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ARTICLE

Electron Spin Resonance and Luminescence Spectroscopic Observation and Kinetic Study of Chemical and Physical Singlet Oxygen Quenching by Resveratrol in Methanol

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Electron spin resonance (ESR) spectroscopy and near-infrared (NIR) fluorescence spectroscopy were performed to observe singlet oxygen quenching by resveratrol. Resveratrol greatly decreased the 2,2,6,6-Tetramethyl-4-piperidone-*N*-oxyl radical signal as determined by ESR spectroscopy. Resveratrol also efficiently decreased luminescence emission at 1268 nm as studied with a NIR spectrofluorometer, showing positive evidence of singlet oxygen quenching by resveratrol. The total singlet oxygen quenching rate constant ($k_r + k_q$) of resveratrol in methanol was determined to be 2.55×10^7 M^{-1} s^{-1}. The singlet oxygen chemical quenching rate constant (k_r) of resveratrol was calculated by measuring its reaction rate with singlet oxygen relative to that of α-terpinene in the same solution under light illumination. The k_r value of resveratrol was 1.15×10^6 M^{-1} s^{-1}. The percent partition of chemical quenching over total singlet oxygen quenching $(k_r \times 100)/(k_r + k_q)$ for resveratrol was 5.11%. The results showed that resveratrol quenches singlet oxygen almost exclusively through the mechanism of physical quenching. Resveratrol showed a protective activity similar to that of BHA on the methylene blue sensitized photooxidation of α-terpinene. This unambiguously explains the mechanism of how resveratrol protects tissues and cells in biological systems or important nutrients in food systems against their photosensitized oxidations.

KEYWORDS: Resveratrol; singlet oxygen; quenching; ESR; near-IR fluorescence spectroscopy

INTRODUCTION

Singlet molecular oxygen ($^1O_2$) in its lowest excited state ($^1\Delta_g$) is known to be extremely reactive to electron-rich compounds such as lipids, vitamins, nucleic acids, and proteins, resulting in fast oxidative losses of these molecules (*1*−*6*). For example, singlet oxygen induces lipid oxidation approximately 1450 times more quickly than ordinary triplet oxygen (*7*). It has been also reported that singlet oxygen plays an important role in the induction of gene expression (*8*−*10*). Singlet oxygen is generated in food systems such as milk and oils (*5*, *6*) and biological systems such as skin, eyes, and plant chloroplasts by photosensitization reactions (*11*, *12*). Metabolic reactions of phagocytes, prostaglandin endoperoxide synthetase, and cytochrome P-450 or during lipid peroxidation catalyzed by plant lipoxygenases are also known routes of singlet oxygen production in biological systems (*13*).

Resveratrol, a class of polyphenolic compounds called stilbenes, is naturally present in grapes and berries of *Vaccinium* species (*14*, *15*). Resveratrol has attracted much attention because of its beneficial health functions including antioxidant activities. Resveratrol has been proposed to have singlet oxygen quenching ability (*16*−*19*). Rimando et al. (*18*) reported that resveratrol and pterostilbene were effective in inhibiting electrolyte leakage caused by acifluorfen-induced photooxidative damage in a plant system. Sparrow et al. (*19*) also reported that resveratrol reduced the formation of pyridinium bisretinoid (A2E)-epoxide from the A2E oxidation with singlet oxygen induced by blue light illumination (430 nm). The authors proposed that the inhibitory action of resveratrol might be due to its singlet oxygen quenching ability (*18*, *19*). There was, however, no previous report of direct evidence of singlet oxygen quenching activity of resveratrol. It has been reported that singlet oxygen can be directly visualized by monitoring its luminescence at around 1270 nm (*11*). Because singlet oxygen is short-lived (few microseconds) and the luminescence signal of singlet oxygen is very weak, it requires a very sensitive detection system (*11*).

