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Caffeic Acid Autoxidation and the Effects of Thiols

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Initial oxygen uptake by *o*-dihydroxybenzenes is slowed by adding thiols like cysteine (Cys) or glutathione (GSH). One molecule of caffeic acid consumed 3.4 atoms of oxygen (O), which increased with thiol addition (Cys 5.5 and GSH 8.5 atoms of O per molecule of phenol). Under the same conditions cysteine or glutathione alone did not consume oxygen. Phloroglucinol did not consume oxygen, but when combined with caffeic acid increased total oxygen uptake (4.9 atoms of O per molecule of caffeic acid). This increased even further with the addition of thiols (Cys 8.1 and GSH 11.4 atoms of O per molecule of phenol) after 4 h. Catechin combined with caffeic acid behaved similarly but had a higher level of oxygen taken up in 3 h (Cys 11.2, GSH 17.0 atoms of O). Reversed-phase HPLC was used to study the reactions of caffeic acid (1 mM) with cysteine (4 mM) during chemical autoxidation. The formation and disappearance of 2-*S*-cysteinylcaffeic acid (2-CCA), 5-*S*-cysteinylcaffeic acid (5-CCA), and 2,5-di-*S*-cysteinylcaffeic acid (DCCA) were investigated. Caffeic acid oxidized correlated with brown (420-nm absorbance) formed. Thiol additions had protective effects on caffeic acid consumption. A golden yellow color measured by 420-nm absorbance is produced at pH 9 and higher which only becomes an objectionable brown once cysteine is depleted. When cysteine is depleted, caffeic acid and the thioethers are rapidly oxidized with formation of brown rather than yellow color. Concentrations of either glutathione or ascorbic acid equimolar with caffeic acid had large protective effects against browning and caffeic acid disappearance at pH 7.0.

The use of antioxidants like sulfur dioxide in foods is limited by legislation, and its replacement would be desirable. It contributes to off-flavors, and a few asthmatic individuals experience severe hypersensitive reactions to SO_2. Even stricter legislation might require its elimination. To be able to find a suitable replacement for sulfur dioxide, it is important to know which oxidation products form and how they are affected by antioxidants to reduce color formation.

The inhibitory action of thiol compounds (cysteine, thioglycolic acid, thiocresol) on browning is caused by combination with the catalyzing quinone. A hydroquinone–sulfite mixture autoxidizes at a lower rate and eventually absorbs more oxygen than hydroquinone alone. If the quinone formed has at least one ring hydrogen, it reacts with the sulfite with formation of the corresponding hydroquinone monosulfonate. The hydroquinone monosulfonates autoxidize and form the disulfonates (James and Weissberger, 1939).

Pyrogallol autoxidizes rapidly in aqueous solution, and this increases at higher pH. However, at pH 7.9 the autoxidation is 99% inhibited by superoxide dismutase (SOD). This indicates a total dependence on the participation of the superoxide anion radical in the reaction. At pH values higher than 9.1 superoxide-independent mechanisms rapidly become dominant. Catalase had no effect on the autoxidation but decreased the oxygen consumption by half, showing that H_2O_2 is a product and that H_2O_2 is not involved in the autoxidation mechanism (Marklund and Marklund, 1974).

The occurrence of oxidative coupling of phenols is well documented with *o*- and *p*-dihydroxyphenols, but usually the products are not well studied in food-related systems (Cha et al., 1986; Cilliers and Singleton, 1989; Ghosh and Misra, 1987; Prutz et al., 1983; Singleton, 1987). It involves the formation of a reactive electrophilic quinone intermediate by PPO, which will readily undergo attack by nucleophiles. The presence of nucleophiles, e.g., thiols like glutathione, leads to the formation of products with addition on different positions of the benzene ring. The 2-position of the benzene ring in caffeic acid is the most electrophilic, and nucleophilic addition occurs preferentially here; in the process the quinone moiety is regenerated to the hydroquinone form (Cheynier et al., 1986; Kalyanaraman, 1987; Singleton, 1987; Singleton et al., 1984; Takahashi, 1987). Upon further oxidation of this addition product by laccase to a quinone, a second addition occurs in the 5-position to give 2,5-di-*S*-glutathionylcaftaric acid (Itahara, 1985; Salgues et al., 1986). These reactions would help explain the increased capacity for oxygen uptake in wines if reaction is slowed and prolonged (Singleton, 1987).

Caffeic acid has been used to model the oxidative reactions since chlorogenic and caftaric acids are major fruit juice phenols. Caftaric acid (caffeoyl tartaric) and other hydroxycinnamic acid–tartaric acid esters are the major phenolic compounds of white grape juice (Singleton et al., 1978). When no special care is taken to avoid oxidation during crushing and pressing, grape PPO causes large losses of these compounds due to their oxidation and conversion into 2-*S*-glutathionyl caftaric acid or "grape reaction product" (GRP) (Cheynier et al., 1986; Salgues et al., 1986; Singleton et al., 1984). In spite of its *o*-dihydroxyphenol structure, GRP alone is not a direct substrate for PPO. The conversion reaction limits the enzymic browning of musts by trapping the *o*-quinone formed by caftaric acid oxidation in colorless form, thus preventing it from proceeding to brown polymers (Singleton et al., 1985). Glu-

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tathione was the limiting factor for this kind of reaction since addition of glutathione increased GRP production, especially in some grape varieties. In fact, the caftaric/glutathione ratio that determines the amount of untrapped quinones may be a parameter to explain varietal differences in browning capacities (Salgues et al., 1986).

In this research we set out to investigate the possible formation of thioethers after chemical oxidative formation (no enzyme involved) of quinones and its effects on oxygen uptake and browning. Specific attention will be given to the rate of oxygen uptake, whether initial uptake is slowed and total uptake is increased, and also whether there are any protective effects on color formation as well as the effects of pH and thiol concentration on the above. The purpose of this research is to investigate in model systems the possibility of replacing sulfites with more desirable thiols to slow or prevent browning reactions of natural polyphenols during oxidation under various pH conditions.

