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INHIBITION OF LIPOXYGENASE-DEPENDENT LIPID PEROXIDATION BY QUERCETIN: MECHANISM OF ANTIOXIDATIVE FUNCTION

UMEO TAKAHAMA

Department of Biology, Kyushu Dental College, Kitakyushu 803, Japan

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Key Word Index—Antioxidant; ascorbate; lipid peroxidation; lipoxygenase; quercetin.

Abstract—Quercetin inhibited soybean lipoxygenase-1-dependent linoleic acid peroxidation. Two to three μM quercetin was required for 50% inhibition. During the inhibition, quercetin was oxidized. The oxidation was observed as an absorbance decrease at about 380 nm and an absorbance increase at about 335 nm. Inhibition of linoleic acid peroxidation by quercetin seems to be due to reduction by the reagent of the linoleic acid radical formed as an intermediate during lipoxygenation. Quercetin oxidation was suppressed by ascorbate under conditions when ascorbate did not affect lipoxygenase-dependent linoleic acid peroxidation. The results suggest that ascorbate can reduce the quercetin oxidized by the linoleic acid radical back to quercetin. Based on the results, the significance of a redox reaction between oxidized quercetin and ascorbate is discussed.

INTRODUCTION

Flavonols are commonly contained in higher plants. Their aglycones are mainly found in lipophilic regions such as oil glands, waxy layers, etc. [1]. Flavonol glycosides are mainly found in vacuoles. To understand the physiological functions of flavonols, their biochemical characteristics must be clarified. In the past, their inhibitory effects on lipid peroxidation have been reported in model systems [2–4] and beef slices [3, 4]. Recently, such effects of flavonols have also been reported in microsomes and mitochondria [5], chloroplasts [6], hepatic cells [7], and erythrocytes [8]. Flavonols are oxidized during inhibition of lipid peroxidation [6]. However, the mechanism of the inhibition is still unclear.

To elucidate the mechanism of the inhibition it must be studied in a lipid peroxidation system the mechanism of which is well understood. Lipoxygenase-dependent linoleic acid peroxidation has been studied in detail and the reaction proceeds as follows [9, 10]: the reaction is initiated by a hydrogen atom abstraction reaction producing a linoleic acid radical. The radical is oxygenated producing a linoleic acid peroxy radical, which is subsequently reduced to form a linoleic acid hydroperoxide.

In this paper, the mechanism of lipid peroxidation inhibition by quercetin is discussed based on the effect of quercetin on lipoxygenase-dependent lipid peroxidation. The interaction between ascorbate and quercetin on the inhibition on lipoxygenase-dependent linoleic acid peroxidation is also discussed.

RESULTS

Time-courses of lipoxygenase-dependent O_2 uptake in the presence of linoleic acid under various conditions are shown in Fig. 1. The rate of O_2 uptake following an initial lag phase was linear in the absence of quercetin (trace A). Lipoxygenase-induced O_2 uptake was suppressed by quercetin and the suppressive effect became greater during the course of the incubation (trace C). When lipoxygenase-dependent linoleic acid hydroperoxide formation was followed at 235 nm, an initial lag phase was observed as described by Smith and Lands [11]. Quercetin inhibited the absorbance increase at 235 nm and the inhibition was greater in the later phase than the initial phase of incubation cf. Fig. 1 (data not shown). These results suggested that quercetin inhibited an oxygenation reaction of linoleic acid peroxidation.

Figure 2 shows quercetin concentration dependence on the inhibition of lipoxygenase-induced O_2 uptake. Half-maximum inhibition was observed at about 3 μM quercetin. More than 100 μM quercetin was required for complete inhibition. About 2 μM quercetin was required for half-maximum inhibition when lipoxygenase-dependent linoleic acid peroxidation was followed spectrophotometrically under conditions similar to those in Fig. 2 (data not shown). The concentration of quercetin for half-maximum inhibition depended on the amount of linoleic acid added to the reaction mixture (cf. Fig. 5).