The total singlet oxygen quenching rate constant ($k_r + k_q$) of resveratrol also has never been previously reported. There are two different singlet oxygen quenching mechanism, physical (k_q) and chemical quenching (k_r) mechanisms (*4*, *7*). If the chemical quenching rate (k_r) of an antioxidant is high, the antioxidant is rapidly consumed along with singlet oxygen quenching, and thus its antioxidant activity cannot last long. Thus, for the evaluation of antioxidant efficiency as a singlet oxygen quencher, it is essential to determine its k_r value along with a $k_r + k_q$ value. However, $k_r/(k_r + k_q)$, the ratio of the chemical quenching rate to the total singlet oxygen quenching rate of resveratrol, also has never been previously reported.

The objectives of this research were (1) to monitor indirectly the singlet oxygen quenching of resveratrol by electron spin

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resonance (ESR) spectroscopy, (2) to directly observe singlet oxygen quenching by resveratrol by monitoring luminescence at 1268 nm with a near-infrared (NIR) spectrofluorometer equipped with a highly sensitive InGaAs detector, (3) to determine the total ($k_r + k_q$) and the chemical (k_r) singlet oxygen quenching rate constants of resveratrol, and (4) to find a dominant quenching mechanism by calculating a ratio of $k_r/(k_r + k_q)$ for resveratrol.

MATERIALS AND METHODS

Materials. Methylene blue (MB), rose bengal, butylated hydroxyanisole (BHA), α-terpinene, resveratrol, and $CDCl_3$ containing teramethylsilane were purchased from Sigma Chemical Co. (St. Louis, MO). Water (HPLC grade) and hexane (HPLC grade) were purchased from Fisher Scientific (Loughborough, LE, U.K.). Methanol (HPLC grade) and acentonitrile (HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ). 2,2,6,6-Tetramethyl-4-piperidone (TMPD) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

ESR Spectroscopy. The formation of water-soluble TMPD-*N*-oxyl radical as a result of the reaction of singlet oxygen with TMPD was monitored by ESR spectroscopy to check the singlet oxygen quenching ability of resveratrol in the TMPD−MB system, as described previously with slight modification (*20*−*22*). For this, a Bruker (EMX−) ESR spectrometer was used at an operating frequency of approximately 9.77 GHz, microwave power of 40 mW, and central magnetic field strength of 3480 G. The scan rate, number of scans, and amplitude modulation for ESR analysis were 18G/min, 1, and 4.00 G, respectively. A total of 800 μL of oxygen-saturated methanol solutions containing TMPD (18.75 mM), methylene blue (8 ppm), and resveratrol (0−1.32 mM) were prepared in duplicate. The prepared solutions (150 μL) were pippetted into ESR tubes. The samples in the ESR tubes were irradiated for 15 min through a condenser lens of (>325 nm) with a light source (tungsten halogen lamp, 40 W) covered with five sheets of overhead projector film. The distance between the condenser lens and sample tube was 15 cm, and the light intensity was 48000 lx. Air was blown to the sample with an electric fan to maintain the temperature at 23 ± 2 °C.

NIR Fluorescence Spectroscopy for Singlet Oxygen Detection. For the direct visualization of singlet oxygen emission, a spectrofluorometer (NanoLog, HORIBA Jobin Yvon, Edison, NJ) equipped with a 450 W xenon lamp, single-grating excitation and emission monochromators, and NIR accessories (InGaAs detector and emission grating at 600 grooves mm^{-1}, blazed at 1000 nm) was used (*11*). The detector was cooled with liquid nitrogen to ensure high sensitivity. A high-pass filter (λ = 780 nm) was used as an order-sorter. The emission spectrum was obtained using an integration time of 30 s (for rose bengal) or 120 s (for methylene blue) and a bandpass of 14 nm. To enhance the yield of $^1O_2$, methanol was saturated with O_2 by purging with pure oxygen for 5 min. Sample solutions containing methylene blue or rose bengal (3.5 μg/mL) as photosensitizers and different concentrations of resveratrol in methanol were prepared.

