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Hydrogen Peroxide-Dependent Oxidation of Flavonols by Intact Spinach Chloroplasts

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ABSTRACT

Externally added quercetin (100 micromolar) was oxidized by intact spinach chloroplasts at a rate of 30 micromoles per mg chlorophyll per hour in the presence of 100 micromolar H_2O_2. The oxidation rate was increased by about 20% in a hypotonic reaction mixture. The thylakoid fraction also oxidized the flavonol in the presence of H_2O_2, and the rate was about 25% of that by intact chloroplasts. The oxidation of quercetin was inhibited by KCN and NaN_3. Ascorbate, which permeates slowly across chloroplast envelope, only slightly suppressed the initial rate of quercetin oxidation by intact chloroplasts, while the oxidation by ruptured chloroplasts was suppressed by ascorbate by about 60%. Quercetin glycosides, quercitrin and rutin, were also oxidized by chloroplasts in the presence of H_2O_2. These results suggest that flavonols are oxidized by peroxidase-like activity in chloroplasts and that externally added flavonols can permeate into the stroma through the envelope of intact chloroplasts.

Flavonols are widely distributed pigments in vascular plants. Their subcellular localization has been investigated and they were found in chloroplasts (12, 14–16) and etioplasts (21) as well as vacuoles (12). Quercetin derivatives have been reported to be localized in spinach chloroplasts (14). However, there are some discrepancies about localization of enzymes which participate in flavonol biosynthesis; some reports suggested the presence of the enzymes in chloroplast fraction (4, 12, 22), but other reports denied the presence of them in chloroplasts (8, 9). If flavonols are localized in chloroplast fraction as reported previously but there are no enzymes which catalyze flavonol synthesis, flavonols are possibly translocated into chloroplasts from the sites where flavonols are synthesized.

Flavonols have been reported to be oxidized by O_2^- (19) and radicals formed during lipid peroxidation (20) which was induced in illuminated chloroplasts, suggesting that flavonols could function as antioxidants in chloroplasts. Since flavonols were also oxidized by peroxidase (2, 20), there was a possibility that peroxidase activity in chloroplasts (5, 10, 13) might oxidize flavonols in the presence of H_2O_2. The experiments described in this paper have been designed to investigate whether flavonols can be oxidized by chloroplasts in the presence of H_2O_2 and whether flavonols are permeable across chloroplast envelope. The obtained results suggest that flavonols can permeate across the chloroplast envelope and be oxidized by chloroplasts in the presence of H_2O_2.

MATERIALS AND METHODS

Intact chloroplasts were isolated from spinach leaves obtained from a local market. Depetiolated spinach leaves were homogenized by a Waring Blendor in a medium which contained 0.5 mM KH_2PO_4, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 10 mM NaCl, 330 mM sorbitol, and 25 mM Mes-NaOH (pH 6.3). The homogenate was filtered through eight layers of gauze. The filtrate was centrifuged at 3,000 rpm for 1 min. The sediment was suspended into the above buffer solution, centrifuged at 3,000 rpm for 45 s, and suspended again into the same buffer solution. To separate intact chloroplasts from broken chloroplasts, the obtained chloroplast fraction was centrifuged in a Percoll density gradient. Percoll and the above buffer solution were mixed to obtain Percoll density gradient. At the bottom of the centrifugation tube, 2 ml of 40% Percoll (v/v) was added. Two ml of 30% and 20% Percoll were layered successively onto the 40% Percoll. Chloroplast suspension was layered onto 20% Percoll. After centrifugation at 4,000 rpm for 10 min, chloroplasts were separated into two bands. Chloroplasts between 20% and 30% Percoll were broken chloroplasts and chloroplasts between 30% and 40% Percoll were intact chloroplasts. The lower band was collected by a syringe and used for experiments. The intactness of the chloroplasts at the lower band was more than 80% by a ferricyanide test (6). Thylakoids were obtained from intact chloroplasts; hypotonically ruptured intact chloroplasts in the buffer solution without sorbitol were collected by centrifugation at 10,000*g* for 10 min, and suspended into the above buffer solution to use in experiments.

Rates of flavonol oxidation were followed at 380 nm with a Hitachi 557 spectrophotometer at 25°C. The basic reaction mixture (1 ml) contained chloroplasts equivalent to 5 to 9 μg of Chl, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, and 50 mM Hepes-NaOH (pH 7.6) with or without 330 mM sorbitol. Reactions were started by adding H_2O_2. The differences of extinction coefficients between flavonols and oxidized flavonols at 380 nm were estimated to be 8.7×10^3 M^{-1} cm^{-1} for quercetin, 5.4×10^3 M^{-1} cm^{-1} for quercitrin, and 7.2×10^3 M^{-1} cm^{-1} for rutin (20).