To elucidate the mechanism of the inhibition by quercetin, it was necessary to establish whether quercetin was oxidized during the inhibition of lipoxygenase-dependent linoleic acid peroxygenation. The oxidation of quercetin had been reported to be accompanied by a decrease in absorbance at 380 nm and an increase at 335 nm [6, 12]. If quercetin was not oxidized, the inhibition could possibly be due to inactivation of lipoxygenase by binding of quercetin to the enzyme. Inactivation of an enzyme by binding of quercetin to it had been reported [13]. The difference spectra of quercetin in the presence of lipoxygenase were recorded. As shown in Fig. 3, bleaching at about 380 nm and an absorbance increase at about 335 nm were observed. In the absence of quercetin, only changes of light scattering due to lipoxygenation were observed. This result suggested that the absorbance changes at about 335 and 380 nm were due to oxidation of quercetin. The rate of quercetin oxidation became faster as the concentration of quercetin was increased and the K_m value for quercetin was about 5 μM (Fig. 4). The data in Figs 3 and 4 suggested that quercetin

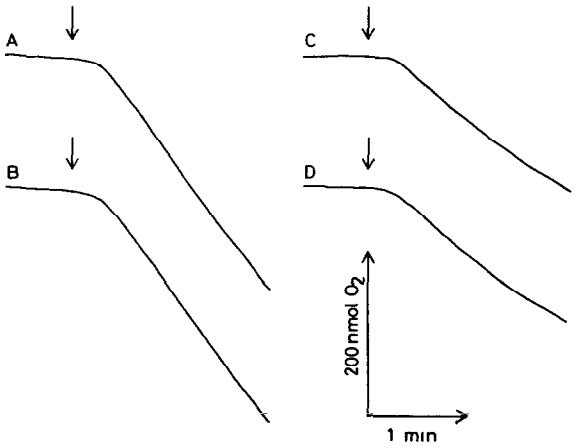

Fig. 1 Time-courses of lipoxygenase-dependent O_2 uptake and the effects of quercetin and ascorbate. The reaction mixture (3.5 ml) contained 0.9 mM linoleic acid, 10 μg lipoxygenase, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl and 50 mM HEPES–NaOH (pH 7.6). Reaction conditions are described in Experimental. Arrows indicate the time when lipoxygenase was added A, no addition; B, 0.1 mM ascorbate; C, 5.7 μM quercetin, D, 0.1 mM ascorbate plus 5.7 μM quercetin.

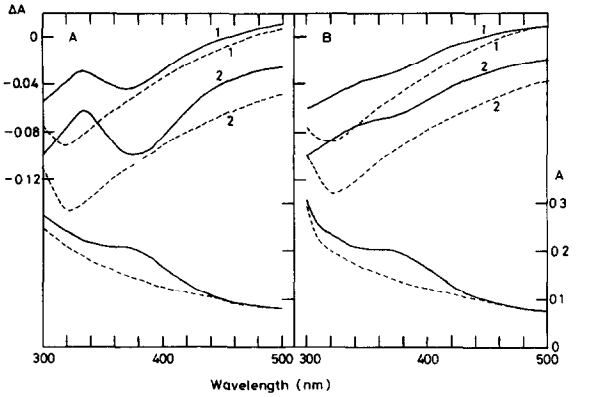

Fig. 3. Quercetin oxidation during inhibition of lipid peroxidation in the presence (B) and absence (A) of ascorbate Reaction mixture (3 ml) contained 10 μg lipoxygenase, 0.2 mM linoleic acid, 3.3 μM quercetin, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl and 50 mM HEPES–NaOH (pH 7.6). Top traces (1 and 2) are difference spectra of quercetin and quercetin oxidized during lipoxygenation Bottom traces are absorption spectra of the reaction mixtures. Solid curves, with quercetin; dashed curves, without quercetin. Trace 1, immediately after the addition of lipoxygenase; trace 2, two min after the addition of lipoxygenase. The concentration of ascorbate was 66 μM.

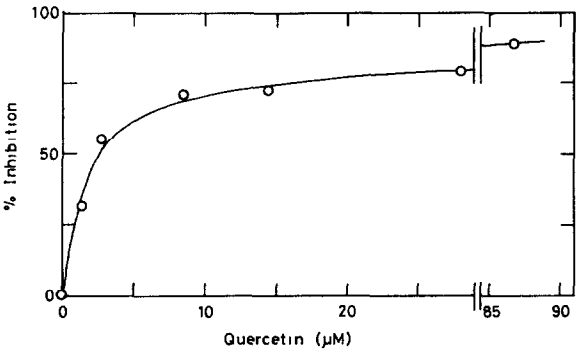

Fig. 2 Effect of quercetin on lipid peroxygenation. Reaction mixture and conditions were the same as that in Fig. 1 except for the amount of lipoxygenase (1 μg) added and the concentration of quercetin Percent inhibition by quercetin was calculated from the initial rate of O_2 uptake.

