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Inhibition of enzymatic browning and protection of sulfhydryl enzymes by thiol compounds

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Abstract

In a reaction between (−)-epicatechin (EC) and 2-mercaptoethanol (2ME), catalyzed by partially purified polyphenol oxidase (PPO) extracted from the style of *Rhododendron mucronatum*, 2′-(2-hydroxyethylthio)–(−)-epicatechin (2′-HETEC), 5′-(2-hydroxyethylthio)–(−)-epicatechin (5′-HETEC), and 2′,5′-bis(2-hydroxyethylthio)–(−)-epicatechin (2′,5′-HETEC) were formed. The rate of formation of 2′,5′-HETEC from 5′-HETEC was faster than that from 2′-HETEC. In the absence of 2ME, the concentration of EC decreased rapidly and the reaction mixture turned brown; 2′-, 5′-, and 2′,5′-HETEC, especially 2′-substituted HETECs, reacted more slowly. These data indicate that 2ME acts both as an inhibitor of the polymerization of *O*-quinone, presumably by binding to it and as a reductant involved in the conversion of *O*-quinone to *O*-dihydroxyphenol. Inhibition of enzymatic browning by other thiol compounds such as cysteine and dithiothreitol was also investigated.

Keywords: Inhibition of enzymatic browning; (−)-Epicatechin; 2′-(2-Hydroxyethylthio)–(−)-epicatechin; 5′-(2-Hydroxyethylthio)–(−)-epicatechin; 2′,5′-Bis(2-hydroxyethylthio)–(−)-epicatechin; Polyphenol oxidase; Polyphenolic compound; *O*-Quinone; Sulfhydryl enzyme; Thiol compound

1. Introduction

In a previous paper (Ozawa et al., 1993) we reported that azalein, (+)-catechin (CA), and (−)-epicatechin (EC) were isolated from the style of *Rhododendron mucronatum* as pollen tube growth promoters. Three EC derivatives, substituted by a 2-mercaptoethanol (2ME) residue, namely 2′-(2-hydroxyethylthio)–(−)-epicatechin (2′-HETEC), 5′-(2-hydroxyethylthio)–(−)-epicatechin (5′-HETEC) and 2′,5′-bis(2-hydroxyethylthio)-(−)-epicatechin (2′,5′-HETEC), were also found. These compounds seemed to be formed by an enzymatic reaction during extraction. 2ME, a reductant, was used for preventing the oxidation of catechins by polyphenol oxidase (PPO) during extraction of the growth promoters.

A considerable number of studies have been done on the conjugates between *O*-dihydroxyphenols and thiol compounds. Sanada et al. (1972) reported the inhibitory effect of sulfhydryl compounds on melanin formation by mushroom tyrosinase. It has also been shown that cysteine inhibits enzymatic browning catalyzed by apple PPO (Richard et al., 1991; Richard-Forget et al., 1992). In their study, cysteine did not inhibit the oxidation of phenolics by PPO; rather, it prevented the subsequent polymerization of phenolics, which results in browning. This is different from the inhibition caused by aromatic carboxylic acids (Kermasha et al., 1993) or by KCN. A mechanism for the inhibition of enzymatic browning by cysteine has been demonstrated. The quinone, which is an oxidation product of PPO, reacts with cysteine non-enzymatically to form colorless conjugates. Sanada et al. (1972) isolated one conjugate between cysteine and catechol and Richard et al. (1991) found one or two conjugates between cysteine and phenolic compounds such as 4-methylcatechol, chlorogenic acid, EC, and CA. Also,

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Ito and Prota (1977) obtained four cysteine adducts from DOPA quinones.

It was presumed that the three HETECs isolated from the style of *R. mucronatum* were similarly formed by the style PPO. In this study, the enzymatic reactions in the presence of thiol compounds were examined in detail. These include reactions leading to the formation of three HETECs and other conjugates between thiol compounds and several phenolics, using PPO prepared from the style of *R. mucronatum*. The mechanism of inhibition of enzymatic browning and of the protection of sulfhydryl enzymes by thiol compounds are also discussed.

