Antioxidant Effect of Riboflavin in Enzymic Lipid Peroxidation

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The antioxidant effect, if any, of riboflavin during lipid peroxidation in an enzyme system was studied. When lipoxygenase was added to a mixture of linoleic acid and riboflavin, absorbance at 234 nm increased and absorbance at 444 nm decreased. Starting about 400 s after the addition of lipoxygenase, however, absorbance at 234 nm tended to decrease and that at 444 nm tended to increase. The decrease at 234 nm suggested that hydroperoxide had decomposed, and the increase at 444 nm suggested that the reduced form of riboflavin had been oxidized. When riboflavin is oxidized, the hydroperoxide in it acts as an electron acceptor and the hydroperoxide itself then decomposes. The extent of the phenomenon may be proportional to the amount of the antioxidant.

Edible oils and fats, especially those that contain much unsaturated fatty acids, readily undergo lipid peroxidation when exposed to light or oxygen (Agarwal et al., 1984; Kellogg and Fridovich, 1975; Yamashoji et al., 1979; Thomas et al., 1985; Truby et al., 1987). Many different antioxidants are used to help prevent such peroxidation. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used synthetic antioxidants, and vitamin E is a widely used natural antioxidant. BHA and BHT are carcinogenic or mutagenic in some organisms (Ito et al., 1985; Ponder and Green, 1985; Witschi and Morse, 1985), and some countries regulate the use of these compounds. Safer antioxidants are needed. Riboflavin has antioxidant effects (Toyosaki et al., 1987a, b, 1988, 1989). Ohama and Yagi (1969) found that irradiation of riboflavin tetrabutyrate and linoleic acid with light inhibits the generation of hydroperoxide. Terada (1975) reported that bacteria that inhibit peroxidation do so with riboflavin. The mechanism of riboflavin antioxidation is not known.

Riboflavin has antioxidant effects in emulsion systems and water systems. Here, I studied whether riboflavin had any antioxidant effect on lipid peroxidation in an enzyme system. If there was such an effect, I wanted to identify the mechanism.

MATERIALS AND METHODS

Materials. Linoleic acid (more than 99% pure) and lipoxygenase (from soybean, type I, lyophilized) were from Sigma Chemical Co. (St. Louis, MO); methylene blue (more than 98.5% pure) was from Tokyo Kasei Co., Ltd. (Tokyo, Japan). Kieselgels 60 G and 60 PF$_{254}$ were from Merck (Darmstadt, Germany). The 0.25-mm thin-layer chromatography (TLC) was done with a TLC apparatus from Yazawa Scientific Apparatus Manufacturing Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of Reagents. The 0.2 M borate buffer was at pH 9.0. The substrate solution of 20 mM linoleic acid contained 0.3% ethanol in borate buffer. The riboflavin solution was diluted with the borate buffer to 10 mM, and the lipoxygenase solution was diluted with the borate buffer to give solutions with activities from 1 to 1000 units.

Preparation of Linoleic Acid Hydroperoxide. Linoleic acid hydroperoxide was prepared according to the procedure of Matsuda et al. (1978) by enzymic peroxidation with lipoxygenase. The standard reaction mixture, containing 32 mM linoleic acid, 0.1% Tween 80, 0.2 M borate buffer (pH 9.0), and 50 units of lipoxygenase in a total volume of 20 mL, was incubated in a conical flask to facilitate its being flushed with pure oxygen. The reaction mixture was stirred mechanically for 40 min at 30 °C under a stream of pure oxygen in a separating funnel. Then hydroperoxide was extracted with diethyl ether and concentrated by removal of the solvent. The formation of linoleic acid hydroperoxide was monitored by measurement of the increase in the absorbance at 234 nm. Absorbance at 234 nm was used as a direct index of the amount of the conjugated diene and as an indirect index of the amount of hydroperoxide. The hydroperoxide was checked by TLC for purity with a mixture of n-hexane, diethyl ether, and acetic acid (60:40:1 v/v/v) as the solvent system and was monitored under UV light.

