Antioxidative Activities of Furan- and Thiophenethiols Measured in Lipid Peroxidation Systems and by Tyrosyl Radical Scavenging Assay

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Malonaldehyde formation from dilinolenoylphosphatidylcholine liposomes was inhibited by 2-methyl-3-furanthiol, 2-thiophenethiol, and furfuryl mercaptan. 2-Methyl-3-furanthiol and 2-thiophenethiol showed strong and comparable inhibitory activities, whereas furfuryl mercaptan showed only slight activity. 2-Methyl-3-furanthiol possessed synergistic activity with α-tocopherol in the lower concentrations. Both 2-methyl-3-furanthiol and 2-thiophenethiol scavenged tyrosyl radicals formed from L-tyrosine in aqueous solutions with activity similar to that of ascorbic acid, whereas furfuryl mercaptan and L-cysteine did not scavenge them. 2-Methyl-3-furanthiol reacted readily with H_2O_2 in aqueous buffer solutions, resulting in bis(2-methyl-3-furyl) disulfide. Scavenging of myoglobin tyrosyl radicals and the decomposition of H_2O_2 are proposed as plausible antioxidative mechanisms for 2-methyl-3-furanthiol and 2-thiophenethiol.

Keywords: *Antioxidant; lipid peroxidation; phospholipid; thiols*

INTRODUCTION

Lipid peroxidation is a major problem associated with the deterioration of food. The oxidation of muscle tissues (i.e., meats), in particular, causes off-flavor, loss of nutritive value, and changes in color and texture (Frankel, 1991). This oxidative process also leads to the production of toxic compounds such as lipid hydroperoxides, epoxides, and aldehydes. Some of the oxidative products have been implicated in human diseases such as atherosclerosis (Steinberg et al., 1989).

The oxidation of muscle tissues involves the peroxidation of unsaturated fatty acids associated with the phospholipids in cell membranes. Myoglobin, an oxygen-binding heme protein found in high concentrations in muscle tissues, has previously been shown to initiate lipid peroxidation in the presence of H_2O_2 (Kanner and Harel, 1985; Rhee et al., 1987). The autoxidation of oxymyoglobin in tissues produces metmyoglobin and the superoxide anion radical ($O_2^{\bullet -}$), which dismutates to H_2O_2 (Wallace et al., 1982). Recently, Davies (1990) demonstrated that the oxidizing equivalent of the globin moiety is associated with an oxygen-centered radical, probably a tyrosine radical. In this context, myoglobin appears to play an important role in mediating oxidative damage to cellular lipids in muscle tissues.

Thiols are thought to play a pivotal role in protecting cell membranes against lipid peroxidation. Sulfhydryl compounds such as glutathione, α-mercaptopropionylglycine, and *N*-acetylcysteine were shown to inhibit the MetMb/H_2O_2-mediated peroxidation of arachidonic acid (Mitsos et al., 1988). Recently, several heteroaromatic thiol compounds such as 2-thiophenethiol, 2-methyl-3-furanthiol, and furfuryl mercaptan formed in Maillard reaction systems were found to possess antioxidative activity in nonaqueous systems (Eiserich and Shibamoto, 1994). However, the antioxidative activity of these thiols may be different in an aqueous condition. In the present study, therefore, the antioxidative activity of 2-methyl-3-furanthiol, 2-thiophenethiol, and furfuryl mercaptan was measured by monitoring malonaldehyde (MA) formation in aqueous lipid peroxidation systems and by tyrosyl radical scavenging assay.