2. Results and discussion

In order to determine if the oxidation of EC and 2ME by PPO was responsible for the formation of three HETECs, three products were separated by HPLC and identified by comparing their retention times and UV spectra with those of the authentic HETECs (Fig. 1). During the reaction (Fig. 2), EC concentration decreased rapidly, and 5′-HETEC and 2′-HETEC were formed immediately, whereas 2′,5′-HETEC formation was delayed. Although 2′-HETEC decreased only slightly even after 4 h of reaction, 5′-HETEC decreased steadily, and 2′,5′-HETEC increased linearly, suggesting that 2′,5′-HETEC is produced mainly from 5′-HETEC. This was confirmed by enzymatic reactions using 2′-HETEC and 5′-HETEC as substrates. The results show that the rate of formation of 2′,5′-HETEC from 5′-HETEC was 18 μM/h under the same experimental conditions as in Fig. 2, about four times faster than its formation from 2′-HETEC and that both 2′- and 5′-HETEC are substrates for this PPO. PPOs from mushroom and grape, however, have been reported to oxidize phenols to form their quinones, necessary for coupled chemical oxidation of thio-substituted phenols (Hansson et al., 1980; Naish-Byfield et al., 1994; Singleton and Cilliers, 1995). In a mixture with EC, 2ME and the style PPO, EC–quinone produced by the PPO may oxidize 2′- or 5′-HETEC to form their quinone, which can accept another 2ME to give 2′,5′-HETEC.

The UV spectra of 2′- and 5′-HETEC (Fig. 1) were similar to those of Cys-2′-EC and Cys-5′-EC, respectively, produced by apple PPO (Richard et al., 1991). Addition of one 2ME residue to EC caused a bathochromic shift to the EC absorption maxima (λ_{max}). The ratio of the absorbance at λ_{max}, A_{258}/A_{292}, was less than 1 for the 2′-adduct, whereas that for 5′-adduct was greater than 1. The reaction between EC and 2ME with the style PPO produced 2′,5′-HETEC in addition to 2′- and 5′-addition compounds; whereas the reaction of the apple PPO (Richard et al., 1991) with EC produced two cysteine addition compounds. The

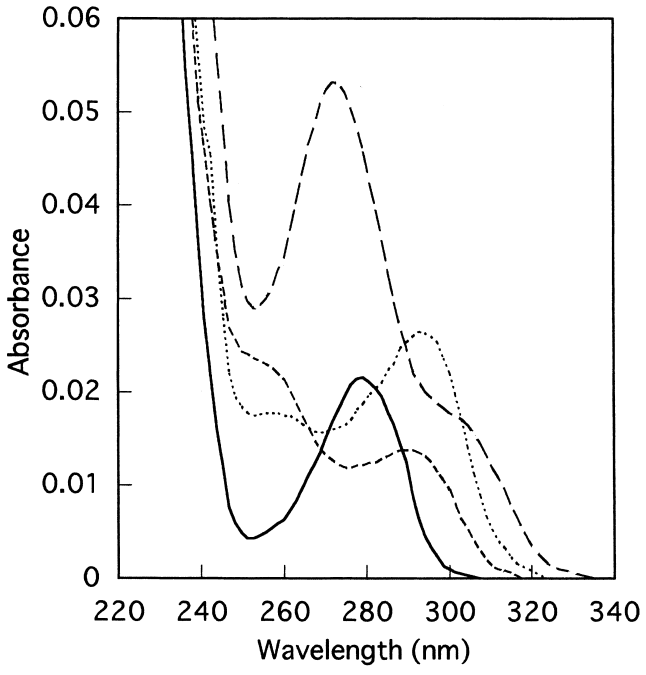

Fig. 1. UV spectra of EC and the three HETECs. EC and the three HETECs were completely separated by HPLC using solvent A, 2% AcOH–MeOH (70:30), and had retention times of EC (11.7 min), 2′-HETEC (9.5 min), 5′-HETEC (16.7 min), and 2′,5′-HETEC (13.6 min), respectively. UV spectra of these peaks were measured with a photodiode array spectrometer. Amount of each authentic sample was 1.2 nmol. ———, EC; · · · · · · · · · · , 2′-HETEC; - - - - - - - , 5′-HETEC; and — — — —, 2′,5′-HETEC.