MATERIALS AND METHODS

The effects of various types of compounds on the oxidation rate of caffeic acid (4 mM) at pH 7.0 and 20 °C were investigated. As strong thiol nucleophiles cysteine (0, 2, 4, 8, 16, 32 mM) and glutathione (4 mM) were used, and as weaker nucleophile alanine (4 mM), to evaluate an effect of an amino group. Ascorbic acid (4 mM) as quinone reductant was tested. Compounds tested but not naturally present in foods were 4 mM dithiothreitol (Cleland's reagent; Calbiochem, La Jolla, CA) and mercaptoethanol (4 mM), individually and in combinations. All these chemicals were from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) unless stated otherwise. Only ultrapure water from a Milli-Q system (Millipore, Milford, MA) was used. Solutions (100 mL) were stirred with turbine-driven magnetic stirrers in a water bath at 20 °C. All solutions were maintained at pH 7.0. Experiments were repeated at pH 10.0 by adding 4.0, 8.0, and 16.0 mM cysteine to 2 mM caffeic acid. The rest of the experimental conditions remained the same. Samples were analyzed by HPLC for caffeic acid, 2-CCA, 5-CCA, and DCCA. Adjustments to pH were done with 0.5% and/or 5% KOH. Oxygen was at a 100% level, and care was taken to ensure that it did not become limiting throughout these experiments. Oxygen in solution was measured with a Clark oxygen electrode and the pH checked after each sampling. Solutions turbid with precipitated cystine were first centrifuged before samples were taken for HPLC or spectrophotometric readings (brown color formation at 420 nm).

Cysteine (4 mM) was added to caffeic acid (1 mM) at pH 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, and 13.0 in 100% oxygen on magnetic stirrers at room temperature. Samples were analyzed for 2-CCA, 5-CCA, DCCA, and caffeic acid by HPLC. This was repeated with 2 mM caffeic acid at pH 8.0, 9.0, 10.0, and 11.0 at 20.0 °C in 100% oxygen.

HPLC. Sampling and HPLC conditions were as described by Cilliers and Singleton (1989). This HPLC method was used to analyze for all reaction and oxidation products, and separation time was 40 min per sample. Detection was at 200 nm, and a C_{18} reversed-phase column was used. When only caffeic acid, 2-CCA, 5-CCA, and DCCA were monitored (200 and 210 nm), mobile phases (A = 92% and B = 8%) were used isocratically to quantitate within 8 min. Only for isocratic separation by HPLC, sampling consisted of acidification of 1.4 mL of sample and 0.1 mL of acetonitrile with sulfuric acid. All other conditions were unchanged. The column was washed with 100% B after each injection and equilibrated before the next 20-μL injection.

Oxygen Uptake. Oxygen uptake was measured in separate experiments with a Gilson differential respirometer (Middleton, WI). Temperatures of the water bath were 0 or 25 °C. KOH (10%, 1.0 mL) in the sidearm was added to 0.5 mL of phenol (2 mM) and 0.5 mL of H_2O with or without thiol. Phenols used were caffeic acid, catechol, phloroglucinol, and gallic acid. Cysteine and glutathione were added in excess amounts, and final concentrations were 4 times higher than that of the phenol (4 mM). Caffeic acid plus phloroglucinol and caffeic acid plus catechin were used as phenolic combinations in equimolar concentrations with and without the thiols, and the effects on oxygen uptake were measured. Additional experiments were done with air and 100% oxygen. Also used were KOH (20%, 1.0 mL) added to the phenol with varied shaker speed and a lower concentration of cysteine (2 mM) with caffeic acid (1 mM). Care was taken to keep shaking speed constant in different experiments. Corrections were made in the oxygen-consumed readings for barometric pressure. These corrections were made on the mean values of samples done in triplicate.

Preparation of 2-*S*-Cysteinylcaffeic Acid (2-CCA). 2-CCA was prepared on gram scale for use as a standard in determining HPLC retention time and in UV–visible spectroscopy. This was done by adding 10 g of caffeic acid and 35 g of cysteine hydrochloride together and making it up to 1000 mL with water. The pH was adjusted to 6.5 with 20% KOH. Polyphenol oxidase (53 mg, mushroom tyrosinase, 4800 units/mg) was dissolved and added to the mixture and stirred on a magnetic stirrer at room temperature. Air was bubbled through the solution with a sintered glass fitting for fine bubbles. The reaction was monitored, and samples were analyzed by HPLC for unreacted caffeic acid and formed 2-CCA.

The reaction mixture was filtered through a 0.45-μm membrane filter (Gelman Sciences Inc., Ann Arbor, MI) to remove precipitated cystine. After acidification with formic acid (5 mL) to pH 3.6, SO_2 (0.2 mL, 5%) was added to destroy the enzyme. After a few minutes, the precipitate was removed with a 0.45-μm membrane filter and yielded 6.4 g of 2-CCA when air-dried of 90% purity (by HPLC at 200 nm). The supernatant still contained 30% of the total 2-CCA, which was recovered by rotary evaporation at 40 °C under reduced pressure (−95 kPa). Recrystallization was done in water by changing the pH to 6.5 under nitrogen atmosphere and then lowering it again to pH 3.6. Finally, the solid was dried in a vacuum oven (−95 kPa) at 35 °C and stored in a desiccator. It appeared stable under these conditions.

2-CCA was identified and characterized by ^{1}H NMR and mass spectrometry with the following information: ^{1}H NMR 8.40 (d, J = 16.2 Hz, Ar CH=), 7.26 (d, J = 8.1 Hz, Ar H, H-6), 6.85 (d, J = 8.5 Hz, Ar H, H-5), 6.40 (d, J = 16.2 Hz, =CH), 3.05 (t, J = 8.3 Hz, CH), 2.83 (d, J = 8.3 Hz, S–CH_2). The FAB mass spectrum in the positive ion mode gave prominent daughter ion peaks at m/z 454 [matrix (154) + 2-CCA (299) + H^+ (1)] and m/z 492 [matrix (154) + 2-CCA (299) + K^+ (39)]. In the negative ion mode a prominent m/z 298 [2-CCA (298) − H (1)] was observed. A mass-analyzed ion kinetic spectrum (MIKES) of the parent ion (m/z 298) gave three prominent peaks at m/z 254 [2-CCA (298) − CO_2 (44)], m/z 211 [2-CCA (298) − CH_2CHNH_2COO (87)], and m/z 167 [2-CCA (298) − CH_2CHNH_2COO (87) − CO_2 (44)].

