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Dimerumic acid as an antioxidant from the mold, *Monascus anka*: the inhibition mechanisms against lipid peroxidation and hemeprotein-mediated oxidation

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Abstract

This study aimed to investigate the antioxidant mechanism of dimerumic acid isolated as the active component with a radical scavenging action from the mold *Monascus anka*, traditionally used for the fermentation of foods. Dimerumic acid inhibited NADPH- and iron(II)-dependent lipid peroxidation (LPO) of rat liver microsomes at 20 and 200 μM, respectively. When ferrylmyoglobin was incubated with dimerumic acid, the myoglobin was scavenged and an electron spin resonance (ESR) signal with nine peaks was observed. The spin adduct was identified as a nitroxide radical by analysis of hyperfine structure. Similar ESR signal was also detected by incubation of dimerumic acid with peroxyl radicals. Thus, it was clarified that the antioxidant action of dimerumic acid is due to one electron donation of the hydroxamic acid group in the dimerumic acid molecule toward oxidants resulting in formation of nitroxide radical.

Keywords: *Monascus anka*; Dimerumic acid; Nitroxide radical; Lipid peroxidation; Ferrylmyoglobin; Electron spin resonance (ESR)

1. Introduction

Reactive oxygen species (ROS) are widely recognized as being involved in the pathogenesis of various diseases and the aging process [1–3]. Thus, much attention has been focused on the investigation of antioxidants that can scavenge ROS, especially natural antioxidants, phenolic and flavonoids from plants which are mostly used as protective agents against free radical-mediated disorders [4–7]. In our laboratory the antioxidant action of traditional foods was examined and dimerumic acid was isolated as a main active component from the mold *Monascus anka*, which has been used traditionally for fermentation of foods in Okinawa, Japan [8–10]. Although, dimerumic acid was once reported as a natural siderophore, characterized by high affinity for iron(III) ions [11,12], its antioxidant property was firstly demonstrated in our laboratory. Dimerumic acid at low concentrations showed strong scavenging action for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and moderate for active oxygen species such as superoxide anion (O_2^-) and hydroxyl ($^\bullet$OH) radicals [10]. The compound also protected against carbon tetrachloride-induced liver toxicity that is mediated by free radicals. However, the antioxidant ability and mechanism of dimerumic acid has not been clarified yet.

In the present study, to elucidate the antioxidant mechanism the inhibitory action of dimerumic acid on LPO in liver nicrosomes prepared from nontreated rats was examined. Moreover, the effect of dimerumic acid on peroxyl radical from 2,2′-azobis (2-amidinopropane)-dihydrochloride (AAPH) and on ferrylmyoglobin (ferrylMb) which is known as biologically significant oxidant to unsaturated fatty acid [13–15], lipoprotein [16] and contribute to post ischemic myocardial injury [17], was also examined by using electron spin resonance spectroscopy. Thus, it was

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)-dihydrochloride; CL, chemiluminescence; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; Mb, myoglobin; ESR, electron spin resonance; LPO, lipid peroxidation; TBA, thiobarbituric acid.

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confirmed that dimerumic acid donates one electron to oxidants resulting in the formation of nitroxide radical.

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), AAPH, ascorbic acid and 2,5-piperazinedione (glycine anhydride) were obtained from Wako Pure Chemicals. Myoglobin (Mb, horse heart), β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase (G6PDH), glucose-6-phosphate (G6P) and $FeSO_4 \cdot 7H_2O$ were purchased from Sigma. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) were obtained from Dojindo Laboratories. All other reagents were of analytical grade. Dimerumic acid (Fig. 1) was isolated from *M. anka* as described previously [10].

2.2. Preparation of liver microsomes

Male Sprague–Dawley rats (300–400 g) were purchased from Nihon SLC, Co. After overnight starvation rats were killed by decapitation. The liver was removed after perfusion with 1.15% potassium chloride solution and the microsomes were prepared by differential centrifugation as reported previously [18] and used for the assay of LPO and chemiluminescence (CL) formation. Protein concentration in microsomes was measured by the method of Lowry *et al.* [19].