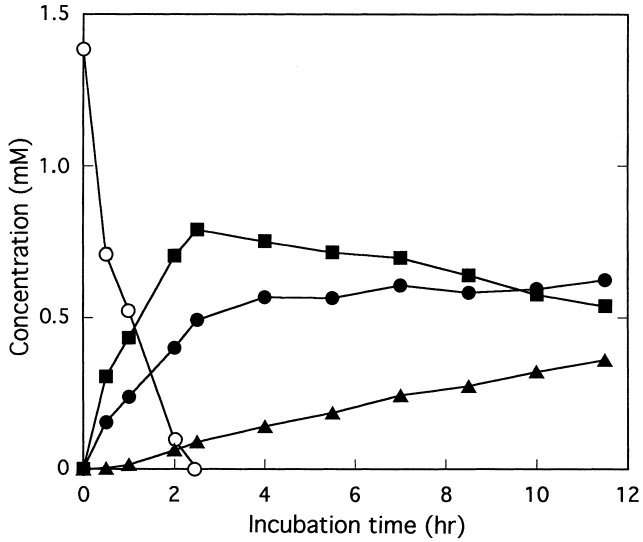

Fig. 2. Time-course of the PPO reaction with EC and 2ME. The reaction mixture contained 0.02 M Na–phosphate buffer (pH 6.5), 1.5 mM EC, 5.7 mM 2ME, and PPO (0.75 nkat) in a total volume of 100 μl. Reaction was carried out at 30°C and at various intervals, 4 μl of the reaction mixture was analyzed by HPLC. ○, EC; ●, 2′-HETEC; ■, 5′-HETEC; ▲, 2′,5′-HETEC.

λ_{max} of 2′,5′-HETEC caused a further bathochromic shift at 258 and 292 nm and a hyperchromic shift at the shorter wavelength, and was thus significantly influenced by the two substitutions.

PPO reactions with CA, chlorogenic acid, (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) were also investigated. During the HPLC analysis, the spectra of the reaction products were measured with a photodiode array UV-VIS detector. In the case of CA, three addition compounds were obtained. Since CA is a stereo-isomer of EC, spectra similar to those of the three HETECs were expected. Three compounds, whose retention times using solvent D were 30.0, 44.3 and 49.4 min, were identified as 2ME-2′-CA, 2ME-5′-CA and 2ME-2′,5′-CA, respectively, by comparing their spectra with those of the three HETECs. Chlorogenic acid is an ester, between quinic acid and caffeic acid, which has an *O*-diphenol group. Three cysteine addition compounds with caffeic acid by autoxidation have been reported (Cilliers and Singleton, 1990). In our experiment with the style PPO, two peaks, whose retention times using solvent B were 22.8 and 35.6 min, were identified as the 2- and 2,5-adduct, respectively, by comparing their spectra with those of the three cysteine–caffeic acid conjugates (Cilliers and Singleton, 1990). The 5-adduct was not detected in this reaction; this is probably because it further reacted with 2ME in the presence of PPO to produce the 2,5-adduct, as in the conversion of 5′-HETEC to 2′,5′-HETEC. As EGC and EGCG are vicinal triphenol compounds, two additional compounds substituted at the 2′- and/or 6′-positions were expected. Accordingly, two 2ME-EGC conjugates with R_t 12.1 and 18.9 min, and 2ME-EGCG conjugates with R_t 14.2 and 17.5 min (solvent A), were obtained from HPLC analysis of reaction mixtures with EGC and EGCG. However, we were not able to identify four compounds from the spectra. There is a possibility of 2ME reacting with the gallic acid moiety of the EGCG.

The effect of 2ME on the enzymatic oxidations of EC and three HETECs were examined. The formation of HETECs was reduced when 2ME was increased to 10 times the amount used in the standard (Fig. 3), probably as a result of the reducing power of 2ME. In the absence of 2ME, the actions of PPO on EC and three kinds of HETECs were also examined. As shown in Fig. 4, almost all the EC was consumed in about 10 min even when the amount of PPO was reduced. On the other hand, the oxidation rates of HETECs, especially 2′-substituted HETECs, were very low, indicating that the