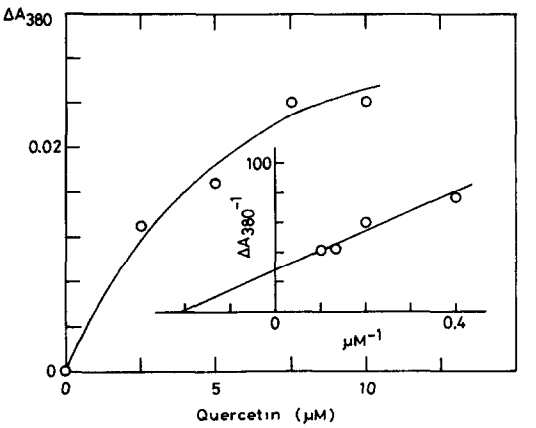

Fig 4. Effect of quercetin concentration on lipoxygenase-dependent quercetin oxidation. The reaction mixture (2 ml) contained 6.6 μg lipoxygenase, 40 μM linoleic acid, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl and 50 mM HEPES–NaOH (pH 7.6). Reactions were stopped by adding 2 ml of methanol 5 min after lipoxygenation was started. Other procedures are described in Experimental The inset shows a double reciprocal plot of the data in Fig. 4

inhibited lipoxygenation by reducing the intermediates of the reaction.

When the effects of linoleic acid concentration on the lipoxygenation inhibition by quercetin and quercetin oxidation were examined, the degree of inhibition of lipid peroxidation and the rate of oxidation became greater as the concentration of the substrate was increased (Fig. 5). Plots of $1/v$ versus $1/[S]$ at a fixed quercetin concentration showed that the slope of the plots in the presence of the inhibitor was nearly the same as that in the absence of the inhibitor (Fig. 5, inset). These kinetics [14] suggested that the inhibition by quercetin was non-competitive inhibition, i.e. the inhibition by quercetin was neither due to inactivation of free enzyme nor interference of its reaction with its normal substrates, but due to reduction of intermediates the concentrations of which increased as the substrate concentration was increased. The linoleic acid radical and linoleic acid peroxy radical were considered as potential intermediates. This consideration was supported by the report that quercetin was oxidized during the degradation of lauroyl peroxide by a radical chain reaction [6]. The oxidation of carotenoids and polyphenols by free radical intermediates during

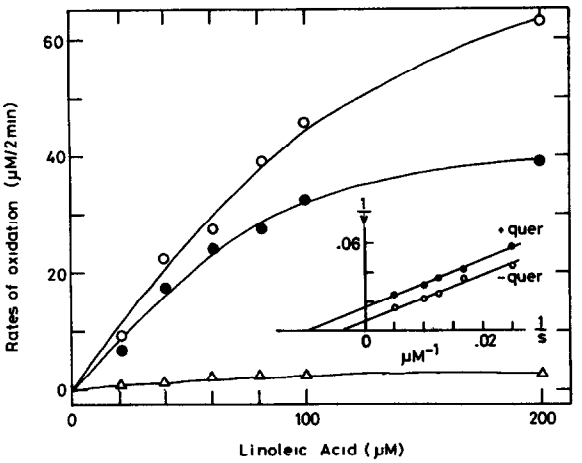

Fig. 5. Effects of linoleic acid concentration on linoleic acid peroxidation and quercetin oxidation. The reaction mixture was the same as that in Fig. 3 except for the amount of lipoxygenase (5 μg) and the concentration of linoleic acid. Rates of lipoxygenation were calculated from the data 2 min after the start of reaction. Rates of quercetin oxidation were determined under the conditions similar to those used to determine the rates of linoleic acid peroxidation ○, Linoleic acid peroxidation; ●, linoleic acid peroxidation in the presence of 3.3 μM quercetin; △, quercetin oxidation. The inset shows double reciprocal plots of the data in Fig. 5.

Table 1. Effect of quercetin on ascorbate oxidation

	Ascorbate oxidized (μM/10 min)
−Quercetin	6.0 ± 0.9 (6)*
+Quercetin (5 μM)	9.2 ± 0.5 (3)

Reactions were run for 10 min at 26° under conditions similar to those used to determine quercetin oxidation (see Experimental) Reaction mixture (2 ml) contained 5 μg lipoxygenase, 0.2 mM linoleic acid, 5 μM quercetin, 25 μM sodium ascorbate, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl and 50 mM HEPES–NaOH (pH 7.6).

