and trihydroxyphenols, as well as diamine. Higher activity was observed using phenylenediamine as the substrate. High activity was also recorded using epicatechin and pyrogallol as substrates. The activity toward tyrosine was 20% of that toward DOPA. No activity was obtained using chlorogenic or gallic acids as substrates.

**Inhibition of Carignan Tyrosinase.** All of the inhibitors tested inhibited the tyrosinase activity (Table 3). The most efficient inhibitors were KCN, showing 52% inhibition at the lowest amended concentration and 100% at the highest, and thiourea, showing 44% and 100% inhibition, respectively. $SO_2$ at its lowest concentration (0.1 $\mu$M) had a very small effect of 10% inhibition, but showed 96% inhibition at its highest concentration (1.87 $\mu$M). Sodium azide was the weakest inhibitor, reaching only 78% inhibition at 5 mM.

## DISCUSSION

In 1990, residents of Toulouse, France, who consume their alcohol largely in the form of red wine, were found to have a very low mortality rate from cardiac diseases, despite a fat consumption rate similar to that in the US (*34*). That phenomenon has since been known as the "French Paradox". It was suggested that red wine reduces mortality significantly more than other alcoholic beverages (*35*−*38*). Many laboratory experiments, using predominantly in vitro approaches, have shown that phenolic constituents present in red wine possess antioxidant, anticancer, and antiinflammatory properties (*13*−*17*, *19*, *20*).

Resveratrol has been found to protect low-density lipoprotein (LDL) from oxidation (*16*), and to be an inhibitor of carcinogenic tumors (*1*, *17*−*20*, *39*, *40*). *Trans*-resveratrol was also reported to have an anticarcinogenic effect when tested in a mouse skin cancer model (*20*), and showed growth inhibition of androgen-responsive prostate cells (*19*).

Resveratrol is found in white and red wines, though generally its levels are higher in the latter, due to skin contact during fermentation (*22*, *24*, *26*, *29*, *41*−*44*). For example, red Zinfandel wines have a higher resveratrol concentration (0.36 $\mu$mol/L) than their white counterparts (0.002 $\mu$mol/L) (*42*). The fermentation of juice on the skins increases the concentration of resveratrol in correlation with higher alcohol levels, as was demonstrated by Pezet and Cuent (*26*), who showed an increase in resveratrol concentration from 0 to 1.58 mg/L after 6 days of fermentation.

Other factors have been found to affect resveratrol concentration in wine. Some factors affect resveratrol in the grape, including maturation, fungal infection, geographical origin, weather, and variety. Resveratrol, like other phytoalexins, is synthesized extensively in the skins as a result of environmental stress (*25*, *42*, *45*) and when conditions support the growth and attack of Botrytis. Some factors that affect resveratrol levels in wine lie within the wine-making process: duration of skin contact (because resveratrol is mainly in the skins), yeast type, malolactic fermentation, and additives (*21*−*25*, *28*, *29*, *43*−*45*). Among the latter, different levels of the yeast $\beta$-glucosidase were suggested to account for the differences of resveratrol levels in wine (*28*). PVPP (polyvinylpolypyrrolidine), a fining agent used in wine production, significantly lowers resveratrol levels (*22*). In Chardonnay wine, the resveratrol level decreased from 28.7 $\mu$g/L without PVPP to 3.4 $\mu$g/L with the addition of 0.96 g/L PVPP.

Since deglycosylation affects final levels of resveratrol in wines, we followed the fate of resveratrol added to grape juice. Its immediate degradation in the grape juice, and its relative stability in de-proteinated juice, suggest that enzymatic activity is involved in the rapid degradation of resveratrol, rather than a spontaneous reaction with substances in the juice.

Early works have shown that pathogenic fungi use laccase to degrade resveratrol (*46*). Laccase belongs to a family of PPO enzymes, which catalyze enzymatic browning in plants and oxidize a range of phenolic substrates to produce reactive quinones (*3*). PPOs have been studied widely in grapes using crude, partially purified, and to a lesser extent, purified enzymes.

This work describes the purification and characterization of a PPO from Carignan juice, with high affinity to resveratrol. The purification of the enzyme enabled its identification as a tyrosinase, as evidenced by its obligatory demand for oxygen (data not shown), its substrate range, and its inhibitors. It showed high substrate specificity to chlorogenic acid and catechol, as reported for other tyrosinases (*47*, *48*). It was inhibited by KCN, thiourea, sodium azide, and $SO_2$. Sodium azide, thiourea, and KCN inhibit the binding of molecular oxygen, which is the final electron acceptor in PPOs.

