Oxidation of Grape Procyanidins in Model Solutions Containing *trans*-Caffeoyltartaric Acid and Polyphenol Oxidase

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Oxidation kinetics of grape seed procyanidin dimers and trimers, galloylated or not, in model solutions containing polyphenol oxidase and caffeoyltartaric acid were studied. Procyanidins were not susceptible to enzymatic oxidation, but they were all oxidized by the enzymatically generated caffeoyltartaric acid *o*-quinones. Galloylated procyanidins underwent condensation reactions faster than the corresponding nongalloylated ones. Coupled oxidation of procyanidin condensation products regenerating caffeoyltartaric acid from its quinone took place.

Hydroxycinnamic acids, and especially caffeoyltartaric and *p*-coumaroyltartaric acid, are the major phenols of white grape juices (Myers and Singleton, 1979; Nagel et al., 1979; Singleton et al., 1978) as well as the best substrates for grape polyphenol oxidase (EC 1.10.3.1) (Gunata et al., 1987).

Enzymatic oxidation of caffeoyltartaric and *p*-coumaroyltartaric acids to caffeoyltartaric acid *o*-quinone is the first step of phenolic compound oxidation which leads to browning in white musts and wines. Most sensitivity toward oxidative browning has been shown to be largely dependent on the hydroxycinnamate to glutathione (and possibly other grape constituents able to trap caffeoyltartaric acid *o*-quinones) ratio (Cheynier et al., 1990). However, extraction of flavans from the solid part of the cluster results in a large increase in must tendency to brown. Also, white wine browning potential is highly correlated with flavanol concentration (Cheynier et al., 1989c; Simpson, 1982). In fact, caffeoyltartaric acid free *o*-quinones react in priority with the available glutathione to form 2-*S*-glutathionylcaffeoyltartaric acid, also referred to as grape reaction product (GRP) (Cheynier et al., 1986; Singleton et al., 1984, 1985). After glutathione depletion, coupled oxidation and condensation reactions take place in musts containing hydroxycinnamic acids in excess, leading to the formation of brown pigments that eventually precipitate. GRP, catechin, procyanidin dimers B1–B4, and epicatechin 3-*o*-gallate are oxidized to the corresponding *o*-quinones in the presence of caffeoyltartaric acid quinone (Cheynier and Van Hulst, 1988; Cheynier et al., 1988). In addition, mixed catechin–caffeoyltartaric acid condensation products formed more easily than caffeoyltartaric acid oligomers (Cheynier et al., 1989a). The purpose of the present study was to investigate the oxidative degradation of various grape procyanidin oligomers in model solutions containing both grape polyphenol oxidase and caffeoyltartaric acid.

MATERIALS AND METHODS

Preparation of Crude Grape Polyphenol Oxidase. Frozen Grenache blanc grapes (400 g) were homogenized in 200 mL of 1.5 M acetate buffer (pH 5) containing 10 g/L ascorbic acid by using a Waring Blendor, filtered on cheesecloth, and centrifuged at 3000*g* for 10 min. The deposit containing polyphenol oxidase was washed with 80% acetone to remove chlorophylls and air-dried. Temperature was maintained below 5 °C throughout the extraction process, and the enzyme extract was stored at −18 °C until used.

Phenolic Substrates. Caffeoyltartaric acid was prepared as described previously (Cheynier and Van Hulst, 1988).

Four procyanidin dimers, B1 [epicatechin-(4β→8)-catechin], B2 [epicatechin-(4β→8)-epicatechin], B5 [epicatechin-(4β→6)-epicatechin], and B7 [epicatechin-(4β→6)-catechin], three procyanidin trimers, C1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin], trimer 2 [epicatechin-(4β→8)-epicatechin-(4β→8)-catechin], and trimer 3 [epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin], and five galloylated compounds, B1 3-*o*-gallate [epicatechin 3-*o*-gallate-(4β→8)-catechin], B2 3-*o*-gallate [epicatechin 3-*o*-gallate-(4β→8)-epicatechin], B2 3′-*o*-gallate [epicatechin-(4β→8)-epicatechin 3-*o*-gallate], B2 3,3′-di-*o*-gallate [epicatechin 3-*o*-gallate-(4β→8)-epicatechin 3-*o*-gallate], and trimer 2,3′-*o*-gallate [epicatechin-(4β→8)-epicatechin 3-*o*-gallate-(4β→8)-epicatechin], purified from a grape seed extract, prepared as described by Bourzeix et al. (1986) and identified following the procedure elaborated by Rigaud et al. (1991), were used in this experiment. Each compound was isolated by means of liquid chromatography over Fractogel TSK HW-40 (Merck, Darmstadt, FRG) using methanol as the eluant, followed by HPLC at the semipreparative scale on a Spherisorb ODS-2 (5-μm packing, 250 × 8 mm i.d.) column, protected with a guard column of the same material (Knauer, Bad Hamburg, FRG), and heated at 41 °C. The HPLC eluting conditions were as follows: flow rate, 2 mL/min; solvent A, 2.5% acetic acid in water; solvent B, 80% (v/v) acetonitrile in A; elution starting with 3% B isocratic for 8 min and continuing with linear gradients from 3 to 30% B in 32 min and from 30 to 100% B in 5 min.