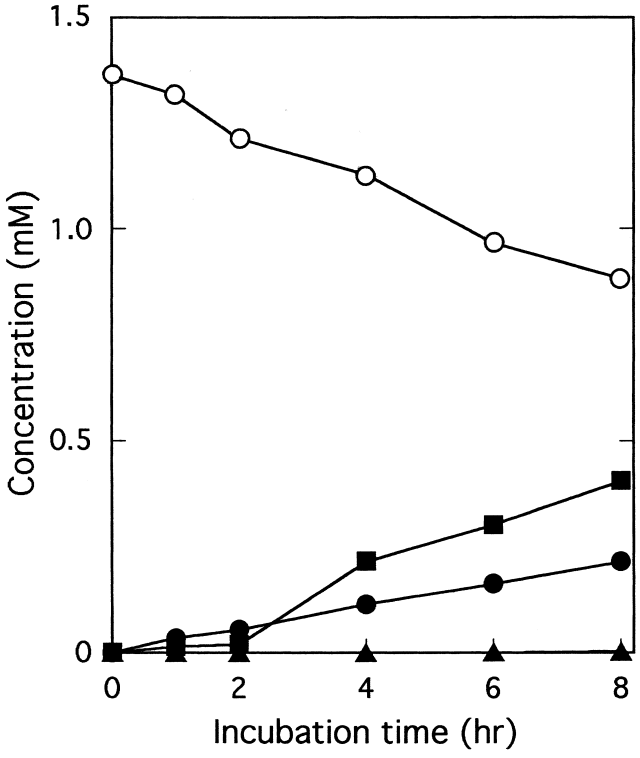

Fig. 3. PPO reaction with EC and a high concentration of 2ME. The reaction was carried out with 10 times as much 2ME (57 mM) as that used in the standard reaction Fig. 2. ○, EC; ●, 2′-HETEC; ■, 5′-HETEC; ▲, 2′,5′-HETEC.

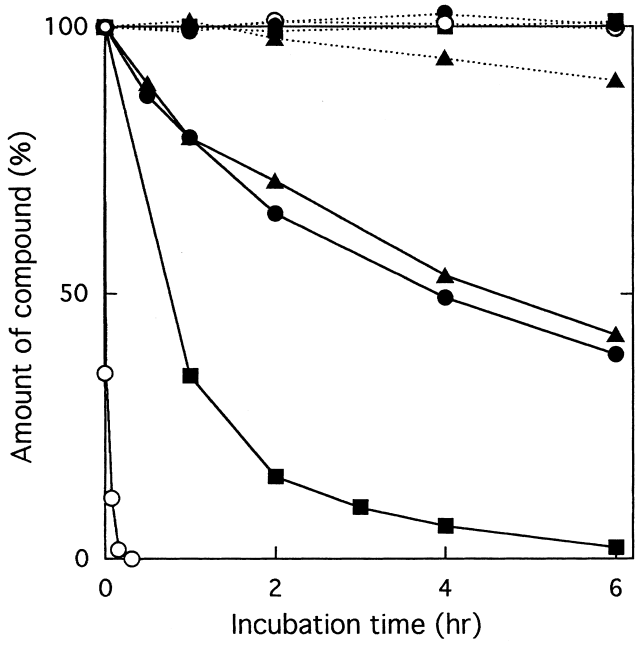

Fig. 4. Oxidation of EC and the three HETECs by PPO in the absence of 2ME. For oxidation of the three HETECs, 0.75 nkat of PPO were used, whereas in the oxidation of EC, the amount of PPO was reduced to one tenth of the amount used in other reactions. In the reaction without PPO there was no decrease of 2′,5′-HETEC in the presence of 2ME. ○, EC; ●, 2′-HETEC; ■, 5′-HETEC; ▲, 2′,5′-HETEC. ———, reaction with PPO; · · · · · · · · · · , reaction without PPO (blank test).

browning reaction was inhibited by conjugation of 2ME to EC. This was in agreement with the results reported by Sanada et al. (1972) and Richard et al. (1991).

The PPO reactions using cysteine and dithiothreitol (DTT) as thiol compounds were also investigated (Fig. 5). In the reaction between EC and cysteine, 2′-, 5′-, and 2′,5′-addition compounds were also obtained (Fig. 5a). These compounds were identified by comparing their spectra with those of the addition compounds (Cys-2′-EC and Cys-5′-EC) formed by apple PPO (Richard et al., 1991), and three 2ME addition compounds with EC were formed by the *R. mucronatum* style PPO. In the reaction with DTT, two main products were obtained by HPLC analysis (Fig. 5b). They are likely to be 2′- and 5′-adducts, judging from their UV spectra, although bridged structures between phenols and quinones have been proposed (Naish-Byfield et al., 1994). It is known that DTT is a more powerful reducing agent than 2ME, which is in turn a stronger reducer than cysteine. The rate of formation of the addition compounds was the fastest with cysteine (Figs. 2 and 5) because, unlike with DTT and 2ME, formation of *O*-quinone was not greatly inhibited by the reducing power of cysteine. Furthermore, in the presence of excess 2ME (Fig. 3) the formation of HETECs, was reduced, suggesting that reduction of EC–quinone by 2ME may prevent the appearance of HETECs' as indicated by the comparison of DTT and cysteine (Fig. 5), although reversible inhibition of the enzyme in the presence of excess 2ME is a possibility (Naish-Byfield et al., 1994).