Mass Spectrometry. A ZAB-HS-2F (VG Analytical, Wythenshawe, U.K.) with ionization FAB (Xe, 8 keV, 1-mA beam current) and accelerating potential of 8 kV and resolution ($M/\Delta M_{10\%\text{valley}}$) 1000 was used. The FAB matrix used was dithiothreitol/dithioerythritol [3:1 (v/v)] in the positive ion mode. A glycerol matrix was used in the negative ion mode, and a mass-analyzed ion kinetic spectrum (MIKES) was obtained of the parent ion (m/z 298).

^{1}H NMR. A Nicolet NT-360 spectrometer was used with ^{1}H frequency 360.065. Proton spectra were accumulated with 16K data points over 8-kHz bandwidth.

RESULTS AND DISCUSSION

Several phenols were analyzed under very alkaline conditions (pH 14), and their total oxygen uptake was measured. The atoms of oxygen (O) consumed per molecule of phenol when the reaction was complete were 3.4 atoms for caffeic acid, caftaric acid 3.2 atoms, 3.2 atoms for pyrogallol, 4.0 atoms for catechol, and 4.8 atoms for gallic acid. Phloroglucinol and coutaric acid did not consume oxygen under these autoxidative conditions, nor did other individual compounds including cysteine, glutathione, tartaric acid, and alanine.

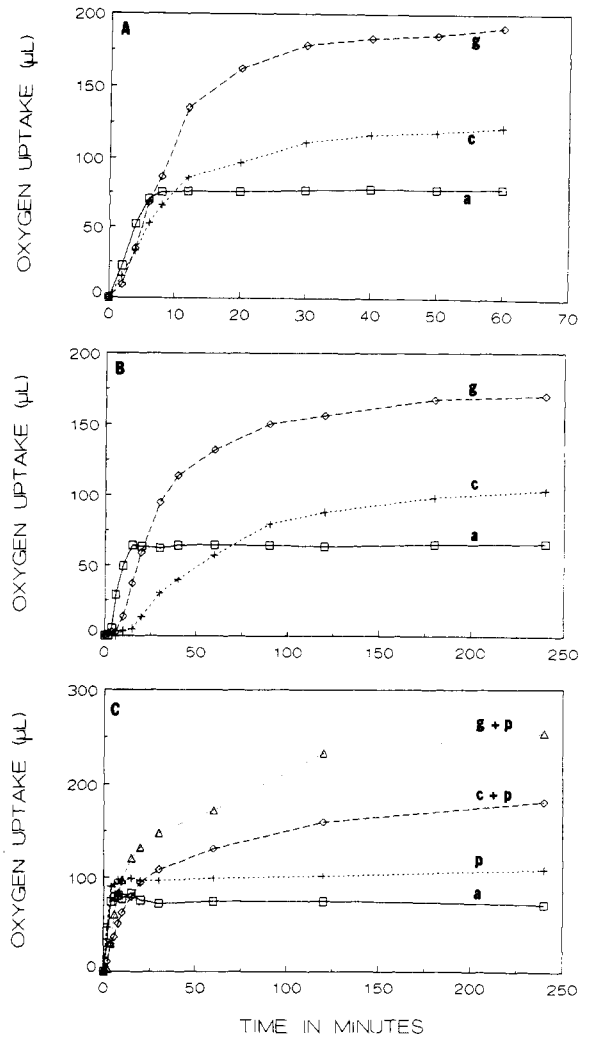

Figure 1. (A) Oxygen consumed after addition of 1.0 mL of 10% KOH to 1.0 mL of caffeic acid (1 mM) and cysteine (4 mM) at 25 °C. (B) Same as in (A) but at 0 °C. (C) Same as in (A) except that phloroglucinol (1 mM) was added. g, reduced glutathione (4 mM) added; c, cysteine (4 mM) added; a, caffeic acid (1 mM); p, phloroglucinol and caffeic acid combination.

Initial oxygen uptake was slowed when thiols were present at 4 times the phenolic molar concentration. Then it increased and consumed more oxygen in total than the phenols alone, e.g., caffeic acid (5.5 atoms of O). For glutathione this value was even greater (8.5 atoms of O). After 15 min, oxygen uptake slowed, but was still continuing after 60 min at 25 °C. These effects on caffeic acid are shown in Figure 1A. Similar effects were observed for caftaric acid and catechol. It is obvious that the thiols are interacting importantly in this oxidation reaction. For the individual phenol alone, the reaction in air is essentially complete in about 10 min and oxygen consumption stops. When thiol compounds are added, oxygen uptake continues long after the reaction stops for the individual phenol. At 0 °C this point was nearly reached after 4 h. At 0 °C the greater slowing of oxygen uptake by cysteine is seen clearly in Figure 1B. At least one reaction during this lag phase appears to be cysteine reducing quinone (with associated cystine production) back to dihydroxyphenol, which then can undergo further oxidation.

When an equimolar amount of phloroglucinol was added to the reaction mixture, oxygen consumption rapidly increased for the combination of caffeic acid and phloroglucinol and then leveled off at a oxygen-consumed value higher (4.9 atoms of O) than 3.4 atoms per caffeic acid (Figure 1C). This was also observed by Tulyathan (1983). This is noteworthy since phloroglucinol by itself did not consume any oxygen. For this an *o*-dihydroxy moiety is necessary, but evidently phloroglucinol combines with the caffeic quinone and regenerates an oxidizable dimer and amplifies the oxygen uptake (Singleton, 1987). With the addition of thiols oxygen uptake increased dramatically (Cys 8.1 and GSH 11.4 atoms of O). After 4 h, oxygen was still consumed at 25 °C. It appears cysteine has two effects, reduction of quinone to caffeic acid while being oxidized to cystine and amplification of caffeic acid oxidation by substitution in the quinone to regenerate the hydroquinone form of 2-*S*-cysteinyl caffeic acid.

