Oxidative Reactions of Caffeic Acid in Model Systems Containing Polyphenol Oxidase

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Enzymatic oxidation products of caffeic acid were monitored by HPLC with diode array detection in model solutions containing different amounts of polyphenol oxidase. Caffeic acid *o*-quinone and a number of secondary products were characterized by their UV–visible spectra. Caffeic acid *o*-quinone was generated enzymatically within the first minutes of incubation, at a maximum concentration which increased at higher enzyme concentrations. It was highly unstable and rapidly replaced by various condensation products. The first and major products were formed by reaction of caffeic acid and its *o*-quinone. These products were themselves enzymatically oxidizable. Their maximum level was higher, but it was reached after longer incubation times with lower enzyme concentrations. Following initial enzymatic oxidation steps, the reactions proceeded further essentially via nonoxidative mechanisms, yielding a series of relatively polar compounds.

INTRODUCTION

Phenolic compounds largely contribute to browning in plant-derived food products (Mathew and Parpia, 1971; Pierpoint, 1966; Singleton, 1972). Oxidation reactions can be catalyzed by polyphenol oxidases or peroxidases or by autoxidation if the enzymes are inactivated or removed during the course of food processing (Singleton, 1972; Cilliers and Singleton, 1991). The fundamental step in browning is the transformation of *o*-diphenols to the corresponding *o*-quinones (Pierpoint, 1966; Mathew and Parpia, 1971). Once formed, the *o*-quinones are very reactive species which may polymerize (Cilliers and Singleton, 1989; Pierpoint, 1966; Rouet-Mayer et al., 1990), oxidize other substrates, and, in the process, be reduced to the original phenol (Mathew and Parpia, 1971; Pierpoint, 1966) or suffer nucleophilic attack by various substances including water (Pierpoint, 1966; Rouet-Mayer et al., 1990), amino acids and proteins (Mason and Peterson, 1965; Matheis and Whitaker, 1984; Pierpoint, 1966), and other polyphenols (Dawson and Nelson, 1938; Singleton, 1972, 1987). Coupled oxidation of substrates whose oxidation–reduction potentials are smaller, such as ascorbic acid (Cheynier et al., 1990; Macheix and Delaporte, 1973; Mathew and Parpia, 1971; Pierpoint, 1966; Rouet-Mayer et al., 1990), sulfite ions (Cheynier et al., 1989a), and other phenolic compounds (Cheynier et al., 1988, 1989a, 1990; Mathew and Parpia, 1971; Prota, 1980) as well as complexation of *o*-quinones with substances possessing amino and thiol groups (Mason and Peterson, 1965; Pierpoint, 1966; Prota, 1980) is well documented. In the absence of reducing or trapping agents, the *o*-quinones may react with one another or condense with hydroquinones, either by a mechanism analogous to a Michael 1,4 addition (Gramshaw, 1970; McDonald and Hamilton, 1973; Singleton, 1987) or through two semiquinone radical intermediates (Cilliers and Singleton, 1991; Singleton, 1987), and polymerize.

Caffeic acid derivatives such as chlorogenic (caffeoylquinic) acid and, in grapes, caftaric (caffeoyltartaric) acid are often among the major ortho-diphenolic compounds in plants and act as good substrates for polyphenol oxidases. The first stages of oxidative browning in grape musts are now well established. Enzymatic oxidation of caftaric acid takes place immediately when the grapes are crushed. The caftaric acid *o*-quinones formed by this process react readily with glutathione, a compound present in grapes in rather large concentration, to yield 2-*S*-glutathionylcaffeoyltartaric acid, also referred to as grape reaction product (GRP) (Cheynier et al., 1986; Singleton et al., 1984, 1985). Following glutathione depletion, the excess caftaric *o*-quinones oxidize other phenolic compounds including GRP (Cheynier and Van Hulst, 1988; Cheynier et al., 1990), catechin (Cheynier et al., 1989a), and procyanidin oligomers (Cheynier and Ricardo da Silva, 1991) to the corresponding *o*-quinones and are simultaneously reduced back to caftaric acid. The enzymatically generated caftaric acid *o*-quinones as well as those formed by coupled oxidation can be assayed in oxidizing solutions by trapping them as benzene sulfones (Cheynier et al., 1989b). In addition, part of the caftaric acid losses cannot be accounted for by these two types of reactions and are therefore attributable to polymerization reactions (Cheynier and Ricardo da Silva, 1991; Cheynier et al., 1989a, 1990).