2.3. Inhibitory action on lipid peroxidation in microsomes

The effect of dimerumic acid on enzymatic or non-enzymatic induced LPO was examined. Enzymatic LPO was induced by incubation of microsomes (1.8 mg protein/mL) in phosphate buffer (100 mM, pH 7.4 including 100 μM DTPA) with the NADPH generating system (0.3 mM G6P, 0.1 unit G6PDH, 6 mM $MgCl_2$ and 9 mM NADP). Dimerumic acid (2, 20 and 200 μM) or ascorbic acid (1, 5 and 10 mM) was added into the reaction mixture before the addition of NADP. In the non-enzymatic LPO, microsomes (3.8 mg protein/mL) in the same phosphate buffer were incubated with $FeSO_4 \cdot 7H_2O$ (100 μM) in the presence or absence of dimerumic acid. After incubation at 37° for 30 min the amount of lipid peroxide in both reaction mixture was estimated by the TBA assay [20]. The inhibition by the antioxidant of enzymatic or non-enzymatic LPO was calculated as follows.

$$\text{Inhibition}\,(\%) = \left(1 - \frac{\text{A1} - \text{A3}}{\text{A2} - \text{A3}}\right) \times 100$$

A_1 and A_2 indicate absorbances at 532 nm obtained from microsomes including LPO system with and without antioxidant, respectively. A_3 is the absorbance in microsomes without the LPO system (blank).

2.4. Measurement of CL from microsomes

Effect of dimerumic acid on cytochrome P450-derived CL was measured with a CL-detector (CLD-110, Tohoku Electronic Co.). CL was generated by mixing microsomes (1.8 mg protein) in phosphate buffer (50 mM, pH 7.4 containing 100 μM DTPA) and dimerumic acid (0.2, 2 and 20 μM) or ascorbic acid (1 mM) followed by the addition of the NADPH generating system (0.3 mM G6P, 0.1 unit G6PDH, 6 mM $MgCl_2$ and 9 mM NADP). The reaction mixture was incubated in the sample chamber of CL-detector at 37° for 15 min. The inhibition of CL-emission by the compounds was calculated as follows.

$$\text{Inhibition}\,(\%) = \left[1 - \left(\frac{\text{B1}}{\text{B2}}\right)\right] \times 100$$

B_1 and B_2 are the total photon counts from microsomes containing the NADPH generating system in the presence and absence of the antioxidant, respectively.

2.5. Measurement of hemoprotein-mediated oxidation

Oxoferryl Mb species, ferrylMb, was generated from the reaction of Mb with H_2O_2. Mb (100 μM) was incubated with H_2O_2 (10 mM) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) at room temperature. Dimerumic acid (2 mM) was added into the solution after ferrylMb formation and then the absorption spectrum (450–650 nm) was recorded using UV–VIS spectrometer (UV-160, Shimazu). The ESR spectra of the reaction mixture were also determined using a JES-FR30 (JEOL) operating at X-band with modulation frequency of 100 kHz. The reaction mixture (200 μL) was transferred to the quartz cell (50 mm long, 1.5 mm thick with 5 mm inside diameter) and ESR signal was measured at 9.4 GHz resonant frequency. Manganese oxide was used as an internal standard to determine

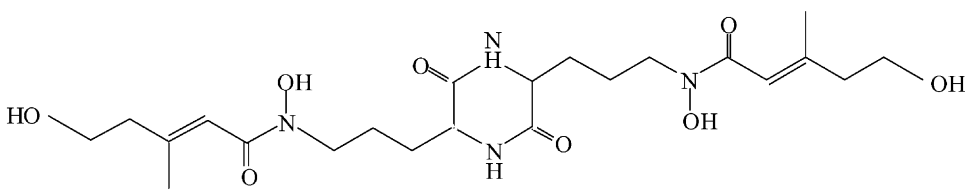


Fig. 1. Chemical structure of dimerumic acid isolated from *M. anka*.

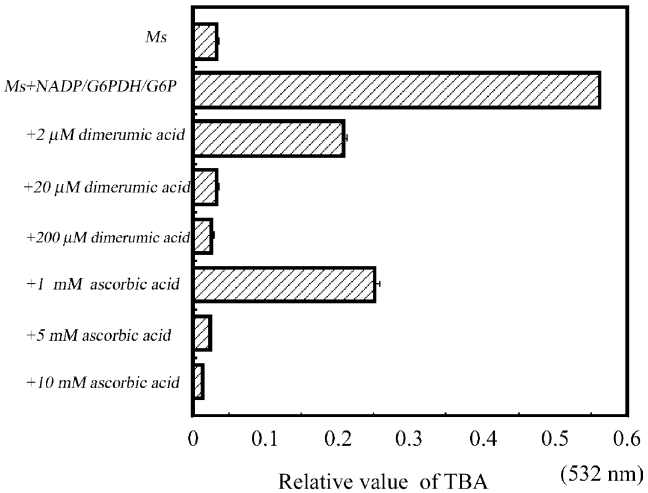


Fig. 2. Effect of dimerumic acid on NADPH-induced LPO in microsomes. Microsomes (1.8 mg protein/mL) were mixed with dimerumic acid (2, 20 and 200 μM) or ascorbic acid (1, 5 and 10 mM) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) followed by addition of the NADPH generating system. The reaction mixture was incubated at 37° for 30 min and then TBA reactive substances were measured as described in Section 2. Each column shows mean ± SD for four experiments.