RESULTS AND DISCUSSION

Figure 1 shows time courses of quercetin oxidation by intact and ruptured chloroplasts and thylakoids in the presence of H_2O_2. Quercetin oxidation was followed at 380 nm since the difference spectrum of oxidized quercetin minus quercetin had a negative peak at about 380 nm (Fig. 5). It has already been reported that quercetin was oxidized by O_2^- and radicals formed during lipid peroxidation and that the oxidation was accompanied by the absorbance decrease at 380 nm (19, 20). The initial rate of quercetin oxidation by ruptured chloroplasts which were obtained by suspending intact chloroplasts into a hypotonic reaction mixture was about 35 μmol mg^{-1} Chl h^{-1} in the presence of 100 μM quercetin and 100 μM H_2O_2. The initial rate of quercetin oxidation by intact chloroplasts was about 80% of that by ruptured chloroplasts under the above conditions. The oxidation rate became slower during incubation. Quercetin was also oxidized by thylakoids, and the rate was about 20% of that by

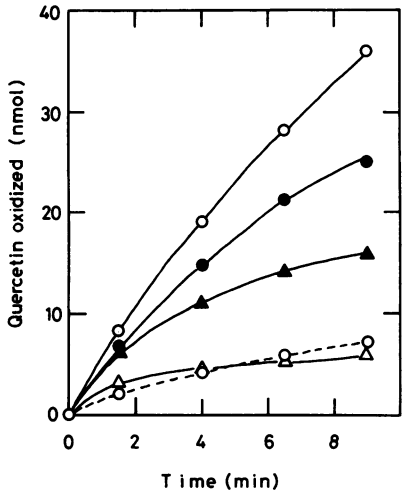

FIG. 1. Time courses of quercetin oxidation and effect of ascorbate. Reaction mixture (1 ml) contained chloroplasts equivalent to 9 μg of Chl, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, 0.1 mM H_2O_2, 0.1 mM quercetin, 330 mM sorbitol, and 50 mM Hepes-NaOH (pH 7.6). Reactions were started by adding H_2O_2. (●——●), Intact chloroplasts; (○——○), ruptured chloroplasts; (○- - -○), thylakoids; (▲——▲), intact chloroplasts plus 0.1 mM ascorbate; (△——△), ruptured chloroplasts plus 0.1 mM ascorbate. Ascorbate was added 1 min prior to the addition of H_2O_2. Ruptured chloroplasts were obtained by suspending intact chloroplasts into the reaction mixture without sorbitol. Thylakoids were prepared as described in "Materials and Methods."

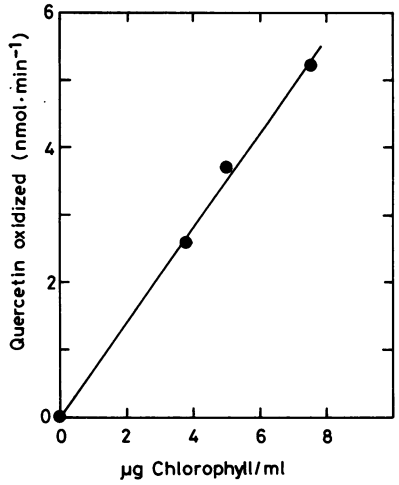

FIG. 2. Effect of Chl concentration on quercetin oxidation. Reaction mixture (1 ml) contained 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, 0.1 mM quercetin, 0.1 mM H_2O_2, and 50 mM Hepes-NaOH (pH 7.6). Reactions were started by adding H_2O_2.

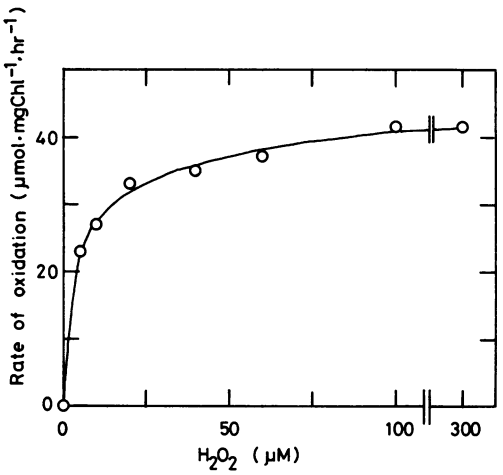

FIG. 3. H_2O_2 concentration dependence on quercetin oxidation. Reaction mixture (1 ml) contained chloroplasts equivalent to 8 μg of Chl, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl, 0.1 mM quercetin, and 50 mM Hepes-NaOH (pH 7.6).

ruptured chloroplasts. Rate of quercetin oxidation in the presence of 100 μM H_2O_2 only was about 20% of that in the presence of both 100 μM H_2O_2 and thylakoids.

