Oxidation of *trans*-Caftaric Acid and 2-*S*-Glutathionylcaftaric Acid in Model Solutions

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The enzymatic oxidation of caftaric acid and the subsequent reactions of its quinone in the presence of different quantities of glutathione and 2-*S*-glutathionylcaftaric acid were monitored by HPLC and polarographic methods. The amount of oxygen consumed and the rate of the oxidation were shown to be dependent on the ratios of the different substrates, which might explain the variation in must-browning potential among grape varieties. An ultrafiltration technique was developed to eliminate the polyphenoloxidase activity from the enzymatic model solutions. 2-*S*-Glutathionylcaftaric acid was oxidized by caftaric acid quinones. Hydrogen peroxide may be formed while the caftaric acid quinones are reduced and involved in an indirect oxidation pathway.

Caftaric (caffeoyltartaric) acid and related products are the major phenols of grape juice prepared with minimal skin contact (Myers and Singleton, 1979; Nagel et al., 1979; Ong and Nagel, 1978; Singleton et al., 1978, 1984, 1985). It is now well established that oxidation of caftaric acid and coutaric acid (its coumaric analogue), catalyzed by the grape polyphenoloxidase (PPO), leads to the formation of 2-*S*-glutathionylcaffeoyltartaric acid, referred to as grape reaction product (GRP) (Cheynier et al., 1986; Singleton et al., 1984, 1985). The reaction involves enzymatic oxidation of caftaric acid (eq 1), followed by spontaneous reaction of the *o*-quinone with glutathione (eq 2).

$$\text{caftaric acid} + 0.5O_2 \rightarrow \text{caftaric acid } o\text{-quinone} + H_2O \quad (1)$$

$$o\text{-quinone} + \text{glutathione} \rightarrow 2\text{-}S\text{-glutathionylcaftaric acid} \quad (2)$$

According to Singleton and co-workers, the glutathione adduct is not a substrate for PPO, in spite of its *o*-dihydroxyphenol structure. Conversion of caftaric acid into GRP is therefore believed to be a way of limiting must-browning by trapping the caftaric acid quinones in the form of the stable glutathione-substituted product and preventing them from proceeding to brown polymers. Thus, the varietal differences in grape susceptibility to browning, which is determined neither by the grape PPO activity (Sapis et al., 1983) nor by the hydroxycinnamic acid content (Romeyer et al., 1985), might depend on the glutathione to caftaric acid ratio.

On the other hand, some instability of GRP following its formation has been noted in grape juice exposed to air (Gunata et al., 1986; Salgues et al., 1986).

The purpose of the present work was to specify the respective roles played by caftaric acid, glutathione, and 2-*S*-glutathionylcaftaric acid in grape juice oxidation and browning. Due to the complexity of the grape juice medium, studies were carried out on model solutions of the submentioned substrates. In some instances, ultrafiltration, a very efficient and simple technique for protein removal (Blanchard, 1981; Nickenig, 1980; Sophianopoulos et al., 1978), was used to separate the enzyme from the reactants after initiation of the oxidation by polyphenoloxidase.

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MATERIALS AND METHODS

Preparation of Crude Grape Polyphenoloxidase. The crude PPO extract was prepared from grape juice as described previously (Singleton et al., 1985).

Isolation of Caftaric Acid. Caftaric acid was isolated from Grenache grapes following the procedure of Singleton (1978) using a preparative HPLC column instead of Sephadex LH-20. The HPLC apparatus was an ISA Jobin-Yvon (Longjumeau, France) system, with an axial compression column (500 × 22 mm), filled with Lichrosorb RP C18 phase (Merck 15–25-μm packing), Modulprep compression module, Modulprep hydraulic module, Modulprep pump, manual injection system, an ISA-SM 25 UV detector set at 313 nm, and a Linseis recorder. Packing pressure was 9 bars, elution pressure 3.5–4.5 bar, and injection pressure 3–5 bar.

Elution was isocratic using 10% methanol in a 3% acetic acid aqueous solution, with a 20 mL/min flow rate. Column fractions were collected and analyzed by HPLC (Cheynier et al., 1986). Solvents and solutions were filtered through a 0.8-μm membrane filter before use on the preparative HPLC column.

The fractions containing *trans*-caftaric acid were combined, evaporated in vacuo at a temperature below 35 °C (Büchni Rotavapor) until the crystalline substance could be scraped from the flask, and finally dried over diphosphorus pentoxide in a vacuum desiccator.

About 600 mg of *trans*-caftaric acid was obtained from 3.6 kg of frozen unripe Grenache grapes.

