Cysteine as an Inhibitor of Enzymatic Browning. 1. Isolation and Characterization of Addition Compounds Formed during Oxidation of Phenolics by Apple Polyphenol Oxidase

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The oxidation of different phenols [4-methylcatechol (MC), chlorogenic acid (CG), (-)-epicatechin (EC), and (+)-catechin (CA)], catalyzed by apple polyphenol oxidase (PPO), was investigated in the presence of an excess of cysteine. The occurrence of one cysteine addition compound with the two former phenols and two cysteine addition products with the latter phenols was demonstrated by HPLC. In all cases, the formation of addition compound(s) was proportional to the degradation of the phenol. After purification by gel filtration on Trisacryl GF05, the structure of each cysteine conjugate was determined by ^{1}H NMR spectroscopy. After additional ^{1}H homonuclear decoupling and 2D homonuclear COSY experiments, it was shown unequivocally that adduct with MC was 5-*S*-cysteinyl-3,4-dihydroxytoluene. With CG, the structure was 2-*S*-cysteinylchlorogenic acid. With catechins, the cysteine was attached on the B ring for the two conjugates. The 2′-position was involved in the first addition compound, whereas it was the 5′-position in the second one. Moreover, the study of UV spectra of the purified compounds allowed the conclusion that the two conjugates of each catechin were formed at the same rate in equivalent amounts.

INTRODUCTION

Browning of damaged tissues of fruits and vegetables during postharvest handling and processing is one of the main causes of quality loss (Mathew and Parpia, 1971). The brown color development is related primarily to the oxidation of phenolic compounds. This reaction, mainly catalyzed by polyphenol oxidase (EC 1.14.18.1; PPO), results in the formation of *o*-quinones which subsequently polymerize, leading to brown pigments (Mayer and Harel, 1979; Vamos-Vigyazo, 1981; Mayer, 1987). The control of enzymatic browning has always been a challenge to the fruit-processing industry (Ponting, 1960). Besides halide salts and aromatic carboxylic acids known to inhibit PPO (Walker and Wilson, 1975; Rouet-Mayer and Phillipon, 1986; Janovitz-Klapp et al., 1990a), numerous chemicals exhibiting reducing properties have been proposed. Among them can be cited sulfites (Embs and Markakis, 1965; Sayavedra-Soto and Montgomery, 1986), ascorbic acid and its derivatives (Sapers and Ziolkowski, 1987; Hsu et al., 1988), and thiol compounds such as cysteine (Walker and Reddish, 1964; Montgomery, 1983; Kahn, 1985; Dudley and Hotchkiss, 1989). When compared on the same material, the former compounds were always found more effective in enzymatic browning inhibition (Muneta, 1981; Golan-Goldhirsh and Whitaker, 1984; Sapers and Douglas, 1987; Janovitz-Klapp et al., 1990a). However, the use of sulfites becomes more and more restricted due to potential hazards (Taylor and Bush, 1986). Therefore, the need for alternative treatments is obvious, and an active research is developed to propose either formulations without sulfites (Langdon, 1987; Santerre et al., 1988) or new products such as stabilized forms of ascorbic acid and β-cyclodextrins (Sapers et al., 1989) and plant sulfhydryl proteases (Taoukis et al., 1990). Surprisingly enough, few studies have been devoted to thiol compounds such as cysteine, although their efficiency in the inhibition of enzymatic browning has been recognized for a long time (Joslyn and Ponting, 1951). Compared to ascorbic acid, which prevents browning by reducing *o*-quinones back to *o*-dihydroxyphenols, it is almost admitted that thiol compounds block the reaction by forming colorless addition compounds with *o*-quinones (Roberts, 1959; Mason and Peterson, 1965; Pierpoint, 1966). However, it is only in a few cases that the structure of these products has been elucidated, namely, cysteine with pyrocatechol or *o*-dihydroxyphenylalanine (Sanada et al., 1972; Dudley and Hotchkiss, 1989), cysteine with caffeic acid, and glutathione with caffeoyltartaric (caftaric) acid (Cheynier et al., 1986). Furthermore, their direct effect on PPO remains questionable. On one hand, although these addition compounds had an *o*-diphenolic structure, they were not substrates of PPO (Sanada et al., 1972; Singleton et al., 1985; Cheynier and Van Hulst, 1988). On the other hand, Sanada et al. (1972, 1976) claimed that the cysteinyl–catechol conjugate gave some inhibitory effect on the browning reaction, whereas Singleton et al. (1985) indicated that the glutathione derivative was not an inhibitor as estimated by the oxygen uptake.