g-value and hyperfine splitting constant (hfsc). The detail conditions for the measurement of ESR signals are described in figure legends.

2.6. Peroxyl radical scavenging activity

Peroxyl radicals were generated by thermal decomposition of AAPH [21,22]. AAPH (125 mM) and dimerumic acid (2 mM) were incubated with or without DMPO (40 mM) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) at 37° for 15 min and then the ESR spectrum was measured as described previously.

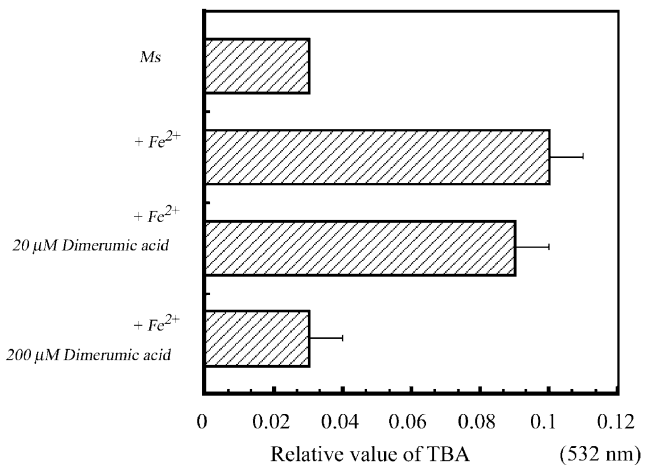


Fig. 3. Effect of dimerumic acid on iron(II)-induced microsomal LPO. Microsomes (3.8 mg protein/mL) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) was mixed with dimerumic acid (20 and 200 μM) followed by addition of $FeSO_4$ (100 μM) and incubated at 37° for 30 min. The TBA reactive substances were measured as described in Section 2. Each column shows mean ± SD for four experiments.

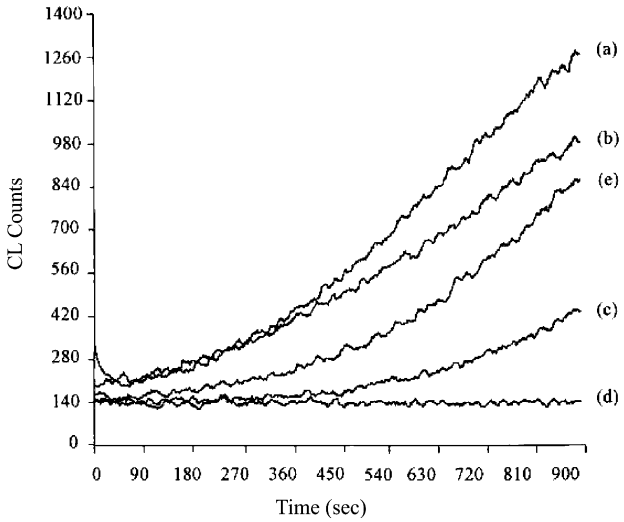


Fig. 4. Effect of dimerumic acid on cytochrome P450-derived CL-emission in microsomes. Microsomes (1.8 mg protein/mL) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) was incubated without antioxidant (a) or with dimerumic acid at 0.2 μM (b), 2 μM (c) and 20 μM (d) or 1 mM ascorbic acid (e) in the presence of the NADPH generating system at 37° for 15 min. Data shows photon counts for 15 min from each reaction mixture.