Preparation of GRP. 2-*S*-Glutathionylcaffeoyltartaric acid was prepared by aerating a solution of the phenolic acid with an excess of the sulfhydryl compound (5/1, w/w) in the presence of 6 g/L of crude grape PPO extract in aqueous 2.5 g/L potassium hydrogen tartrate at room temperature. GRP crystals were obtained after purification on the preparative HPLC column, evaporation, and drying performed as for caftaric acid.

Preparation and Incubation of Model Solutions. All the enzymatic reactions were carried out with 1 mg/mL crude grape PPO at 30 °C using air agitation, unless otherwise specified. The molarity of the model solutions of caftaric acid, glutathione, and GRP in a pH 3.6 aqueous potassium hydrogen tartrate buffer varied from 0 to 0.4 mM, concentrations in the same range as those of grape juice.

Elimination of Enzyme by Ultrafiltration. In order to determined the mechanism of GRP oxidation by the caftaric acid–PPO system, enzyme-free caftaric acid quinone solutions were prepared by enzymatic oxidation of

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caftaric acid followed by removal of the PPO from the oxidized solution by means of ultrafiltration.

An 0.4 mM caftaric acid–2 mg/mL PPO solution was pumped through a 0.22-μm GS filter (Millipore, Molsheim, France) which served as a prefilter and then through a 10000-Da SM 14539 ultrafilter (Sartorius, Palaiseau, France). A P3 peristaltic pump (Pharmacia Fine Chemicals, Sweden) was used to provide enough pressure, and both types of filters were held in a 25-mm-diameter Swinnes support (Millipore, Molsheim, France). Over a period of 60 min, the ultrafiltered caftaric acid solution was gradually led into 4 mM glutathione, 0.4 mM GRP, 0.4 mM GRP + NaN_3 (200 mg/L final concentration), or 0.4 mM GRP + catalase (2 mg/mL) model solutions. The catalase was beef liver catalase (65000 units/mg) obtained from Boehringer (Mannheim, West Germany) as a suspension. The moment of the first ultrafiltered drip (time 0) was 5.05, 4.55, 4.55, and 5.00 min, and the final volume was 9.5, 9.4, 8.9, and 9.3 mL, respectively, starting with 2.5 mL each.

In the ultrafiltration experiments, 0.5 mM benzoic acid was added to the GRP and glutathione model solutions to serve as an internal standard. Benzoic acid was neither a substrate for PPO nor an inhibitor, nor did it give any reactions with the used substrates.

To test the efficiency of the ultrafilters to remove PPO activity, a 1 mg/mL PPO solution was ultrafiltered and added to 4 mM chlorogenic acid and 0.2 mM caftaric acid model solutions. The reaction was followed with HPLC for 6 h. The ultrafilters were 99–100% effective.

Hydrogen Peroxide Detection. Hydrogen peroxide analyses were performed following a method adapted from that of Bergmeyer (1965), using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (Boehringer, Mannheim, West Germany) as the reagent (Colonna et al., 1981).

The peroxidase was type I horseradish peroxidase purchased from Sigma (St. Louis, MO).

The molarity of the GRP solution, while testing its sensitivity for peroxidase, was 0.5 mM, the concentration of hydrogen peroxide 0.01%, and that of peroxidase was 1 g/L.

Measurement of Oxygen Consumption. Oxygen consumption was measured at 30 °C on an Oxi-191 oxymeter (WTW, Weilheim, West Germany) with an EO-190 Clarck electrode connected to a LKB Bromma 2210 recorder. A closed cell with variable volume was used to measure the oxygen uptake and to take samples at the same time without modifying the incubation conditions. The consumption of 1 g/L PPO solution in aqueous 2.5 g/L potassium hydrogen tartrate was measured and used as a base line.

HPLC Analyses. Samples of HPLC analyses were filtered through a 0.45-μm membrane filter immediately before use. The concentrations of the reagents were suitable for direct injection of 10–30 μL. The HPLC apparatus was a Waters Chromatography Division (Millipore Corp., Milford, MA) system including a 720 controller, a 730 data module, M 510 pumps, a 710B autoinjector, and a 440 double-wavelength absorbance detector set at 280 and 313 nm. The column was reversed-phase Lichrospher C-18 (5-μm packing, 4 × 250 mm), protected with a guard column of the same material (KNAUER, Bad Homburg, West Germany) heated at 31 °C. With constant volumes, an isocratic development was used with 10% of eluant A (80% acetonitrile in water), 90% of eluant B (2.5% acetic acid in water), and 1 mL/min flow rate. Known dilutions of *trans*-caftaric acid and GRP were applied to determine the response factor (mass/unit peak area).