The exact mechanism of inhibition by $SO_2$ is not known. However, two theories have been suggested. In the first, PPO is actually inhibited by $SO_2$, whereas the

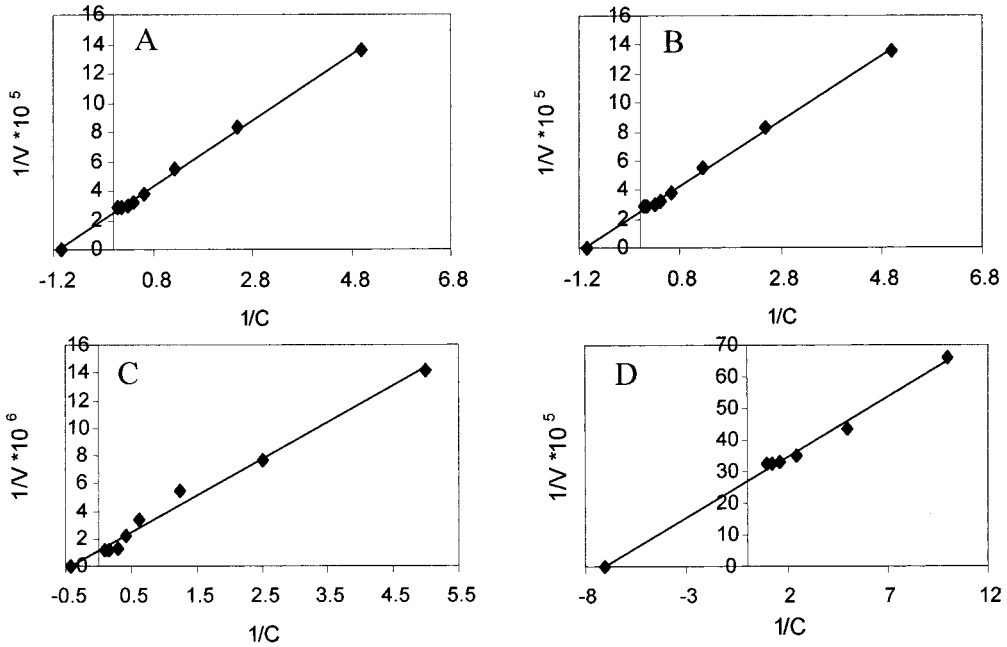


**Figure 6.** Lineweaver-Burk plots of mushroom tyrosinase activity toward DOPA (A) and resveratrol (B), and of Carignan tyrosinase activity toward DOPA (C) and resveratrol (D).

**Table 2. Substrate Specificity of Carignan Tyrosinase for Various Polyphenols**

| substrate | substrate type | wavelength measured (nm) | relative activity (%) |
|---|---|---|---|
| guaiacol | monohydroxy | 385 | 84 |
| tyrosine | monohydroxy | 475 | 20 |
| DOPA | dihydroxy | 475 | 100 |
| chlorogenic acid | dihydroxy | 420 | 0 |
| caffeic acid | dihydroxy | 410 | 74 |
| epicatechin | dihydroxy | 395 | 154 |
| catechol | dihydroxy | 385 | 82 |
| gallic acid | trihydroxy | 385 | 0 |
| pyrogallol | trihydroxy | 300 | 124 |
| resveratrol | di-/monohydroxy | HPLC[a] | 172 |
| phenylenediamine | diamine | 610 | 251 |

[a] Resveratrol was determined by HPLC as described.

**Table 3. Inhibition of Carignan Tyrosinase by Various Inhibitors**

| inhibitor | concentration (mM) | inhibition (%) |
|---|---|---|
| sodium azide | 0.5 | 19 |
| | 1.25 | 31 |
| | 2.5 | 57 |
| | 5 | 78 |
| KCN | 0.5 | 52 |
| | 1.25 | 74 |
| | 2.5 | 89 |
| | 5 | 100 |
| $SO_2$ | 0.1 | 10 |
| | 0.47 | 45 |
| | 0.94 | 93 |
| | 1.87 | 96 |
| thiourea | 0.5 | 44 |
| | 1.25 | 71 |
| | 2.5 | 88 |
| | 5 | 100 |

other suggests that the *o*-quinones formed during the enzymatic reaction are reduced to the original phenols by $SO_2$ (*4*).