3. Results

3.1. Effect of dimerumic acid on microsomal lipid peroxidation

Antioxidant action of dimerumic acid on NADPH- or iron(II)-induced LPO in rat liver microsomes was evaluated. As shown in Fig. 2, dimerumic acid at 2 and 20 μM

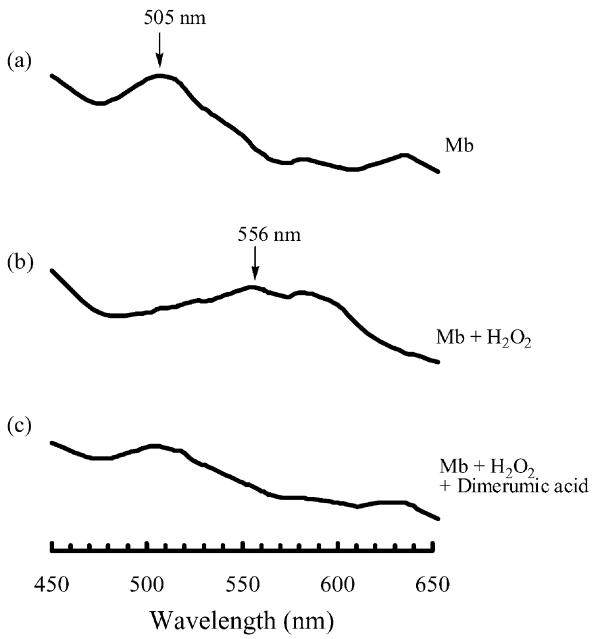


Fig. 5. Effect of dimerumic acid on Mb spectra. Mb was incubated with H_2O_2 in the absence or presence of dimerumic acid and the absorption spectra were recorded. (a) Mb (100 μM) alone, (b) Mb and H_2O_2 (10 mM) and (c) Mb with H_2O_2 (10 mM) and dimerumic acid (2 mM).

inhibited NADPH-induced LPO by 66.9 and 100%, respectively whereas ascorbic acid inhibited it by 58.3% at 1 mM and by 100% at 5 mM. Dimerumic acid at 200 μM also completely inhibited iron(II)-induced LPO (Fig. 3). These results indicate that dimerumic acid effectively acts as an antioxidant at low concentrations.

3.2. CL assay

Cytochrome P450-derived CL-emission in liver microsomes was used as the assay for an antioxidant activity of dimerumic acid. When various concentrations of dimerumic acid (0.2, 2, and 20 μM) were incubated with microsomes in the presence of the NADPH generating system, the inhibition of CL-emission was 15.1, 64.8 and 77.6%, respectively whereas inhibition by ascorbic acid at 1 mM was 39.1% (Fig. 4). Dimerumic acid at 50 μM also suppressed CL-emission even though it was given 5 min after the incubation of microsomes with the NADPH generation system (data not shown).

3.3. Scavenging of ferrylMb and peroxyl radicals

Fig. 5 showed changes in absorption spectra of Mb treated with H_2O_2 or H_2O_2 and dimerumic acid. The spectrum of Mb alone showed a peak at 505 nm whereas a peak at 556 nm, a characteristic spectrum of ferrylMb was detected after treatment of Mb with H_2O_2 (Fig. 5a and b). When dimerumic acid was added into the solution of Mb and H_2O_2, similar spectrum as seen in Mb alone was observed (Fig. 5c). It means that ferrylMb generated with H_2O_2 was reduced by dimerumic acid, showing that dimerumic acid can act as a scavenger of the strong biological oxidant, ferrylMb.

