Cysteine as an Inhibitor of Enzymatic Browning. 2. Kinetic Studies

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The effect of cysteine and cysteine–quinone addition compound (CQAC) on apple polyphenol oxidase (PPO) activity was investigated by HPLC and polarography, using 4-methylcatechol (4MC), chlorogenic acid (CG), and (−)-epicatechin (EC) as substrate. With 2.2 mM 4MC, in the presence of cysteine at thiol to phenol ratios above 1, there was no effect in the degradation rates of 4MC, while CQAC formation was proportional to the 4MC loss but without color formation. For cysteine to phenol ratios below 1, the degradation rate of 4MC was slowed down; the lower the cysteine content, the earlier the decrease in 4MC degradation was observed. In the latter case, the CQAC content increased and then decreased but developed a strong color. Similar results were obtained with CG or EC as substrate. In the presence of CQAC, polarography experiments showed that they were not substrate but competitive inhibitors of apple PPO with a slightly higher affinity than their phenol precursors. HPLC analysis showed that Cys-5-MC (the CQAC derived from 4MC) was rapidly degraded, whereas 4MC degradation was slowed with a strong violet color formation. Nonenzymatic cooxidation reactions between CQAC and *o*-quinone leading to the regeneration of phenol and the formation of pigments were observed. Cysteine at a higher concentration prevented color development by trapping *o*-quinones as colorless CQAC, while at low amounts the *o*-quinones formed in excess can cooxidize CQAC, leading to a phenol regeneration with a deep color formation.

INTRODUCTION

Browning of damaged tissues of fruits and vegetables is often an undesirable reaction (Lee, 1991). The oxidative browning of fruits is mainly due to polyphenol oxidase (EC 1.14.18.1; PPO). In the presence of oxygen, this enzyme catalyzes the oxidation of phenols into *o*-quinones that subsequently polymerize, leading to brown pigments (Zawistowski et al., 1991). Because enzymatic browning is in most cases detrimental to the quality, considerable effort has been devoted to its control (Ponting, 1960). Since heat treatments, such as a blanching process, are sometimes not desirable (Vamos-Vigyazo, 1981), a wide range of chemical compounds has been proposed to inhibit PPO (Mayer and Harel, 1991). These chemicals are often divided in two classes, those acting on the enzyme and those acting on the reaction products (Mayer and Harel, 1979). To the first class belong halide salts (Martinez et al., 1986; Rouet-Mayer and Philippon, 1986; Janovitz-Klapp et al., 1990a), carboxylic acids (Walker and Wilson, 1975; Janovitz-Klapp et al., 1990a), and some chelating agents such as cyanide, azide, diethyl dithiocarbamate (Mayer and Harel, 1979). In the second class are mainly found the reducing agents among which ascorbic acid and its derivatives, thiol-containing compounds, and sulfites are the most widely studied (Muneta, 1981; Sayavedra-Soto and Montgomery, 1986; Sapers and Ziolkowsky, 1987; Martinez-Cayuela et al., 1988; Janovitz-Klapp et al., 1990a; Molnar-Perl and Friedman, 1990a,b). These compounds either act by reducing *o*-quinones to *o*-diphenols or react with *o*-quinones to yield colorless compounds, but some of them exhibit also a direct inhibitory effect on the enzyme (Embs and Markakis, 1965; Golan-Goldhirsh and Whitaker, 1984; Hsu et al., 1988). Because of potential hazards, the use of sulfiting agents becomes more restricted (Taylor and Bush, 1986), and numerous attempts to develop alternative processes without sulfites have been made (Langdon, 1987; Santerre et al., 1988, 1991; Sapers et al., 1989a–c).

Compared to other reducing agents, few studies have been carried out on thiol agents. There is still some debate on their mechanism of action. It is generally known that they act primarily with *o*-quinones and form colorless addition compounds (Pierpoint, 1966), the structures of which have been elucidated (Sanada et al., 1972; Cheynier et al., 1986; Dudley and Hotchkiss, 1989; Richard et al., 1991). However, some authors indicated that cysteine also reduces the *o*-quinones to their phenol precursors (Walker, 1977; Cilliers and Singleton, 1990). The direct inhibition of PPO by cysteine through the formation of stable complexes with copper has also been proposed by Kahn (1985). Moreover, the direct effect of addition compounds on PPO is in dispute. Sanada et al. (1972, 1976) claimed that the cysteinyl–catechol conjugate showed some inhibitory effect on the browning reaction, whereas Singleton et al. (1985) indicated that the glutathione derivative of caftaric (caffeoyltartaric) acid was not an inhibitor as estimated by the oxygen uptake.