The purpose of this study was to develop a rapid method of preparation and purification of cysteine adduct compounds using different phenols, namely, 4-methylcatechol (MC), chlorogenic acid (CG), (+)-catechin (CA), and (-)-epicatechin (EC), with a purified PPO from apple (Janovitz-Klapp et al., 1989) to prove their structure and subsequently to investigate their role as effectors in the enzymatic reaction.

MATERIALS AND METHODS

Materials. Apples from the variety of Red Delicious picked at commercial maturity were used as an enzyme source. The PPO was 120-fold purified from the cortex in three steps: extraction, fractional precipitations by ammonium sulfate, and

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hydrophobic chromatography with phenyl-Sepharose CL4B (Pharmacia) according to the method of Janovitz-Klapp et al. (1989). Trisacryl GF05 was from IBF Biotechnics (Villeneuve-La-Garenne, France). CG, EC, and CA were from Extrasynthèse (Genay, France), and all other chemicals were of reagent grade from Sigma (St. Louis, MO).

Methods. *Assay for PPO Activity.* PPO activity was assayed polarographically according to the method of Janovitz-Klapp et al (1990b) using MC as substrate. Activity was expressed as nanomoles of oxygen consumed per second (nanokatals) in the assay conditions.

Cysteine Addition Products Preparation. The cysteine conjugates were enzymically prepared in a McIlvaine buffer at pH 4.5 and 30 °C. Purified apple PPO (100 nkat) was added to 10 mL of the buffer solution containing 4 mM phenol, 8 mM cysteine (with the exception of MC for which concentrations were doubled), and 0.2 mM vanillic acid. The latter compound was used as internal standard in the subsequent quantification by HPLC. In spite of its very weak inhibition properties (Janovitz-Klapp et al., 1990a), its effect on velocity was negligible. Aliquots of 0.5 mL were periodically withdrawn and mixed with 0.5 mL of stopping solution containing NaF (2 mM). Sodium fluoride was shown to be a very strong inhibitor of apple PPO (Janovitz-Klapp et al., 1990a). The solvent used for the stopping solution was either water–acetonitrile (80/20 v/v) or water–methanol (82/18 v/v) depending on the eluent system used for the HPLC analysis. Just before injection, samples were acidified by a drop of orthophosphoric acid.

HPLC Analysis. A Varian liquid chromatograph (9010 pump and 9050 detector driven by a 9020 workstation) was used with detection at 280 nm. Samples of 0.01 mL were injected into a Chromspher (Chrompack) C18 reversed-phase cartridge (guard column 1 cm long and column 10 cm long, 3 mm i.d., particle size 5 μm). Two isocratic elution systems were used at a 1 mL min^{-1} flow rate. System 1 was water (acidified at pH 2.6 by orthophosphoric acid)–acetonitrile (90/10 v/v) for CG and EC. System 2 was water (2.5% in glacial acetic acid)–methanol (91/9 v/v) for MC and CA. The relative area at 280 nm, with vanillic acid as internal standard, was used for the quantification of each detected peak.

Gel Filtration. For preparative purposes, vanillic acid was omitted in the buffer solution at pH 4.5. After the total degradation of phenol and its transformation in conjugate(s) have been checked by HPLC, the resulting solution (not more than 8 mL) was applied onto a Trisacryl GF05 column (70-mL bed volume, 35 cm long) previously equilibrated with distilled water at 40 mL h^{-1}. For the separations of cysteine conjugates from catechins, a longer column (190-mL bed volume, 95 cm long) and a slower flow rate (20 mL h^{-1}) were used. Absorbance measurement at 290 nm and HPLC analysis were performed on each 5-mL fraction. Furthermore, the presence of free amino acid group was assayed by ninhydrin in the following way: 0.1 mL of the fraction was added to 1 mL of ninhydrin (1% in methanol) and heated at 60 °C for 15 min, and the absorbance was determined at 560 nm. Fractions containing the cysteine conjugate(s) were pooled, analyzed by HPLC, and lyophilized.

NMR Spectroscopy. Routine ^1H NMR spectra, ^1H homonuclear decoupling, and 2D homonuclear COSY experiments were performed by using a Bruker 250-MHz instrument, in D_2O with *tert*-butyl alcohol as internal reference (δ = 1.27).

UV Spectroscopy. UV spectra of the phenols and their corresponding cysteine conjugate(s) were performed in the HPLC solvent system in which they were separated with a Varian Techtron DMS90 spectrophotometer.