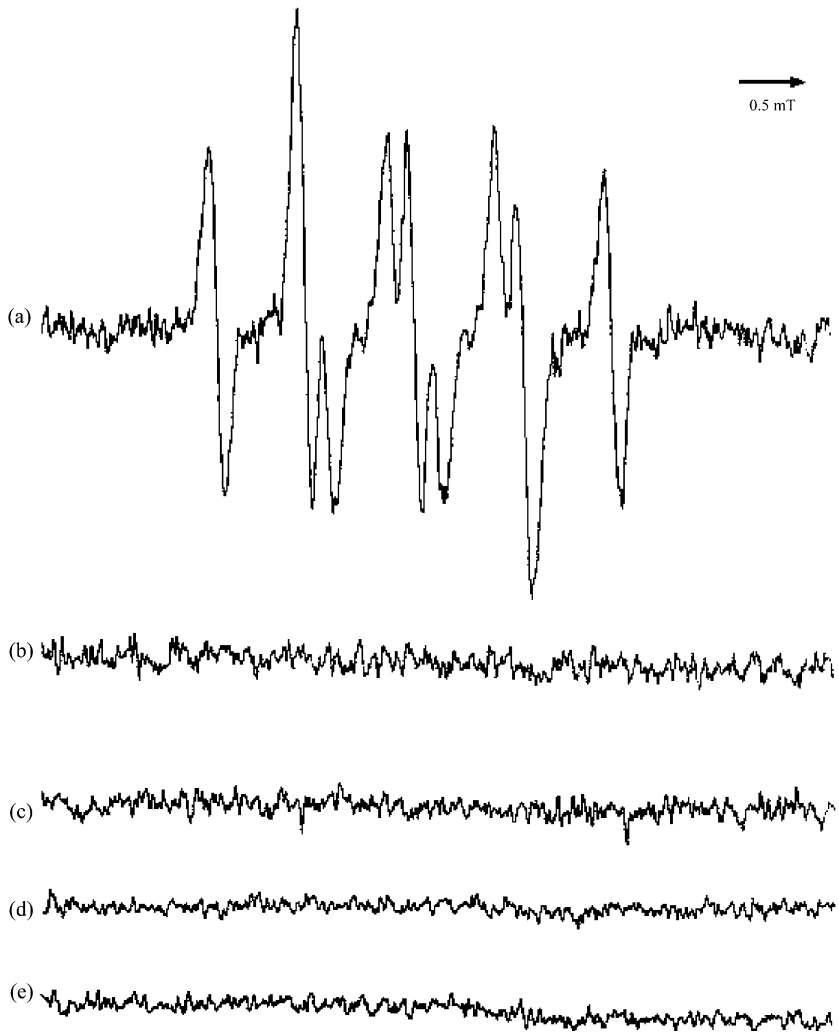


Fig. 6. ESR spectra of dimerumic acid treated with Mb and H_2O_2. Dimerumic acid was mixed in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) with Mb or H_2O_2 or both and then ESR spectra were recorded. (a) The reaction mixture contained Mb (100 μM), dimerumic acid (2 mM) and H_2O_2 (10 mM). (b) The solution was the same as (a) except dimerumic acid. (c) The solution was the same as (a) except H_2O_2. (d) The solution was the same as (a) except Mb (100 μM). (e) The reaction mixture was the same as (a) except that it contained glycine anhydride (2 mM) instead of dimerumic acid. ESR settings were as follows: microwave power, 4 mW; modulation width, 0.1 mT; gain, 500; scan time, 2 min; time constant, 0.1 s.

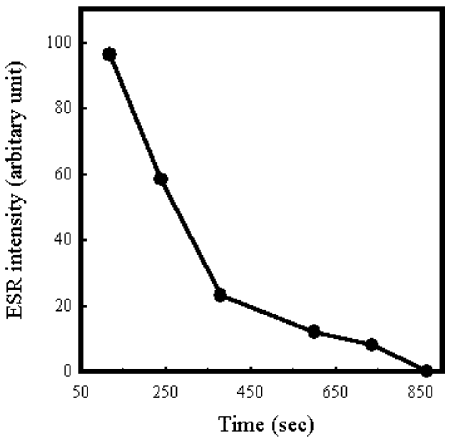


Fig. 7. The lifetime of nitroxide radical generated from dimerumic acid in the presence of Mb and H_2O_2. ESR spectrum from dimerumic acid incubated with Mb and H_2O_2 was monitored until the ESR signal was completely decayed. The ESR intensity was determined from the ratio to Mn^{2+} in mgO. The nitroxide radical was generated from Mb (100 μM), dimerumic acid (2 mM) and H_2O_2 (10 mM) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA). ESR settings were as follows: microwave power, 4 mW; modulation width, 0.1 mT; gain, 500; scan time, 2 min; time constant, 0.1 s.

To gain insight into the reactions, the ESR spectra of these solutions were measured. A nine-line of ESR signal was obtained after mixing of dimerumic acid with Mb and H_2O_2 (Fig. 6a). The ESR parameters of the signal were determined as follows: $g = 2.0084$, $\alpha N = 0.82$ mT and $\beta H = 0.66$ mT, indicating that the spin adduct generated from the reaction of dimerumic acid with ferrylMb is a nitroxide radical. This radical was not observed when dimerumic acid was incubated with Mb or H_2O_2 alone (Fig. 6c and d). As shown in Fig. 7, nitroxide radical completely decayed until 850 s, suggesting that the lifetime of the radical is short compared with that of a five member nitroxide compound such as tempol.