In a previous work (Richard et al., 1991), we developed a method for the preparation and purification of cysteine–quinone addition compounds (CQAC) from different phenols. The purpose of this study was to investigate their roles as effectors in the enzymatic reaction.

MATERIALS AND METHODS

Materials. Apples from the variety Red Delicious, picked at commercial maturity, were used as enzyme source. The PPO was 120-fold purified from the cortex in three steps: extraction, fractional precipitations by ammonium sulfate, and hydrophobic chromatography with phenyl-Sepharose CL4B (Pharmacia) according to the method of Janovitz-Klapp et al. (1989). Chlorogenic acid (CG) and (−)-epicatechin (EC) were from Extrasynthèse (Genay, France); 4-methylcatechol (4MC) and all other chemicals were of reagent grade from Sigma (St. Louis, MO).

Methods. *Preparation of Cysteine–Quinone Addition Compounds.* Cys-5-MC (CQAC with 4MC), Cys-2-CG (CQAC with CG), Cys-2′-EC, and Cys-5′-EC [the two CQAC with (−)epi-

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catechine] were prepared and purified following the method described by Richard et al. (1991).

Assay for PPO Activity. PPO activity was assayed polarographically according to the method of Janovitz-Klapp et al. (1990b) using 20 mM 4MC as substrate. Activity was expressed as nanomoles of oxygen consumed per second (nanokatals) in the assay conditions. For inhibition studies with CQAC at pH 4.5, the three phenolic substrates were varied between 0.3 and 5 mM in the control and two concentrations of CQAC. All assays were performed in duplicate and apparent V_m and K_m values were determined by using a nonlinear regression data analysis program developed for IBM PC by Leatherbarrow (1987).

Phenol Oxidation by Apple PPO in the Presence of Cysteine or CQAC. All of the enzymatic reactions were carried out with purified apple PPO (between 10 and 20 nkat mL^{-1}) in a reaction vessel at pH 4.5 and 30 °C, in the presence of 0.2 mM vanillic acid (internal standard for HPLC analysis) using air agitation unless otherwise specified. The concentrations of phenolic substrates, cysteine, and CQAC varied from 0.5 to 2.2 mM, from 0 to 5 mM, and from 0 to 2 mM, respectively. For each time tested, 0.5 mL was withdrawn from the reaction vessel and immediately mixed with an equal amount of stopping solution containing 2 mM NaF (Richard et al., 1991). The latter compound was shown to be a very potent inhibitor of apple PPO (Janovitz-Klapp et al., 1990a). The residual phenols and CQAC were separated and quantified by HPLC (9010 pump and 9050 UV detector driven by a 9020 workstation from Varian) on 10-μL samples using the isocratic conditions described by Richard et al. (1991). The relative response factors at 280 nm of phenols and their respective CQAC were determined by injection of pure compounds.

RESULTS AND DISCUSSION

Phenol Oxidation in the Presence of Cysteine. Both the 4MC oxidation and the CQAC (Cys-5-MC) formation catalyzed by apple PPO were monitored by HPLC (Figure 1).

In the first 2 min of the reaction, no lag period was observed and the 4MC degradation rate was unaffected by cysteine for concentrations up to 5 mM. These findings rule out, in this period, the reduction of *o*-quinone to the phenol precursor by cysteine with the concomitant formation of cystine. In addition, they are in agreement with the results obtained by polarography by Janovitz-Klapp et al. (1990a). After 2 min, the 4MC degradation rate slowed for cysteine concentrations lower than 2 mM. The lower the cysteine content between 0.5 and 2 mM, the earlier the decrease in the 4MC degradation rate was observed. After 30 min, the 4MC was totally degraded when the cysteine concentration was higher than 2.5 mM (i.e., higher than the phenol concentration), and between 2 and 0.5 mM, the residual amount of 4MC increased as the cysteine content decreased. In the absence of cysteine, 4MC was not fully degraded, meaning that PPO was inactivated probably through a suicide inactivation mechanism (Golan-Goldhirsh and Whitaker, 1985) involving secondary reaction products.