RESULTS AND DISCUSSION

Occurrence of Addition Compounds. When apple PPO was incubated with MC, a yellow coloration rapidly developed together with oxygen uptake. When the enzymatic reaction was monitored by HPLC, a new peak appeared corresponding to *o*-quinones, connected with the decrease of the MC peak. When an excess of cysteine was initially present in the reaction medium, there was still an oxygen uptake but no color development. Moreover, the

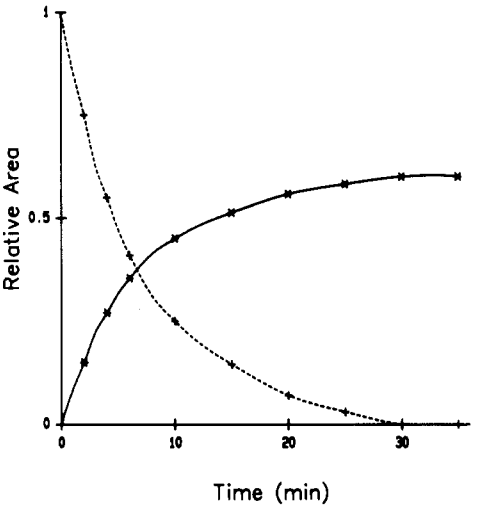

Figure 1. MC (- -+- -) disappearance and Cys-5-MC (–*–) formation by HPLC during oxidation of 8 mM MC in the presence of 16 mM cysteine at pH 4.5 with PPO (100 nkat). Results are expressed in percent of the initial relative area of MC with vanillic acid as internal standard.

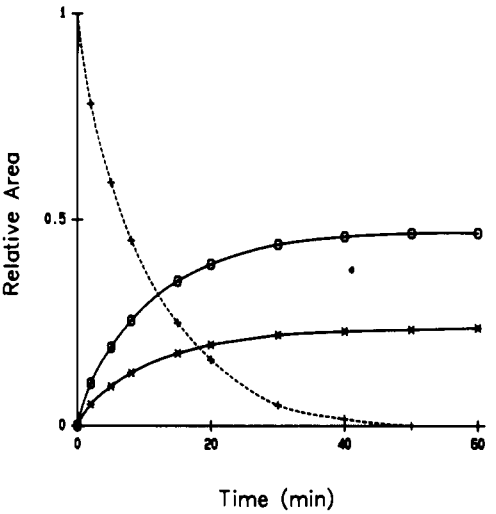

Figure 2. EC (- -+- -) disappearance, Cys-2′-EC (–*–) formation, and Cys-5′-EC (–o–) formation by HPLC during oxidation of 4 mM EC in the presence of 8 mM cysteine at pH 4.5 with PPO (100 nkat). Results are expressed in percent of the initial relative area of EC with vanillic acid as internal standard.

HPLC showed the absence of the *o*-quinone peak, the decrease of the MC peak, and a new peak, the importance of which increased as that of MC peak decreased. This new peak was shown to be the adduct of cysteine with the MC–*o*-quinones, Cys-5-MC. (The structure of the different conjugates will be established in the following paragraph.) With a sufficient amount of PPO and after a certain time (e.g., 30 min with 100 nkat of PPO for 10 mL of 8 mM MC), all the MC was consumed, and although the enzyme was still active, the Cys-5-MC peak did not change (Figure 1). It may be concluded that the adduct was not a substrate for apple PPO. A similar result was obtained with CG which gave one addition compound, Cys-2-CG.

However, when EC was used as substrate, HPLC showed the presence of *two* adducts, Cys-2′-EC and Cys-5′-EC. Moreover, as for MC and CG, the increase of each peak, Cys-2′-EC and Cys-5′-EC, was proportional to the decrease in EC. When the latter was exhausted, the two peaks corresponding to the addition compounds were stable (Figure 2). In a proper elution system (system 2, Table I), a similar result was obtained with CA as substrate, i.e.,

Table I. Retention Times of the Phenols and Their Cysteine Conjugate(s) in the Two HPLC Isocratic Elution Systems (Relative to That of Vanillic Acid)[a]

compd	rel retention time		absorbance ratio at 280 nm, conjugate/phenol consumed
	system 1[b]	system 2[c]	
CG	0.75	0.92	
Cys-2-GC	0.61	1.17	0.61
EC	1.74	1.76	
Cys-2′-EC	0.42	0.49	0.24
Cys-5′-EC	1.5	2.21	0.46
CA	0.84	0.65	
Cys-2′-CA	0.58	0.55	0.29
Cys-5′-CA	0.58	0.65	0.41
MC	1.71	1.3	
Cys-5-MC	1	1.5	0.63
vanillic acid[d]	1	1	

[a] Absorbance at 280 nm of the conjugate formed relative to that of phenol consumed. [b] System 1: water (pH 2.6 with orthophosphoric acid)–acetonitrile (90/10). [c] System 2: water (2.5% in acetic acid)–methanol (91/9). [d] Retention times for vanillic acid were 2.6 and 3.4 min in system 1 and system 2, respectively.

two adducts, Cys-2′-CA and Cys-5′-CA. In a single isocratic elution system, all the phenolics were not always well resolved from their addition compound(s) or from the internal standard that we used, namely vanillic acid. The relative retention times of all compounds to that of vanillic acid are given in Table I for each phenol and their cysteine conjugate(s) for the two isocratic elution systems used together with the area ratios at 280 nm of the addition compound formed to the phenol consumed.