Determination of Total Singlet Oxygen Quenching Rate ($k_r + k_q$). The singlet oxygen quenching rate constant of resveratrol in methanol was determined according to the previously reported method with a slight modification (*23*−*25*). In this study, singlet oxygen was generated in a photochemical way in methanol containing methylene blue. The sample solutions containing 0.2×10^{-3} M α-terpinene (as a singlet oxygen trap), resveratrol (as a singlet oxygen quencher, $(0-4) \times 10^{-3}$ M), and methylene blue (as a sensitizer, 13.2 μg/mL) in oxygen-saturated methanol were prepared. Then, 10 mL of the prepared sample solutions was transferred into 20 mL capacity glass vials with screw caps. The prepared sample vials were stored in a light storage box and illuminated at 25 ± 1 °C for 20 min under the condition of 3000 lx fluorescence light (*26*, *27*). An aliquot (200 μL) of sample solution was taken at 4 min intervals. The contents of α-terpinene in the solutions were measured by gas chromatography (GC). Triplet determinations of singlet oxygen quenching rates for resveratrol were carried out.

Determination of Chemical Singlet Oxygen Quenching Rate (k_r) of Resveratrol. For the determination of the k_r value of resveratrol, sample solutions containing α-terpinene (0.25×10^{-3} M), resveratrol (0.25×10^{-3} M), and methylene blue (13.2 μg/mL) in oxygen-saturated methanol were prepared. Ten milliliters of the prepared sample solutions were transferred into 20 mL capacity glass vials with screw caps. The prepared sample vials were illuminated at 25 ± 1 °C for 24 min under the condition of 3000 lx fluorescence light. An aliquot (200 μL) of sample solution was taken at 4 min intervals. The contents of α-terpinene and resveratrol in the solutions were measured by GC and HPLC, respectively. The k_r value of resveratrol was determined, in triplicate, by measuring the relative oxidation rate of resveratrol as compared to the oxidation rate of α-terpinene, the k_r value of which is known, in the same solution (*23*).

Determination of Antiphotooxidative Activity As Compared with BHA. Sample solutions containing α-terpinene (0.2×10^{-3} M), methylene blue (13.2 μg/mL), and resveratrol or BHA (4.0×10^{-3} M) in oxygen-saturated methanol were prepared. Ten milliliters of the prepared sample solutions was transferred into 20 mL capacity glass vials with screw caps in triplicate. The prepared sample vials were stored in a light storage box at 25 ± 1 °C for 15 min as described previously. Then, the contents of α-terpinene in the solutions were measured by GC. The light intensity of the sample level was 3000 lx. The temperature within the light box was 20 ± 1 °C during light storage.

GC for the Determination of α-Terpinene Contents. The contents of α-terpinene were determined by a gas chromatograph (GC-2120, Shimadzu, Japan) equipped with a flame ionization detector. The injector and detector temperatures were 140 and 250 °C, respectively. The oven temperature was programmed with an initial temperature of 70 °C for 1 min and then an increase at the rate of 7 °C/min to 180 °C. The column used was a nonpolar capillary column (ZB-5 ms, 30 m × 0.25 mm, 0.25 μm film thickness, Phenomenex, Torrance, CA). Helium gas was used as a carrier gas with a head pressure of 100 kPs. The GC peak was identified by GC-MS and comparison of its retention time with that of authentic sample under identical GC conditions. The ratio of its peak area to that of known amounts of authentic α-terpinene was used for the calculation of quantity of α-terpinene.

Gas Chromatography−Mass Spectrometry (GC-MS). The identification of major oxidation products of α-terpinene by methylene blue sensitized photooxidation was carried out with a gas chromatograph−mass spectrometer (Perkin-Elmer). The electron ionization of mass spectra was 70 eV. The GC conditions for GC-MS such as carrier gas and column used, carrier gas head pressure, injector and detector temperatures, and oven temperature programs were identical as those used for α-terpinene as described before.

NMR Spectroscopy. For NMR spectroscopy, the photoinduced reaction product of α-terpinene was extracted from the light-illuminated sample. Two hundred milliliters of the light-illuminated samples was concentrated to about 5 mL by nitrogen flushing. Then, 20 mL of double-distilled water was added to the sample. The reaction product including compound **A** was extracted twice from the solution with 20 mL of hexane. Then the hexane was removed by nitrogen flushing. ^1H and ^{13}C NMR spectra were measured on a JEOL JNM-X400 spectometer in $CDCl_3$ with teramethylsilane as internal standard, and chemical shifts are given as δ values.