Similar observations were made for equimolar concentrations of catechin and caffeic acid. Since catechin has both the *m*- and *o*-dihydroxy moieties, more oxygen was consumed than by phloroglucinol because the ortho moiety would oxidize as is the case with catechol. After 3 h at 25 °C, caffeic acid and the catechin combination had consumed 8.0 atoms of O per molecule of the sum of catechin and caffeic acid. Thiol addition to this phenolic combination increased oxygen uptake (Cys 11.3 and GSH 17.0 atoms of O per phenol molecule) after 3 h. These did not stabilize but slowly increased to 13.2 (Cys) and 19.3 (GSH) atoms of O per phenol after 9 h.

Oxygen solubility was limiting in some of these oxygen uptake studies with the Gilson respirometer. The rate of oxygen consumption corresponded to shaking speed and also increased ca. 5-fold when the system was equilibrated with 100% oxygen. The use of 20% KOH also slowed the rate of oxidation. Its higher viscosity would affect the rate of diffusion of oxygen into the solution. The above parameters were kept constant to obtain comparable data. The use of 2 mM instead of 4 mM cysteine did not change the rate or the amount of oxygen consumed per mole of phenol. This indicates that cysteine is not reacting with oxygen but with the oxidized phenol and that cysteine solubility is not a factor.

In all cases it was found that the addition of thiol slowed the initial rate of oxygen uptake. This is attributed to regenerative reaction in which the quinone is reduced back to the *o*-dihydroxyphenol. The thio adduct has a lower oxidation potential and through coupled oxidation can reduce its unsubstituted originating quinone. It is also easier to autoxidize, which is seen as a later increase in the rate of oxygen consumption. Ring opening of the caffeic acid quinone to form the muconic acid derivative will be slowed due to thiol addition competition.

To further study the effects of thiol compounds on phenols under chemical oxidative conditions, caffeic acid was used as a model for naturally occurring chlorogenic and caftaric acids and other *o*-dihydroxyphenols. Cysteine was used as a model for thiols. Lowering the pH from 14 to as low as 7 slowed the reaction and allowed therefore more detailed interim studies. Four peaks were separated with the HPLC conditions applied (Figure 2), namely, caffeic acid, 2-CCA, the 5-S alternate form of 2-CCA (5-CCA), and 2,5-di-*S*-cysteinyl-caffeic acid (DCCA). The latter two were identified with UV–visible spectra (DCCA similar to 2,5-di-*S*-glutathionyl caftaric acid; Salgues et al., 1986) from the diode array detector. Caffeic acid and 2-CCA were known from spectra and retention time from authentic standards. Spectra of these four compounds are given in Figure 3.

The effects of pH 8.0, 9.0, 10.0, and 11.0 on the rate of 2 mM caffeic acid oxidation in the presence of 8 mM cysteine and the formation of color (420-nm absorbance) are presented in Figure 4. Color formation correlated well with caffeic acid oxidized [$P \leq 0.001$ for pH 9 (degrees freedom

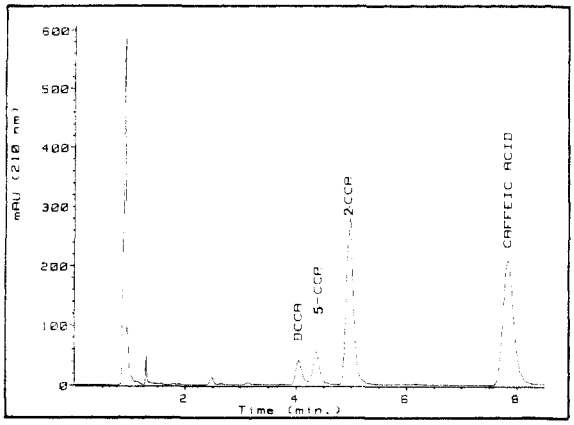

Figure 2. HPLC chromatogram of reaction products of cysteine (4 mM) and caffeic acid (1 mM) oxidation in 100% oxygen after 4 h at pH 10.0 and room temperature. Flow rate was 1.0 mL/min at 40 °C on a 10-cm 3-μm reversed-phase column with detection by diode array detector at 210 nm. Separation is isocratic by mixing 92% A (pH 2.6, 0.05 M ammonium phosphate buffer) with 8% B (80% acetonitrile and 20% A). 2-CCA, 2-*S*-cysteinyl caffeic acid; 5-CCA, 5-*S*-cysteinyl caffeic acid; DCCA, 2,5-di-*S*-cysteinyl caffeic acid.

(df) = 9, r = 0.957), pH 10 (df = 10, r = 0.915), and pH 11 (df = 5, r = 0.957) and 4 mM (df = 5, r = 0.953), 8 mM (df = 6, r = 0.953), and 16 mM (df = 8, r = 0.901) cysteine added at pH 10]. The rate of caffeic acid oxidation was increased at increased pH even with cysteine present: pH 9.0 = 4820 × 10^{-5} h^{-1} (df = 8, r = 0.947); pH 10.0 = 7570 × 10^{-5} h^{-1} (df = 9, r = 0.984); pH 11.0 = 24 760 × 10^{-5} h^{-1} (df = 4, r = 0.992) at 20 °C. At all these pH values cysteine had a protective effect. Instead of the formation of a dark brown color observed with caffeic acid oxidation with no additions, in the presence of cysteine a yellow color formed that persisted until all caffeic acid was oxidized or until the cysteine was consumed. The colors, although measured at 420-nm absorbance, were visually quite different.

