Mechanism of *trans*-Caffeoyltartaric Acid and Catechin Oxidation in Model Solutions Containing Grape Polyphenoloxidase

Véronique Cheynier,* Nathalie Basire, and Jacques Rigaud

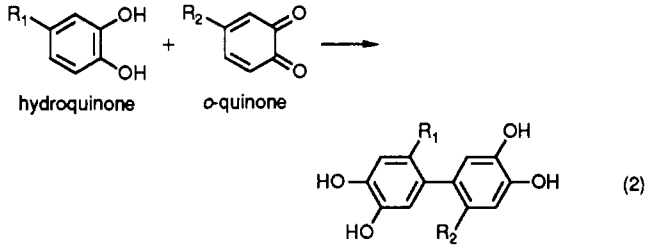
Oxidation of *trans*-caffeoyltartaric acid, catechin, and various mixtures of these compounds, in the presence of grape polyphenoloxidase, was studied in model solutions. A derivatization method, using benzenesulfinic acid, was developed to analyze caffeoyltartaric acid and free catechin *o*-quinones. The concentrations of phenolic substrates and of the corresponding free *o*-quinones were monitored by HPLC and the kinetics of oxidation and condensation reactions determined. The enzymically generated caffeoyltartaric acid *o*-quinones oxidize catechin to catechin *o*-quinone by coupled oxidation and are simultaneously reduced back to caftaric acid. The catechin *o*-quinones react rapidly with hydroquinones to form condensation products.

Hydroxycinnamic acids and especially *trans*-caffeoyltartaric (caftaric) acid are the major phenols of grape musts prepared with minimal skin contact (Myers and Singleton, 1979; Nagel et al., 1979; Ong and Nagel, 1978; Singleton et al., 1978, 1984, 1985) as well as the best substrates for grape polyphenoloxidase (PPO) (Gunata et al., 1987). However, the sensitivity of white musts toward oxidative browning is limited neither by their hydroxycinnamic acid content (Romeyer et al., 1985) nor by their polyphenoloxidase activity (Sapis et al., 1983). On the other hand, white wine browning potential is significantly correlated with the concentration of flavanols (Cheynier et al., 1989; Simpson, 1982).

The enzymically generated caftaric acid *o*-quinones were shown to oxidize other phenols, such as 2-*S*-glutathionylcaftaric acid (Cheynier and Van Hulst, 1988) and flavans (Cheynier et al., 1988), by coupled oxidation mechanisms (reaction 1), with reduction of the caftaric acid quinones back to caftaric acid.

caftaric acid quinone + hydroquinone → caftaric acid + *o*-quinone (1)

The *o*-quinone formed by enzymatic or coupled oxidation can also react with a hydroquinone to yield a condensation product (reaction 2) (Singleton, 1987).



(2)

condensation product (hypothetical formula)

In addition, other condensation products may be formed by covalent binding of the *o*-quinones with proteins of the polyphenoloxidase extract (Matheis and Whitaker, 1984).

Caftaric acid oxidation rate was enhanced by the presence of (epi)catechin, presumably meaning that caftaric acid–flavan mixed condensation products form more readily than caftaric acid oligomers (Cheynier et al., 1988). However, in these studies, sodium metabisulfite was systematically added to the samples to inhibit the enzyme. As a consequence, all the free quinones eventually present in the solutions were instantly reduced back to the corresponding hydroquinones so that oxidation and condensation reactions could not be distinguished.

The purpose of the present work was to study, for caftaric acid, catechin, and various mixtures of these compounds, the evolution of their hydroquinones, free *o*-quinones, and condensation products, in the presence of grape PPO, in order to specify the mechanisms and respective kinetics of oxidation and condensation reactions.

MATERIALS AND METHODS

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

Phenolic Substrates. Caffeoyltartaric acid was extracted from Grenache blanc grapes following the procedure of Singleton et al. (1985) and purified by preparative HPLC (Cheynier and Van Hulst, 1988). (+)-Catechin was purchased from Flucka A.G. (Buchs, Switzerland).