Based on these results, the following mechanism is proposed for inhibition of enzymatic browning by thiol compounds. *O*-Diphenol is oxidized by PPO, in the absence of 2ME, whence the quinone radicals polymerize by oxidative coupling reaction (Brown, 1967), resulting in the 'browning' phenomenon (Fig. 6). Since 2ME is a reductant, it reduces part of the *O*-quinone back to the original structure, O-dihydroxyphenol (denoted as a reversible reaction in Fig. 6). On the other hand, as shown in the previous reaction, radicals from 2ME or other thiol compounds immediately react with O-quinone radical to form addition compounds such as HETECs and cysteinyldopa (Ito and Prota, 1977); consequently, the browning by PPO will be inhibited. In food processing, the use of cysteine will be more effective than halide salts or aromatic carboxylic acids for prevention of browning. Also, cysteine is safer than sulfites (Vamos-Vigyazo, 1981).

The mechanism of inhibition of enzymatic browning leads to the identification of another role for thiol compounds, in light of the reports by Weaver et al. (1970, 1972), which describe enzyme inhibition by γ-L-glutaminyl–3,4-benzoquinone. For example, when we prepared the enzyme solution in this or other experiments, especially from plants containing high levels of phenolic compounds (an excess of 2ME was included

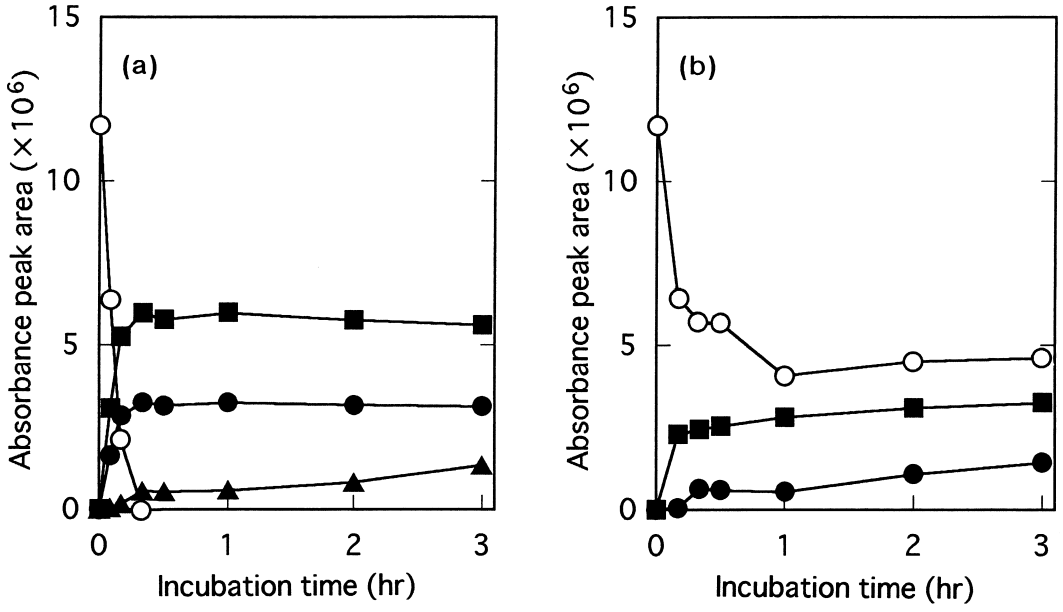

Fig. 5. PPO reactions with EC in the presence of cysteine or DTT. The reactions were performed as described in the Experimental section. In the reactions with 6 mM cysteine (a) and 6 mM DTT (b), 0.15 nkat and 0.75 nkat of PPO were used, respectively. The retention times of the substrates and the reaction products separated by HPLC were as follows: EC (30.4 min), Cys-2′-EC (6.6 min), Cys-5′-EC (16.1 min), Cys-2′,5′-EC (5.3 min), and DTT–2′-EC (33.9 min) using solvent C; DTT–5′-EC (2 peaks, 22.0 and 23.2 min) using solvent A. The amounts of the products were represented by the absorbance peak area at 280 nm. ○, EC; ●, 2′-adduct ; ■, 5′-adduct; ▲, 2′,5′-adduct.

as a reducing agent), it was observed that thiol compounds underwent addition reactions during the extraction of the enzyme and inhibited the browning of the solution. These results suggest that an excess of thiol compounds including reductants may prevent inhibition of sulfhydryl enzymes, such as, dehydrogenases by quinone compounds, that is, the reaction which couples SH-groups in the active site of the enzymes with quinones produced by PPO (Fig. 6).