Measurements of Reduced and Oxidized Riboflavin and of Hydroperoxide. The reaction mixture contained 10 mM riboflavin, 20 mM linoleic acid or 15 mM hydroperoxide, 0.2 M
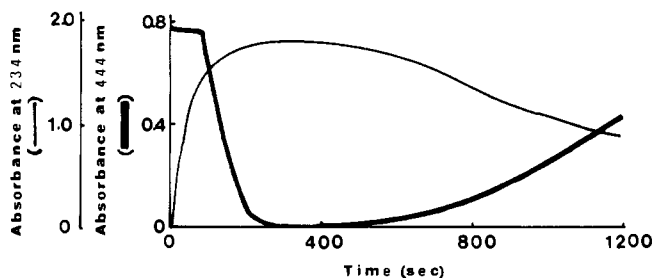
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Figure 1. Changes over time in absorbance at 234 (conjugated diene of linoleic acid) and 444 nm (isoalloxazine ring of riboflavin). Lipoxygenase (500 units) was added to 4.0 mL of a 0.2 M borate buffer solution containing 10 mM riboflavin, 20 mM linoleic acid, and 1% ethanol. Then absorbances at the two wavelengths were monitored over time. The amount of hydroperoxide was calculated from the amount of the conjugated diene. The amount of hydroperoxide that had been produced by 400 s after the start of the reaction and the amount of hydroperoxide that had decomposed by 2000 s after the start of the reaction were calculated and expressed as percentages (about 100% in both cases). The decrease in absorbance from just before lipoxygenase was added until 400 s after it was added was expressed as a percentage, and this was used to indicate the percentage of reduced riboflavin produced. The percentage of oxidized riboflavin produced was calculated from the increase in absorbance from 400 (0%) to 2000 s. The percentage of reduced riboflavin produced was 100%, and the percentage of oxidized riboflavin produced was no greater than 80%.

borate buffer (pH 9.0), and 1% ethanol; the total reaction volume was 4.0 mL. The reaction was started by the addition of different amounts of lipoxygenase activity. Reduced and oxidized forms of riboflavin were monitored by a spectrophotometer that recorded the increase (oxidized form) or decrease (reduced form) in absorbance at 444 nm for 20 min. Hydroperoxide was monitored by the measurement of the decrease (generation) or increase (decomposition) in absorbance at 234 nm for 20 min. The concentration of oxygen before the reaction was measured with an oxygen analyzer (Oxigraph 8, Central Kagaku Co., Tokyo, Japan).

TLC of Samples after Reaction. The formation of linoleic acid hydroperoxides and other products was checked by TLC with a mixture of n-hexane, diethyl ether, and acetic acid (60:40:1 v/v/v) as the solvent system. The TLC plate was sprayed with 50% sulfuric acid, heated at 110 °C for 20 min, and checked under a fluorescent lamp.

RESULTS AND DISCUSSION

I set out to monitor the lipoxygenase-induced changes in the conjugated diene of linoleic acid (absorbance at 234 nm) and in the isoalloxazine ring of riboflavin (absorbance at 444 nm). The absorbance at 234 nm increased for the first 400 s after lipoxygenase was added, and then it gradually decreased. This suggested that the hydroperoxide that was generated in the peroxidation reaction later decomposed. About 100 s after lipoxygenase was added, the absorbance at 444 nm decreased rapidly. It gradually increased starting about 400 s after the reaction had begun (Figure 1). This phenomenon is consistent with the conversion of reduced riboflavin to its oxidized form.

I calculated the percentage of reduced riboflavin that was generated and the percentage of hydroperoxide that was generated when different amounts of lipoxygenase activity were put in a solution containing both linoleic acid and riboflavin. With 30 units of lipoxygenase activity or more, both reduced riboflavin and hydroperoxide were generated at nearly the 100% level (Figure 2).

I next calculated the percentage of hydroperoxide that decomposed and the percentage of reduced riboflavin that returned to its oxidized form. Almost 100% of the hydroperoxide decomposed at lipoxygenase activity levels
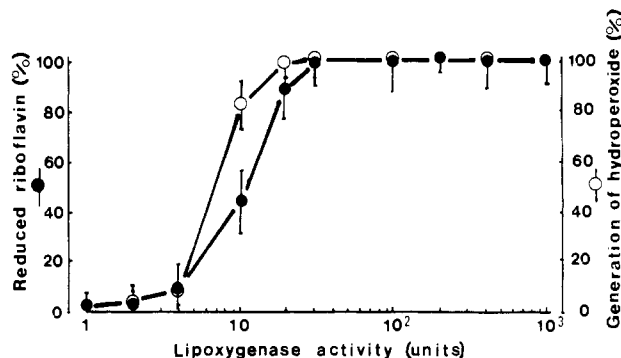