Ito and Prota (1977) obtained four cysteine adducts from Dopa quinones, the major product being the 5-S conjugate besides the 2-S, 6-S, and 2,5-di-S conjugates. Recently, Cilliers and Singleton (1990) showed the formation of three addition compounds by autoxidation of caffeic acid in the presence of cysteine, the major product being the 2-S conjugate besides the 5-S and the 2,5-di-S conjugates. For pyrocatechol with cysteine, Sanada et al. (1972) and Dudley and Hotchkiss (1989) isolated one addition compound, *S*-(2,3-dihydroxyphenyl)cysteine. In the same way, Cheynier et al. (1986) elucidated the structure of a single reaction product of glutathione with caftaric acid. Alternatively, with CA, Roberts (1959) postulated the formation of one conjugate with cysteine (or glutathione), the structure of which was not given. Piretti et al. (1977) indicated that in the presence of sodium benzenesulfinate, mushroom tyrosinase catalyzed the formation of 6′-phenylsulfonyl-CA as the sole product. These results prompted us to develop a method for the preparation and purification of sufficient amounts of each of the conjugates to elucidate their structure and assess their spectral properties.

Purification of Addition Compounds. Each phenol was fully degraded by PPO in the presence of an excess of cysteine. The reaction was then stopped by adding NaF (2 mM), and the resulting solution was loaded onto a Trisacryl GF05 column and eluted with water. This gel filtration allowed the separation of the conjugate(s) from (i) the enzyme protein and (ii) both the cysteine in excess and the salts of the buffer solution. A typical chromatogram is shown in Figure 3 with MC as substrate. If we consider first the ninhydrin-reactive fractions, we observe a small peak in the dead volume of the column corresponding to the PPO protein. The latter was followed by a large peak close to the total volume which did not absorb at 290 nm corresponding to the cysteine which has not reacted with *o*-quinones. Finally, another large peak was observed at a K_{av} close to 1.65, which absorbed at 290 nm and which was shown to be the conjugate Cys-5-MC by HPLC analysis of the corresponding fractions. Therefore,

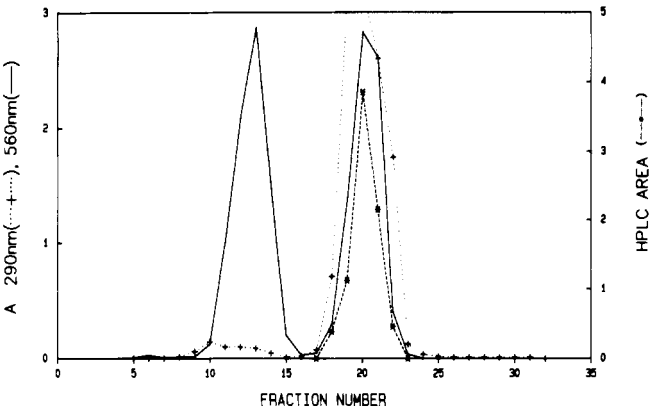

Figure 3. Elution profile on Trisacryl GF05 of fully degraded MC in the presence of an excess of cysteine by apple PPO: absorbance at 290 nm (full line); absorbance at 560 nm after reaction with ninhydrin (+); HPLC area of Cys-5-MC peak (*, arbitrary units).

Table II. K_{av} of MC, CG, EC, and CA and Their Cysteine Conjugate(s) on Trisacryl GF05

compd	K_{av}	column type	flow rate
MC	1.6	smaller	40 mL min^{-1}
Cys-5-MC	1.65	70-mL bed volume	
CG	1.4	35 cm long	
Cys-2-CG	1.3		
EC	2.4	longer	20 mL min^{-1}
Cys-2′-EC	1.8	190-mL bed volume	
Cys-5′-EC	2.2	95 cm long	
CA	2.6		
Cys-2′-CA	1.9		
Cys-5′-CA	2.1		