With varying volumes, benzoic acid was used as an internal standard, to calculate the volume of the solution and the amount of *trans*-caftaric acid and GRP present in each sample. Gradient development was carried out starting with 10% A for 10 min, continuing with a linear gradient from 10% to 20% A in 10 min, from 20% to 60% A in 5 min, and from 60% to 100% A in 5 min. Retention times of *trans*-caftaric acid, GRP, and benzoic acid were 5.0, 7.6, and 26.0 min, respectively.

All samples taken were added with 5% of a 4.5% SO_2 solution unless otherwise specified, so that the quinones eventually present were reduced and integrated in the measured GRP and *trans*-caftaric acid amounts.

RESULTS AND DISCUSSION

PPO Oxidation of Caftaric Acid and Glutathione Mixtures. Caftaric acid oxidation and GRP formation were monitored by HPLC. The resulting data for molar ratios of glutathione to caftaric acid of 0, 0.5, 1, and 1.5 are presented in Figure 1.

As expected, the glutathione to caftaric acid ratio determined the amount of GRP formed. At the above 1 mol of glutathione/mol of caftaric acid, the yield of conversion of caftaric acid into GRP was very high (up to 92.5%), which indicates a very good affinity of the caftaric acid quinone for glutathione (Figure 1C,D). No discoloration was observed in the solutions.

Below 1 mol of glutathione/mol of caftaric acid, GRP was formed rapidly at first. After a few minutes, its concentration reached a maximum, depending on the amount of glutathione available, and then decreased steadily (Figure 1B). This can be related to our previous observations on grape juice (Salgues et al., 1986) and shows that although GRP is not oxidized by grape PPO, it can undergo other degradation reactions. The observed decrease does not involve hydrolysis as none of the hydrolysis products (Cheynier et al, 1986) could be detected in the solutions. Furthermore, it is likely to be due to oxidation since a red brown color, different from the yellow brown color of oxidized *trans*-caftaric acid, developed following GRP disappearance.

The rate of caftaric acid oxidation was also influenced by glutathione. The glutathione-free medium showed the slowest rate (see Figure 1A) whereas, in all the solutions containing the sulfhydryl compound, the amount of caftaric acid decreased very fast as long as that of GRP increased, i.e. until all the glutathione was consumed. Then the remaining caftaric acid was oxidized at a much slower rate, close to that observed in the glutathione-free medium.

Thus, oxidation of caftaric acid into the corresponding quinone is apparently faster when the quinone is trapped as the glutathione adduct. This might mean either that the free quinone acts as a retroinhibitor for PPO or that the reverse reaction, i.e. reduction of the caftaric quinone back to caftaric acid, occurs concurrently with the enzymatic oxidation.

PPO Oxidation of Caftaric Acid and GRP Mixtures. The evolution of caftaric acid and GRP concentration in various synthetic media was monitored by HPLC. The results of the analyses are presented in Figure 2 for molar ratios of caftaric acid to GRP of 0, 0.5, and 1.

The amount of GRP remained constant in the nonenzymatic solution. It showed a small decrease in the presence of PPO (Figure 2A), indicating either that PPO is slightly active on GRP or that it is contaminated by other enzymes or oxidizing agents, as mentioned previously (Salgues et al., 1986). The polarographic method, using hydroquinone as the specific substrate for laccase (Dubernet, 1974), was carried out to check that the crude PPO