To establish identification of the pure enzyme as tyrosinase, we compared it with other grape PPOs and found that the optimal pHs and temperatures were similar. It is interesting to note that tyrosinases function efficiently even at temperatures as low as 4 °C. Tyrosinase activity was constant between pH 5 and pH 9, in agreement with that of other grape PPOs (*31*, *47*, *49*).

We also compared the Carignan tyrosinase to a mushroom tyrosinase (EC 1.14.18.1). $K_m$ describes the affinity of an enzyme to its substrate. Thus, the affinity of Carignan tyrosinase to resveratrol was higher than its affinity to DOPA. The fungal enzyme showed a different order of substrate specificity. It is well recognized that some fungi are able to attack the berry when it produces resveratrol, although others are inhibited by the phytoalexin (*22*−*27*, *29*, *42*, *45*, *50*). Differential abilities of various PPOs to degrade resveratrol may explain the above.

To study the factors affecting resveratrol concentration in wine, we used Carignan juice as a model for must production in the wine-making process. While the grape is intact, compartmentalization exists which protects resveratrol in the grape skin from oxidizing enzymes. Later, $SO_2$ is added to the juice in order to protect it from oxidation, enhance the wine yeast, and inhibit microbial deterioration. Between these two steps, the grapes are crushed to produce the juice, in which resveratrol is exposed to tyrosinase and to molecular oxygen. As shown here, $SO_2$ is a potent inhibitor of Carignan tyrosinase. The higher concentrations of $SO_2$ used in this work resemble those used in wine-making, suggesting that the early spraying of grapes with $SO_2$ is an important factor affecting the final amount of resveratrol in wine. We also show that the concentrations of tyrosinase are higher in the flesh than in the skins and this may preserve resveratrol before crushing. Being a potent antioxidant, resveratrol is susceptible to dissolved molecular oxygen. Molecular oxygen is required also for the activation of PPO's. Thus, once oxygen is omitted from the juice (during fermentation), resveratrol is stable not only to chemical, but also to enzymatic, oxidation.

LITERATURE CITED


(1) Whitehead, T. P.; Robinson, D.; Allaway, S.; Syms, J.; Hale, A. Effect of red wine ingestion on the antioxidant capacity of serum. *Clin. Chem.* **1995**, *41*, 32−35.

(2) Vaughn, D. C.; Duke, S. O. Function of polyphenol oxidase in higher plants. *Physiol. Plant.* **1984**, *60*, 106−112.

(3) Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* **1979**, *18*, 193-215.

(4) Singleton, V. L.; Zaya, J.; Trousdale, E. White table wine quality and polyphenol composition as affected by must $SO_2$ [sulfur dioxide] content and pomace contact time. *Am. J. Enol. Vitic.* **1980**, *31*, 14−20.

(5) Hooper, R. L.; Collins, G. G.; Rankine, B. C. Catecholase activity in Australian white grape varieties. *Am. J. Enol. Vitic.* **1985**, *36*, 203−206.

(6) Lamikanra, O.; Kirby, S. D.; Musingo, M. N. Muscadine grape polyphenoloxidase: partial purification by high-pressure liquid chromatography and some properties. *J. Food Sci.* **1992**, *57*, 686−689.

(7) Sanchez-Ferrer, A.; Bru, R.; Valero, E.; Garcia-Carmona, F. Changes in pH-dependent grape polyphenoloxidase activity during maturation. *J. Agric. Food Chem.* **1989**, *37*, 1242−1245.

(8) Valero, E.; Varon, R.; Garcia-Carmona, F. Characterization of polyphenol oxidase from Airen grapes. *J. Food Sci.* **1988**, *53*, 1482−1485.

(9) Yokotsuka, K.; Makino, S.; Singleton, V. L. Polyphenol oxidase from grapes: precipitation, resolubilization and characterization. *Am. J. Enol. Vitic.* **1988**, *39*, 293−302.

(10) Kidron, M.; Harel, E.; Mayer, A. M. Catechol oxidase activity in grapes and wine. *Am. J. Enol. Vitic.* **1978**, *29*, 30−35.

(11) Traverso-Rueda, S.; Singleton, V. L. Catecholase activity in grape juice and its implications in winemaking. *Am. J. Enol. Vitic.* **1973**, *24*, 103−109.