Preparation and Incubation of Model Solutions. Enzymatic incubations were carried out in 2.5 g/L potassium bitartrate (pH 3.65) with 1 g/L crude grape PPO extract, at 30 °C, with air agitation unless otherwise specified. All model solutions contained 0.2 mM of either caftaric acid or catechin, and, in two-component mixtures, the molarity of the second substrate equalled 0.1, 0.2, or 0.4 mM.

Oxygen uptake was measured by an oxymeter equipped with a Clark electrode in a closed system of variable volume as described previously (Cheynier and Van Hulst, 1988).

Analysis of Free *o*-Quinones. The free caftaric acid and catechin *o*-quinones were analyzed in the form of their benzenesulfinic acid adducts (Pierpoint, 1966; Piretti et al., 1977). The reaction of caftaric acid and catechin *o*-quinones with benzenesulfinic acid was extremely fast and yielded a single sulfone for each substrate. These derivatives were stable and were easily analyzed by HPLC. Caftaric acid and catechin benzene sulfones prepared by aerating, respectively, 2 mM caftaric acid and 2 mM catechin with 10 mM benzenesulfinic acid in the presence of grape polyphenoloxidase were used as standards for the HPLC analyses.

HPLC Analyses. Samples were taken at regular intervals throughout the incubations and immediately added with benzenesulfinic acid crystals to trap the free quinones eventually present as the corresponding sulfones. After a few seconds, they were added with 5% of a 4.5% sodium metabisulfite solution to stop the enzymatic reaction, filtered through 0.45 μM membrane filters, and analyzed by HPLC. The HPLC apparatus was a Waters Chromatography Division (Millipore Corp., Milford, MA) system including a 720 controller, two M 510 pumps, a 710 B automatic injector, a 440 double-wavelength absorbance detector set at 280 and 313 nm, and an Enica 21 (Delsi, France) integrator. The column was an ODS 2 Spherisorb (5 μM packing) 4 × 250 mm column protected with a guard column of the same material (Knauer, RFA). Elution conditions: solvent A, 2.5% acetic acid in water; solvent B, 80% acetonitrile in A; flow rate, 1 mL/min, 5% B isocratically for 4 min, and then linear gradients from 5% to 20% B in 16 min and from 20% to 30% B in 10 min, followed

Laboratoire des Polymères et des Techniques Physicochimiques, Institut des Produits de la Vigne, Institut National de la Recherche Agronomique, 9, Place Viala, 34060 Montpellier Cedex, France.

0021-8561/89/1437-1069$01.50/0

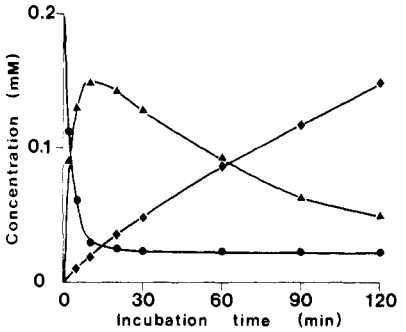


Figure 1. Evolution of caftaric acid hydroquinones (●), *o*-quinones (▲), and condensation products (♦) in the presence of 1 g/L grape polyphenoloxidase.

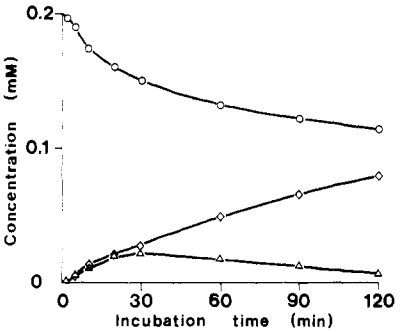


Figure 2. Evolution of catechin hydroquinones (○), *o*-quinones (△), and condensation products (◇) in the presence of 1 g/L grape polyphenoloxidase.

by washing and reconditioning of the column. The response factors were determined for each compound by injection of known dilutions.