3. Experimental

3.1. Plant and chemicals

Styles with stigma were picked from fresh flowers of *R. mucronatum* in Tsukuba city and stored at −20°C. EC, EGC, EGCG, and CA were prepared from green tea and catechu (Takino and Imagawa, 1963). Chlorogenic acid and 4-methylcatechol were purchased from Tokyo Chemical Industry. The three HETECs were obtained, as described previously (Ozawa et al., 1993). Their physico-chemical properties and spectral data are as follows,

2′-HETEC: mp 205–207°C; Anal. found: C, 55.28; H, 4.95; and S, 8.83%; Calcd. for $C_{17}H_{18}O_7S$: C, 55.73; H, 4.92; S, 8.74%; $[\alpha]_D^{23}$ −64.4°; (acetone; *c* 1.0); IR ν_{max} (KBr) cm^{-1}: 3370, 1613, 1490, 1460, 1420, 1260, 1230, 1130, 1080, 1030, 1000, 870, 820, 780; ^{1}H-NMR spectral data (270 MHz, acetone-d_6) δ: 2.8–3.0 (4H, *m*, –S–CH_2–$C\underline{H}_2$–OH), 3.6–3.7 (2H, *m*, –S–$C\underline{H}_2$–CH_2–OH), 4.27 (1H, *br s*, H-3), 5.55 (1H, *s*, H-2), 5.92 (1H, *d*, $J = 2.31$ Hz, H-6), 6.04 (1H, *d*, $J = 2.31$ Hz, H-8), 6.90 (1H, *d*, $J = 8.25$ Hz, H-5′), 7.21 (1H, *d*, $J = 8.25$ Hz, H-6′); ^{13}C-NMR spectral data (67.5 MHz, acetone-d_6) δ: 29.9 (CH_2, C-4), 39.7 (CH_2, –CH_2–OH), 60.8 (CH_2, –S –CH_2 –), 66.0 (CH, C-3), 77.8 (CH, C-2), 95.8 (CH, C-6), 96.2 (CH, C-8), 99.8 (C, C-4a), 116.4 (CH, C-5′), 118.2 (C, C-2′), 120.7 (CH, C-6′), 135.0 (C, C-1′), 145.5, 147.4 (C, C-3′, C-4′), 157.50, 157.54, 157.77 (C, C-5, C-7, C-8a); and FABMS *m*/*z*: 367 ($M^+ + H$), 349, 289, 273, 166 and 138.

5′-HETEC: mp 201°C; Anal. found: C, 54.74; H, 5.04; and S, 8.68%; Calcd. for $C_{17}H_{18}O_7S{\cdot}1/2H_2O$: C, 54.39; H, 5.10; and S, 8.54%; $[\alpha]_D^{25}$ −59.8° (acetone; *c* 1.0); IR ν_{max} (KBr) : 3470, 3300, 1600, 1485, 1455, 1360, 1315, 1265, 1190, 1170, 1135, 1090, 1055, 1010, 910, 820 and 790 cm^{-1}; ^{1}H-NMR spectral data (270 MHz, acetone-d_6) δ: 2.75 (1H, *dd*, $J = 16.83$, 3.30 Hz, H-4), 2.83 (1H, *dd*, $J = 16.83$, 4.62 Hz, H-4), 2.95 (2H, *t*, $J = 6.27$ Hz, –S–CH_2–$C\underline{H}_2$–OH), 3.68 (2H, *t*, $J = 6.27$ Hz, –S–$C\underline{H}_2$–CH_2–OH), 4.21–4.24 (1H, *m*, H-3), 4.89 (1H, *s*, H-2), 5.94 (1H, *d*, $J = 2.31$ Hz, H-6), 6.03 (1H, *d*, $J = 2.31$ Hz, H-8), 7.05 (1H, *d*, $J = 1.65$ Hz, H-2′), and 7.10 (1H, *d*, $J = 1.65$ Hz, H-6′); ^{13}C-NMR spectral data (67.5 MHz, acetone-d_6) δ: 29.9 (CH_2, C-4), 38.4 (CH_2, –CH_2–OH), 61.1 (CH_2, –S–CH_2–), 66.8 (CH, C-3), 79.1 (CH, C-2), 95.7 (CH, C-6), 96.2 (CH, C-8), 99.7 (C, C-4a), 115.3 (CH, C-2′), 120.4 (C, C-5′), 124.2 (CH, C-6′), 132.2 (C, C-1′), 145.6, 145.7 (C, C-