Preparation and Incubation of Model Solutions. Enzymatic incubations were carried out in 2.5 g/L potassium hydrogen tartrate (pH 3.65) with 0.01 mg/mL crude enzymatic extract. The enzyme suspension was sonicated for 10 min before use to break the protein aggregates and homogenize the suspension. Each of the listed compounds (0.1 mM initial concentration) was incubated individually in the presence of (A) grape polyphenol oxidase and (B) grape polyphenol oxidase and caffeoyltartaric acid (0.1 mM initial concentration). The reaction was started by addition of the phenolic substrates solution. Model solutions (20 mL initial volume in 50-mL Erlenmeyer flasks) were maintained at 30 °C in a thermostated water bath and oxidized thoroughly by using air agitation on a magnetic stirrer. One-milliliter aliquots were withdrawn at regular intervals throughout the incubation period, sodium benzenesulfinate (2.5 mg) was added to trap the free *o*-quinones eventually present (Cheynier et al., 1989b), and the samples were stabilized by addition of a sodium metabisulfite solution (0.1% w/v).

HPLC Analyses. Samples were filtered through 0.45-μM membrane filters prior to injection (injection volume 40 μL) onto the HPLC column. The HPLC apparatus was a Millipore-Waters

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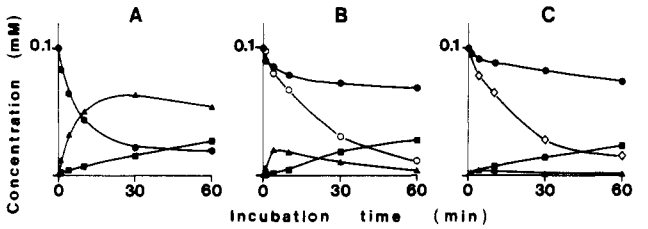

Figure 1. Concentration of caffeoyltartaric acid hydroquinones (●), *o*-quinones (▲) and condensation products (■), of procyanidin B2 (○) and of procyanidin B2 3′-*o*-gallate (◇) in solutions containing (A) caffeoyltartaric acid, (B) caffeoyltartaric acid and procyanidin B2, and (C) caffeoyltartaric acid and procyanidin B2 3′-*o*-gallate, oxidized by grape polyphenol oxidase.

(Milford, MA) system including a 710B autoinjector, a 720 system controller, two M510 pumps, and a Lambda Max 481 spectrophotometer set at 280 nm and connected to an Enica 21 integrator (Delsi, France). The column was reverse-phase Lichrospher 100 RP-18 (5-μm packing, 250 × 4 mm i.d.) protected with a guard column of the same material (Merck). The elution conditions were as follows: flow rate, 1 mL/min; column temperature, 30 °C; solvent A, 2.5% acetic acid in water; solvent B, acetonitrile–solvent A (80/20 v/v); elution starting with 5% B isocratically for 4 min and then from 5 to 20% B in 16 min, from 20 to 30% B in 10 min, and from 30 to 100% B in 5 min, followed by washing and reconditioning of the column. The response factors were determined for each phenolic compound by injection of known dilutions.

RESULTS AND DISCUSSION

Under our experimental conditions, none of the oligomers tested was oxidized by the grape polyphenol oxidase crude extract in the absence of caffeoyltartaric acid. Thus, the slight losses of procyanidin dimers observed previously (Cheynier et al., 1988) may have resulted from adsorption of the procyanidin molecules on the protein extract, used at a much higher concentration because of its lower specific polyphenol oxidase activity, rather than from their enzymatic oxidation.

In contrast, the concentration of all procyanidins decreased rapidly in the presence of both polyphenol oxidase and caffeoyltartaric acid. The procyanidin losses measured after 1 h of incubation averaged 82% (SD 9.5%) for the 12 compounds tested. Besides, caffeoyltartaric acid was oxidized slower in two-component mixtures (36.5 ± 9% loss after 1 h vs 74% when incubated alone with the enzyme), confirming that coupled oxidations of the flavans, regenerating caffeoyltartaric acid by reduction of its *o*-quinone, took place.