When AAPH was incubated with DMPO, a four-line of ESR signal was observed, showing DMPO-carbon centered radical which was formed by the reaction of DMPO with peroxyl radicals from AAPH decomposition (Fig. 8a). The four-line ESR signal was reduced to 24.1% by addition of dimerumic acid (2 mM) (Fig. 8c) but not by glycine anhydride, a part of the ring in dimerumic acid molecule (Fig. 8d). These results suggested that competitive reaction occurs between dimerumic acid and DMPO for the AAPH derived peroxyl radicals and the hydroxamic acid group in dimerumic acid could be the functional group of the antioxidant. When dimerumic acid was incubated with AAPH in the absence of DMPO, the nine-line ESR signal (marked with closed circle in Fig. 8e) as seen in ferrylMb in Fig. 6a was detected, suggesting a nitroxide radical formation. It was therefore, clarified that dimerumic acid could scavenge peroxyl radicals resulting in the formation of nitroxide radical.

4. Discussion

The effect of dimerumic acid on enzymatic and non-enzymatic LPO was examined. In the enzymatic LPO, NADPH-cytochrome P450 reductase/cytochrome P450 system generates O_2^- and H_2O_2 followed by formation of $^\bullet$OH radical leading to LPO [23,24]. In the non-enzymatic LPO iron(II) can take part in electron-transfer reactions with oxygen followed by $^\bullet$OH radical formation [25]. Thus, OH radical produced or perferryl radical may cause LPO which involves various reactive intermediates such as peroxyl radicals, singlet oxygen and exited carbonyls. CL-detector counts photon (hv) from singlet oxygen and exited carbonyls as shown in Scheme 1a. In this study dimerumic acid inhibited enzymatic LPO and CL-emission at 20 μM but non-enzymatic LPO at 200 μM. Since dimerumic acid is a siderophore, characterized by high affinity for iron(III) ions in fungus [11,12], its metal chelating ability may contribute to an inhibition of LPO. However, we added DTPA, a metal chelator, in our assay system, it was therefore, suggested that the inhibitory action of LPO by dimerumic acid mostly comes from anitoxidant ability other than metal chelating action. The low concentration of dimerumic acid needed to inhibit enzymatic LPO compared with that of non-enzymatic LPO suggests that an inhibition of cytochrome P450 enzymes by dimerumic acid might be involved because the crude extract of the mold *M. anka* inhibits P450 [9]. It was also demonstrated that peroxyl radicals are scavenged by dimerumic acid (Fig. 8). In a previous report, we showed that dimerumic acid also has a moderate scavenging action for $^\bullet$OH and O_2^- radicals [10]. Taken together it was confirmed that dimerumic acid scavenges free radicals associated with LPO resulting in inhibition of the peroxidation.

The oxoferryl moiety formed by reaction of oxygen with hemoproteins such as Mb, catalase and peroxidase is known to possess strong oxidizing abilities which can induce biological disorders such as cell damages in myocardium [17]. FerrylMb can act as a potent oxidant for xenobiotics such as the hydoxylamine derivatives which are, in turn, converted to nitroxide radical or nitric oxide [26–29]. It has been reported that desferrioxamine, a hydroxamic acid derivative, is converted to nitroxide radical by H-abstraction of hydroxamic acid group with oxidants such as O_2^-, $^\bullet$OH and oxoferryl moiety of peroxidase [30]. Nitroxide radical formation was also observed when dimerumic acid was incubated with ferrylMb or AAPH (Figs. 6 and 8). To confirm the site of nitroxide radical formation in dimerumic acid, glycine anhydride, a part of the ring in dimerumic acid was incubated with Mb and H_2O_2 or AAPH (Figs. 6e and 8d). Signals for nitroxide radical were not observed by the glycine derivative, confirming that the nitroxide radical is formed by H-abstraction of the hydroxamic acid group in dimerumic acid. These results demonstrated that the hydroxamic acid group in dimerumic acid acts as key function for its antioxidant