Few studies on polymerization reactions are available, in spite of their considerable interest with regard to browning, probably because the intermediate condensation products are highly labile and, in particular, oxidizable by the parent *o*-quinones (Singleton, 1987). However, a number of oxidation and/or condensation products of caffeic and chlorogenic acids have recently been reported in various oxidizing model systems. According to Oszmianski and Lee (1990), all of the chlorogenic acid derivatives formed by enzymatic oxidation at pH 3.5 and 6.5 exhibited UV–visible spectra resembling that of their precursor, with a maximum absorbance at or near 320 nm. On the other hand, Cilliers and Singleton (1991) demonstrated that products of nonenzymatic oxidation of caffeic acid exhibited characteristic UV–visible spectra with two absorption maxima at 290 and 326 nm. The 326-nm absorbance relative to that at 290 nm was about half that of the original caffeic acid, indicating the loss of some of the side-chain conjugation during oxidative condensation. In fact, the isolated products were shown to be dimers and trimers of caffeic acid formed by reactions involving the side chain of at least one of the caffeic acid units, as

0021-8561/92/1440-2038$03.00/0

expected from the UV–visible data. The primary oxidation products, i.e., the *o*-quinones of chlorogenic acid and caffeic acid, were observed in none of the above-mentioned studies, whereas Rouet-Mayer et al. (1990) were able to detect catechin *o*-quinone, which they claimed to be less stable than chlorogenic acid *o*-quinone, among the peaks obtained by HPLC analysis of solutions oxidized enzymatically at pH 4.

The purpose of our work was to study the kinetics of formation and/or degradation of the major condensation products obtained by enzymatic oxidation of caffeic acid and, in particular, to determine the influence of different levels of polyphenol oxidase activity on these kinetics.

MATERIALS AND METHODS

Preparation of Crude Grape Polyphenol Oxidase. The crude grape polyphenol oxidase extract was prepared as described previously (Cheynier and Ricardo da Silva, 1991) and stored at −18 °C until used.

Oxidation of Caffeic Acid. Caffeic acid was purchased from Fluka (A.G. Buchs, Germany). Enzymatic incubations were carried out in 0.013 M potassium hydrogen tartrate (pH 3.6), at 30 °C, with air agitation on a magnetic stirrer. The enzyme extract (2000 mg/L of protein) was suspended in the potassium hydrogen tartrate solution and sonicated for 5 min before use to break the protein aggregates and homogenize the suspension. The reaction was started by addition of the enzyme suspension (concentration of 25, 100, or 200 mg/L of protein). All model solutions contained initially 0.5 mM caffeic acid and 0.2 mM benzoic acid (Aldrich, Strasbourg, France), used as an internal standard, unless otherwise specified.

Sampling. One-milliliter aliquots were withdrawn at regular intervals throughout the incubation period. The enzymatic reaction was stopped by addition of 0.25 mL of sodium fluoride (0.12 M), a very strong polyphenol oxidase inhibitor (Janovitz-Klapp et al., 1990). Samples were immediately filtered through 0.45-μm membrane filters and injected (injection volume 25 μL) onto the HPLC system.

For each enzyme concentration, three series of incubations, with sampling, respectively, at 5 min and 1, 2, 4, 8, and 24 h, 10 min and 1, 2, 16, and 24 h, and 30 min and 2, 4, 8, and 24 h, were performed in duplicate so that each data point is the mean of two to six measurements.

HPLC Analyses. The HPLC apparatus was a Waters Chromatography Division (Millipore Corp., Milford, MA) system, including a 720 controller, two M510 pumps, and a 710 B automatic injector. The oxidation kinetics were monitored using a Kontron 430 (Zürich, Switzerland) detector set at 280 nm and connected to an Enica 21 (Delsi, France) integrator. The UV–visible spectra of oxidation products were recorded from 240 to 500 nm, and the purity of the peaks was checked by means of a Millipore-Waters photodiode array detector connected on-line to the HPLC system described above. The column was a reversed-phase Lichrospher C-18 (5-μm packing, 250 × 4 mm i.d.) column, protected with a guard column of the same material (Merck, Darmstadt, Germany). The elution conditions were as follows: flow rate, 1 mL/min; column temperature, 26 °C; solvent A, 2.5% acetic acid in water; solvent B, acetonitrile–solvent A (80/20 v/v); linear gradients from 5 to 20% B in 20 min and from 20 to 40% B in 20 min, followed by washing and reconditioning of the column. HPLC on the semipreparative scale was performed under the same elution conditions but using a Spherisorb ODS-2 (5-μm packing, 250 × 8 mm i.d.) column (Knauer, Bad Hamburg, Germany) and 2 mL/min as the flow rate.

A calibration curve was constructed and the response factor of caffeic acid determined by injection (20 μL) of eight standards containing different concentrations of caffeic acid ranging from 0.01 to 1 mM and benzoic acid (0.2 mM). The latter was used as an internal standard to account for eventual variations of sampling volumes or concentrations of the reaction media in the course of incubation. Under our experimental conditions, it was neither a substrate nor an inhibitor for polyphenol oxidase, nor did it give any reaction with caffeic acid oxidation products.