Effects of ascorbate on the quercetin oxidation are shown in Figure 1, too. Ascorbate (100 μM) significantly suppressed the initial rate of quercetin oxidation by ruptured chloroplasts. The inhibition by ascorbate was nearly saturated at 100 μM. In intact chloroplasts, 100 μM ascorbate slightly suppressed the initial rate of quercetin oxidation and the suppression became significant during incubation. The inability of ascorbate to suppress the initial rate of quercetin oxidation by intact chloroplasts may be due to its lower permeability across chloroplast envelope (1, 3, 13); the rate of ascorbate permeation through chloroplast envelope was estimated to be less than 1 μmol mg^{-1} Chl h^{-1} at 100 μM ascorbate from the data in References 1 and 3. The value is much lower than the initial rate of quercetin oxidation by H_2O_2 in intact chloroplast suspension. The results in Figure 1 suggest that quercetin is mainly oxidized by peroxidase-like activity in stroma and thylakoids. The data that quercetin oxidation by H_2O_2 in the presence of chloroplasts was inhibited by 1 mM KCN and 5 mM NaN_3 by about 80% also support the above consideration. The peroxidase-like activity seems not to be localized on the outer surface of the chloroplast envelope since ascorbate, which may be easily accessible to the outer surface of the chloroplast envelope, did not suppress the initial rate of quercetin oxidation by intact chloroplasts (Fig. 1). Nakano and Asada (13) did not observe peroxidation of externally added ascorbate by intact chloroplasts. The results in Figure 1 also suggest that quercetin can permeate into stroma through chloroplast envelope. The suppression of quercetin oxidation by ascorbate may be due to the competition to the peroxidase-like activity or reduction of oxidized quercetin by ascorbate.

The rate of quercetin oxidation by ruptured chloroplasts was linear as a function of chloroplast concentration (Fig. 2). When the effect of H_2O_2 concentration on quercetin oxidation was examined, the oxidation rate was nearly saturated at 100 μM H_2O_2 (Fig. 3). Double reciprocal plots showed that K_m and V_{max} values for H_2O_2 in the presence of 100 μM quercetin were 4 μM and 44 μmol mg^{-1} Chl h^{-1}, respectively.

Quercetin glycosides, quercitrin and rutin, were also oxidized by H_2O_2 in the presence of chloroplasts (Figs. 4 and 5). The rate of oxidation of 100 μM quercitrin and rutin by ruptured chloroplasts in the presence of 100 μM H_2O_2 were about 9 and 4 μmol mg^{-1} Chl h^{-1}, respectively. The rates of oxidation of quercitrin and rutin by intact chloroplasts were about 5 and 1.5 μmol mg^{-1} Chl h^{-1} under the above conditions. The K_m and V_{max} values for quercetin, quercitrin, and rutin oxidation by ruptured chloroplasts in the presence of 100 μM H_2O_2 were 30 μM and 50 μmol mg^{-1} Chl h^{-1}, 142 μM and 33 μmol mg^{-1} Chl h^{-1}, and 142 μM and 13 μmol mg^{-1} Chl h^{-1}, respectively. The data indicate that quercetin glycosides can also be oxidized by peroxidase-like activity in spinach chloroplasts and that permeability of quercetin glycosides across chloroplast envelope seems to be slower than that of their aglycone.

Difference spectra of oxidized flavonols minus flavonols (Fig. 5) indicate that during bleaching at 380 nm, an absorbance increase at about 330 nm was observed, *i.e.* flavonols which had an absorption maximum at about 380 nm were transformed to compounds which had an absorption maximum at about 330

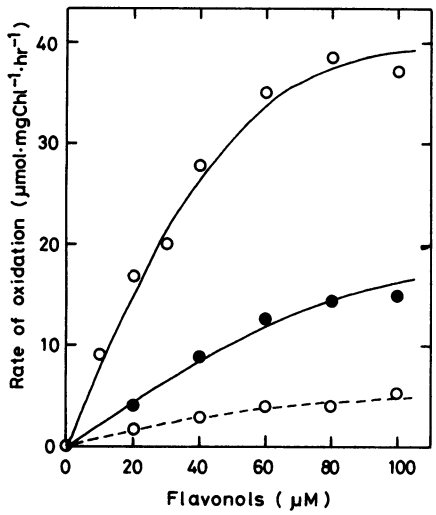

FIG. 4. Flavonol concentration dependences on their oxidation. Reaction mixture was the same as that in Figure 3 except for flavonol concentration. (O——O), Quercetin; (●——●), quercitrin; (O- - -O), rutin. Reactions were started by adding 0.1 mM H_2O_2.