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EXPERIMENTAL PROCEDURES

Materials. 2-Methyl-3-furanthiol, furfuryl mercaptan, benzothiazole, and 2-methylpyrazine were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-Methylhydrazine was bought from Fluka Chemical Co. (Buchs, Switzerland). 2-Thiophenethiol was purchased from Lancaster (Windham, NH). L-Tyrosine, superoxide dismutase (SOD; from bovine erythrocytes, 3000 units/mg), thymol-free catalase (purified powder from bovine liver, 11 000 units/mg), H_2O_2, α-tocopherol, butylated hydroxytoluene (BHT), and horse heart myoglobin were purchased from Sigma Chemical Co. (St. Louis, MO). Dilinolenoylphosphatidylcholine (DLPC) was bought from Avanti Polar Lipids (Alabaster, AL) and was stored under nitrogen at −20 °C. Standard 1-methylpyrazole was synthesized according to a method previously reported (Umano et al., 1988).

Preparation of Phospholipid Liposomes. The method of liposome preparation was as described by Kagan et al. (1992) with slight modification. A chloroform solution of DLPC was evaporated to dryness under a nitrogen stream. The residual material was brought up in phosphate buffer (10 mM, pH 7.4, at 37 °C) under nitrogen at 4 °C. The lipid dispersion was then sonicated at 4 °C under a nitrogen stream to form multilamellar liposomes for immediate use in oxidation assays.

Oxidation of Phospholipid Liposomes. Solutions (5.0 mL) containing 10 mM phosphate buffer (pH 7.4), 54 μM myoglobin, 36 μM H_2O_2, and 255 μM DLPC (liposomes) were incubated in the presence or absence of antioxidants at 37 °C for 2 h. Each antioxidant was added in an ethanol solution (the ethanol content never exceeded 0.5% of the final volume), at concentrations of 12.5, 25, and 50 μM with or without α-tocopherol. Control samples without added antioxidants also included ethanol (0.5%). The oxidation was terminated with the addition of BHT (40 μg) and catalase (100 μg).

Determination of Malonaldehyde (MA) in Oxidized Phospholipid Liposomes. Samples were analyzed for MA according to the method reported by Wong et al. (1991) and Ichinose et al. (1989) with a slight modification. Each 5.0-mL sample was mixed with 1.0 mL of 6 N HCl and 50 μL of *N*-methylhydrazine and allowed to stand for 1 h at room

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temperature with stirring. The pH of the mixture was adjusted to 7.0 with 6 N NaOH, and then the mixture was centrifuged at 10000*g* and 4 °C for 10 min to remove precipitated proteins. The aqueous supernatent was collected, and the *N*-methylpyrazole was cleaned using C_{18} solid-phase extraction cartridges (Varian Sample Preparation Products, Harbor City, CA). 1-Methylpyrazole was eluted from the cartridge with 4 mL of dichloromethane, and the eluate was concentrated to 1 mL under a nitrogen stream. 2-Methylpyrazine (15 nmol/mL) was added as a gas chromatographic internal standard.

A Hewlett-Packard (HP) Model 5890 gas chromatograph (GC) equipped with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) bonded-phase DB-Wax fused silica capillary column (J&W Scientific, Folsom, CA) and a nitrogen–phosphorus detector (NPD) was used for routine quantitative analysis. The oven temperature was held at 60 °C for 2 min and then programmed to 190 °C at 3 °C/min. Injector and detector temperatures were 250 °C. Linear helium carrier gas flow rate was 30 cm/s with a split ratio of 1:25. The GC peak areas were integrated with a System Instruments Model 7000B integrator. A GC standard calibration curve for 1-methylpyrazole was prepared according to the method reported previously (Ettre, 1967).

Tyrosyl Radical Scavenging Assay. The fluorescence-based tyrosyl radical scavenging assay developed by Holler and Hopkins (1989) was used without modification. L-Tyrosine (400 μM), SOD (20 μg/mL), and catalase (20 μg/mL) were mixed in a 100 mM phosphate buffer solution (pH 7), and the final volume of the solution was adjusted to 1 mL in a fluorescence cuvette. Fluorescence emission was observed at 405 nm, with a slit width of 2 nm. Ascorbic acid and L-cysteine were added as 10 mM phosphate buffer solutions. 2-Methyl-3-furanthiol, 2-thiophenethiole, and furfuryl mercaptan were added as 2 μM ethanolic solutions. Fluorescence measurements were made with a Shimadzu Model RF-540 spectrofluorophotometer every 15 s. The excitation wavelength used was 275 nm, with a slit width of 20 nm.