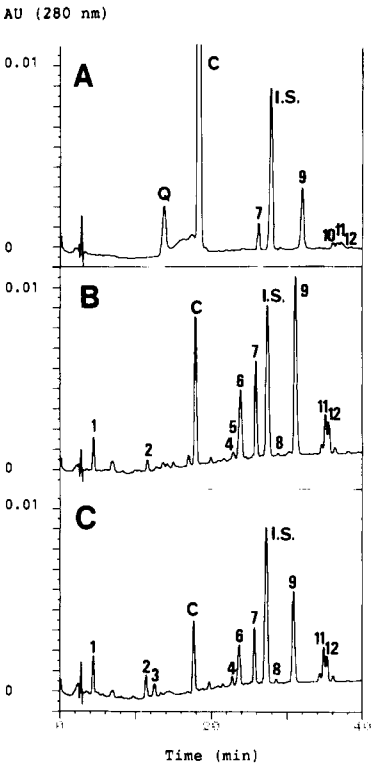

Figure 1. HPLC chromatograms of a caffeic acid solution oxidized by polyphenol oxidase (200 mg/L protein) for 5 min (A), 4 h (B) and 24 h (C). C, caffeic acid; Q, caffeic acid *o*-quinone; I.S., internal standard (benzoic acid 0.2 mM); 1–12, reaction products. Analysis was done by C-18 reversed phase with mobile phase A, 2.5% acetic acid in water, and mobile phase B, 80% acetonitrile in mobile phase A. Detection was at 280 nm. Linear gradients from 5 to 20% mobile phase B in 20 min and from 20 to 40% mobile phase B in 20 min were applied.

Caffeic acid *o*-quinones were measured directly by HPLC. An additional series of experiments was carried out to determine their response factor. For this purpose, a solution of caffeic acid (0.5 mM) and polyphenol oxidase (500 mg/L of protein extract) was prepared. After 10 min of incubation, samples were taken in duplicate and sodium fluoride was added to them to inhibit enzymatic oxidation. After filtration, one sample was immediately injected as described above and the second one stabilized by addition of glutathione (4 mM) to trap caffeic *o*-quinones as 2-*S*-glutathionylcaffeic acid (Cheynier et al., 1986). The response factor of caffeic acid free *o*-quinones was then calculated from that of 2-*S*-glutathionylcaffeic acid, itself determined by injection of known dilutions of the glutathione adduct. The latter was prepared by incubation of caffeic acid (2 mM) with glutathione (10 mM) and grape polyphenol oxidase and purified by preparative HPLC as described previously (Cheynier and Van Hulst, 1988).

The amounts of other oxidation products are expressed as peak area units at 280 nm.

Oxidation of Caffeic Acid Reaction Products. Further studies on caffeic acid reaction products were performed, at least in duplicate, directly on the fractions collected from the semipreparative column and immediately after elution, to avoid degradation of the isolated compounds during concentration or storage. For enzymatic oxidation experiments, each fraction was diluted four times in 0.013 M potassium hydrogen tartrate to minimize eventual inhibition of polyphenol oxidase by the chromatographic solvents and incubated at room temperature, with continuous aeration by rapid stirring, in the presence of benzoic acid (0.2 mM) and the enzyme suspension (100 mg/L), prepared as described above. Two control solutions, respectively enzyme-free and containing 100 mg/L of the polyphenol oxidase extract inactivated by sodium fluoride (0.024 M), were also prepared for each compound to evaluate losses due to autoxidation and/or interactions with proteins of the enzyme extract rather than to enzymatic oxidation.

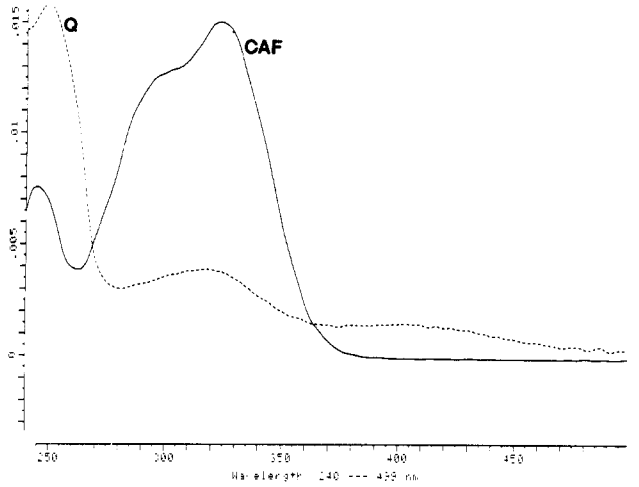

Figure 2. Overlayed UV–visible spectra of caffeic acid (C) and its *o*-quinone (Q).

RESULTS AND DISCUSSION

The disappearance of caffeic acid and the formation of its oxidation products were monitored by HPLC. Linear calibration curves were obtained over the concentration range 0.01–1.0 mM with coefficients of variation of 1.7 and 3% (n = 8), respectively, for caffeic acid and 2-*S*-glutathionylcaffeic acid response factors.

Figure 1 shows typical chromatographic profiles obtained with 200 mg/L polyphenol oxidase crude extract after 5 min (A) and 4 (B) and 24 h (C) of incubation. Several oxidation products were detected 5 min after initiation of the reaction. The first eluting peak (Q) exhibited a UV–visible spectrum (Figure 2) similar to that published by Macheix and Delaporte (1973) for chlorogenic acid *o*-quinone. When collected after elution from the HPLC column, it was respectively reduced to caffeic acid and converted to 2-*S*-glutathionylcaffeic acid in the presence of sodium metabisulfite (10 mM) and glutathione (5 mM) and therefore identified as caffeic acid *o*-quinone, which is the primary product of enzymatic oxidation.