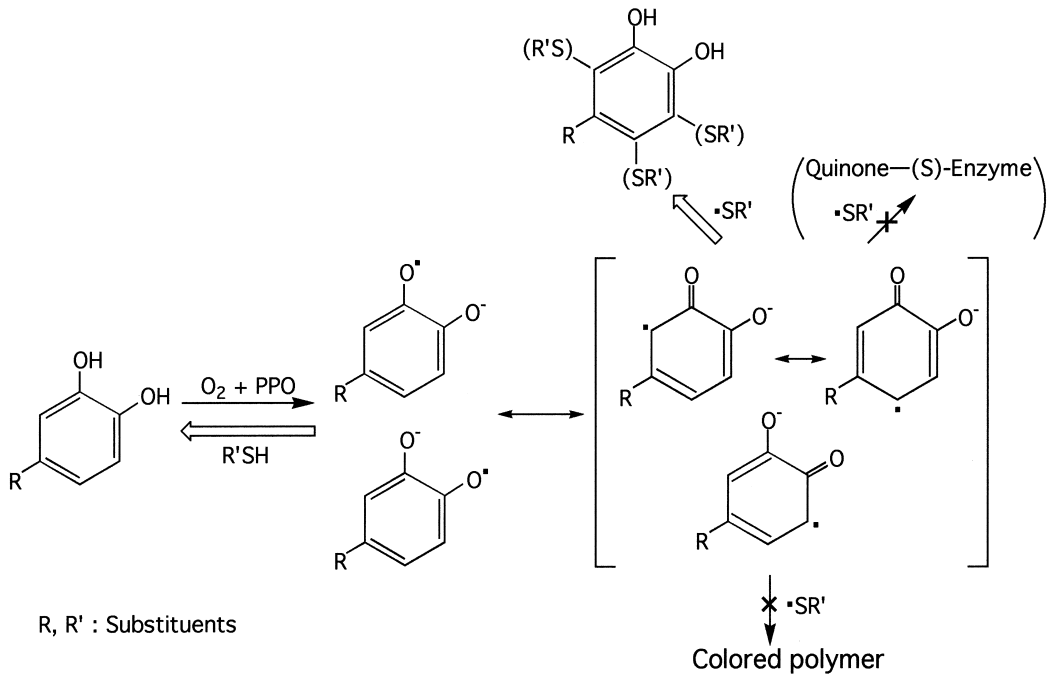

Fig. 6. Mechanism of inhibition of enzymatic browning by thiol compounds and protection of active sites of sulfhydryl enzymes.

3′, C-4′), 157.0 and 157.5 (C, C-5, C-7, C-8a); and FABMS *m/z*: 367 ($M^+ + H$), 349, 323, 288, 257, 228, 181, 160 and 139.

2′,5′-HETEC: mp 115–120°C; Anal. found: C, 50.91; H, 5.06; and S, 14.27%; Calcd. for $C_{19}H_{22}O_8S_2 \cdot 1/2H_2O$: C, 50.54; H, 5.13; S, 14.20%; $[\alpha]_D^{23}$ −99.1° (acetone; *c* 1.0); IR ν_{max} (KBr): 3350, 1625, 1600, 1510, 1460, 1410, 1280, 1235, 1140, 1090, 1065, 1010, 930, 860 and 810 cm^{-1}; ^{1}H-NMR spectral data (270 MHz, acetone-d_6) δ: 2.8–3.1 (6H, *m*, H-4, –S–CH_2–C<u>H</u>$_2$–OH × 2), 3.6–3.8 (4H, *m*, –S–C<u>H</u>$_2$–CH_2–OH × 2), 4.27 (1H, *br s*, H-3), 5.55 (1H, *s*, H-2), 5.94 (1H, *d*, $J = 2.31$ Hz, H-6), 6.04 (1H, *d*, $J = 2.31$ Hz, H-8), and 7.38 (1H, *s*, H-6′); ^{13}C-NMR spectral data (67.5 MHz, acetone-d_6) δ: 30.0 (CH_2, C-4), 36.8 and 39.6 (CH_2, –CH_2–OH × 2), 60.7 and 61.3 (CH_2, –S–CH_2– × 2), 65.8 (CH, C-3), 77.5 (CH, C-2), 95.8 (CH, C-6), 96.2 (CH, C-8), 99.7 (C, C-4a), 117.2 (C, C-2′), 122.8 (CH, C-6′), 123.0 (C, C-5′), 134.7 (C, C-1′), 144.7, 147.1 (C, C-3′, C-4′), 157.3, 157.5 and 157.7 (C, C-5, C-7, C-8a); and FABMS *m/z*: 443 ($M^+ + H$), 424, 407, 379, 361, 329, 289, 273, 166, 138.