*Standard deviation, with number of measurements in parentheses.

lipoxygenase-dependent lipid peroxidation had been reported [15].

The effects of ascorbate on lipoxygenase-dependent lipid peroxidation and quercetin oxidation were examined. Under the conditions used in this study, 0.1 mM ascorbate did not affect O_2 uptake by lipoxygenase both in the absence and presence of quercetin (Fig. 1, traces B and D). No effect of ascorbate was observed, either, when lipoxygenation was followed at 235 nm (data not shown). However, ascorbate suppressed lipoxygenase-induced quercetin oxidation in the presence of linoleic acid (Fig. 3). The results suggested that the suppression was not due to inhibition of lipoxygenase-dependent linoleic acid peroxidation by ascorbate, but due to reduction of oxidized quercetin. During suppression of the lipoxygenase-dependent quercetin oxidation by ascorbate, ascorbate oxidation proceeded at a rate similar to that of quercetin oxidation (compare Table 1 and Fig. 5). In the absence of quercetin, the rate of ascorbate oxidation was slower than that in the presence of quercetin (Table 1), as expected from the finding (Fig. 1) that ascorbate did not affect lipoxygenation. This result also supported the possibility that ascorbate can reduce oxidized quercetin.

Removal of superoxide anion radical by ascorbate for the reason of the suppression was excluded since superoxide dismutase did not affect quercetin oxidation. The formation of a superoxide anion radical during lipoxygenation by lipoxygenase [16] and the oxidation of quercetin by superoxide anion radical [12] had been reported.

DISCUSSION

In previous papers, it has been shown that quercetin can suppress lipid peroxidation which is induced by the oxidants accumulated on the oxidizing side of photosystem II of spinach chloroplasts [6] and by a photosensitized reaction in the presence of hematoporphyrin [8]. In these studies, lipid peroxidation was followed by a thiobarbituric acid test. Therefore, it is not clear whether quercetin can suppress the initial reactions of lipid peroxidation or not.

When linoleic acid is peroxidized by lipoxygenase, linoleic acid hydroperoxide is formed via a three step reaction, i.e. hydrogen atom abstraction to form a linoleic acid radical, oxygenation of the radical to form a linoleic acid peroxide radical, and addition of a hydrogen atom to the peroxy radical [9]. If quercetin is oxidized during lipoxygenase-dependent linoleic acid peroxidation, it is probable that quercetin is oxidized by the linoleic acid radical or the linoleic acid peroxy radical. The observations that quercetin is oxidized in the presence of both linoleic acid and lipoxygenase (Figs 3–5) and that quercetin inhibits lipoxygenase-dependent O_2 uptake (Fig. 1) and linoleic acid peroxidation (data not shown) indicate that the inhibition of lipoxygenase-dependent linoleic acid peroxidation is due to reduction of the linoleic acid radical by quercetin although the reduction of the linoleic acid peroxy radical by quercetin cannot be excluded. Carotenoid radicals which are formed in illuminated chloroplasts are also reduced by quercetin [17, 18].

When quercetin is oxidized by enzyme extracted from *Cicer arietinum*, a compound which has an absorption maximum at 335 nm is formed [19]. Hösel and Barz [19] have identified the compound as a flavanone corresponding to quercetin. Although the oxidized quercetin also had an absorption maximum at about 330 nm (Fig. 3), the compound remains to be identified.

The inhibition of quercetin oxidation by ascorbate (Fig. 3) and oxidation of ascorbate in the presence of quercetin (Table 1) suggest that ascorbate can reduce quercetin oxidized by the linoleic acid radical. The redox reaction between ascorbate and oxidized quercetin might be important in the physiological functions of flavonols as antioxidants. Such a redox reaction between oxidized quercetin and ascorbate has been suggested [18, 20]. Ascorbate may be oxidized to dehydroascorbate. Glutathione can regenerate ascorbate from dehydroascorbate *in vivo* [21].

As a physiological function of flavonols in plants, scavenging of lipid radicals as discussed previously [22] is

possible. The localization of flavonol aglycones, which are more potent inhibitors of lipid peroxidation than their glycosides [6], in lipophilic regions such as oil glands, waxy layer, etc. [1] provides circumstantial support for the function of flavonols as antioxidants *in vivo*.

EXPERIMENTAL

Superoxide dismutase was obtained from Sigma. Quercetin and linoleic acid were from Wako Pure Chem. Industries Co. Soybean lipoxygenase was obtained from Serva Feinbiochem. Co. It was estimated that this enzyme preparation contained mainly lipoxygenase-1, since rate of linoleic acid peroxidation by the preparation at pH 7.8 was about twofold of that at pH 6.5 [cf. 23].