Concerning Cys-5-MC (Figure 1B), its formation was proportional to the 4MC degradation for cysteine concentrations higher than 2.5 mM. For lower cysteine concentrations (less than 1 mM), the Cys-5-MC content increased for the first 2 min and then decreased to zero at 15 min. When the Cys-5-MC began to decrease, the solution developed a strong violet color different from that observed with oxidized 4MC, which was orange-yellow. The time to reach the maximum in Cys-5-MC increased with the increased amount of cysteine. This change appeared to be related to the decrease in the 4MC degradation rates (Figure 1A). Similar results have been obtained by Cheynier et al. (1990) during the oxidation of caftaric acid in the presence of glutathione (GSH). They

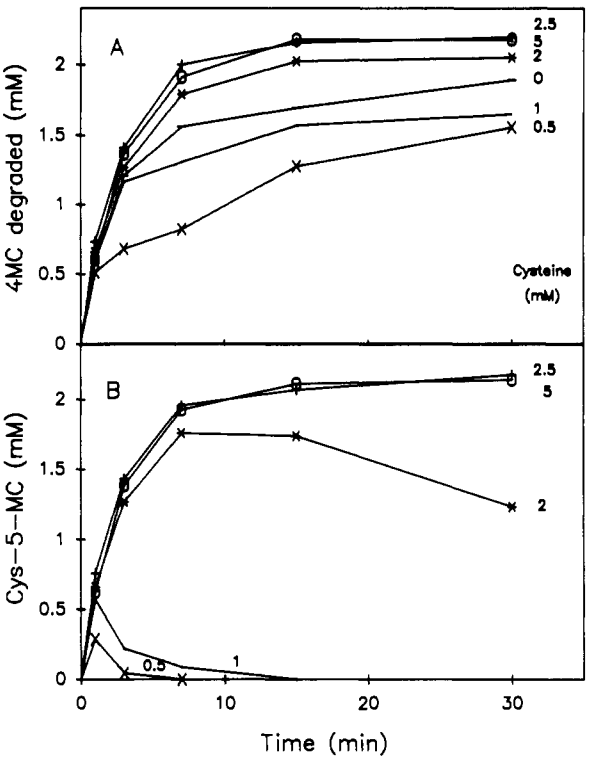

Figure 1. Oxidation of 4-methylcatechol by apple PPO in the presence of variable amounts of cysteine: (A) 4MC degradation; (B) Cys-5-MC formation. Conditions: 2.2 mM 4MC was oxidized by 20 nkat mL^{-1} of purified apple PPO in the presence of cysteine (between 0 and 5 mM) in a McIlvaine buffer solution at pH 4.5 with air agitation. For each experimental point, 0.5 mL was withdrawn from the reaction vessel and immediately mixed with 0.5 mL of NaF (2 mM) in acidified water (pH 2.6)–acetonitrile (80/20 v/v) before analysis by HPLC.

proposed that at ratios of GSH to caftaric acid below 1, the quinones in excess (not trapped as addition compound) were able to cooxidize the thiol adduct with regeneration of the phenol precursor (Cheynier and Van Hulst, 1988).

To confirm that the decrease in 4MC degradation rate and the increase in Cys-5-MC formation were related to cooxidation reactions, the oxidation process of 4MC (0.5 mM) was followed spectrophotometrically at 253 nm in the presence of different amounts of cysteine between 0 and 0.3 mM. This wavelength was selected because, according to Richard et al. (1991), absorbance was at a minimum for 4MC and at a maximum for Cys-5-MC (ϵ_{4MC} = 340 M^{-1} cm^{-1} and $\epsilon_{Cys\text{-}5\text{-}MC}$ = 3520 M^{-1} cm^{-1}). When cysteine was present, a kinetic pattern with two linear phases of increasing absorbance was observed (Figure 2A), a rapid one, which was colorless, followed by a slower one, during which a violet color developed. When the absorbance values corresponding to the interpolated breaking point between the two linear phases were related to the initial amount of cysteine, a straight line passing through the origin was obtained (Figure 2B). Its slope was equal at 3200 M^{-1} cm^{-1}, a value which is close to the difference in the molar extinction coefficients of Cys-5-MC and 4MC at 253 nm. This suggests that the first period corresponded to the exclusive formation of Cys-5-MC until cysteine was exhausted. After this phase, the *o*-quinones in excess reacted with Cys-5-MC, leading to the formation of violet pigments.