The rate of caffeic acid oxidation was slowed at pH 8.0 in the presence of 8 mM cysteine [no cysteine added 11334 × 10^{-5} h^{-1} (df = 4, r = 0.982); with cysteine 3480 × 10^{-5} h^{-1} (df = 9, r = 0.909) at 20 °C]. Cystine precipitated after 8 h. Very little color was produced in this time. Caffeic acid oxidation at pH 9 and 11 is very rapid in 100% oxygen. However, with cysteine added the rate of browning was greatly reduced. A yellow color that increased in intensity, but with no brown, was observed until all the caffeic acid was oxidized or all cysteine consumed, at which point brown was rapidly produced, especially at pH 9.0. At the higher pH values a yellow color with some brown persisted. At pH 11.0 the color was bright yellow with absorbance of 2.5 at 420 nm and no brown after 4 h at pH 11.0. At pH 9.0, cysteine had a shorter protective effect than at pH 10.0, which might be due to being below the phenolic pK_a value. The objectionable dark brown differed from the yellow-golden color spectrophotometrically with the former having more absorbance in the visible region.

The effects of different concentrations (1, 2, 4, 8, and 16 mM) on caffeic acid (2 mM) oxidation at pH 10.0 and formation of color (420 nm) are shown in Figure 5. Cysteine concentration can affect caffeic acid oxidation. The rates of caffeic acid disappearance at pH 10 were independent of cysteine concentration [4 mM = 6630 × 10^{-5} h^{-1} (df = 5, r = 0.957); 8 mM = 6570 × 10^{-5} h^{-1} (df = 5, r = 0.990); 16 mM = 8109 × 10^{-5} h^{-1} (df = 7, r = 0.996)], indicating an unchanged mechanism of protection. However, the time it takes for caffeic acid to become fully oxidized is dependent on the cysteine concentration. Caffeic acid with no added cysteine will be oxidized to a very brown color within a few minutes at pH 10.0. This was slowed to at least 3 h with half and an equimolar amounts of cysteine. The protective effect against browning and caffeic acid disappearance increased with increasing cysteine concentration.

Once cysteine is fully depleted, oxidation of caffeic acid and formation of brown color are rapid. The yellow color increases in intensity until cysteine is depleted. At this point the concentrations of 2-CCA, 5-CCA, and DCCA are all at a maximum. Disappearance of these compounds by further oxidation is rapid. In Figures 6 and 7 these effects on caffeic acid oxidation, the formation of intermediates, and color (420 nm) are shown for 4, 8, and 16 mM cysteine concentrations at pH 10.0. Caffeic acid oxidized correlated well with 2-CCA formed ($P \leq 0.001$ for pH 8–11 and 4, 8, and 16 mM cysteine added at pH 10.0). 2-CCA also correlated with color formed ($P \leq 0.001$ for pH 8–10 and $P \leq 0.01$ for the different cysteine concentrations at pH 10.0).

Cysteine concentration also affected the concentration of the thioether products. A large effect was observed for 2-CCA and 5-CCA (parts A and B of Figure 7, respectively) between ca. equimolar concentrations and more than twice the molar concentrations. Approximately equally high concentrations formed for 4, 8, and 16 mM cysteine, and their rates of formation were also the same. However, upon depletion of cysteine they are rapidly oxidized and removed. This occurred at different time intervals.

DCCA had different rates of formation, formed maximally after 2-CCA and 5-CCA were formed, and different concentrations that formed which increased with increasing cysteine concentration (Figure 7C). This reinforces the postulation (based on the UV–visible spectrum) and literature comparison that this compound is the 2,5-di-*S*-cysteinyl thioether of caffeic acid since its formation would be more dependent on cysteine concentration and it could form only from either 2-S- or 5-S-CCA.

Over the pH range 9.0–13.0 in 100% oxygen, with constant caffeic acid and cysteine concentrations, 2-CCA was formed at equal, high maximum concentrations (not shown). The time to reach maximum concentration varied and was fastest at pH 13.0 (0.5 h) and slowest at pH 9.0 (8 h). This phenomenon did not occur at pH 8.0 and pH 7.0, and the 2-CCA concentrations were much lower at these pH values. This suggests that the quinone concentration needs to be high enough for this addition reaction to take place and form 2-CCA. We previously found the oxidation of caffeic acid to be dependent on its phenolate ion concentration. The latter would be a calculated 3.1% at pH 8.0 and 24% at pH 9.0 for a pK_a value of 9.5. This could also apply for quinone concentration. It should also be considered that the pK_a for the sulfhydryl group of cysteine is 8.3. This would mean that most of the cysteine sulfur is ionized in the high-pH reactions mentioned. 5-CCA reacted similarly to 2-CCA and DCCA had its highest peak area at pH 10.0. The protective effect is possible with the formation of oxidation products with a lower redox potential, e.g., 2-CCA, which is preferentially oxidized compared to caffeic acid. Through regenerative polymerization, quinones formed by chemical autoxidation under alkaline conditions become again hydroquinones either by dimerization with phenol or by thioether formation.

The effect of cysteine concentration was also investigated at pH 7.0 in 100% oxygen at 20 °C, and results are presented in Figure 8. Caffeic acid oxidation showed a lag