The peak area of caffeic acid free *o*-quinone was highly correlated (r = 0.999, n = 6) with that of 2-*S*-glutathionylcaffeic acid in the same samples stabilized by addition of glutathione. The response factor (concentration/peak area unit) of caffeic acid *o*-quinone was thus calculated from that of 2-*S*-glutathionylcaffeic acid.

Concentration changes of caffeic acid and of its *o*-quinone during incubation at the three polyphenol oxidase levels tested are shown in Figure 3.

Caffeic acid concentration decreased rapidly at the beginning of incubation and leveled off after 4 h, as observed earlier with caftaric acid (Cheynier et al., 1989a). This might be due to inactivation of the enzyme by the oxidation products. However, in the case of caftaric acid (Cheynier et al., 1989a), oxidation could be resumed by addition of glutathione and caftaric acid, but not polyphenol oxidase, which seems to deny the inhibition hypothesis. Oxidation was slower with the lowest enzyme content (25 mg/L) and similar for the other two concentrations (100 and 200 mg/L). In contrast, the maximum level of caffeic acid *o*-quinone, reached after 5–10 min, was proportional to the enzyme content. A rapid decay was observed in all three solutions, and no free quinone could be detected after 2 h. Note that the amount of caffeic acid *o*-quinones produced was much lower than that of caftaric acid *o*-quinones in caftaric acid solutions oxidized under similar conditions (Cheynier et al., 1989a). This suggests that caffeic acid *o*-quinone is more reactive than caftaric acid *o*-quinone and proceeds to condensation products faster, as observed earlier for catechin quinone (Cheynier et al., 1989a; Rouet-Mayer et al., 1990).

Additional oxidation products, which increased in number as the reaction proceeded further, were formed along with the quinone (Figure 1). The major ones (peaks 6, 7, 9, 11, 12) eluted after caffeic acid in the reversed-phase chromatographic system and should therefore be less polar. This is in agreement with the findings of Cilliers and Singleton (1989, 1991) and, according to these authors, indicates that the oxidation products are not ring-opened products. As well, the compounds obtained by enzymatic oxidation of chlorogenic acid had longer retention times than their precursors, indicating that they were less polar and/or had higher molecular weight than their precursors (Oszmianski and Lee, 1990).

Examination of the kinetic behavior of the various compounds formed with the three enzyme concentrations allowed us to distinguish between primary (formed directly from the quinone) and secondary (formed in several reaction steps) condensation products. An example of such kinetics is presented in Figure 4 for the solution containing 200 mg/L polyphenol oxidase. Compounds **5**, **7**, and **9** accumulated rapidly at the beginning of incubation, as the concentration of caffeic acid *o*-quinone dropped, and then disappeared gradually from the oxidizing media. They were formed in larger amounts, although slower, and were more stable with lower enzyme concentrations (e.g., compound **9**, Figure 5), suggesting that they were susceptible to oxidation either directly by polyphenol oxidase or enzymatically by generated products. They were progressively replaced by other compounds, including in particular **4**, **6**, and a number of more polar products, some of which also behaved like transitory products. In contrast, peaks 11 and 12, although formed earlier and at lower concentrations, appeared relatively stable under our experimental conditions.

After separation by HPLC, UV–visible spectra were recorded for each compound from 240 to 500 nm by means of a photodiode array detector. It was found that the spectra of certain peaks were similar, suggesting that they might be structural analogues (Cilliers and Singleton, 1991). Actually, caffeic acid oxidation products could be separated into three main classes with regard to their UV–visible spectra.

The spectrum of compound **7** (Figure 6) is representative of the first class and similar to that of caffeic acid. As the relative absorbances at 290 and 326 nm are not modified, it is believed that the side-chain conjugations of the original caffeic acid units still exist in the molecule. Thus, it might be a caffeic acid oligomer and, since it was one of the first condensation products formed, in fact, is probably a dimer resulting from Michael 1,4 addition between the quinone and another caffeic acid molecule (Gramshaw, 1970; McDonald and Hamilton, 1972; Singleton, 1987). Similar spectra were obtained for several other compounds (**4**, **8**), again suggesting that they were caffeic acid derivatives formed by reactions not involving the side chain, although they had a 9–12-nm bathochromic shift.

The second class of compounds (**1**, **2**, **10**–**12**) exhibited characteristic spectra with two absorption maxima at 290 and 326 nm (e.g., compounds **10** and **11**, Figure 6), indicating that they were modified oligomers of caffeic acid generated by reactions involving the side chain of at least one of the caffeic acid units (Cilliers and Singleton, 1991). Structures of that type have been established for the major products of nonenzymatic autoxidative reactions of caffeic acid, called caffeicins by Cilliers and Singleton