steric hindrance was not the only factor explaining the chromatographic behavior of Cys-5-MC in the Trisacryl GF05 gel. A similar behavior was observed with phenols and their conjugates, since they all exhibited a K_{av} higher than 1 (Table II). Such a result has been already obtained for phenolic compounds with Sephadex LH 20, a hydroxypropylated dextran gel, by Concin et al. (1980) and Lattanzio and Marchesini (1981). According to these authors, the retardation of phenols can be related to the existence of hydrogen bonds between the solute and either the ether or the hydroxyl groups of the Sephadex LH 20. The chemical structure of Trisacryl GF05 includes the presence of one secondary amine group and three primary hydroxymethyl groups. Therefore, it is highly probable that hydrogen bonds between this matrix and phenols (including their conjugates) occurred, explaining their chromatographic behaviors. From Table II, it can be seen that CG, MC, and their corresponding conjugates were less retarded than the two catechin isomers and their corresponding addition compounds. Since, in the two former cases, only one conjugate was present, a relatively small column was sufficient to obtain rapidly a high amount of the addition product in a purified form (checked by HPLC). After gathering and lyophilization of the most ninhydrin reactive fractions, yields as high as 90% were easily reached, meaning that in one chromatographic step, 15 mg of pure conjugate can be obtained. Alternatively, a longer column and a slower flow rate were necessary for the separation of the two catechin conjugates (either from EC of from CA) due to their close K_{av}. Thus, these chromatographies were much more time-consuming, and due to an overlap between the two peaks, the overall yields (sum of the two conjugates) were less than 60%.

Structure of the Addition Compounds. For ease of comparison among the other substituted *o*-diphenols, the 1-position of MC was assigned to the carbon with the me-

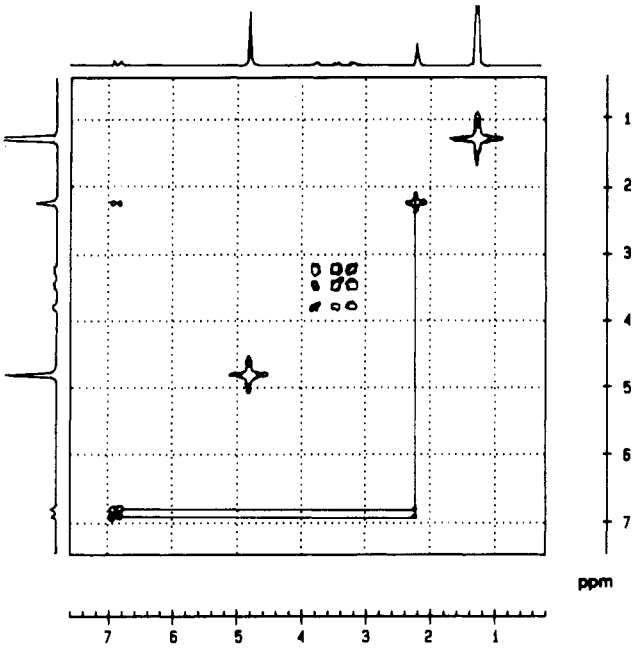

Figure 4. COSY spectrum of Cys-5-MC: couplings among aromatic (H2 and H6) and methyl group protons (full lines).

thyl group; therefore, MC becomes 3,4-dihydroxytoluene. In the NMR spectrum of its conjugate, the presence of only two aromatic protons in the 7 ppm region indicated that substitution was on the ring. Due to the lack of AB pattern in the same region, this substitution did not occur on the 2-position. Moreover, the lack of coupling constant between the two remaining protons seemed to indicate a para position and therefore a substitution in the 6-position for cysteine. However, a meta position for the two remaining protons cannot be ruled out, since the coupling constant was only J = 1.4 Hz (δ H-2 = 7.16, δ H-6 = 7.11) in the spectrum of CG and was undistinguishable in the spectra of the B ring of EC (δ H-2′ = 7.05, δ H-6′ = 6.95) and of MC (δ H-2 = 6.77, δ H-6 = 6.62). Therefore, additional experiments are required to determine on which carbon, 5 or 6, the cysteine was substituted.

A ^{1}H decoupling experiment was performed on the methyl resonance of MC and Cys-5-MC. For MC, both broad singlet (H-2) and doublet (H-5) split in doublet of doublet with a meta constant of J = 2 Hz. For Cys-5-MC, both broad singlets (δ = 6.94 and 6.81) were similarly affected and split into two doublets with a coupling constant of J = 1.9 Hz. This obviously indicated a meta position and therefore the presence of cysteine in the 5-position. These results were confirmed by the 2D homonuclear COSY experiment. The COSY spectrum showed the coupling pattern between the two lower field resonances (H-2 and H-6) and between these two protons and the upper field signal of the methyl group (Figure 4).

Substitution by the cysteine sulfur can be ascertained by the presence of a single α proton with a broad signal at 3.76 ppm and two nonequivalent β protons at 3.19 and 3.47 ppm as a pair of doublets of doublets. Basically, the protons of the cysteine moiety exhibited the same pattern in all the conjugates studied, indicating the formation of a thioether linkage between the cysteine and the ring. These observations are in agreement with the NMR data reported by Cheynier et al. (1986) and Dudley and Hotchkiss (1989) for the glutathionylcaftaric adduct and the cysteinylcatechol adduct, respectively.