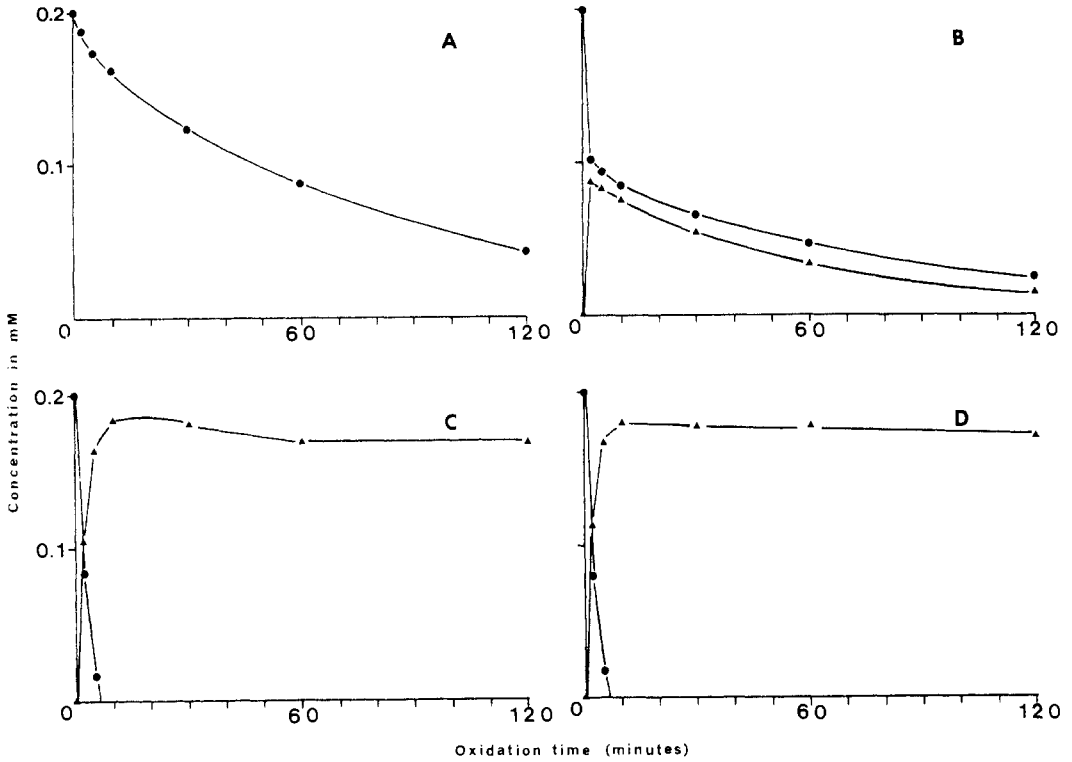

Figure 1. Time course of *trans*-caftaric acid (●) oxidation and GRP (▲) formation in 0.2 mM *trans*-caftaric acid and 1 g/L crude PPO model solutions containing 0 (A), 0.1 (B), 0.2 (C), and 0.3 mM (D) glutathione (initial molar ratios of glutathione to *trans*-caftaric acid equal to 0, 0.5, 1.0, and 1.5, respectively).

contained no laccase activity. Note that a similar loss had been observed in the PPO, glutathione, and caftaric acid solution for the glutathione to caftaric acid molar ratio of 1 (Figure 1C) but not for the higher ratios (Figure 1D). This might mean that GSH is more readily oxidized than GRP by the submentioned contaminants and can therefore protect it when present in excess. Addition of caftaric acid to the PPO–GRP solutions induced complete degradation of the glutathionyl derivative, probably owing to co-oxidation phenomenon, involving enzymatic oxidation of caftaric acid into the corresponding quinone followed by oxidation of GRP by the caftaric quinone, according to eq 1 and 3.

caftaric acid + $0.5O_2$ → caftaric acid *o*-quinone + H_2O (1)

caftaric acid *o*-quinone + GRP → caftaric acid + GRP quinone (3)

The relative amounts of caftaric acid and GRP did not have an important influence on the oxidation rate of either substrate. The rate of caftaric acid oxidation was only slightly lower than that observed previously in the case of caftaric acid alone (Figures 2C and 1A), which seems to indicate that spontaneous reduction of the caftaric acid quinone back to caftaric acid does not require the presence of GRP. The rate of GRP oxidation was the same with both caftaric acid concentrations (Figure 2B,C). Thus, the availability of caftaric acid quinones was not a limiting factor for GRP oxidation, even when the initial molarity of GRP was twice that of caftaric acid, meaning that reaction 1 was much faster than reaction 3, at least under our experimental conditions, i.e. with excess PPO.

Oxygen Uptake. The amounts of oxygen consumed per mole of caftaric acid oxidized in the presence of 1 g/L PPO and various quantitites of glutathione are given in Table I.

When excess glutathione was available to trap the caftaric quinones formed, 0.4 mol of molecular oxygen was consumed/mol of caftaric acid oxidized. This is lower than the theoretical 0.5 value expected, according to 1 and 2. This consumption was, however, equal to the oxygen uptake measured by Pierpoint (1966) for the PPO oxidation of chlorogenic acid in the presence of cysteine.