Similar results were obtained with other phenols except the final colors were different. Thus, after oxidation, CG was pale orange in the absence of cysteine and became bright orange in its presence (for thiol to CG ratios below 1), whereas with EC it turned from bright yellow to pink.

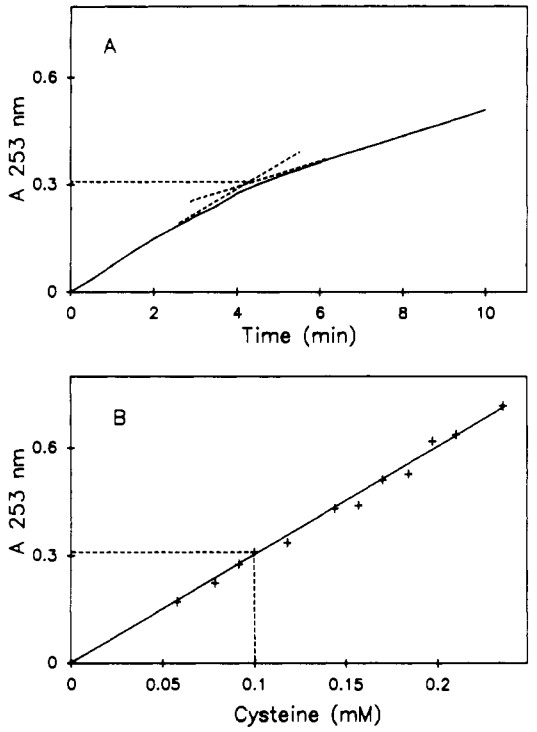

Figure 2. Kinetics of 4-methylcatechol oxidation by apple PPO in the presence of cysteine followed by spectrophotometry at 253 nm: (A) absorbance evolution (cysteine = 0.1 mM); (B) relationship between the absorbance at the breaking point interpolated between the two linear phases of the kinetic and cysteine content. Conditions: 0.5 mM 4MC was oxidized by 10 nkat mL^{-1} of apple PPO in the presence of cysteine (between 0 and 0.3 mM) in a McIlvaine buffer solution at pH 4.5.

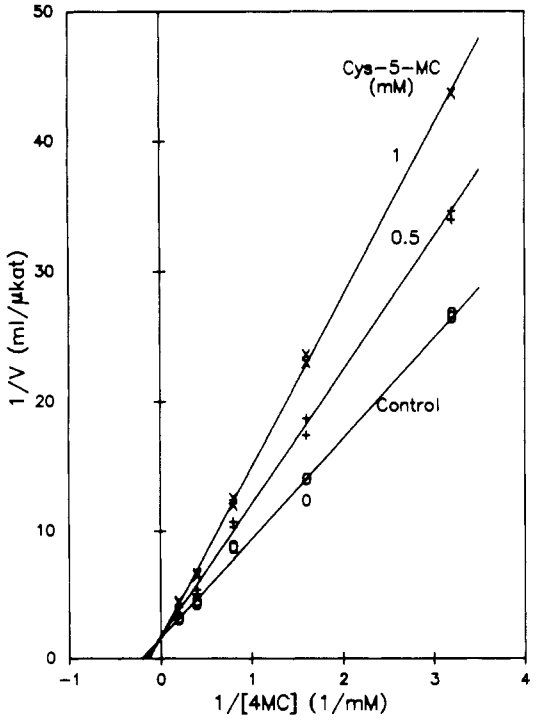

Figure 3. Inhibition of purified apple PPO by Cys-5-MC with 4MC as substrate: (O) control; (+) 0.5 mM and (×) 1 mM in Cys-5-MC.

In the latter case, according to Richard et al. (1991), two CQACs were formed, Cys-2′-EC and Cys-5′-EC, in the first phase. In the second phase, after cysteine was exhausted, Cys-5′-EC was degraded 1.5 times faster than Cys-2′-EC.

Phenol Oxidation in the Presence of Addition Compounds. *Polarographic Studies.* Although the

Table I. Inhibition Constants of CQAC Using 4MC, CG, and EC as Substrates of Apple PPO at pH 4.5[a]

	substrate K_i, mM		
CQAC	4MC	CG	EC
Cys-5-MC	1.61	1.63	1.61
Cys-2-CG	1.89	1.82	1.81
Cys-2′-EC	2.86	2.92	2.94
Cys-5′-EC	2.90	2.91	2.87

[a] All compounds were competitive inhibitors.