(12) Wissemann, K. W.; Lee, C. Y. Polyphenoloxidase activity during grape maturation and wine production. White grape varieties grown in the northeastern United States. *Am. J. Enol. Vitic.* **1980**, *31*, 206−211.

(13) Das, D. K.; Sato, M.; Ray, P. S.; Maulik, G.; Engelman, R. M.; Bertelli, A. A. E.; Bertelli, A. Cardioprotection of red wine: Role of polyphenolic antioxidants. *Drugs Exp. Clin. Res.* **1999**, *25*, 115−120.

(14) Cushman, M.; Nagarathnam, D.; Gopal, D.; Chakraborti, A. K.; Lin, C. M.; Hamel, E. Synthesis and evaluation of stilbene and dihydrostilbene derivatives as potential anticancer agents that inhibit tubulin polymerization. *J. Med. Chem.* **1991**, *34*, 2579−2588.

(15) Carbo, N.; Costelli, P.; Baccino, F. M.; Lopez-Soriano, F. J.; Argiles, J. M. Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. *Biochem. Biophys. Res. Comm.* **1999**, *254*, 739−743.

(16) Fremont, L.; Belguendouz, L.; Delpal, S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life Sci.* **1999**, *64*, 2511−2521.

(17) Fuhrman, B.; Lavy, A.; Aviram, M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation [see comments]. *Am. J. Clin. Nutr.* **1995**, *61*, 549−554.

(18) Goldberg, D. M.; Hahn, S. E.; Parkes, J. G. Beyond alcohol: beverage consumption and cardiovascular mortality. *Clin. Chim. Acta* **1995**, *237*, 155−187.

(19) Hsieh, T. C.; Wu, J. M. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp. Cell Res.* **1999**, *249*, 109−115.

(20) Jang, M. S.; Cai, E. N.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.

(21) Mattivi, F.; Reniero, F.; Korhammer, S. Isolation, characterization, and evolution in red wine vinification of resveratrol monomers. *J. Agric. Food Chem.* **1995**, *43*, 1820−1823.

(22) Threlfall, R. T.; Morris, J. R.; Mauromoustakos, A. Effect of variety, ultraviolet light exposure, and enological methods on the *trans*-resveratrol level of wine. *Am. J. Enol. Vitic.* **1999**, *50*, 57−64.

(23) Castellari, M.; Spinabelli, U.; Riponi, C.; Amati, A. Influence of some technological practices on the quantity of resveratrol in wine. *Z. Lebensm.−Unters.−Forsch. A (Food Research and Technology)* **1998**, *206*, 151−155.

(24) Jeandet, P.; Bessis, R.; Gaugheron, B. The production of resveratrol (3,5,4′-trihydroxystilbene) by grape berries in different developmental stages. *Am. J. Enol. Vitic.* **1991**, *42*, 41−46.

(25) Jeandet, P.; Bessis, R.; Maume, B. F.; Meunier, P.; Peyron, D.; Trollat, P. Effect of enological practices on the resveratrol isomer content of wine. *J. Agric. Food Chem.* **1995**, *43*, 316−319.

(26) Pezet, R.; Cuenat, P. Resveratrol in wine: Extraction from skin during fermentation and post-fermentation standing of must from Gamay grapes. *Am. J. Enol. Vitic.* **1996**, *47*, 287−290.

(27) Vrhovsek, U.; Eder, R.; Wendelin, S. The occurrence of *trans*-resveratrol in Slovenian red and white wines. *Acta Aliment.* **1995**, *24*, 203−212.

(28) Vrhovsek, U.; Wendelin, S.; Eder, R. Effects of various vinification techniques on the concentration of *cis*- and *trans*-resveratrol and resveratrol glucoside isomers in wine. *Am. J. Enol. Vitic.* **1997**, *48*, 214−219.

(29) Okuda, T.; Yokotsuka, K. *Trans*-resveratrol concentrations in berry skins and wines from grapes grown in Japan. *Am. J. Enol. Vitic.* **1996**, *47*, 93−99.

(30) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(31) Cash, J. N.; Sistrunk, W. A.; Stutte, C. A. Characteristics of Concord grape polyphenoloxidase involved in juice color loss. *J. Food Sci.* **1976**, *41*, 1398−1402.

(32) Benjamin, N. D.; Montgomery, M. W. Polyphenol oxidase of Royal Ann cherries: purification and characterization. *J. Food Sci.* **1973**, *38*, 799−806.