Determination of the Condensation Kinetics. The amounts of each substrate incorporated in condensation products (either phenolic polymers or protein-bound *o*-diphenols) were estimated as the difference between the substrate initial concentration and the sum of its reduced form (hydroquinone) and oxidized form (free *o*-quinone) concentrations.

RESULTS AND DISCUSSION

The evolution of caftaric acid, caftaric acid free *o*-quinone, and polymerized caftaric acid concentrations in the presence of grape polyphenoloxidase are presented in Figure 1. Enzymatic oxidation of caftaric acid to caftaric acid *o*-quinone was much faster than condensation. After 5 min, the amount of caftaric acid free quinones represented 75% of total caftaric acid. Thus, addition of sodium metabisulfite when sampling induced a very large bias in the determination of the oxidation rate. In fact, caftaric acid oxidation rate was at first similar to that measured in the presence of excess glutathione (Cheynier and Van Hulst, 1988). However, the amount of caftaric acid remained constant after 10 min whereas removal of the quinones by formation of the glutathione adduct led to complete depletion of caftaric acid. This equilibrium can be displaced by addition of glutathione, which traps the free quinones, and of caftaric acid but not of PPO, indicating that it is not due to inactivation of the enzyme by the quinone or the condensation products. It may result from the reduction of caftaric acid quinones back to caftaric acid in coupled oxidation of condensation products.

Catechin enzymatic oxidation (Figure 2) was much slower than that of caftaric acid, in agreement with the results of Gunata et al. (1987). The amount of free catechin quinones remained very low, meaning that oxidation and condensation rates are approximately the same.

The evolution of caftaric acid reduced (hydroquinone), oxidized (*o*-quinone), and condensed forms in the presence

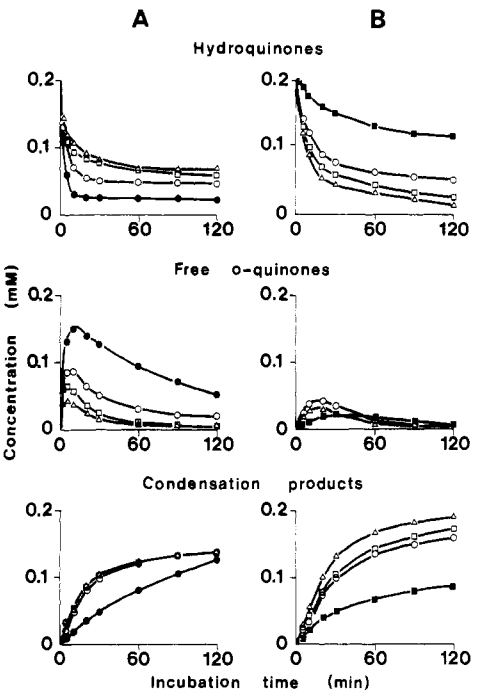


Figure 3. (A) Evolution of caftaric acid hydroquinones, *o*-quinones, and condensation products in the presence of grape polyphenoloxidase and catechin: 0 (●), 0.1 mM (○), 0.2 mM (□), and 0.4 mM (△). (B) Evolution of catechin hydroquinones, *o*-quinones, and condensation products in the presence of grape polyphenoloxidase and caftaric acid: 0 (■), 0.1 mM (○), 0.2 mM (□), and 0.4 mM (△).

of PPO and various catechin concentrations and that of the three catechin forms in the presence of PPO and various caftaric acid concentrations are presented in Figure 3, parts A and B, respectively. The rate of caftaric acid oxidation and the concentration of caftaric acid free *o*-quinones decreased with increasing amounts of catechin whereas catechin oxidation rate increased with increasing amounts of caftaric acid. This is in favor of a coupled oxidation mechanism (reaction 1) in which the enzymically generated caftaric acid *o*-quinone oxidizes catechin to catechin *o*-quinone and is simultaneously reduced.

However, the amount of free catechin *o*-quinones remained very low, whereas catechin condensation rate increased with increasing amounts of caftaric acid, meaning that the rate of catechin quinone formation is the limiting factor for catechin condensation.