For CG and its conjugate Cys-2-CG, the substitution pattern was different from that of Cys-5-MC, since the two remaining aromatic protons at 7.13 and 6.9 ppm

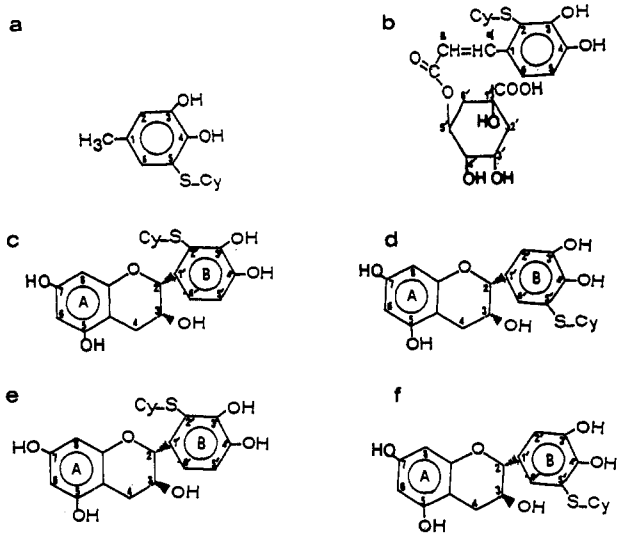

Figure 5. Proposed structures for Cys-5-MC (a), Cys-2-CG (b), Cys-2′-EC (c), Cys-5′-EC (d), Cys-2′-CA (e), and Cys-5′-CA (f).

displayed a 8.5-Hz coupling constant in accordance with an ortho configuration. Therefore, the cysteine is linked to carbon 2. Moreover, substitution results in a downfield chemical shift of 0.58 ppm for the vinyl α proton but has a negligible effect for the vinyl β proton. These two protons displayed a large coupling constant (J = 15.9 Hz), indicating that the trans configuration was maintained in the Cys-2-CG compound. A similar observation was reported by Cheynier et al. (1986) on the glutathionylcaftaric adduct.

Concerning EC, the NMR data indicated that only one cysteine moiety was present in each of the isolated conjugates. Moreover, the substitution occurred on the B ring of the molecule but at a different position for the two conjugates, Cys-2′-EC and Cys-5′-EC. For the former, the presence of a high coupling constant (J = 8.9 Hz) between the two aromatic protons at 7.22 and 7.09 ppm indicates an ortho configuration as well as their location on carbons 5′ and 6′ of the B ring. Thus, cysteine is attached on carbon 2′. As with Cys-2-CG, this 2′-*S*-cysteinyl substitution causes a large downfield chemical shift of 0.73 ppm of the α proton (position 2 on the chromane ring), whereas the β proton (position 3 on the chromane ring) was not affected. For the second conjugate, the aromatic protons of the B ring exhibited a pattern similar to that of Cys-5-MC, i.e., lack of coupling constant. Again, this indicates a substitution either in the 5′- or in the 6′-position of the B ring. Compared to Cys-2′-EC (and Cys-2-CG), this substitution did not affect the chemical shift of the α proton (position 2 on the chromane ring). By analogy with the results obtained with Cys-5-MC, a substitution in the 5′-position can be proposed for Cys-5′-EC.

The NMR data obtained with CA and its first conjugate were very similar to those of EC and Cys-2′-EC. Therefore, the same arguments can be used to propose that substitution was on the 2′-position of the B ring for Cys-2′-CA. Due to the low recovery in the second conjugate, its NMR study was not performed, but the examination of the NMR spectrum of the mixtures of the two CA conjugates indicated that Cys-5′-CA has a structure equivalent to Cys-5′-EC. Thus, the difference of the NMR spectrum of the mixture and that of Cys-2′-CA gave an NMR spectrum similar to that of Cys-5′-EC. The proposed structures of all the conjugates are given in Figure 5.