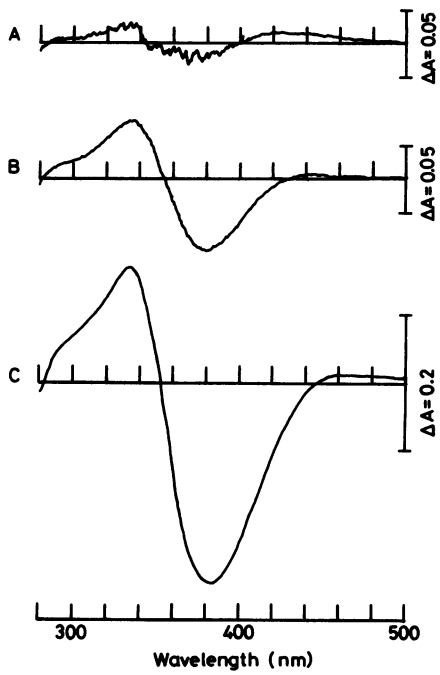

FIG. 5. Difference spectra of oxidized flavonols minus flavonols. Reaction mixture was the same as that in Figure 3 except for flavonols used. The difference spectra were measured 7 min after the addition of 0.1 mM H_2O_2. A, Rutin; B, quercitrin; C, quercetin. H_2O_2-induced absorbance decrease at 380 nm was less than 0.002 in the presence of chloroplasts only under the above conditions.

nm by H_2O_2. Oxidation of the flavonols was also observed by horseradish peroxidase in the presence of H_2O_2. The oxidation was accompanied by the bleaching at about 380 nm and the absorbance increase at about 330 nm (data not shown).

The data described above suggest that flavonols are oxidized by spinach chloroplasts in the presence of H_2O_2. The rate of H_2O_2 formation by intact chloroplasts was about 1 μmol mg^{-1} Chl h^{-1} (17) and the rate of flavonol oxidation by spinach chloroplasts was about 35 μmol mg^{-1} Chl h^{-1} in the presence of 100 μM quercetin and 100 μM H_2O_2. These data suggest that flavonols can function as effective scavengers of H_2O_2 in chloroplasts if they are localized at about 100 μM. The lower K_m value for H_2O_2 of flavonol peroxidase-like activity (4 μM) than that of ascorbate peroxidase activity (40 μM) (13) may indicate that flavonol peroxidase-like activity mainly function at lower H_2O_2 concentrations to scavenge H_2O_2.

The difference spectra of O_2^-- and lipid peroxy radical-oxidized flavonols minus flavonols had a negative and a positive peaks at about 330 and 380 nm (19, 20) as well as the difference spectra of H_2O_2-oxidized flavonols minus flavonols (Fig. 5). These results suggest that flavonols are converted to the same compound independent of the used oxidative systems.

Barz and Wierman (2) have proposed that flavonols were transformed to the corresponding 2,3-dihydroxyflavanones by action of peroxidase, which had an absorption maximum at about 330 nm (7). However, the compound formed from flavonols by chloroplasts in the presence of H_2O_2 seems not to be the flavanones since the flavanones which have been proposed by Barz *et al.* (2, 7) were formed by the addition of water to flavonols but not by oxidation. Quercetin has also been reported to be decomposed to a depside and CO by singlet molecular oxygen generated in organic solvents (11). The product formed from quercetin in this experiment (Fig. 5) seems not to be the depside and CO because the product which has an absorption maximum at about 330 nm was partially reduced to quercetin by ascorbate under certain conditions (Takahama *et al.*, submitted).

As have been discussed (18–20), flavonols seem to function as antioxidants by scavenging O_2^- and terminating lipid peroxidation and carotenoid photobleaching. The results obtained in this paper also support the antioxidative function of flavonols in chloroplasts by scavenging H_2O_2. The reports that flavonols are localized in the chloroplast fraction (12, 14–16) and that the concentration of flavonols in chloroplasts increased by illumination (12) might support the function of flavonols as antioxidants in chloroplasts, too.

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