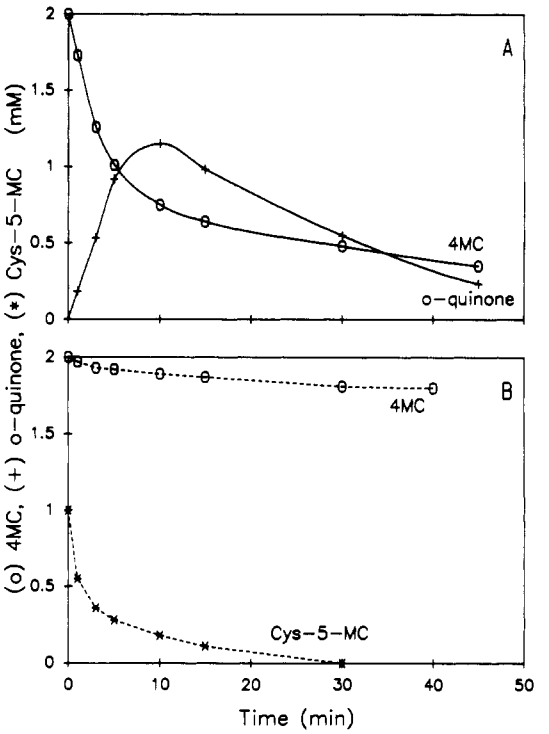

Figure 4. Oxidation of 4-methylcatechol by apple PPO with and without Cys-5-MC: (A) without Cys-5-MC; (B) with Cys-5-MC (1 mM) added. Conditions: 2 mM 4MC was oxidized by 10 nkat mL^{-1} of purified apple PPO in the absence or in the presence of Cys-5-MC (1 mM) in a McIlvaine buffer solution at pH 4.5 with air agitation. For each experimental point, 0.5 mL was withdrawn from the reaction vessel and immediately mixed with 0.5 mL of NaF (2 mM) in acidified water (pH 2.6)–acetonitrile (80/20 v/v) before analysis by HPLC (in the absence of Cys-5-MC, the NaF solution was supplemented by 2 mM cysteine to quantify *o*-quinone as CQAC).

CQACs had an ortho-diphenolic structure, they were not substrates of apple PPO (Richard et al., 1991), in agreement with the behavior of other thiol adducts with other PPOs (Sanada et al., 1972; Singleton et al., 1985). However, their direct effect on the enzyme has never been demonstrated. Therefore, we have studied the effects of four different CQACs (Cys-5-MC, Cys-2-CG, Cys-2′-EC, and Cys-5′-EC) on apple PPO by polarography, using successively the three phenolic precursors (4MC, CG, and EC) as substrate. In each case, the results were deduced from Lineweaver–Burk double-reciprocal plots. Good straight lines were obtained, and a typical example is shown Figure 3 for Cys-5-MC with 4MC as substrate. The obtained result corresponded to a competitive inhibition with an inhibition constant K_i of 1.6 mM. Similar behaviors were obtained for the four CQAC, and the corresponding K_i values are given in Table I for the three substrates tested. For a given adduct, K_i appeared to be independent of the substrate, which is consistent with a competitive inhibition. The different K_i ranged between 1.5 and 3 mM at pH 4.5, showing that inhibition properties of CQAC were not negligible. If they were weaker inhibitors than the