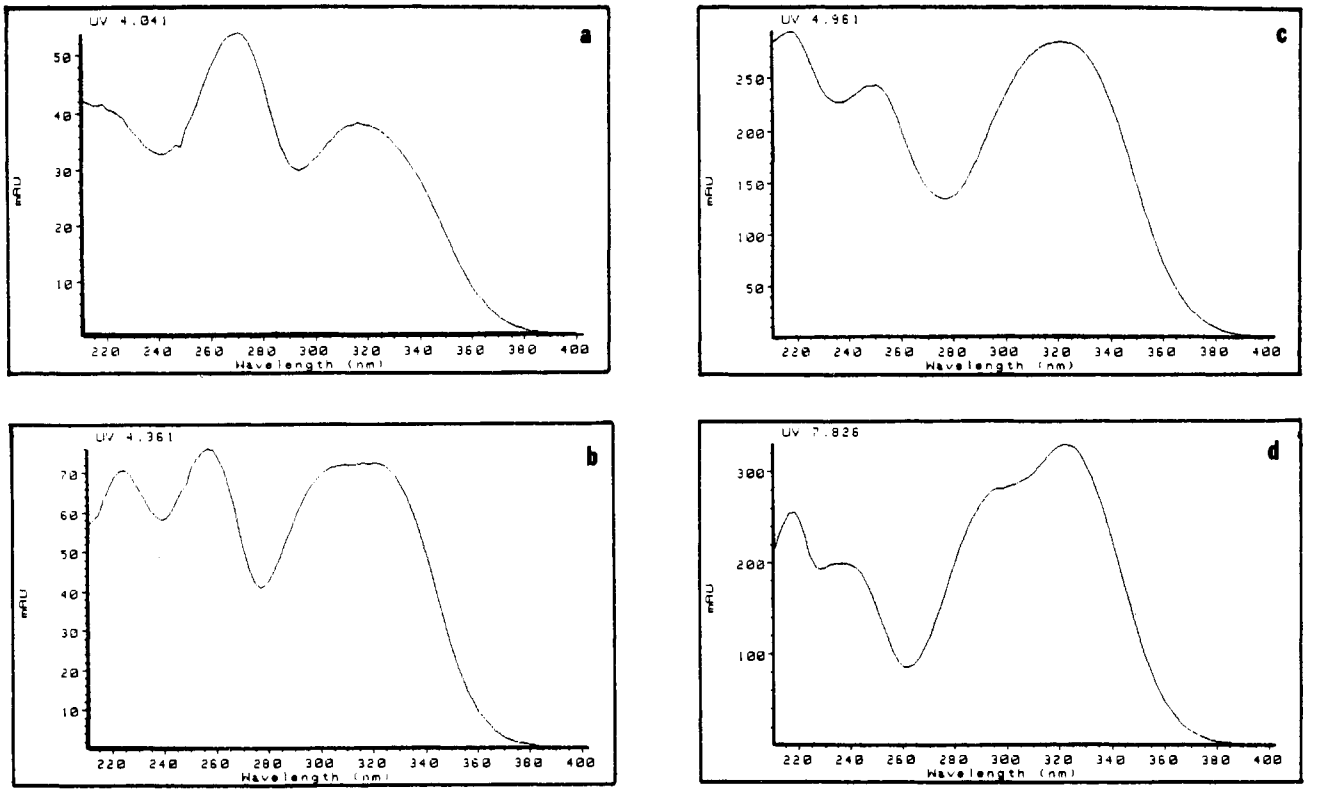

Figure 3. UV-visible spectra obtained by diode array detector, scanning from 210 to 400 nm. The displayed spectra were taken at HPLC peak apices. a, DCCA; b, 5-CCA; c, 2-CCA and d, caffeic acid.

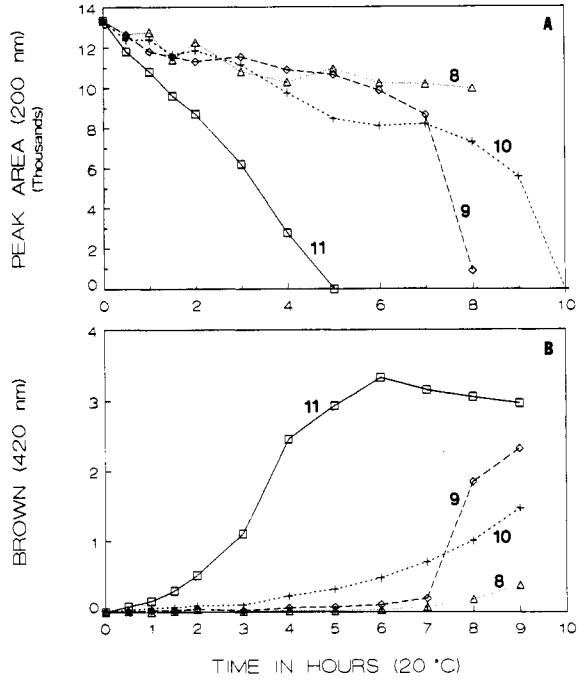

Figure 4. Caffeic acid disappearance by HPLC (A) and color production at 420 nm (B) as affected by pH values 8 (△), 9 (◇), 10 (+), and 11 (□) during oxidation of 2 mM caffeic acid in the presence of 8 mM cysteine at 20 °C in oxygen (100%).

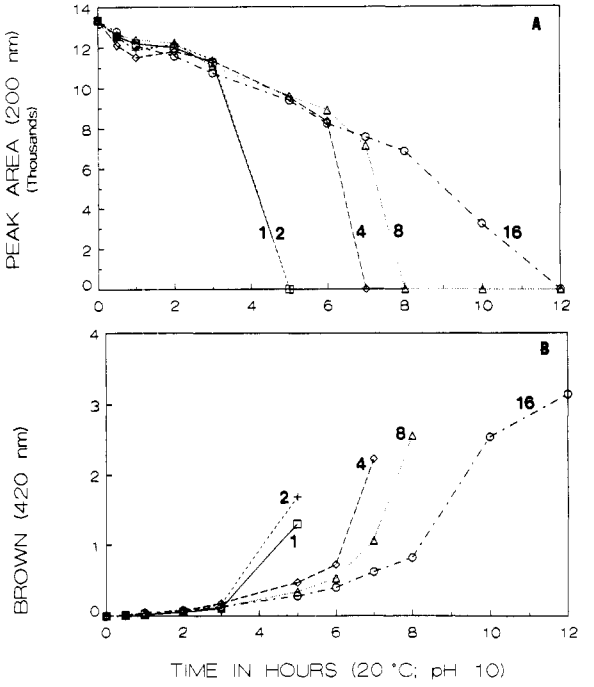

Figure 5. Effects of cysteine concentration (1, 2, 4, 8, and 16 mM) on (A) caffeic acid (2 mM) disappearance at pH 10.0 and 20 °C in 100% oxygen atmosphere. HPLC with 200-nm detection was used for caffeic acid determination. (B) Formation of brown (420 nm) under the same experimental conditions described in (A).

with cysteine present. However, this effect is small and inconsistent with cysteine concentration. Precipitation of cystine occurred after 12 h of oxidation, which ended the protective effect on the formation of brown (420 nm). At pH 7.0, cystine started to precipitate after 12 h. Cystine, cysteine, and alanine had little long-term effect on slowing the rate of caffeic acid oxidation and in preventing color formation at pH 7.0, but cysteine did have an initial protective effect. In an attempt to reduce brown by using cysteine, more was added after 12 h to counteract the precipitation problem, but with no success.