Reaction of 2-Methyl-3-furanthiol with H_2O_2. Solutions (5 mL) containing 10 mM phosphate buffer (pH 7.4), 1 mM 2-methyl-3-furanthiol, and 0.5 mM H_2O_2 were allowed to stand at room temperature for various periods of time (0–5 min) with stirring. The reactions were terminated by the addition of catalase (1 mg) to consume unreacted H_2O_2. The aqueous samples were immediately cleaned using C_{18} solid-phase extraction cartridges with 4 mL of dichloromethane. The eluates were adjusted to a final volume of 5 mL with dichloromethane, and then benzothiazole (0.5 mM) was added as a gas chromatographic internal standard.

Monitoring the Reaction between 2-Methyl-3-furanthiol and H_2O_2. The loss of 2-methyl-3-furantiol and formation of the corresponding reaction products were monitored by an HP Model 5890 GC equipped with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB-1 bonded phase fused silica capillary column and a flame photometric detector (FPD) set in the sulfur mode. The relative amounts of compounds were determined using a relative peak area (RPA). The RPA of each compound was calculated by dividing the peak area of the compound by the peak area of the internal standard (benzothiazole). The injector and detector temperatures were 250 °C. The GC oven temperature was programmed from 80 to 250 °C at 5 °C/min. The linear velocity of the helium carrier gas flow was 27 cm/s with a split ratio of 1:25.

Mass spectral (MS) identification of the reaction product, bis(2-methyl-3-furyl) disulfide, was performed on an HP Model 5971 series mass selective detector (MSD) interfaced to an HP Model 5890 gas chromatograph. Mass spectra were obtained by electron impact ionization at 70 eV and a source temperature of 250 °C. The capillary column and GC conditions were as described above.

RESULTS AND DISCUSSION

Myoglobin (Mb) reacts with H_2O_2 to form reactive oxygen-centered radicals that can induce lipid peroxidation as summarized in Figure 1. Metmyoglobin, the ferric state of the heme protein, reacts initially with H_2O_2 to form a ferri–peroxide complex (reaction 1) that decomposes to either the ferryl myoglobin radical and/or the hydroxyl radical (reaction 2) (Davies, 1990; Puppo and Halliwell, 1988). Both radicals induce lipid peroxidation (reaction 3). The reduction of ferryl myoglobin (Fe^{4+}) to metmyoglobin (Fe^{3+}) via electron transfer has been observed for various thiol-containing compounds including glutathione and dihydrolipoic acid (Romero et al., 1992).

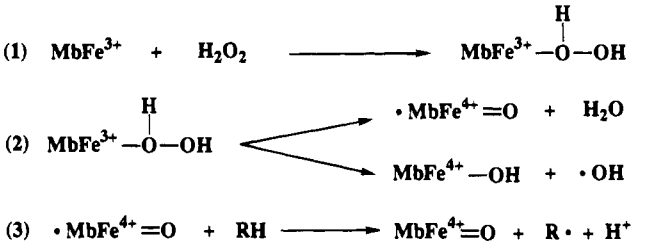

Figure 1. Formation mechanisms of oxidative species produced from the interaction of H_2O_2 with metmyoglobin ($MbFe^{3+}$).