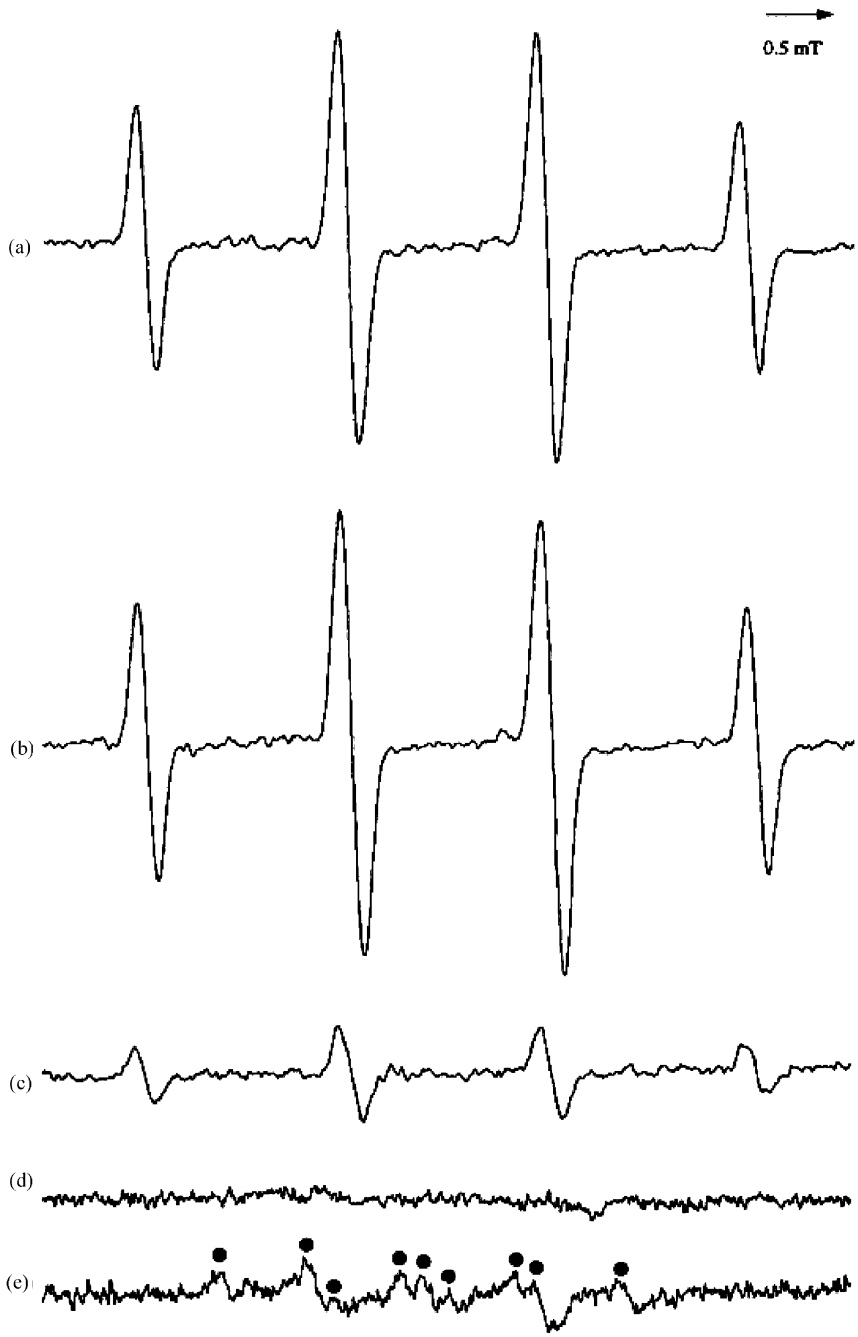
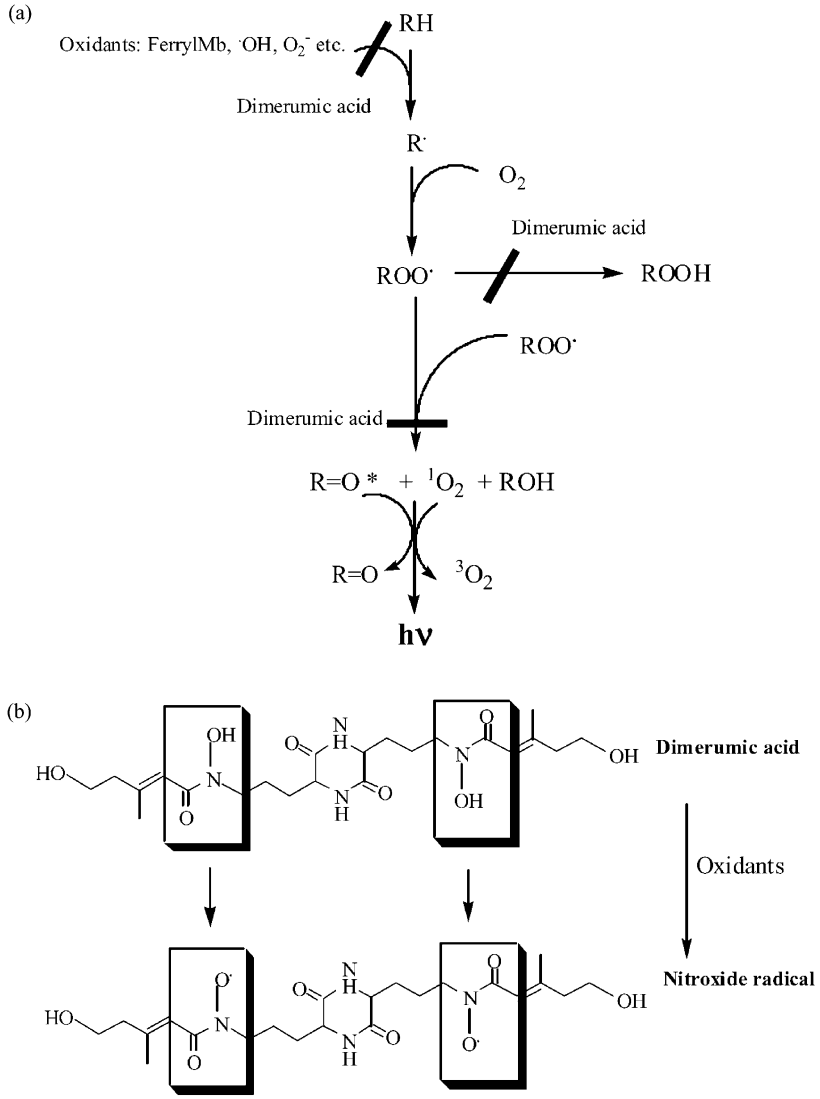