3.2. Extraction and partial purification of PPOs

All procedures were carried out at 4°C. Styles with stigma (200 g) were homogenized in a blender with 60 g of washed Polyclar AT and 1 l of 0.1 M Na_2HPO_4 solution (pH 7.0) containing 0.6% ascorbic acid and 5 mM 2ME. The homogenate was squeezed through cheesecloth and the residue was washed twice with the same solution. The PPOs were extracted from the resulting residue, using the same solution containing 1% Tween 20. The precipitate, between 50 and 90% saturation with $(NH_4)_2SO_4$, was collected by centrifugation (30,000 × *g*, 10 min) and dissolved in the Na_2HPO_4–ascorbic acid solution without Tween 20. After centrifugation, the supernatant was dialyzed for 12 h against 0.02 M Na–phosphate buffer (pH 7.0).

The enzyme solution was loaded onto a DEAE–cellulose column (DE-52, Whatman, 1.6 × 35 cm) equilibrated with 0.02 M Na–phosphate buffer (pH 7.0), and the column was washed with the same buffer. A linear gradient elution with NaCl from 0 to 0.5 M in the same buffer gave two fractions A and B, which eluted at 0 and 0.07 M NaCl, respectively. After exchange of the buffer with 0.02 M Na–acetate buffer (pH 5.0), each fraction was loaded onto a CM–Toyopearl 650M column (Tosoh Corporation, 1.6 × 20 cm) equilibrated with 0.02 M Na–acetate buffer (pH 5.0), and eluted as for DEAE–cellulose column chromatography. Fractions A and B were further separated into three activities (PPOs I, II, and III) and two activities (PPOs IV and V), respectively. For further purifications of the relatively stable PPOs II and III, CM-Toyopearl 650 M column (1.0 × 25 cm, under the same conditions as described above) and hydroxyapatite (BIO-RAD, 1.0 × 15 cm, a linear gradient elution with Na–phosphate buffer from 0.1 to 0.4 M, pH 6.8) column chromatography were used. PPOs II and III were eluted from the hydroxyapatite column with 0.26–0.29 and 0.28–0.31 M Na–phosphate buffer, respectively. PPO II was used in this study since it was more stable, had a higher specific activity, and was obtained in higher yield than other PPOs. The specific activity and yield of PPO II were 12 μkat/mg and 1.9%, respectively. One katal was defined as the amount of enzyme which reduced 1 mol of 2-nitron–5-thiobenzoic acid anion per second using 4-methylcatechol as a substrate (Esterbauer et al., 1977; Okamoto et al., 1988). Protein concentration was measured by the method of Bradford (1976).

3.3. Enzyme reaction

Oxidation reaction by PPO was carried out both in presence and absence of 2ME, as well as, other thiol compounds at 30°C. Standard reaction mixture contained 0.02 M Na–phosphate buffer (pH 6.5), 3 mM substrate, 5.7 mM 2ME, and the enzyme solution in a total volume of 40 μl. Reaction was started by the addition of the enzyme solution (0.75 nkat) and was stopped after intervals of 0, 0.5, 1, 2, 3, and 4 h by the addition of 4 μl of 2 N HCl. Aliquots of the reaction mixtures were analyzed with HPLC.

3.4. HPLC analysis

The reaction products of PPO were analyzed by HPLC (TRI ROTOR, Japan Spectroscopic Co. Ltd.) with Inertsil prep-ODS column (250 × 6.0 mm ID, 10 μm, GL Sciences, Japan) at 23°C. The following solvent systems were used to separate the addition compounds: A, 2% AcOH–MeOH (70:30) for the reactions of EC (R_t: 11.7 min) and with 2ME, EGC (R_t: 8.8 min) with 2ME, EGCG (R_t: 10.0 min) with 2ME, and EC with DTT; B, 2% AcOH–MeOH (75:25) for the reaction of chlorogenic acid (R_t: 14.0 min) with 2ME; C, 2% AcOH–MeOH (80:20) for the reaction of EC (R_t: 30.4 min) with cysteine, and EC with DTT; and D, 2% AcOH–MeOH (85:15) for the reaction of CA (R_t: 21.5 min) with 2ME. The flow rate was 1 ml/min and the eluates were monitored at 280 nm. The spectra of eluates were also measured using a photodiode array UV-VIS detector, SPD-6MA (Shimadzu Corporation, Japan).

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