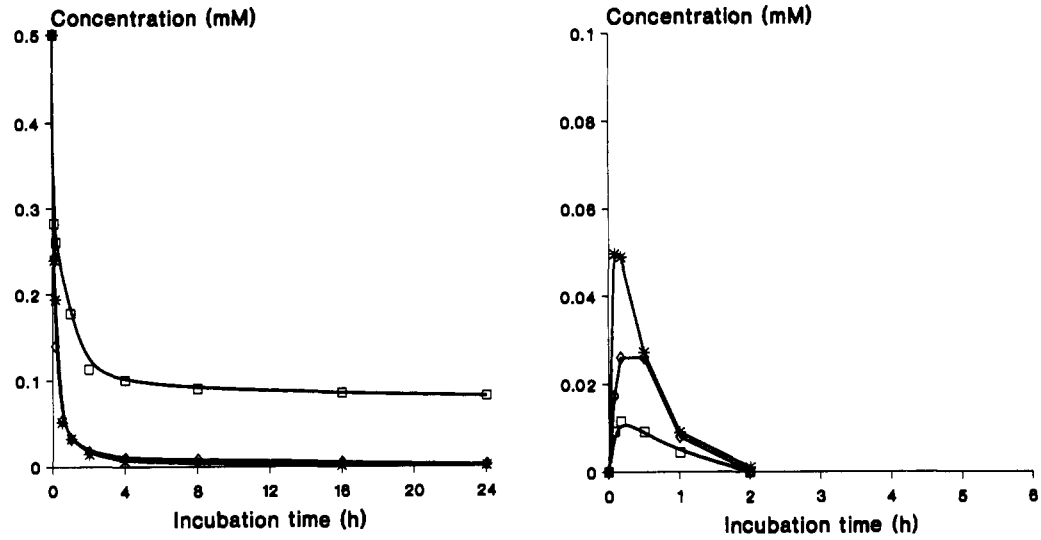

Figure 3. Concentration of caffeic acid (left) and caffeic acid *o*-quinone (right) in the course of incubation with 25 (□), 100 (◊), or 200 mg/L (*) of polyphenol oxidase extract.

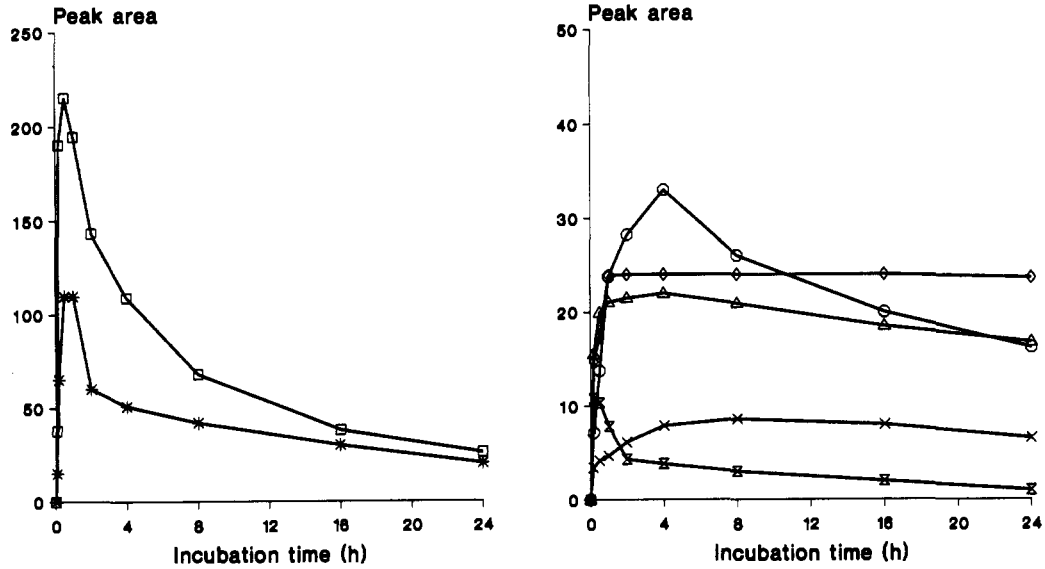

Figure 4. Relative amounts of reaction products 7 (*), 9 (□), 4 (×), 5 (▽), 6 (○), 11 (◊), and 12 (△) in the course of incubation of a caffeic acid (0.5 mM) solution with 200 mg/L polyphenol oxidase extract. All values are expressed as peak areas at 280 nm.

(1991). The relative absorbances at 290 and 326 nm were highly variable within this class of compounds, suggesting that different proportions of side-chain conjugation might be lost in the course of enzymatic oxidative polymerization. In particular, the increase of the 290-nm absorbance relative to that at 326 nm in the spectra of compounds 11 and 12 was lower than that published for the caffeicins. Therefore, and given their relatively late elution times, these compounds appear likely to be trimers—or larger oligomers—of caffeic acid in which more than half of the side-chain conjugation still exists. In contrast, a minor product (10) exhibited a UV-visible spectrum similar to that of the identified caffeicins and was thus expected to be one of them. However, the autoxidation products prepared by oxidizing a caffeic acid solution at pH 8.5 as described by Cilliers and Singleton (1989, 1991) coeluted with none of those formed by enzymatic oxidation, with the exception of compound 7, which was present in trace amounts in the autoxidized solution.