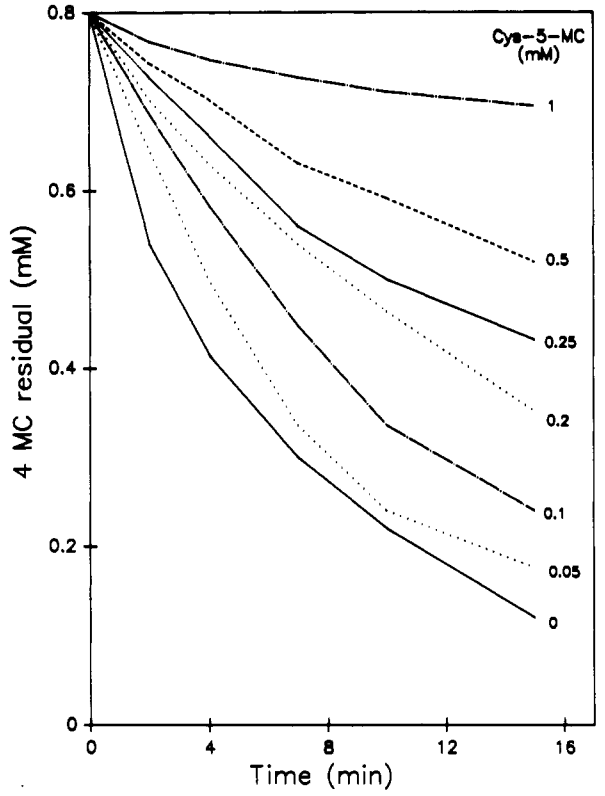

Figure 5. Oxidation of 4-methylcatechol by apple PPO in the presence of variable amounts of Cys-5-MC. Conditions: 0.8 mM 4MC was oxidized by 10 nkat mL^{-1} of purified apple PPO in the presence of Cys-5-MC (between 0 and 1 mM) in a McIlvaine buffer solution at pH 4.5 with air agitation. For each experimental point, 0.5 mL was withdrawn from the reaction vessel and immediately mixed with 0.5 mL of NaF (2 mM) in acidified water (pH 2.6)–acetonitrile (80/20 v/v) before analysis by HPLC.

aromatic carboxylic acids of the benzoic (excepted vanillic and syringic acids) and cinnamic (excepted sinapic acid) series, they were close to the *p*-hydroxyphenylpropionic and phenylpropionic acids (Janovitz-Klapp et al., 1990a). Moreover, they exhibited a greater affinity for apple PPO than their corresponding phenolic precursors, the K_m values of which ranged between 4.2 and 6 mM (Janovitz-Klapp et al., 1990b). Lastly, close values of K_i were obtained for the two CQACs derived from EC, meaning that the position of the thiol substitution did not affect their inhibitory properties.

HPLC Studies. The effect of Cys-5-MC on the oxidation of 4MC catalyzed by apple PPO was studied by HPLC. Degradation of 4MC (2 mM) with and without Cys-5-MC (1 mM) is shown in Figure 4. In the presence of Cys-5-MC, a strong inhibition of the 4MC oxidation was observed (Figure 4B): the initial degradation rate was less than 10% of that observed in the system without Cys-5-MC, and a violet color developed. At the same time, Cys-5-MC was rapidly degraded and disappeared after 30 min of reaction. The difference in the oxidation rates with and without Cys-5-MC cannot be ascribed to the sole competitive inhibition since, with a K_i close to 1.6 mM, the initial rate will represent approximately 70% of the control.

The inhibition of 4MC (0.8 mM) oxidation was apparent as soon as Cys-5-MC was added (Figure 5). Thus, with 1 mM in Cys-5-MC, there was less than 10% of 4MC degraded after 15 min of enzymatic oxidation, compared to the 85% loss without Cys-5-MC. This strong inhibition was also associated with a rapid degradation of Cys-5-MC (data not shown).

Similar results were obtained with CG and EC in the presence of their respective CQACs (Cys-2-CG, Cys-2′-EC, and Cys-5′-EC). Thus, it appeared that CQACs, which were not substrates, were degraded by secondary reactions. This degradation was not observed in the presence of free cysteine and therefore could be attributed to *o*-quinones.

Table II. Effect of Addition of Different Amounts of Cys-5-MC on Oxidized 4MC by Apple PPO after the Enzymatic Reaction Was Stopped by NaF[a]

residual amounts before addition [solution 1 (NaF + cysteine)]		amount of added Cys-5-MC	residual amounts after addition [solution 2 (NaF + Cys-5-MC)]		
4MC	*o*-quinone[b]		Cys-5-MC	4MC	*o*-quinone
0.64	0.89	1.54	1.15	1.44	
0.78	0.76	1.16	0.66	1.46	
0.58	0.93	0.68	0.25	1.42	
0.60	0.93	0.39	0.02	1.36	
0.76	0.76	0.20		1.32	

[a] The NaF solution contained cysteine (2 mM) for the determination of residual amounts of 4MC and *o*-quinone before addition of Cys-5-MC (solution 1) or contained the indicated amount of Cys-5-MC for the determination of residual amounts of 4MC, *o*-quinone, and Cys-5-MC after addition of Cys-5-MC (solution 2); HPLC analyses were carried out immediately after the addition of the NaF solutions. [b] Determined as Cys-5-MC.