Figure 2. Effects of lipoxygenase activity on the generation of hydroperoxide and reduced riboflavin. The reaction conditions were the same as those described in the legend to Figure 1, but the amount of lipoxygenase added was varied. The amounts of hydroperoxide and reduced riboflavin produced were measured 400 s after the reaction had begun, as shown in Figure 1. Each point represents the mean of four trials, and standard deviations are shown.
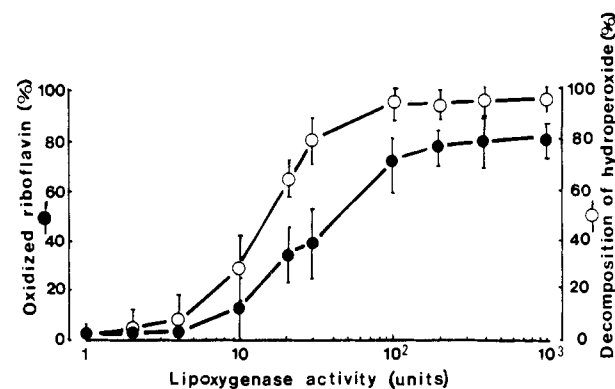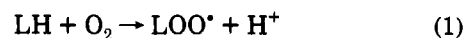

Figure 3. Effects of lipoxygenase activity on the decomposition of hydroperoxide and oxidized riboflavin measured 2000 s after the reaction had begun. The reaction conditions were the same as those described in the legend to Figure 1. Each point represents the mean of four trials, and standard deviations are shown.
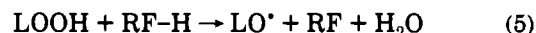
of 100 units or higher. The largest proportion of reduced riboflavin that returned to its oxidized form was 80% (Figure 3). The remaining 20% may have decomposed.

This phenomenon and the delay in the change in absorbance at 444 nm suggested that the reactions shown in Scheme I occur when lipoxygenase is added. When

Scheme I

$$LH + O_2 \rightarrow LOO^{\bullet} + H^+ \quad (1)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L \quad (2)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (3)$$

once a certain amount of LOOH is produced

$$LH + O_2 + RF \rightarrow LOO^{\bullet} + RF-H \quad (4)$$

$$LOOH + RF-H \rightarrow LO^{\bullet} + RF + H_2O \quad (5)$$

lipoxygenase is added, linoleic acid (LH) combines with oxygen, which produces peroxy radicals (LOO$^{\bullet}$). Then hydrogen is removed from LH and combines with the peroxy radicals, which produces hydroperoxide (LOOH). Peroxy radicals are then produced again, as shown in eq
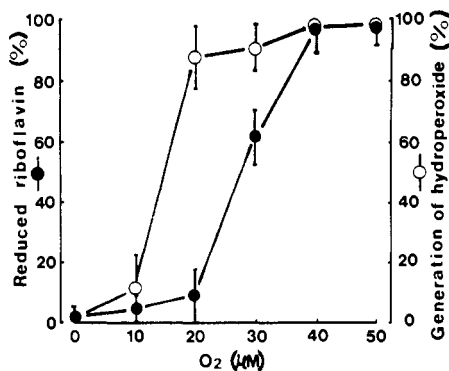
Figure 4. Effects of oxygen on the generation of hydroperoxide and reduced riboflavin. The reaction took place in a custom-built Thunberg cell, equipped with an electrode for measuring oxygen. First the amount of oxygen in the solution was adjusted by deaeration of the cell. Then the reaction was begun under the conditions described in the legend to Figure 1. Each point represents the mean of four trials, and standard deviations are shown.

3, and these peroxy radicals can react with LH (eq 2). Thus, there can be a chain reaction (eqs 2 and 3), which cases LOOH to accumulate. Once a certain amount of LOOH accumulates, LH can also react with oxygen and riboflavin (RF) (eq 4), which would produce reduced riboflavin (RF–H). The RF–H can then react with LOOH and revert to RF. In that reaction (eq 5), LOOH accepts an electron and decomposes.

Lipoxygenase generates hydroperoxide by the addition of oxygen to unsaturated fatty acids with the basic structure of 1(Z),4(Z)-pentadiene (Hellwing et al., 1990). The increase in absorbance at 234 nm recorded here was consistent with that mechanism. The effect of oxygen in the reactions shown in Scheme I seemed to be substantial. However, the question here is why absorbance at 444 nm decreased. It was not clear whether oxygen played any part in this decrease. Thus, the next step was to determine if oxygen participated in the reduction of riboflavin, in the generation of hydroperoxide, or in both. Dissolved oxygen did affect both (Figure 4). It is possible that dissolved oxygen did not contribute directly to the reduction of riboflavin, because when lipoxygenase was added, riboflavin may have received a hydrogen from the lipid (eq 4 in Scheme I), which in this experiment was linoleic acid, so the riboflavin was probably in its reduced state.