Both ascorbic acid and glutathione exerted large effects in preventing browning and on the caffeic acid disappearance (Figure 9). The effect of ascorbic acid would be to reduce the caffeic quinone as fast as it formed, preventing both caffeic loss and browning. Like cysteine, glutathione would be expected to both reduce some

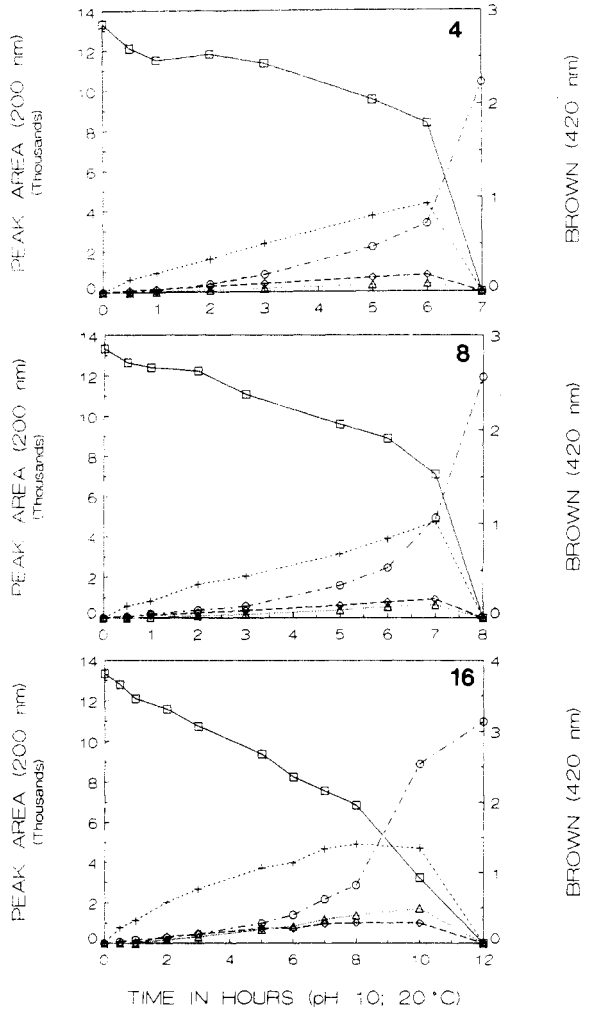

Figure 6. Effects of cysteine concentration (4, 8, and 16 mM) on the formation of brown at 420 nm (○), 2-CCA (+), 5-CCA (◇), and DCCA (△) from the oxidation of 2 mM caffeic acid (□) at pH 10.0 and 20 °C. HPLC with 200-nm detection was used for compound determination.

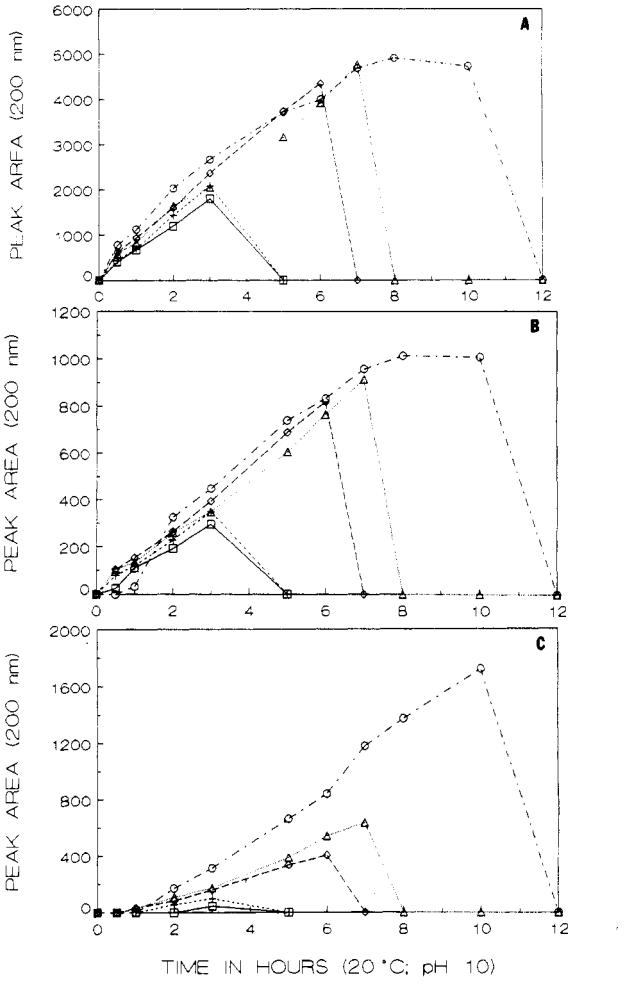

Figure 7. Effects of cysteine concentrations 1 (□), 2 (+), 4 (◇), 8 (△), and 16 mM (○) and caffeic acid (2 mM) oxidation on the formation of (A) 2-CCA, (B) 5-CCA, and (C) DCCA. HPLC with 200-nm detection was used for compound determination.

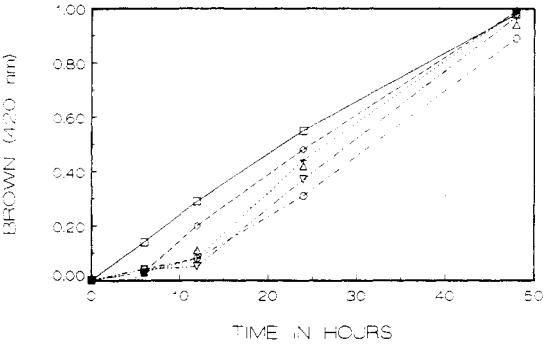

Figure 8. Effect of cysteine concentration on the formation of brown at 420 nm. Caffeic acid (4 mM) was oxidized at pH 7.0 and 20.0 °C in 100% oxygen with different cysteine concentrations from 0 to 32 mM as indicated. Cysteine: □, 0 mM; +, 2 mM; ◇, 4 mM; △, 8 mM; ○, 16 mM; ▽, 32 mM.

of the quinone, forming oxidized glutathione (GSSG), and substitute to caffeic quinone. Since caffeic acid did not disappear in the presence of ascorbic acid and GSH (until color started to form), reduction is the major reaction.