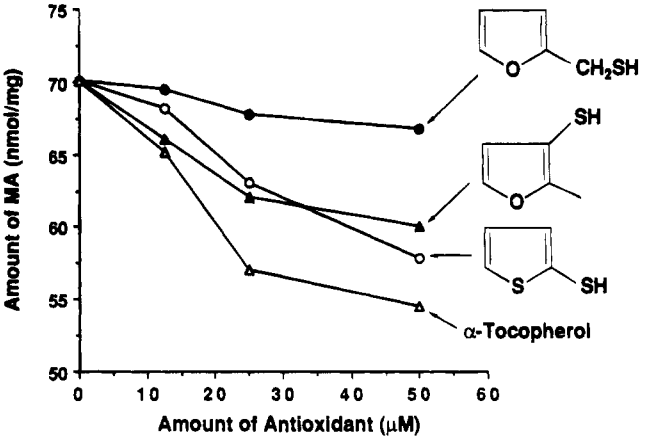

Figure 2. Inhibitory activity of furfuryl mercaptan (●), 2-methyl-3-furanthiol (▲), 2-thiophenethiol (○), and α-tocopherol (△) to MA formation in DLPC liposomes oxidized with Mb/H_2O_2. MA concentration is shown per milligram of phospholipid.

Inhibitory Effects of Thiols. Figure 2 shows the inhibitory activity of 2-methyl-3-furanthiol, 2-thiophenethiol, furfuryl mercaptan, and α-tocopherol in various concentrations. All compounds displayed a dose–response inhibition in the formation of MA. 2-Methyl-3-furanthiol, 2-thiophenethiol, and α-tocopherol at 50 μM showed some antioxidative activity, inhibiting MA formation by 17, 19, and 23%, respectively. Direct attachment of the thiol group to the aromatic ring may provide moderate antioxidative activity due to the formation of a stable intermediate aromatic thiyl radical generated upon H• abstraction (Eiserich and Shibamoto, 1994). On the other hand, furfuryl mercaptan (50 μM), which produced aliphatic thiyl radical, inhibited MA formation only slightly (5%).

When 2-methyl-3-furanthiol (12.5 μM) and α-tocopherol (12.5 μM) were tested for antioxidative activity separately, 2-methyl-3-furanthiol and α-tocopherol inhibited the formation of MA by 5.9 and 7.3%, respectively, whereas a combination of the two (12.5 μM of each) resulted in a 26.5% inhibition of MA formation. Total effects would be 13.2%, but a synergistic effect resulted in 26.5%. Food and biological systems are very complex in both the number and the type of chemicals

$$\text{Tyr-OH} \xrightarrow{h\nu} \text{Tyr-OH}^*$$

$$\text{Tyr-OH}^* \longrightarrow \text{Tyr-O}\cdot + \text{H}^+ + \text{e}^-$$

$$2\ \text{Tyr-O}\cdot \longrightarrow \text{Dityrosine}$$

Figure 3. Photooxidative formation mechanism of the fluorescent dityrosine used in the tyrosyl radical scavenging assay [adapted from Holler and Hopkins (1989)].

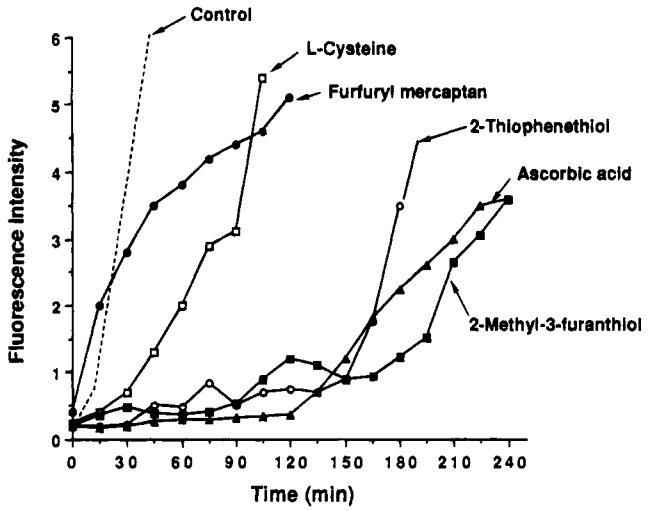

Figure 4. Effect of 2 μM concentrations of 2-thiophenethiol, furfuryl mercaptan, L-cysteine, ascorbic acid, and 2-methyl-3-furanthiol on the formation of dityrosine. The control sample contained no added antioxidants. Fluorescence intensity is based on a relative scale.

present. There may be many compounds possessing a synergistic activity of inhibition toward lipid peroxidation.