Table I. Oxygen Uptake during *trans*-Caftaric Acid Oxidation by Crude PPO for Different Glutathione to *trans*-Caftaric Acid Ratios

time, min	oxygen uptake, molecule/molecule of *t*-caft ox.		
	0.2 mM caft	0.2 mM caft + 0.1 mM GSH	0.2 mM caft + 0.2 mM GSH
5	1.76	0.64	0.36
10	1.61	0.77	0.39
30	1.06	0.81	0.40
60	0.90	0.81	0.40
120	0.69	0.80	0.40
180	0.72	0.80	0.40

In the case of caftaric acid in PPO alone, 0.7 mol of oxygen was consumed/mol of caftaric acid, in agreement with the oxygen-uptake data published for the browning of other dihydroxyphenols (Esterbauer et al., 1977; Harel et al., 1966; Pierpoint, 1966). However, at the beginning of the incubation, the oxygen decreased much faster than the caftaric acid. A possible explanation is that the measured loss of caftaric acid is in fact equal to the actual quantity of caftaric acid oxidized minus the amount of caftaric acid regenerated by reduction of the quinone, either by spontaneous means or by the SO_2 added when sampling. Since the reduction does not release molecular oxygen, its occurrence leads to an overestimation of the ratio of oxygen consumed to oxidized caftaric acid. This overestimation is proportional to the amount of caftaric acid present both in the reduced and oxidized (quinone) forms and thus especially important at the beginning of the incubation.

In the presence of GRP, the oxygen uptake per mole of oxidized phenol was slightly lower (Table II). Caftaric

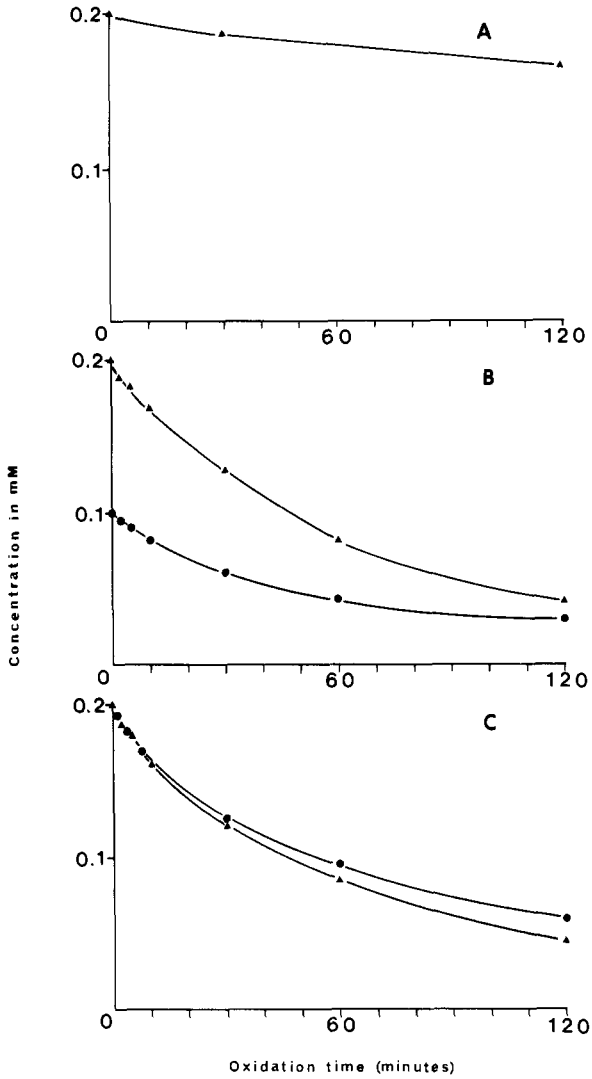

Figure 2. Time course of *trans*-caftaric acid (●) and GRP (▲) oxidation in 0.2 mM GRP and 1 g/L crude PPO model solutions containing 0 (A), 0.1 (B), and 0.2 mM (C) *trans*-caftaric acid (initial molar ratios of *trans*-caftaric acid to GRP equal to 0, 0.5, and 1.0, respectively).

Table II. Oxygen Uptake during *trans*-Caftaric Acid and GRP Oxidation (in the Presence of PPO) for Different *trans*-Caftaric Acid to GRP Ratios[a]

time, min	oxygen uptake, molecule/molecule of *t*-caft or GRP ox.	
	0.2 mM GRP + 0.1 mM caft	0.2 mM GRP + 0.2 mM caft
5	1.42	1.40
10	1.03	1.29
30	0.79	0.90
60	0.58	0.59
120	0.47	0.53
180	0.42	0.49

[a] No measurable oxygen consumption at 0.2 mM GRP.

acid can be regenerated from the quinone by a mechanism involving oxidation of GRP into GRP quinone (see equ 3), so that some of caftaric acid quinones reduced are taken into account in the calculation of the GRP loss. Spontaneous reduction of the GRP quinone is apparently less important than that of caftaric acid quinone, probably owing to stabilization of the orthoquinone structure by the glutathione substituent.