The third class of compounds, namely compounds 3, 6, and 9, was characterized by spectra with a single absorbance peak at 283 nm (e.g., peak 9, Figure 6), indicating the loss of all the side-chain conjugation in the process of their formation.

Moreover, compound 5 (Figure 6) exhibited an absorption maximum at 360 nm and was the only individual colored product detected beside caffeic acid *o*-quinone. However, it is not a quinoid, since it was not reduced by addition of sodium metabisulfite or ascorbic acid. In fact, caffeic acid *o*-quinone was the only reducible compound detected in the oxidizing solutions, meaning that secondary quinones, if formed, are highly unstable.

Additional experiments were performed to study the reactivity of the major oxidation products and establish filiations among them. For this purpose, the main transitory products, i.e., compounds 7 and 9, were isolated by means of HPLC on the semipreparative scale (1.5-mL injection volume) from a solution of caffeic acid (5 mM) and polyphenol oxidase (500 mg/L) incubated for 1 h. The isolated compounds were kept in solution in the chromatographic eluant, either alone or in mixture with caffeic acid free *o*-quinone. Eventual changes were then monitored by HPLC on the analytical scale. Their enzymatic oxidation was also studied. The quinone was isolated by injection (1.5-mL injection volume) on the semipreparative column of a 1 mM caffeic acid solution oxidized for 10 min with 1000 mg/L enzymatic extract and used immediately.

The chromatogram obtained by immediate reinjection

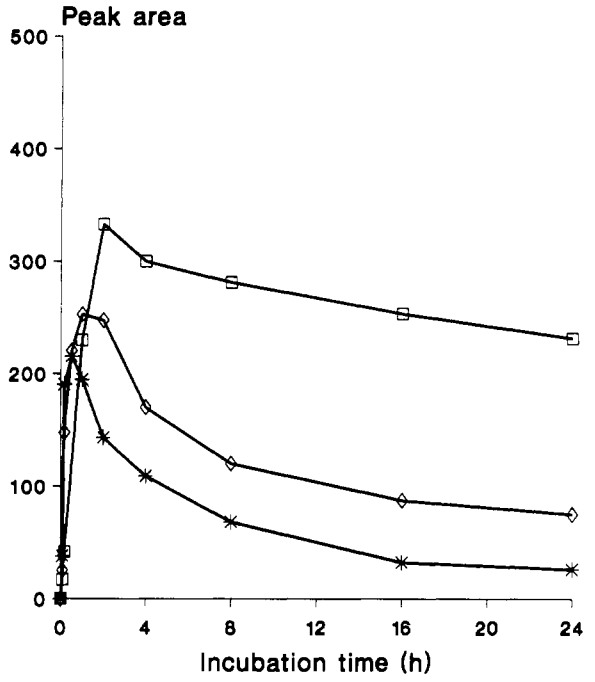

Figure 5. Kinetic behavior of compound 9 in 0.5 mM caffeic acid solutions oxidized by 25 (□), 100 (◇), or 200 mg/L (*) polyphenol oxidase extract.

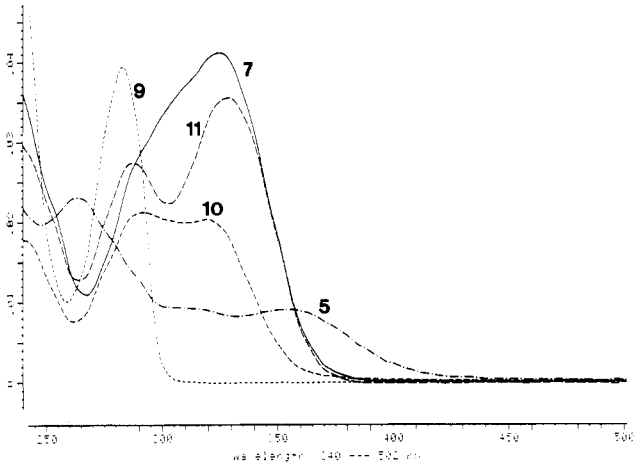

Figure 6. Overlayed UV–visible spectra of the major products formed in polyphenol oxidase catalyzed oxidation of caffeic acid solutions.

of the quinone in its elution solvent showed the presence of caffeic acid along with the expected quinone peak, meaning that some reduction of the quinone had taken place. As the reaction time prior to injection increased, the concentration of free quinones decreased gradually and a new compound (X) eluting at 22.5 min and showing two absorption maxima at 290 and 326 nm accumulated. Trace amounts of more polar products and of compound 9 were also detected. The level of caffeic acid remained constant, but, as observed earlier in oxidizing caftaric acid solutions (Cheynier et al., 1989a), this equilibrium was shifted by addition of glutathione as long as some free quinone remained in the solution, suggesting that it resulted from a competition between reactions consuming caffeic acid and its regeneration from the quinone.