Tyrosyl Radical Scavenging Assay. The predominant radical formed at relatively low H_2O_2/Mb ratios (i.e., when H_2O_2 is not in excess) is associated with the globin moiety and, more specifically, centered on a tyrosine residue (Davies, 1990). The tyrosyl radical may be responsible for initiating lipid peroxidation. In the absence of any other radicals, tyrosine phenoxyl radicals form dityrosine, which can be monitored by its characteristic fluorescence at 405 nm (Holler and Hopkins, 1989). The formation mechanisms involved in these reactions are shown in Figure 3. Reduction or "repair" of tyrosyl radicals by 2-thiophenethiol, 2-methyl-3-furanthiol, furfuryl mercaptan, L-cysteine, and ascorbic acid at 2 μM is shown in Figure 4.

The formation of the fluorescent dityrosine is extremely rapid in the absence of any antioxidant. Ascorbic acid inhibited this transformation within approximately 2.5 min. 2-Methyl-3-furanthiol and 2-thiophenethiol showed activity similar to that of ascorbic acid. L-Cysteine and furfuryl mercaptan (both aliphatic thiols) did not suppress the formation of the dityrosine significantly. The aromatic character of 2-methyl-3-furanthiol and 2-thiophenethiol may be necessary for reduction of the tyrosyl radical. These results are consistent with the previous report. Aliphatic thiols (such as glutathione) suppressed just slightly the formation of dityrosine residues, whereas ovothiol A (an aromatic mercaptohistidine derivative) suppressed formation considerably (Holler and Hopkins, 1989). Holler and Hopkins (1990) also observed that ovothiol A was much more effective compared to glutathione in scavenging a variety of oxygen-centered free radicals including Fremy's salt and Banfield's radical.

The transfer of an electron from aromatic thiols to oxygen-centered radicals such as the tyrosyl radical is more thermodynamically favorable than that for an aliphatic thiol (Holler and Hopkins, 1989). Further-