The following experiment was carried out to demonstrate the occurrence of nonenzymatic reaction involving *o*-quinone and Cys-5-MC. An aliquot of 1.6 mM 4MC was oxidized by purified apple PPO (20 nkat mL^{-1}) with air agitation. After 5 min (corresponding to a maximum in the *o*-quinone formation), the reaction was stopped by two stopping solutions, one (solution 1) with NaF containing cysteine (2 mM) and the other (solution 2) with NaF containing various amounts of Cys-5-MC (between 0.2 and 1.54 mM). The contents of the two solutions were analyzed by HPLC (Table II). Before addition of Cys-5-MC (solution 1), the residual amounts of 4MC ranged between 0.58 and 0.78 mM with an *o*-quinone content ranging between 0.76 and 0.93 mM. After addition of Cys-5-MC (solution 2), the *o*-quinone immediately disappeared, the 4MC content increased, and part of the Cys-5-MC was degraded. In all cases, a strong violet coloration appeared. With the exception of the two lowest concentrations in Cys-5-MC, which probably were too low in the ratio to the *o*-quinone content and therefore were fully degraded, the stoichiometries in the phenol regenerated were for the *o*-quinone degraded close to 1 and for the Cys-5-MC degraded close to 2.

Thus, these experiments clearly demonstrated that *o*-quinone and CQAC were able to react nonenzymatically, leading to regeneration of phenol and formation of pigments. However, the stoichiometry of 2 mol of 4MC regenerated for 1 mol of Cys-5-MC degraded cannot be attributed to the sole cooxidation reaction

4MC *o*-quinone + Cys-5-MC → 4MC + Cys-5-MC *o*-quinone

which corresponds to a 1:1 stoichiometry. Therefore, one can admit other mechanisms of phenol regeneration from *o*-quinone. This could be either the protonation of two semiquinone free radicals formed from *o*-quinone (Singleton, 1987) or the reaction of an *o*-quinone with a water molecule leading to a triphenol which then, by cooxidation with another *o*-quinone molecule, regenerates one phenol and forms a *p*-quinone (Richard-Forget et al., 1992). It could be also the result from more complex reactions involving cooxidation of eventual condensation products.

Nevertheless, the strong inhibition of phenol degradation by apple PPO in the presence of CQAC is explained

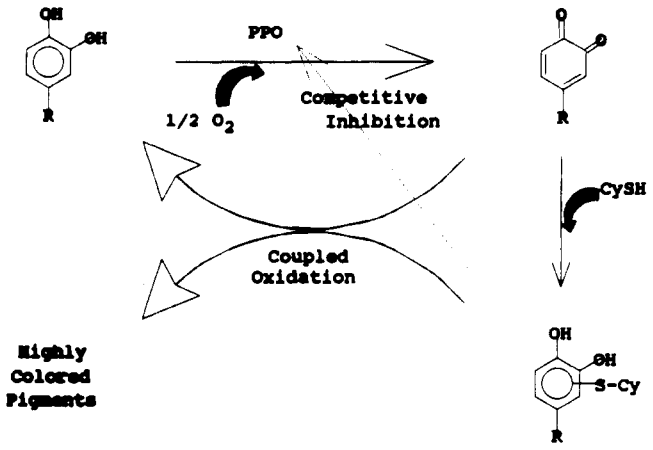

Figure 6. Effect of cysteine and cysteinyl addition compounds with *o*-quinones on the enzymatic oxidation of *o*-diphenols.

both by the primary effect of the competitive inhibition on the enzyme and by the secondary effect of the coupled oxidation by *o*-quinone, leading to phenol regeneration (Figure 6).

In conclusion, cysteine has no direct effect on apple PPO, at least in the concentration range examined in this study. During enzymatic oxidation, it traps the *o*-quinone by forming cysteinyl adducts. These CQACs are not substrates, but they show competitive inhibition properties with an affinity for the enzyme higher than that of their precursors. With a sufficient amount of cysteine (i.e., for cysteine to phenol ratios above 1), the phenol is fully degraded in CQAC without color formation. For cysteine to phenol ratios below 1, *o*-quinones are formed in excess and are able to react with the CQAC with regeneration of phenols (i.e., substrate for the enzymatic reaction) and formation of highly colored pigments. Therefore, provided this critical level in cysteine concentration is exceeded, this thiol could in theory allow a permanent protection against enzymatic browning. However, this level may often be nonconsistent with good organoleptic properties of the final product. Therefore, formulations including other innocuous additives have to be optimized to succeed in the control of enzymatic browning.

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