Unexpectedly, polymerization of the GRP quinones to form brown products does not seem to require any oxygen since approximately one atom only of oxygen per mole of

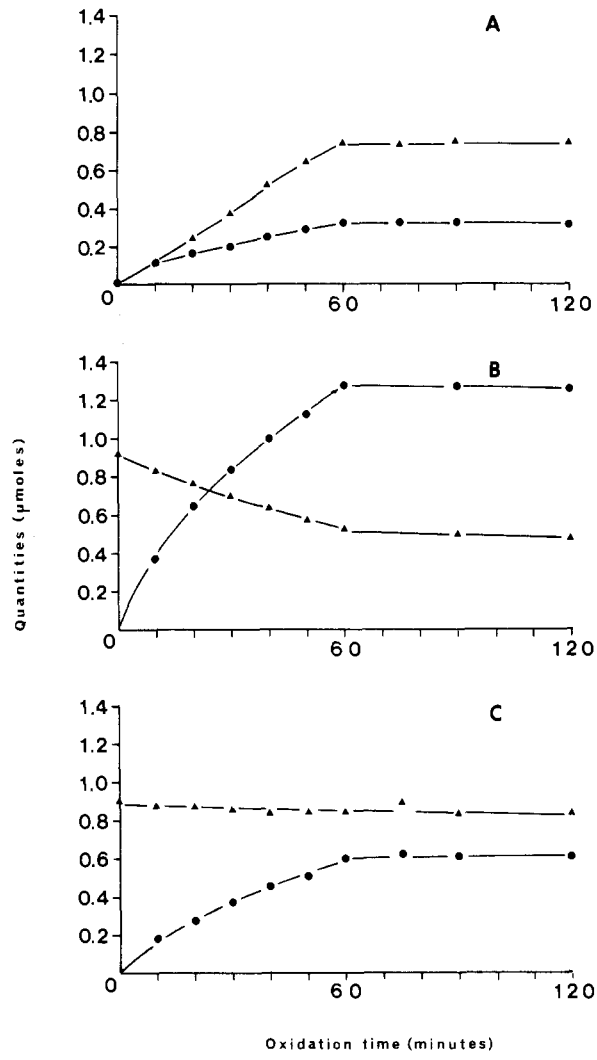

Figure 3. Measured amounts of GRP (▲) and *trans*-caftaric acid (●) in PPO-free media prepared by gradual addition of an ultrafiltered crude PPO (2 g/L) and *trans*-caftaric acid (0.4 mM initial concentration) solution: A, 2.5 mL of a glutathione solution (4 mM); B, 2.5 mL of a GRP solution (0.4 mM); C, 2.5 mL of a GRP (0.4 mM) + NaN_3 (200 mg/L final concentration) solution. Addition was stopped after 60 min, and reactions were allowed to continue for another 60 min. (Final volumes were 9.5, 9.4, and 8.9 mL, respectively.)

phenol was consumed by the end of the enzymatic oxidation. This suggests that the additional oxygen uptake measured in the case of caftaric acid alone was in fact necessary to reoxidize the spontaneously reduced caftaric acid quinones rather than to further oxidize them to brown polymers.

Obtention of Free Caftaric Acid Quinones and Studies of Their Nonenzymatic Reactions. The results of the caftaric acid and GRP analyses in the ultrafiltration experiments are presented in Figure 3A,B.

Less caftaric acid was found in the solution containing glutathione (see Figure 3A) than in the one containing GRP (see Figure 3B), although the same ultrafiltered mixture of nonoxidized caftaric acid and caftaric acid quinones was added simultaneously to both solutions. An additional ultrafiltration experiment in which the caftaric acid and PPO solution was pumped simultaneously into 2.5 mL of 0.5 mM benzoic acid and into 2.5 mL of 0.5 mM benzoic acid + 0.4 mM GRP confirmed that the rate of caftaric acid oxidation did not depend on the presence of GRP, as already observed in the enzymatic solutions (Figures 1A and 2C).

Caftaric acid disappearance was faster in the presence of glutathione. Since, with the ultrafiltration experimental setup, the quinone cannot act as an enzyme inhibitor, this provides evidence that PPO oxidation of caftaric acid is extremely fast and that reduction of the quinone back to caftaric acid takes place simultaneously, unless the quinone is removed from the solution, for instance when trapped by reaction with glutathione (see Figure 3A).