Addition of caffeic acid to the *o*-quinone solution yielded essentially compound 9, along with smaller amounts of 6, 7, and X. The decrease of caffeic acid quinone concentration was much faster in the presence of caffeic acid than in the pure quinone solution, suggesting that the hydroquinone participates in the degradation of its quinone, as observed by Dawson and Nelson (1938) in aqeuous solutions of *o*-benzoquinone and catechol. The relative amounts of the major reaction products (X, 7, 9, 6) in a quinone solution (55 μM) containing various concentrations of caffeic acid (0, 33, 67, 250, and 500 μM) are presented in Table I. The amounts of compounds 7 and 9 formed were positively correlated (r = 0.816 and 0.818, respectively, $n = 10$, $p < 0.01$) and that of compound X negatively correlated (r = −0.932, n = 10, $p < 0.001$) with the initial concentration of caffeic acid. This means that formation of compounds 7 and 9 proceeded via a reaction between caffeic acid and its quinone and competed with polymerization or hydroxylation of the latter leading to compound X. Peak areas of compounds 7 and 9 were also highly correlated together (r = 0.993, n = 10, $p <$ 0.001) and with that of compound 6 (r = 0.979 and 0.98, n = 10, $p < 0.001$).

Figure 7 shows the degradation of reaction products 7 and 9 in solutions containing polyphenol oxidase (100 mg/L of protein extract), polyphenol oxidase inactivated with 0.024 M sodium fluoride, or no enzyme (control). Both compounds disappeared much faster when incubated individually with polyphenol oxidase. The rates of degradation in solutions containing inactivated enzyme and in enzyme-free solutions were similar, confirming that the losses observed in the presence of active enzyme were not due to interactions with proteins of the enzymatic extract but to enzymatic oxidation. In the absence of enzyme, compound 7 appeared to be relatively stable, whereas spontaneous degradation of compound 9 took place. The latter was also observed in solutions stored under helium atmosphere or in the presence of sodium metabisulfite and is therefore believed to be nonoxidative.

Losses measured after 1 h of incubation with caffeic acid *o*-quinone were the same as those in enzyme-free solutions, meaning that compounds 7 and 9 were not oxidized by the *o*-quinone, contrary to our expectations (Singleton, 1987).

The products obtained from compounds 7 and 9 were monitored by HPLC with diode array detection and compared to the products of caffeic acid enzymatic oxidation on the basis of retention times and UV–visible spectra. Enzymatic oxidation of 7 (Figure 8A) and of 9 (Figure 8B) gave essentially one new compound (a), eluting at 14.9 min and exhibiting an absorption maximum at 280 nm. Nonenzymatic reactions of 9 first yielded compound 6 (Figure 8C) and, with longer incubation times (Figure 8D), a number of additional compounds, including 3, 4, and 8. Conversely, when isolated by semipreparative HPLC, compound 6 spontaneously gave compound 9. Actually, in all initially pure solutions of 6 or 9, the peak area of compound 6 consistently equalled 33% (±2%, n = 8) of that of compound 9, after a few hours of storage, suggesting that they might be isomers.

Once this equilibrium was established, the concentration of both decreased gradually while the other products accumulated. Note that the spectra of some of them (4, 8) belong to the first group mentioned above, indicating that the conjugation between the phenolic group and the side-chain double bond was restored in the course of the reactions. The products generated from 9 by nonenzymatic reactions were also formed in the presence of polyphenol oxidase. However, the enzymatically oxidized solution of 9 contained only compound a after 24 h of incubation. As well, when polyphenol oxidase was added to a 1-day-old solution of 9, the products of nonenzymatic reactions disappeared. This probably means that they were enzymatically oxidized, as the concentration of all of

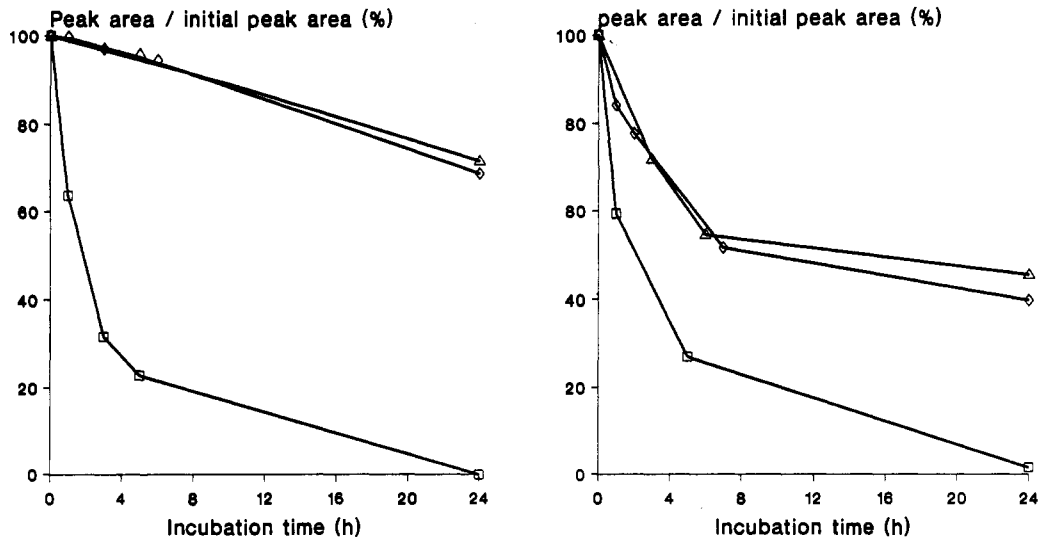

Figure 7. Relative amounts of compounds 7 (left) and 9 (right) during the course of incubation in pure solution (◊), with 100 mg/L enzyme extract (□), and with 100 mg/L enzyme extract inactivated by sodium fluoride 0.12 M (△). All incubations were carried out in duplicate in the semipreparative eluent diluted four times in 0.013 M potassium hydrogen tartrate.