On the other hand, caftaric acid condensation rate was faster in the presence of catechin but independent of catechin concentration, indicating that caftaric acid–catechin copolymers form more readily than pure caftaric acid oligomers, probably by addition of catechin *o*-quinones with caftaric acid.

Consequently, oxidation of mixtures of catechin and caftaric acid in the presence of grape polyphenoloxidase is likely to proceed via enzymatic oxidation of caftaric acid, followed by coupled oxidation of catechin by caftaric acid quinone. The catechin quinones formed by coupled oxidation react readily with hydroquinones, which can be either catechin or caftaric acid to yield condensation products. As well, the latter can be oxidized in the same way by caftaric acid quinones and polymerize further.

In all model solutions, one atom of oxygen was consumed throughout the incubation per molecule of phenolic substrate oxidized. This corresponds to the enzymatic oxidation stoichiometry and, therefore, indicates that, under our experimental conditions, i.e. acidic medium and relatively short incubation times, condensation reactions do

not require molecular oxygen. The much higher consumptions measured previously (Cheynier and Van Hulst, 1988) actually resulted from the reduction of the free quinones by the sulfite ions added when sampling to inhibit the enzymatic reactions.

The oxidation sequence established in model solutions is consistent with the reactions of phenolic compounds observed in musts. Under normal processing conditions, caftaric acid is oxidized very fast. Its quinones react primarily with the available glutathione to form 2-*S*-glutathionylcaffeoyltartaric acid (Cheynier et al., 1986; Singleton et al., 1985). Following glutathione depletion, the excess caftaric acid quinones are involved in coupled oxidation mechanisms in which the glutathionyl adduct or catechin can serve as the reductant. Among phenolic compounds, epicatechin gallate, procyanidins, and condensation products, which have lower redox potentials than their monomer precursors (Singleton, 1987), should be the best targets for these coupled oxidations. However, other must components such as ascorbic acid and when sulfur dioxide is used, sulfite ions, should be more readily oxidized and, therefore, protect phenolic compounds from oxidation.

Registry No. Polyphenoloxidase, 9002-10-2.

LITERATURE CITED

Cheynier, V.; Van Hulst, M. W. J. Oxidation of *trans*-Caftaric Acid and 2-*S*-Glutathionylcaftaric Acid in Model Solutions. *J. Agric. Food Chem.* **1988**, *36*, 10–15.

Cheynier, V.; Trousdale, E.; Singleton, V. L.; Salgues, M.; Wylde, R. Characterization of 2-*S*-Glutathionyl Caftaric Acid and its Hydrolysis in relation to Grape Wines. *J. Agric. Food Chem.* **1986**, *34*, 217–221.

Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of Grape Juice Phenolic Compounds in Model Solutions. *J. Food Sci.* **1988**, *53*, 1729–1732, 1760.

Cheynier, V.; Rigaud, J.; Souquet, J. M.; Barillère, J. M.; Moutounet, M. Effect of Pomace Contact and Hyperoxidation on the Phenolic Composition of Grenache and Chardonnay Wines. *Am. J. Enol. Vitic.* **1989**, *40*, 36–42.

Gunata, Y. Z.; Sapis, J. C.; Moutounet, M. Substrates and Aromatic Carboxylic Acid Inhibitors of Grape polyphenoloxidase. *Phytochemistry* **1987**, *26*, 1573–1575.

Matheis, G.; Whitaker, J. R. Modifications of Proteins by Polyphenoloxidase and Peroxidase and their Products. *J. Food Biochem.* **1984**, *8*, 137–162.

Myers, T. E.; Singleton, V. L. The non Flavonoid Phenolic Fraction of Wine and its Analysis. *Am. J. Enol. Vitic.* **1979**, *30*, 98–102.

Nagel, C. W.; Baranowoski, J. D.; Wulf, L. W.; Powers, J. R. The Hydroxycinnamic Acid Tartaric Acid Esters Content of Musts and Grape Varieties Grown in the Pacific Northwest. *Am. J. Enol. Vitic.* **1979**, *30*, 198–201.