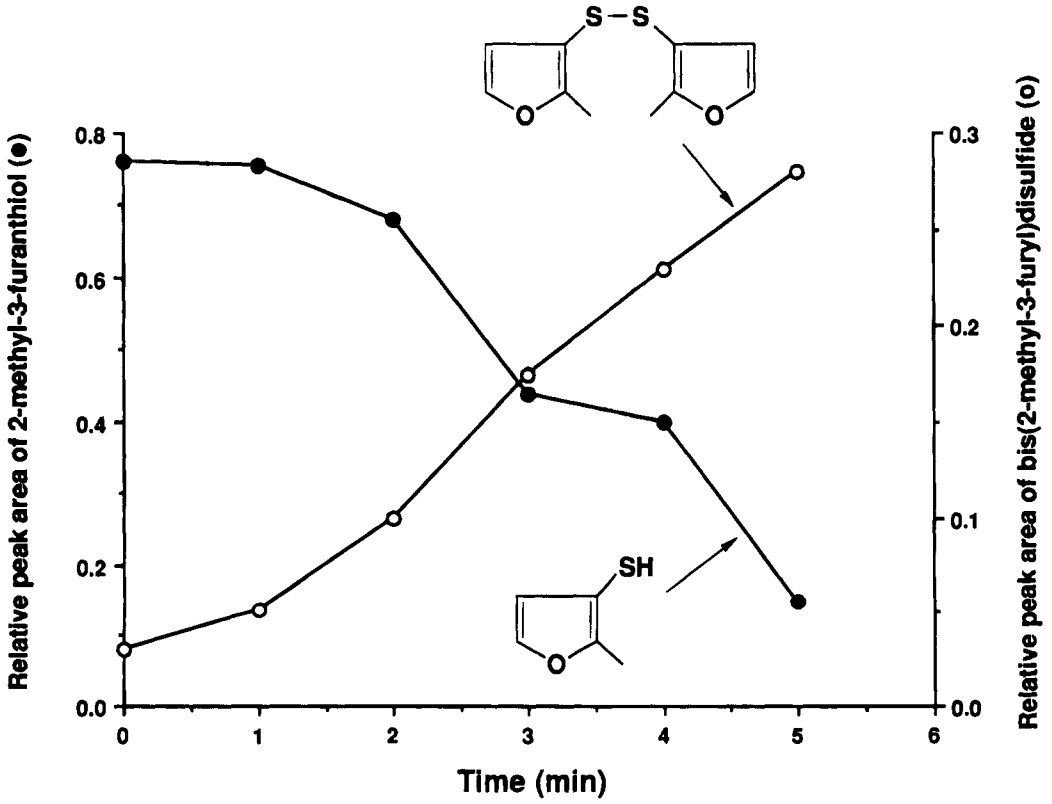

Figure 5. Depletion of 2-methyl-3-furanthiol upon reaction with H_2O_2 and the subsequent formation of the bis(2-methyl-3-furyl) disulfide.

more, the aromatic thiolate anion is more nucleophilic than the aliphatic thiol and may explain its high reactivity with H_2O_2. The superiority of aromatic thiols (such as 2-methyl-3-furanthiol, 2-thiophenethiol, and ovothiol A) compared to aliphatic thiols (such as cysteine, glutathione, and furfuryl mercaptan) is also due to the inherent advantage of forming a stable aromatic (delocalized) radical rather than a reactive aliphatic thiyl radical.

Reaction of 2-Methyl-3-furanthiol and H_2O_2. The decomposition of H_2O_2 in oxidizing systems is an important path by which thiol-containing compounds exert antioxidative effects (Aruoma et al., 1989). The possible reaction between H_2O_2 (0.5 mM) and 2-methyl-3-furanthiol (1 mM) was, therefore, investigated. Thiol compounds interfere with the peroxidase-based assay for H_2O_2 because they are substrates for horseradish peroxidase (Halliwell and De Rycker, 1978) and, hence, H_2O_2 decomposition could not be directly measured. The reaction, therefore, was followed by the loss of 2-methyl-3-furanthiol and subsequent formation of the corresponding bis(2-methyl-3-furyl) disulfide. Nearly complete oxidation of 2-methyl-3-furanthiol to the disulfide occurred within 5 min as shown in Figure 5. The same phenomenon was observed in an organic solution system (Eiserich et al., 1992). These results are consistent with the observation made by Turner et al. (1988) that the aromatic ovothiol is rapidly oxidized to the ovothiol disulfide by H_2O_2 affording an antioxidative protective mechanism.

Conclusions. Scavenging of oxygen-centered radicals such as the biochemically relevant tyrosine phenoxyl radical may be a reasonable antioxidative mechanism for the aromatic thiols studied. The nucleophilic thiol group scavenges peroxyl and alkoxyl radicals. The five-membered heteroaromatic rings such as thiophene and furan can scavenge a hydroxy radical (Eiserich et al., 1992). The aromatic thiols investigated in the present study may reduce the high oxidation state of the heme iron in myoglobin, and they deserve further attention. The present study proved that these thiols have antioxidative activity in an aqueous system as well as in an organic system (Eiserich and Shibamoto, 1994). Moreover, numerous aliphatic and aromatic thiols are formed in foods as a result of the Maillard reaction (Shibamoto, 1980, 1984). Further investigation of their impact on foods and biological systems is in order.

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