Mercaptoethanol was effective in slowing brown formation, and a combination of mercaptoethanol and dithiothreitol did not produce any brown. However, it has to be kept in mind that the total sulfhydryl concentration in the latter experiment was 12 mM compared to 4 mM used in the other experiments. This also applies to the use of dithiothreitol and cysteine since the former contains two sulfhydryl groups. A combination of cysteine with dithiothreitol had a marked reduction in color. Dithiothreitol should prevent cysteine production, and precipitation was not observed.

Once glutathione is consumed either by addition to the quinone or by forming GSSG, color production is rapid. If glutathione is used in excess, it substitutes and reduces all formed quinone and very little or no color is formed. Theoretically this should be the same for cysteine. However, this happened only partially in practice. Cysteine is reactive (Thompson et al., 1985), it formed cystine in the presence of oxygen and oxidizing phenol which is insoluble, and it precipitates from solution at low pH. Oxidized glutathione remained soluble and may contribute with other side reactions to exert additional protective effects to prevent quinones from forming "polymers" which would give color. It is also possible that the 2-*S*-glutathionylcaffeic acid that forms has a higher oxidation potential than 2-CCA and is slower to be further oxidized to colored products (Carstam et al., 1986). Correlation coefficients of caffeic acid consumed and brown (420 nm) produced were very highly significant at all treatments ($P \leq 0.001$) with correlation coefficients higher than 0.960 ($n = 8$). Similar patterns were observed in graphs of all treatments for caffeic acid consumed and brown (420 nm) formed and are thus not presented. With no caffeic acid consumed no color at 420 nm formed.

An equimolar concentration of ascorbic acid as an antioxidant had the largest protective effect on the formation

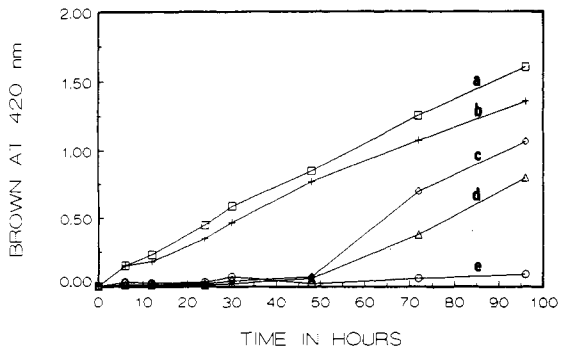

Figure 9. Effects of addition of equimolar concentrations to caffeic acid of an amine, a thiol, and an antioxidant on the formation of brown at 420 nm. Caffeic acid (4 mM) was oxidized at pH 7.0 and 20 °C in 100% oxygen. a, caffeic acid; b, alanine; c, glutathione; d, mercaptethanol; e, ascorbic acid.

of brown. Thiols like glutathione and mercaptoethanol (not a food additive) had similar large effects up to 48 h under the same conditions (Figure 9). Alanine had only a little anti-browning effect and no lag protective effect. It thus appears that the production of cystine is the biggest factor with cysteine, making it effective for only 12 h. Ascorbic acid can reduce the quinone back to the *o*-dihydroxyphenol, thus preventing coupling of phenols which would lead to increased color upon further oxidation. In all these treatments caffeic acid consumed (as measured by HPLC) correlated significantly ($P \leq 0.001$) with the color (420 nm) produced.

It has been stated that enzymic oxidation produces quinones much more rapidly at lower pH than does autoxidation, but once quinone is formed, the remainder of the reaction is the same whether originally enzymic or nonenzymic oxidation was occurring. These data justify that conclusion. This research showed that thiol addition takes place under autoxidative conditions on the benzene ring once the quinone is formed. The formation of 2-CCA under nonenzymic autoxidation was proven. The good correlation of caffeic acid consumption with brown formed indicates that the protective effect of additives lies in the formation of intermediates with little or no color. The thiol adducts, e.g., 2-CCA, have lower oxidation potentials than caffeic acid and through coupled reduction have a protective effect on the original phenol by reducing its unsubstituted quinones as they form. Once the thiol additive is consumed and its adducts are in the oxidized form, brown color formation starts when *o*-dihydroxyphenol is being oxidized.

This research showed that 2-CCA prepared enzymically could also be produced under chemical autoxidative (no enzyme) conditions with the concomitant formation of 5-CCA and DCCA. The speed and specificity of the enzyme-catalyzed reaction only applies to quinone formation. Subsequent coupling with cysteine (or other compounds including glutathione, phloroglucinol, and catechin) is straight chemistry between the quinone as an electrophile and the thiol or other nucleophile. In the course of the reaction the quinone moiety is "regenerated" to the hydroquinone form, and as a consequence the total amount of oxygen consumed is increased as the new hydroquinone is autoxidized. This is hypothesized to result from the cysteine rapidly removing any quinone formed and thus minimizing the level of semiquinone free radicals and slowing the initial reaction. These results help explain observations in wine and food autoxidation regarding high capacity of natural phenols for oxygen and increasing capacity if the reaction is slowed but prolonged.

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Registry No. 2-CCA, 100045-72-5; 5-CCA, 127184-13-8; DCCA, 127206-82-0; L-Cys, 52-90-4; L-ala, 56-41-7; GSH, 70-18-8; caffeic acid, 331-39-5; catechin, 120-80-9; phloroglucinol, 108-73-6; ascorbic acid, 50-81-7; mercaptoethanol, 60-24-2; cysteine hydrochloride, 52-89-1.