Ong, B. Y.; Nagel, C. W. Hydroxycinnamic Acid – Tartaric Acid Ester Content in Mature Grapes and during the Maturation of White Riessling Grapes. *Am. J. Enol. Vitic.* **1978**, *29*, 277–281.

Pierpoint, W. S. The Enzymic Oxidation of Chlorogenic Acid and Some Reactions of the Quinones Produced. *Biochem. J.* **1966**, *98*, 567–579.

Piretti, M. W.; Serrazanetti, G. P.; Paglione, G. The Enzymatic Oxidation of (+)Catechin in the Presence of Sodium Benzenesulphinate. *Ann. Chim.* **1977**, *67*, 395–406.

Romeyer, F. M.; Sapis, J. C.; Macheix, J. J. Hydroxycinnamic Esters and Browning Potential in Mature Berries of some Grape Varieties. *J. Sci. Food Agric.* **1985**, *36*, 728–732.

Sapis, J. C.; Macheix, J. J.; Cordonnier, R. E. The Browning Capacity of Grapes. II. Browning Potential and Polyphenoloxidase Activities in Different Mature Grape Varieties. *Am. J. Enol. Vitic.* **1983**, *34*, 157–162.

Simpson, R. F. Factors Affecting Oxidative Browning of White Wine. *Vitis* **1982**, *21*, 233–239.

Singleton, V. L. Oxygen with Phenols and related Reactions in Musts, Wines, and Model Systems: Observations and Practical Implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69–77.

Singleton, V. L.; Timberlake, C. F.; Lea, A. G. H. The Phenolic Cinnamates of White Grapes and Wines. *J. Sci. Food Agric.* **1978**, *29*, 403–410.

Singleton, V. L.; Zaya, J.; Trousdale, E.; Salgues, M. Caftaric Acid in Grapes and Conversion to a Reaction Product during Processing. *Vitis* **1984**, *23*, 113–120.

Singleton, V. L.; Salgues, M.; Zaya, J.; Trousdale, E. Caftaric Acid Disappearance and Conversion to Products of Enzymic Oxidation in Grape Must and Wine. *Am. J. Enol. Vitic.* **1985**, *36*, 50–56.

Received for review September 19, 1988. Accepted February 27, 1989.

Deamination of Lysine as a Marker for Nitrite–Protein Reactions

Edyth L. Malin,* Rae Greenberg, Edwin G. Piotrowski, Thomas A. Foglia, and Gerhard Maerker

The deamination of ϵ-amino groups in poly-L-lysine by nitrite at low pH produces two amino acid isomers, δ- and ϵ-hydroxynorleucines, which elute with acidic amino acids as separate peaks during amino acid analysis. Chlorinated derivatives of the hydroxynorleucines, formed during HCl hydrolysis, elute later with neutral amino acids but are absent in mercaptoethanesulfonic acid hydrolysates. Nitrite treatment of proteins at low pH followed by HCl hydrolysis produces identical deaminated and chlorinated derivatives of lysine residues. The results indicate the potential for lysine deamination and suggest a way to monitor the fate of nitrite as well as to detect reactions that proteins may undergo in nitrite-processed foods. Hydroxynorleucines and their chlorinated derivatives are readily detected by routine amino acid analysis; in contrast, special amino acid analysis techniques are required to observe changes in all other protein amino acid residues that can react with nitrite.

The fate of nitrite used in food processing and the mechanism of nitrosamine production have been difficult to assess partly because of the lack of specific information on the chemistry of nitrite interactions with reactive groups in food components. Reactions of nitrite with nearly all of the protein amino acids having reactive side chains have been extensively characterized (Bonnet and Nicolaidou, 1977), but deamination reactions at the lysine ϵ-amino group have not been rigorously examined. Moreover, only

Eastern Regional Research Center, U.S. Department of Agriculture—Agricultural Research Service, Philadelphia, Pennsylvania 19118.