The increase in the absorbance at 444 nm (starting about 400 s after the reaction had begun) probably reflected the return of reduced riboflavin to its oxidized state. To determine if oxygen contributed to this phenomenon, its concentration was varied. Dissolved oxygen contributed nothing to the reversion of oxidized riboflavin to its reduced form or to the decomposition of hydroperoxide (data not shown). These results were consistent with the reactions shown in Scheme I, which occurred when lipoxygenase was added to a solution containing both linoleic acid and riboflavin. Although the absorbance at 234 nm increased while the absorbance at 444 nm decreased, it is possible that the riboflavin was reduced as a result of the hydroperoxide, provided that hydroperoxide was generated more rapidly than reduced riboflavin. Hydroperoxide could have acted as an electron acceptor when reduced riboflavin returned to its oxidized state. An experiment was done to determine if hydroperoxide participates in the reduction of riboflavin, or in the oxidation of reduced riboflavin, or in both. The concentration of hydroperoxide had no effect on the reduction of riboflavin, but it did affect the oxidation of reduced riboflavin (Figure 5). The lack of a relationship
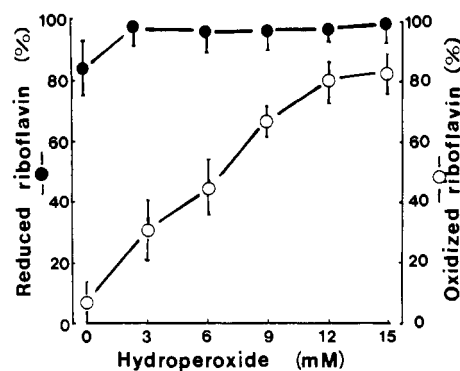

Figure 5. Effects of hydroperoxide on reduced and oxidized riboflavin. Under the conditions described in the legend to Figure 1, known concentrations of hydroperoxide were added. The percentages of reduced and oxidized riboflavin produced were measured 400 and 2000 s, respectively, after the reaction had begun, as described in the legend to Figure 1. Each point represents the mean of four trials, and standard deviations are shown.
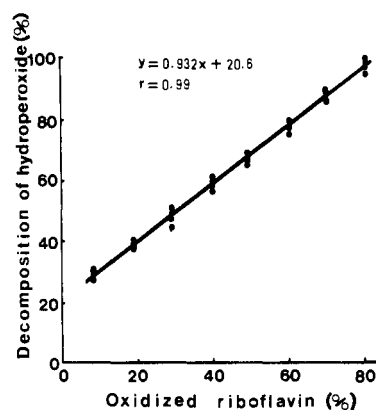

Figure 6. Correlation between the percentage of oxidized riboflavin and the percentage of decomposed hydroperoxide. The reaction conditions were the same as those described in the legend to Figure 1. First the times required to reach certain percentages of oxidized riboflavin production were measured. Then the decrease in absorbance at 234 nm was measured at various times, when a known amount of oxidized riboflavin had been produced. The amount of hydroperoxide was calculated from the amount of the conjugated diene. Then the decomposition of hydroperoxide was calculated as a percentage of the amount of hydroperoxide 400 s after the start of the reaction. These values were plotted against the corresponding values of oxidized riboflavin.

between the concentration of hydroperoxide and the reduction of riboflavin (Figure 5) is consistent with eq 4 of Scheme I. Similarly, the positive association between the concentration of hydroperoxide and the oxidation of reduced riboflavin (Figure 5) is consistent with eq 5 of Scheme I.

If Scheme I is correct, then the hydroperoxide generated in eq 2 decomposes and acts as an electron acceptor in eq 5. As the concentration of oxidized riboflavin increases, more hydroperoxide should decompose. There was a linear relationship between the percentage of hydroperoxide that decomposed and the percentage of riboflavin that oxidized (Figure 6; $r = 0.99$), which is consistent with this hypothesis.

In foods, lipid peroxidation can be caused by exposure to light. Riboflavin is photosensitive, and when it is irradiated, various forms of active oxygen may be generated. These may cause lipid peroxidation, during which the riboflavin is reduced. In the dark, the hydroperoxide generated during the reduction of riboflavin acts as an electron acceptor. Hydroperoxide may then decompose as it causes the reduced riboflavin to revert to its oxidized

form, and these reactions are quite similar to those shown in Scheme I.

In summary, the present data show that riboflavin acted as an antioxidant in this enzyme system. The mechanism proposed to account for this effect includes reversion of riboflavin from its reduced to oxidized form and decomposition of hydroperoxide. Thus, the reactions involving riboflavin in a water system (Toyosaki et al., 1989) may be similar to those in the enzyme system described here.

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