High-Performance Liquid Chromatography (HPLC) for the Determination of Resveratrol Contents. The contents of resveratrol were determined by a HPLC (SCL-10A vp, Shimadzu, Kyoto, Japan). The column used was a reverse phase column (μ-Bondapak C18, 30 mm × 0.25 mm, 0.25 μm film thickness, Waters). A fluorescence detector (RF-10A XL, Shimadzu) with excitation at 300 nm and emission at 390 nm was used to quantify the resveratrol in the solutions. An isocratic mobile phase (water/acetonitrile, 75:25) was used. After each injection, the column was cleaned with 100% acetonitrile for 5 min. The HPLC peak was identified by comparison of its retention time with that of authentic sample under the identical gas chromatographic conditions. The ratio of its peak area to that of known amounts of authentic resveratrol was used for the calculation of quantity of resveratrol in the sample

Statistical Analysis. Analysis of variance (ANOVA) and Duncan's multiple-range test (a post hoc test) were used to ascertain the effects of antioxidants on the methylene blue-sensitized photooxidation of α-terpinene at α = 0.05 by using a SPSS statistical analysis program (SPSS 14.0K, SPSS, Chicago, IL).

RESULTS AND DISCUSSION

ESR Spectroscopy. The TMPD-*N*-oxyl radical peaks were monitored with ESR spectroscopy to indirectly detect singlet oxygen induction in the TMPD−MB system. The ESR signal

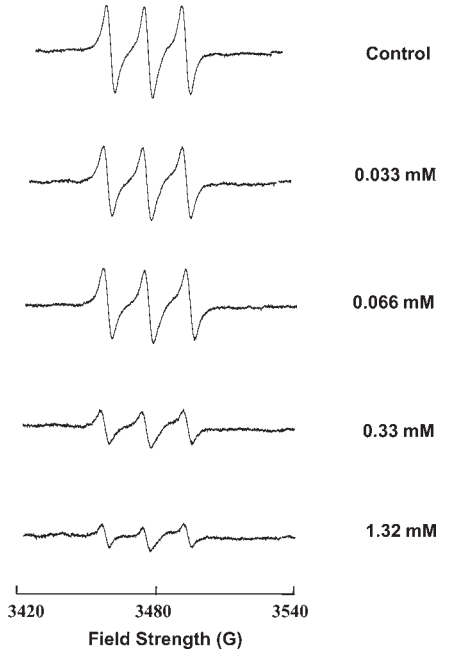

Figure 1. ESR spectra of 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TMPD-*N*-oxyl) radical in a TMPD−MB system after light illumination as affected by resveratrol.

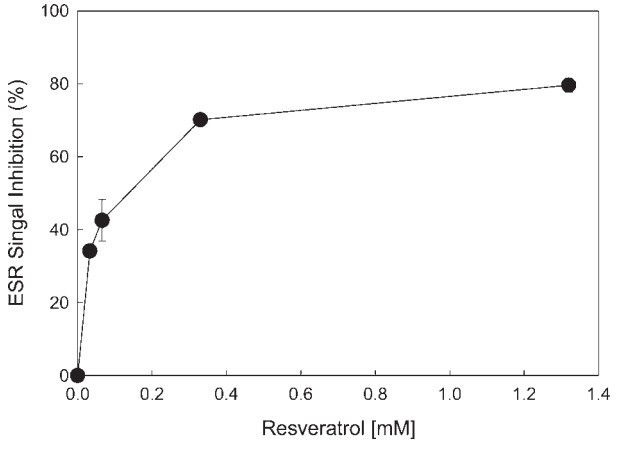

Figure 2. Effect of different concentrations of resveratrol on the ESR signal intensity in methanol containing methylene blue (8 ppm) and TMPD (18.75 mM).

in **Figure 1** was at $g = 2.00901$ with $\alpha_N = 1.53$ mT and line width = 4.1G. The result showed clear TMPD-*N*-oxyl radical signals induced by light illumination and a dose-dependent reduction of ESR signals by resveratrol. It has been reported that the formation of water-soluble TMPD-*N*-oxyl radical from TMPD is indirect evidence of singlet oxygen involvement in the system (*20*). The induction of a TMPD-*N*-oxyl radical signal suggested the formation of singlet oxygen in the system. Resveratrol treatments at 0.033, 0.066, 0.33, and 1.32 mM inhibited 34.2 ± 1.3, 42.6 ± 5.7, 70.2 ± 1.0, and 79.6 ± 1.9% TMPD-*N*-oxyl radical formation, respectively (**Figure 2**). The results suggested that resveratrol may quench singlet oxygen, and its quenching activity was concentration-dependent. Resveratrol at the tested concentrations up to 1.32 mM was not enough to scavenge all of the singlet oxygen formed. Thus, the complete quenching of singlet oxygen may require much higher concentrations of resveratrol.