UV Spectrophotometry of Addition Compounds. The enzymatic oxidation of each phenol at pH 4.5 in the

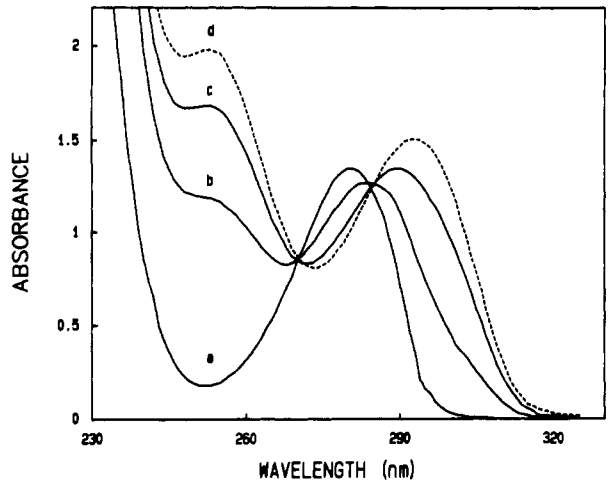

Figure 6. UV spectra of MC oxidation by apple PPO in the presence of an excess of cysteine at four times of reaction: (a) 0, (b) 1, (c) 8, and (d) 30 min of enzymatic reaction. Initial conditions: MC (0.5 mM), cysteine (1 mM), and PPO (30 nkat) in 3 mL of McIlvaine buffer at pH 4.5.

presence of an excess of cysteine was followed by UV spectrophotometry between 230 and 340 nm. As an example, the spectra corresponding to 0, 1, 8, and 30 min of reaction with MC as substrate are given in Figure 6. The initial absorption maximum at 280 nm of MC was gradually replaced by two maxima at 255 and 293 nm, which clearly appeared after 30 min of reaction. After this time, it was shown by HPLC that MC has disappeared and that only Cys-5-MC was apparent. If it is assumed that 1 mol of phenol consumed gave 1 mol of conjugate and that MC and Cys-5-MC were the only compounds absorbing at 280 nm, then at pH 4.5 the ratio of absorbances Cys-5-MC/MC was equal to 0.72 at this wavelength. By HPLC, this ratio was found to be close to 0.63 (Table I). Only a small part of this divergence can be attributed to the difference in solvents used. When the spectra of MC and Cys-5-MC were recorded in water–methanol (HPLC solvent system 2), the ratio of absorbances became equal to 0.69 (Figure 7a). This probably means that either a small amount of *o*-quinones of MC was not trapped by cysteine to form Cys-5-MC or a small amount of conjugate was consumed by side reactions, e.g., with MC–*o*-quinones, or both of these possibilities. The last assumption cannot be ruled out since it has been shown by Valero et al. (1988) that *o*-quinones of MC can react with the addition product of MC with proline. Likewise, Cheynier et al. (1988) and Cheynier and Van Hulst (1988) postulated and later on (Cheynier et al., 1990) demonstrated an oxidation of 2-*S*-glutathionylcaftaric acid by caftaroylquinones when the latter were present in excess. Lastly, we have observed that the ratio of 0.63 found during the HPLC experiments was always slightly lower in the beginning than at the end of the reaction. This could be due to a higher amount of *o*-quinones compared to conjugate at the beginning which thus would favor side reactions between these two compounds. A similar phenomenon was found for CG since the absorbance ratio Cys-2-CG/CG at 280 nm was 0.73 (Figure 7b) compared to the 0.61 value obtained by HPLC (Table I). In this case, the formation of another adduct is probable since in our HPLC experiments a small peak, representing a constant percentage (4%) of Cys-2-CG, was always apparent just before Cys-2-CG. Due to the very low amount formed, its structure has not been determined. It can only be speculated that it is an adduct with either a substitution on the 5-position by analogy with catechins or a double substitution on the 2- and 5-positions by