Lipoxygenase-dependent O_2 uptake was followed with a Clark type oxygen electrode at 25°. The basic reaction mixture (3.5 ml) contained 0.9 mM linoleic acid, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM NaCl and 50 mM HEPES–NaOH (pH 7.6). The mixture was preincubated for about 5 min before addition of lipoxygenase Lipoxygenase-dependent linoleic acid hydroperoxide formation was followed also at 235 nm with a Hitachi 557 spectrophotometer under conditions similar to those used to follow O_2 uptake except for the concentrations of linoleic acid and lipoxygenase. A molar extinction coefficient of 25 000 $M^{-1}cm^{-1}$ at 235 nm was used for linoleic acid hydroperoxide [24] Reactions were started as described above. In the quercetin-containing reaction mixtures, the quercetin was bleached at 235 nm However, the rate of the absorbance change was about 1% of that of linoleic acid hydroperoxide formation. The absorbance change of quercetin at 235 nm was estimated from the absorbance change of quercetin at 380 nm. At this wavelength, linoleic acid hydroperoxide did not show any absorbance

To determine quercetin oxidation during lipoxygenation, 2 ml of MeOH was added after a definite time of incubation to the 2 ml of reaction mixture to inactivate lipoxygenase and to clarify the reaction mixture. After this treatment, the $A_{380\ nm}$ was determined. Quercetin has an absorption maximum around this wavelength. The reaction mixture without lipoxygenase was also treated as described above to calculate the amount of quercetin oxidized Ascorbate oxidation was followed under conditions similar to those just described. Unreacted ascorbate was estimated using 2,6-dichloroindophenol.

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REFERENCES

1 Wollenweber, E. and Dietz, V H. (1982) *Phytochemistry* **20**, 869.
2. Letan, A. (1966) *J. Food Sci.* **31**, 518.
3. Pratt, D. E. and Watts, B. M. (1964) *J. Food Sci.* **29**, 27
4. Pratt, D E. (1965) *J. Food Sci.* **30**, 737
5. Cavallini, L., Bindoli, A. and Siliprandi, N. (1978) *Pharmacol. Res Commun.* **10**, 13.
6. Takahama, U. (1983) *Photochem. Photobiol.* **38**, 363.
7. Younes, M and Siegers, C. P. (1981) *Planta Med.* **43**, 240.
8. Sorata, Y., Takahama, U and Kimura, M. (1984) *Biochim. Biophys. Acta* **799**, 313.
9. de Groot, J. J. M. C., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., Wever, R., and van Gelder, B. F. (1975) *Biochim. Biophys. Acta* **377**, 71.
10. Pistorius, E. K., Axelrod, B. and Palmer, G. (1976) *J. Biol. Chem.* **251**, 7144.
11. Smith, W. L. and Lands, W E. M. (1972) *J. Biol. Chem.* **247**, 1038.
12. Takahama, U. (1983) *Plant Physiol.* **71**, 598.
13. Cantley, L. C., Jr. and Hammes, G. G. (1976) *Biochemistry* **15**, 1.
14. Lehninger, A. L. (1978) in *Biochemistry*, p. 200. Worth, New York.
15. Tappel, A. L (1961) *Methods Enzymol.* **5**, 539.
16. Veldink, G. A., Garssen, G. J., Slappendel, S., Vliegenthart, J. F. G. and Boldingh, J. (1977) *Biochem. Biophys. Res. Commun.* **78**, 424.
17 Takahama, U. (1982) *Plant Cell Physiol.* **23**, 859.
18. Takahama, U. (1984) *Plant Cell Physiol.* **25**, 1181.
19. Hösel, W and Barz, W. (1972) *Biochim. Biophys. Acta* **261**, 294.
20. Takahama, U., Youngman, R. J. and Elstner, E. F. (1984) *Photobiochem. Photobiophys.* **7**, 175
21. Foyer, C. H. and Halliwell, B. (1976) *Planta* **133**, 21.
22. McClure, J. W (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds), pp. 970–1055. Chapman & Hall, London.
23. Christopher, J., Pistorius, E. and Axelrod, B. (1970) *Biochim. Biophys. Acta* **198**, 12.
24. Johnston, A E., Zilch, K T., Selke, E. and Dutton, H. J. (1961) *J Am. Oil Chem. Soc.* **38**, 367.