(33) Zhou, P.; Smith, N. L.; Lee, C. Y. Potential purification and some properties of Monroe apple peel polyphenol oxidase. *J. Agric. Food Chem.* **1993**, *41*, 532−536.

(34) Seigneur, M.; Bonnet, J.; Dorian, B.; Benchimol, D.; Drouillet, F.; Gouverneur, G.; Larrue, J.; Crockett, R.; Boisseau, M. R.; Ribereau Gayon, P.; Bricaud, H. Effect of the consumption of alcohol, white wine, and red wine on platelet function and serum lipids. *J. Appl. Cardiology* **1990**, *5*, 215−222.

(35) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(36) Gronbaek, M.; Deis, A.; Sorensen, T. I.; Becker, U.; Schnohr, P.; Jensen, G. Mortality associated with moderate intakes of wine, beer, or spirits. *BMJ [Br. Med. J.]* **1995**, *310*, 1165−1169.

(37) Gronbaek, M. Wine and mortality. *Biofactors* **1997**, *6*, 377−383.

(38) Gronbaek, M. Type of alcohol and mortality from cardiovascular disease. *Food Chem. Toxicol.* **1999**, *37*, 921−924.

(39) Goldberg, D. M. Does wine work? *Clin. Chem.* **1995**, *41*, 14−16.

(40) Maxwell, S.; Cruickshank, A.; Thorpe, G. Red wine and antioxidant activity in serum. *Lancet* **1994**, *344*, 193−194.

(41) de Lima, M. T. R.; Waffo Teguo, P.; Teissedre, P. L.; Pujolas, A.; Vercauteren, J.; Cabanis, J. C.; Merillon, J. M. Determination of stilbenes (*trans*-astringin, *cis*- and *trans*-piceid, and *cis*- and *trans*-resveratrol) in Portuguese wines. *J. Agric. Food Chem*. **1999**, *47*, 2666−2670.
(42) Siemann, E. H.; Creasy, L. L. Concentration of the phytoalexin resveratrol in wine. *Am. J. Enol. Vitic.* **1992**, *43*, 49−52.
(43) McMurtrey, K. D.; Minn, J.; Pobanz, K.; Schultz, T. P. Analysis of wines for resveratrol using direct injection high-pressure liquid chromatography with electrochemical detection. *J. Agric. Food Chem*. **1994**, *42*, 2077−2080.
(44) Goldberg, D. M.; Yan, J.; Ng, E.; Diamandis, E. P.; Karumanchiri, A.; Soleas, G.; Waterhouse, A. L. A global survey of *trans*-resveratrol concentrations in commercial wines. *Am. J. Enol. Vitic.* **1995**, *46*, 159−165.
(45) Jeandet, P.; Bessis, R.; Sbaghi, M.; Meunier, P.; Trollat, P. Resveratrol content of wines of different ages: relationship with fungal disease pressure in the vineyard. *Am. J. Enol. Vitic.* **1995**, *46*, 1−4.
(46) Sbaghi, M.; Jeandet, P.; Bessis, R.; Leroux, P. Degradation of stilbene-type phytoalexins in relation to the pathogenicity of *Botrytis cinerea* to grapevines. *Plant Pathol.* **1996**, *45*, 139−144.
(47) Okuda, T.; Pue, A. G.; Fujiyama, K.; Yokotsuka, K. Purification and characterization of polyphenol oxidase from Muscat Bailey A grape juice. *Am. J. Enol. Vitic.* **1999**, *50*, 137−143.
(48) Kong, K. H.; Hong, M. P.; Choi, S. S.; Kim, Y. T.; Cho, S. H. Purification and characterization of a highly stable tyrosinase from *Thermomicrobium roseum*. *Biotechnol. Appl. Biochem*. **2000**, *2*, 113−118.
(49) Nakamura, K.; Amano, Y.; Kagami, M. Purification and some properties of a polyphenol oxidase from Koshu grapes Japan. *Am. J. Enol. Vitic.* **1983**, 122−127.
(50) Langcake, P.; McCarthy, W. V. The relationship of resveratrol production to infection of grapevine leaves by *Botrytis cinerea*. *Viti [Siebeldingen, Bundesforschungsanstalt fur Rebenzuchtung Geilweilerhof]* **1979**, *18*, 244−253.

Received for review September 8, 2000. Revised manuscript received December 19, 2000. Accepted December 19, 2000. This work was supported by the Zevi Herman Shapira Research fund.

JF0011079