Fig. 8. ESR spectra of nitroxide radical generated from dimerumic acid in the presence of AAPH. AAPH (125 mM) and dimerumic acid (2 mM) or glycine anhydride (50 mM) were incubated with or without DMPO (40 mM) in phosphate buffer (100 mM, pH 7.4 containing 100 μM DTPA) at 37° for 15 min. (a) AAPH and DMPO. (b) AAPH, DMPO and glycine anhydride. (c) AAPH, DMPO and dimerumic acid. (d) AAPH and glycine anhydride. (e) AAPH and dimerumic acid. Closed circles (●) on the ESR signal correspond to that of nitroxide radical. ESR spectra were measured as follows: microwave power, 4 mW for (a), (b), (c) and 16 mW for (d) and (e); modulation width, 0.1 mT; gain, 500; scan time, 2 min; time constant, 0.1 s.

action. Thus, the mechanism of antioxidant action of dimerumic acid could be proposed as depicted in Scheme 1.

Nitroxide radicals have been applied in biological and medicinal fields as biophysical tools to study cell membrane fluidity and to observe free radical reactions *in vivo* [31]. As a biological activity it is known that a stable nitroxide radical, tempol has superoxide dismutase mimic function and facilitates catalase-like activity of Mb [32,,33]. Recently, it was evidenced that pyrroline carboxamide derivatives as an antiarrhythmic drug are oxidized *in vivo* followed by conversion to nitroxide radical which show defense against oxidative stress caused by H_2O_2, O_2^-, ferrylMb and peroxyl radicals [34]. Thus, our data suggested that dimerumic acid and its oxidized form, nitroxide radical could be significant antioxidant in biological system.



Scheme 1. The proposed mechanisms of antioxidant action of dimerumic acid. (a) The scheme shows the process of LPO. Each bold line indicates the sites inhibited by the antioxidant. RH, lipids; R•, alkyl radical; RO•, alkoxyl radical; ROO•, peroxyl radical; ROH, alcohol and R=O*, excited carbonyls. (b) Nitroxide radical formation from dimerumic acid. In the presence of oxidants the hydrogen atom of the hydroxamic acid group in dimerumic acid is abstracted resulting in nitroxide radical.

Desferrioxamine is an iron(III) chelating agent, which can inhibit iron-dependent free radical reaction seen in animal models of disease or toxins [35–38]. Dimerumic acid is also produced by the soil fungus *Epicoccum purpurascens* as a siderophore (iron-chelator) which plays physiological role in the fungus as conveyer of essential iron [11,,12]. Therefore, a chelating action of dimerumic acid would be expected *in vivo* as well as desferrioxamine treatment. Further studies for a chelating action of dimerumic acid are in progress in our laboratory.

In conclusion dimerumic acid is one of the antioxidant compounds in *M. anka* and can scavenge oxidants such as •OH, O_2^-, ferrylMb and peroxyl radicals, and strongly inhibit LPO. It was clarified that the antioxidant action of dimerumic acid is due to one-electron donation toward oxidants from the hydroxamic acid group in the structure.

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