H_2O being the most probable hydrogen donor in the GRP free solution, the following reduction mechanism was suggested:

caftaric acid *o*-quinone + $2H_2O \rightarrow$ caftaric acid + H_2O_2 (4)

In agreement with the proposed mechanism, analyses showed the presence of hydrogen peroxide in the ultrafiltered caftaric acid–PPO solution, but not in the same solution after addition to glutathione. Detection of hydrogen peroxide did not require addition to peroxidase. Therefore, a check was made to show that the crude enzyme preparation exhibited peroxidase activity and that it was not removed by ultrafiltration. As one would expect peroxidase to be retained by the 10000-Da ultrafilters, the activity remaining in the filtrate is probably carried by smaller molecules such as heme proteins. We also verified that no hydrogen peroxide was detected either in the caftaric acid or in the enzyme extract alone.

The association of hydrogen peroxide released by spontaneous reduction of caftaric acid quinone with peroxidase from the grape enzymatic extract is a very powerful oxidizing system. Hence, it appeared necessary to study whether GRP could be oxidized directly by the caftaric acid quinone or whether a two-step reaction involving hydrogen peroxide formation and peroxidasic oxidation of GRP was an obligate pathway. Addition of sodium azide, a peroxidase inhibitor (Schreier and Miller, 1985; Vamos-Vigyazo, 1981) to the GRP solution, prior to oxidation by the ultrafiltered caftaric acid–PPO solution, was used to distinguish between the peroxidasic and the nonenzymatic mechanisms (Figure 3C).

The loss of GRP in the solution containing the enzyme inhibitor was only 10% of that observed in the presence of peroxidase activity, which seems to indicate that both mechanisms can occur simultaneously but that the hydrogen peroxide peroxidase catalized pathway is considerably faster than the direct oxidation of GRP by the caftaric acid quinone. When the peroxidasic pathway was blocked, a smaller amount of caftaric acid was found in the samples taken, which probably indicates that more caftaric acid quinones were lost by oxidative polymerization.

Furthermore, adding hydrogen peroxide and peroxidase to a GRP solution resulted in an immediate formation of a bright red color and conversion of GRP into a new peak eluted at 5.5 min, which might be the corresponding quinone (Pierpoint, 1970; Delaporte and Macheix, 1972). Note that when SO_2 was added to the sample, the red color faded, the amount of GRP appeared unchanged, and no new peak could be detected.

However, addition of catalase to the GRP and benzoic acid solution induced no significative change in the GRP and caftaric acid oxidation rates (curves identical with those of Figure 3B), meaning that hydrogen peroxide was not a compulsory intermediate for the oxidation of GRP although the peroxidasic mechanism may be important in normal grape juice conditions. Therefore, sodium azide probably interfered with GRP oxidation in ways other than peroxidase inhibition. One of the hypotheses is that it may have reacted with the caftaric acid quinone, thus competing with GRP oxidation as well as with the reduction of the quinone back to caftaric acid.

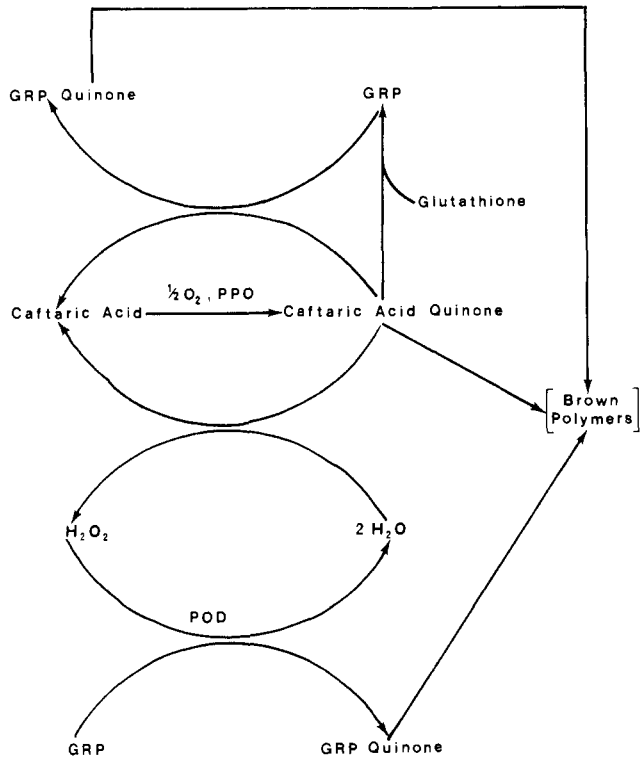

Figure 4. Proposed reaction mechanism for the coupled oxidation of *trans*-caftaric acid and GRP.

The complete scheme proposed for the PPO oxidation of caftaric acid and subsequent reactions in the presence of glutathione and/or 2-*S*-glutathionylcaffeoyltartaric acid is presented in Figure 4.