Procyanidin free *o*-quinones could not be determined directly as benzene sulfones because several derivatives were formed for each compound.

Examination of the oxidation kinetics allowed us to distinguish between galloylated and nongalloylated procyanidins. Examples of such kinetics are presented in Figure 1B (nongalloylated procyanidins, e.g., B2) and Figure 1C (galloylated procyanidin, e.g., B2 3′-*o*-gallate), along with the kinetics of caffeoyltartaric enzymatic oxidation (Figure 1A). Although the rate of caffeoyltartaric acid oxidation was similar in all two-component mixtures (no significant difference between the two groups of solutions; $p < 0.05$), the level of free caffeoyltartaric acid *o*-quinones was significantly lower ($p < 0.01$) in the presence of galloylated compounds. The mean maximum caffeoyltartaric acid quinone concentration (±SD) was 11.5 ± 4.8 μM in solutions containing nongalloylated compounds and 2.5 ± 0.8 μM in solutions containing galloylated procyanidins, suggesting that more coupled oxidation took place in the latter. However, the oxidation rate of

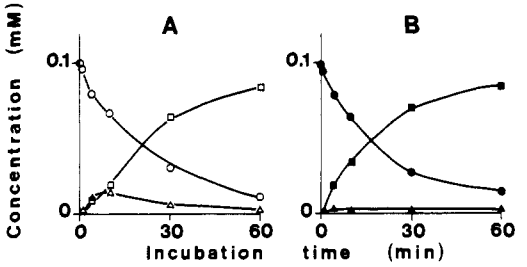

Figure 2. (A) Oxidation of procyanidin B2 (○) and formation of its *o*-quinone (△) and condensation products (□) in the presence of 0.1 mM caffeoyltartaric acid and grape polyphenol oxidase. (B) Oxidation of procyanidin B2 3′-*o*-gallate (●) and formation of its *o*-quinone (▲) and condensation products (■) in the presence of 0.1 mM caffeoyltartaric acid and grape polyphenol oxidase.

galloylated procyanidins did not exceed that of nongalloylated procyanidins, indicating that they were not the main targets of these enhanced coupled oxidation reactions. Note that, unlike that of catechin (Cheynier et al., 1989a), the presence of procyanidins, whether galloylated or not, did not seem to increase incorporation of caffeoyltartaric acid into polymers since the total amount of caffeoyltartaric acid in the reduced (hydroquinone) and oxidized (*o*-quinone) forms was approximately the same in all model solutions.

Additional experiments were carried out on B2–caffeoyltartaric acid and B2 3′-*o*-gallate–caffeoyltartaric acid mixtures to evaluate the amount of free procyanidin *o*-quinones present in the solutions throughout the incubations (Figure 2).

This was achieved by taking samples in duplicate and stabilizing one of them by addition of ascorbic acid (2 mM) to reduce free *o*-quinones back to the corresponding hydroquinones and the second one by addition of benzenesulfinic acid to trap quinones in the form of sulfones as previously described (Cheynier et al., 1989b). The amount of free procyanidin quinones was then evaluated as the difference between hydroquinone concentration in the ascorbic acid sample (i.e., hydroquinone plus reduced free *o*-quinone) and in the benzenesulfinate sample.

A large difference, which could not be accounted for by procyanidin B2's faster oxidation rate, was observed between the concentration of B2 free *o*-quinones and that of B2 3′-*o*-gallate quinones, suggesting that the latter underwent condensation faster, although it may also mean that B2 3′-*o*-gallate quinones react less readily with benzenesulfinic acid than B2 quinones. The rate of incorporation of B2 3′-*o*-gallate into polymers (either phenolic polymers or protein-bound phenols), calculated as the total amount of substrate lost (i.e., initial substrate concentration minus remaining concentrations of reduced and oxidized forms), exceeded that of procyanidin B2 at the beginning of the incubation period, in accordance with the former hypothesis. This increased reactivity is presumably due to the extra *o*-diphenol units present in the galloyl moiety.

Whether the kinetic differences observed between galloylated and nongalloylated procyanidins are significant in wine-making technology is unknown. However, the above results confirm that, under normal must obtention conditions, i.e., in the presence of caffeoyltartaric acid, grape polyphenol oxidase, and air, procyanidins are easily oxidized and involved in condensation reactions. This leads to increased must browning but, on the other hand, lowers procyanidin concentration and should therefore improve wine color stability.

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