Table I. Influence of Caffeic Acid Concentration on the Formation of Reaction Products from Its *o*-Quinone (Initial Concentration of Caffeic Acid *o*-Quinone 55 μM)

initial amount of caffeic acid, μM	amounts of reaction products formed, peak area units × 10^{-3a}				
	CAF, μM	X	7	9	6
0	14.8	20.0	0	8.8	0
0	14.2	18.7	0	8.0	0
33		13.4	8.2	28.9	8.5
33		14.1	6.3	21.5	6.5
67		12.8	8.8	33.8	10.8
67		11.9	6.6	25.3	7.8
250		8.7	9.6	37.8	11.8
250		8.1	9.7	35.2	9.3
500		3.4	12.3	42.7	11.5
500		3.4	13.7	44.4	15.0

[a] Except as noted for CAF.

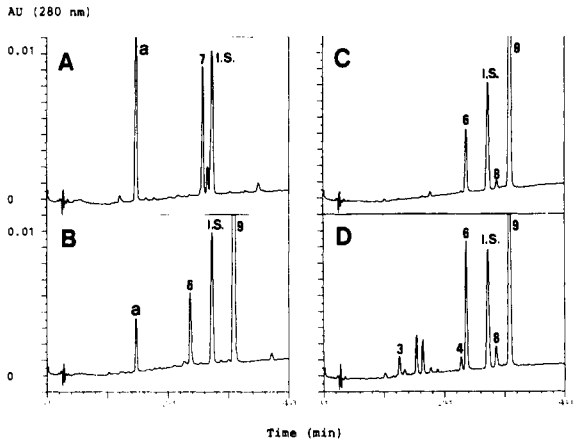

Figure 8. HPLC chromatograms at 280 nm of a solution of compound 7 and benzoic acid (0.2 mM) incubated for 5 h with polyphenol oxidase (100 mg/L) (A) and of a solution of compound 9 and benzoic acid (0.2 mM) incubated for 1 h with polyphenol oxidase (100 mg/L) (B), stored for 2 h (C), and stored for 24 h (D). Chromatographic conditions are the same as in Figure 1.

them, except 6, continued to increase in the same solution stored without enzyme for another 24 h.

Thus, it seems that the *o*-quinone generated by enzymatic oxidation of caffeic acid reacts primarily with its precursor to yield compound 7, which is presumably a caffeic acid dimer formed by C–C linkage between the phenolic rings, and compound 9. Both are themselves substrates for polyphenol oxidase but not oxidizable by caffeic acid *o*-quinone. Besides, compound 9 spontaneously proceeds, by nonoxidative mechanisms, to a number of other derivatives, including peaks 4 and 6. These nonoxidative reactions play an unexpectedly important part in the degradation of enzymatic oxidation products in the caffeic acid model systems studied. However, nonoxidative products were presumably oxidizable by polyphenol oxidase and did not accumulate in the solutions containing higher ratios of enzyme to phenol.

The major product (a) formed enzymatically from 7 and 9 could not be detected in the oxidizing caffeic acid solutions. This suggests that the competition between enzymatic reactions (leading to a) and nonenzymatic reactions (leading to 6 and 4) is in favor of the latter. However, this may also mean that compound a or an eventual intermediate—possibly a quinoid—in the mechanism of its formation interacts with other components of the medium.

Although only the very first reaction steps were actually oxidative, the products formed in enzymatically oxidized caffeic acid solutions differed from those obtained by autoxidation in alkaline medium. Since it has been shown by Cilliers and Singleton (1989) that the nature of caffeic acid autoxidation products in nonenzymatic systems is independent of the pH, this suggests that the reactions of the enzymatically generated *o*-quinones and those of the semiquinone radicals resulting from nonenzymatic oxidation yield different products. Although oxidizing caffeic acid solutions browned considerably, most reaction products were colorless. This probably indicates that pigments are higher molecular weight compounds which cannot be analyzed under our chromatographic conditions, but it may also mean that many different colored substances are formed in small amounts (Cilliers and Singleton, 1989).

Identification of the major products obtained by enzymatic oxidation of caffeic acid should provide complementary information on the mechanisms responsible for their formation. Their isolation in quantities sufficient to perform NMR and mass spectrometry studies and achieve their structural characterization is under way. However, it has proved very difficult so far, because of their high reactivity and fast interconversions during storage.

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Received for review March 26, 1992. Revised manuscript received August 5, 1992. Accepted August 21, 1992.

Registry No. Caffeic acid, 331-39-5; polyphenol oxidase, 9002-10-2; caffeic acid *o*-quinone, 15416-77-0.