Substitution of caftaric acid by glutathione has been considered as a way of limiting must-browning. In our model solutions, this was true but only if the quantities of glutathione present were sufficient to allow complete conversion of caftaric acid into GRP; that is to say, irreversibly trap all the caftaric acid quinones formed. When glutathione was the limiting factor for this reaction (for ratios of glutathione to caftaric acid below 1), which is probably the case in most grape varieties, formation of GRP actually led to increased oxygen consumption and browning. Moreover, oxidized GRP developed a dark red-brown color whereas oxidized caftaric acid was rather yellow.

Ultrafiltration was shown to be a very efficient technique to eliminate the polyphenoloxidase activity. The caftaric acid quinones isolated by this method proved a lot more stable than expected and could be used to oxidize GRP. This ultrafiltration method should open new ways to investigate non-enzymatic oxidation reactions of polyphenols.

One can reasonably assume that the mechanisms proposed in Figure can occur during normal must preparation. Glutathione analysis and determination of the grape glutathione to caftaric acid ratio should provide complementary information on the importance of the described reactions in relation to must browning.

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Registry No. GRP, 110826-68-1; caftaric acid, 67879-58-7; glutathione, 70-18-8; caftaric acid quinone, 110826-69-2.

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Prostaglandin-like Substances, Precursors of Red Pigment, Formed during Autoxidation of Methyl Arachidonate

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Red pigment forming substances (RPS) formed during autoxidation of methyl arachidonate were purified and the chemical structures determined. The production of RPS showed a good relation with lipid peroxidation, until the peroxide value reached a maximum. The RPS were purified successively by gel chromatography on Sephadex LH-20, column chromatography on silica gel 60, and high-performance liquid chromatography on μ-Porasil. Four purified RPS fractions were analyzed by IR spectrometry and by GC–MS after reduction with $NaBH_4$ or $NaBD_4$. Two RPS out of the four predicted by theory were identified as the stereoisomers of 3-(5-hydroxy-3-oxo-2-pentylcyclopentyl)-2-propenal and methyl 4-[2-(2-formylvinyl)-3-hydroxy-5-oxocyclopentyl]butanoate.

The interaction of peroxidized lipids with nitrogenous compounds, such as amino acids and proteins, results in the browning of foods, "rusting" so to speak (Gardner, 1979; Pokorny, 1981), or in the formation of age pigment in vivo (Porta and Hartroft, 1969; Mead, 1976; Hirai et al., 1982). These browning reactions originate from the same Schiff base condensation or carbonyl–amine condensation as do the Maillard-type amine–sugar browning in foods (Reynolds, 1969; Mester et al., 1981). However, there is little documentation on the red coloration induced by autoxidation of lipids. In recent work, we found that autoxidized lipids containing polyunsaturated fatty acids turned reddish brown when reacting with amino acids (Nakamura, 1984) and that development of the reddish color depended on formation of certain conjugated carbonyls in the autoxidized lipids. The carbonyls, red pigment forming substances (RPS), were isolated from autoxidized linolenate and were determined to be 3-(2-ethyl-5-hydroxy-3-oxocyclopentyl)-2-propenal and methyl 8-[2-(2-formylvinyl)-3-hydroxy-5-oxocyclopentyl]octanoate (I and II, respectively) in Figure 1 (Nakamura, 1985, 1986).

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The RPS (I) had a single intense λ_{max} at 226–227 nm in ethanol (ϵ 14 000), and the red pigment obtained by the reaction with glycine had λ_{max} 515 nm in methanolic solution ($E^{1\%}_{1cm}$ = 400). The coloration occurred rapidly at high temperature but was rather unstable. Although the physiological significance of this prostaglandin-like substance has not been determined, some of these RPS may have biological effects.

According to the mechanism of formation, deduced from findings with methyl linolenate (Nakamura 1985, 1986), at least four RPS (III–VI in Figure 1) could be produced from methyl arachidonate. We now report separation and identification of these compounds, formed during autoxidation of methyl arachidonate.

EXPERIMENTAL SECTION

Coloration and Preparation of Red Pigment Forming Substances (RPS). *Autoxidation.* Methyl arachidonate (99% grade; Sigma Chemical Co., St. Louis, MO) was oxidized at 40 °C in the dark with stirring and with occasional bubbling of air. Aliquots of the autoxidized methyl arachidonate taken at regular intervals were stored at −40 °C until use for assays.

Coloration. Twenty milligrams of the autoxidized methyl arachidonate was dissolved in 2 mL of MeOH, and 1 mL of 0.5 M glycine (pH 7.0) was added to the solution.

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