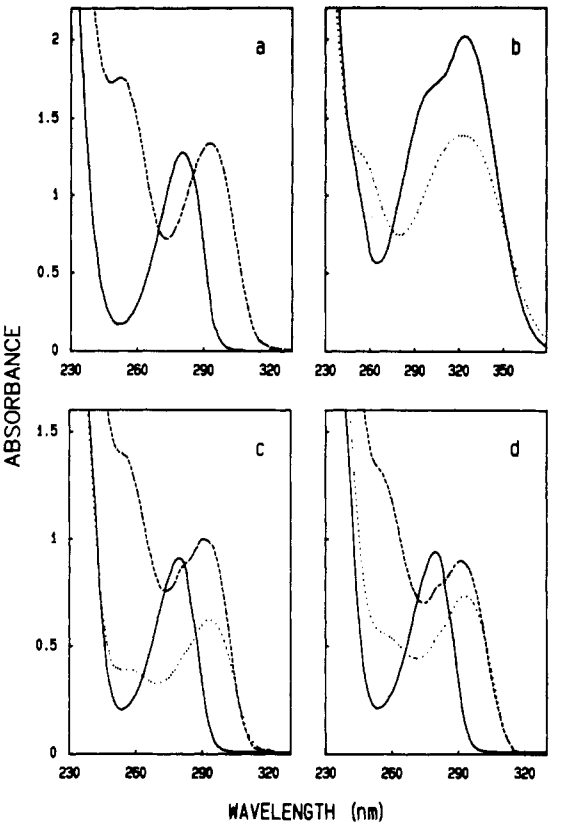

Figure 7. UV spectra of phenols and their cysteine conjugate(s): (a) MC (full line) and Cys-5-MC (dashed line) both 0.5 mM; (b) CG (full line) and Cys-2-CG (dotted line) both 0.1 mM; (c) EC (full line), Cys-2′-EC (dotted line) and Cys-5′-EC (dashed line) all 0.25 mM; (d) CA (full line), Cys-2′-CA (dotted line) and Cys-5′-CA (dashed line) all 0.25 mM. Spectra a and d were in water (2.5% in glacial acetic)–methanol (91/9). Spectra b and c were in water (made pH 2.6 by orthophosphoric acid)–acetonitrile (90/10).

analogy with the findings of Salgues et al. (1986) on the adducts of caftaric acid with glutathione.

The spectrum of Cys-2CG exhibited a maximum close to 325 nm in the same region as that of CG, but the shoulder at 300 nm has disappeared and another maximum at shorter wavelengths was apparent at 255 nm (Figure 5b). These results are in agreement with those given by Cheynier et al. (1986) for the 2-*S*-glutathionylcaftaric acid. For the two conjugates of EC (Figure 7c), the maximum at 280 nm was shifted toward longer wavelengths, namely 295 nm for Cys-2′-EC and 291 for Cys-5′-EC, with a new absorbance maximum close to 255 nm. The latter was much higher for Cys-5′-EC than for Cys-2′-EC. Moreover, the absorbance at 280 nm of Cys-5′-EC was roughly twice that of Cys-2′-EC. By HPLC, it was shown all along the enzymatic degradation of EC in the presence of an excess of cysteine that the relative area at 280 nm of Cys-2′-EC was approximately half that of Cys-5′-EC. Hence, it can be concluded that the two addition compounds of EC were formed at the same rate. The two conjugates of CA exhibited UV spectra roughly similar to those of conjugates of EC of similar structure. The relative absorbance at 280 nm was found close to 1.5 for the two conjugates (Cys-5′-CA/Cys-2′-CA) of CA (Figure 7d), which again indicates that they were formed in equivalent amounts. For the second maximum at 255 nm, absorbance of Cys-5′-CA was more than twice that of Cys-2′-CA. Thus, the cysteine addition on the ring seemed to promote a much more intense absorbance in the 260-nm region, compared to the 290–320-nm region, when sulfur was on the 5-position instead of the 2-position. A similar result can be observed

on the spectra given by Salgues et al. (1986) for the 2-*S*-glutathionylcaftaric acid and the 2,5-di-*S*-glutathionylcaftaric acid. For the same absorbance at 320 nm, the absorbance at 265 nm of the latter was approximately twice the former.

In conclusion, among the four phenols tested, the thiol substitution was either mainly on the 2-position (CG) or mainly on the 5-position (MC) or on both positions in roughly equal amounts (both catechins). The arguments of Cheynier et al. (1986) for the exclusive substitution in the 2-position with caftaric acid, namely double activation of this position through conjugative delocalization of an electron to the side-chain carbonyl on one hand and to an oxygen on the ring on the other hand, hold for CG. In contrast, the methyl group of MC, which is less electron attracting, does not activate the 2-position and hence favors the 5-position. Catechins which have an oxygen in the β-position (in the chromane ring) of the β ring could be intermediate and give substitution in the two positions in equal amounts. We have tested (*o*-dihydroxyphenyl)propionic and (*o*-dihydroxyphenyl)acetic acids, two other substrates of apple PPO. In HPLC, one main peak was apparent accompanied by a minor peak (representing 5.5 and 2.5% of the main peak, respectively) in each case. Moreover, the UV spectra of the conjugate(s) showed a higher absorbance at 255 nm than at 295 nm, suggesting that cysteine was mainly substituted on the 5-position. It can be proposed that the carboxyl group of the lateral chain was too far from the ring to favor the cysteine substitution on the 2-position.

In the second part of this work, the cysteine addition compounds will be studied as effectors of the phenol oxidation catalyzed by apple PPO.

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Registry No. PPO, 9002-10-2; MC, 452-86-8; CG, 327-97-9; EC, 490-46-0; CA, 154-23-4; 2-*S*-cysteinyl-CG, 133071-65-5; 2′-cysteinyl-EC, 133071-66-6; 5′-cysteinyl-EC, 133161-80-5; 2′-cysteinyl-CA, 133161-81-6; 5′-cysteinyl-CA, 133161-82-7; 5-*S*-cysteinyl-3,4-dihydroxytoluene, 17054-97-6; cysteine, 52-90-4.