NIR Fluorescence Spectroscopy for Direct Monitoring of Singlet Oxygen Quenching. For direct visualization of singlet oxygen,

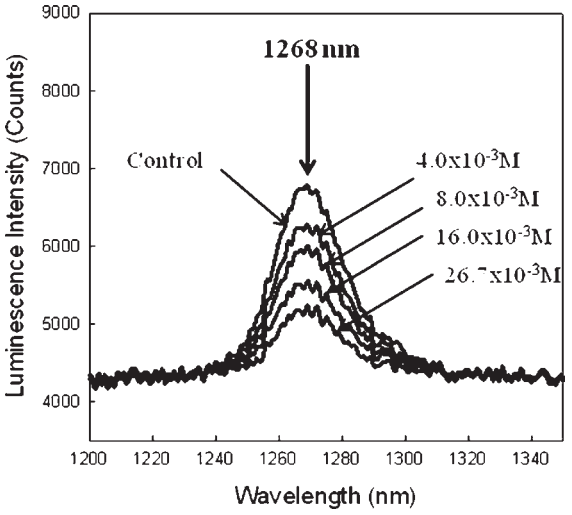

Figure 3. Emission spectra of singlet oxygen in methanol generated by rose bengal with light excitation at 559 nm as affected by resveratrol.

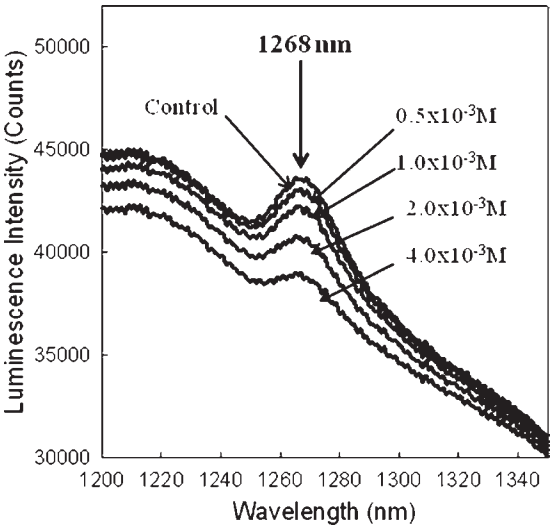

Figure 4. Emission spectra of singlet oxygen in methanol generated by methylene blue with light excitation at 665 nm as affected by resveratrol.

a spectrofluorometer equipped with a highly sensitive InGaAs detector was used (*11*). The detector was cooled with liquid nitrogen to ensure high sensitivity. In this system, rose bengal or methylene blue was used as sensitizer for the photochemical production of singlet oxygen. Singlet oxygen was detected by monitoring the luminescence emission at 1268 nm due to the dimole singlet oxygen energy release. Singlet oxygen, a reactive oxygen species, is in an excited state with a 94.3 kJ/mol higher energy than normal oxygen. When the excited state of singlet oxygen returns to its ground state, its high energy releases with an emitting luminescence at 1268 nm (*11*). **Figure 3** shows the effects of resveratrol on the emission spectra at the NIR region in methanol containing rose bengal by light excitation at 559 nm. The result shows that light excitation at 559 nm in methanol containing rose bengal induced a high-intensity light emission with maximum wavelength at 1268 nm. There was no light emission at around 1268 nm in methanol without the addition of rose bengal (data not shown). This result clearly showed that rose bengal efficiently produced singlet oxygen through light excitation at 559 nm. The addition of resveratrol in methanol decreased the emission signal height at the wavelength

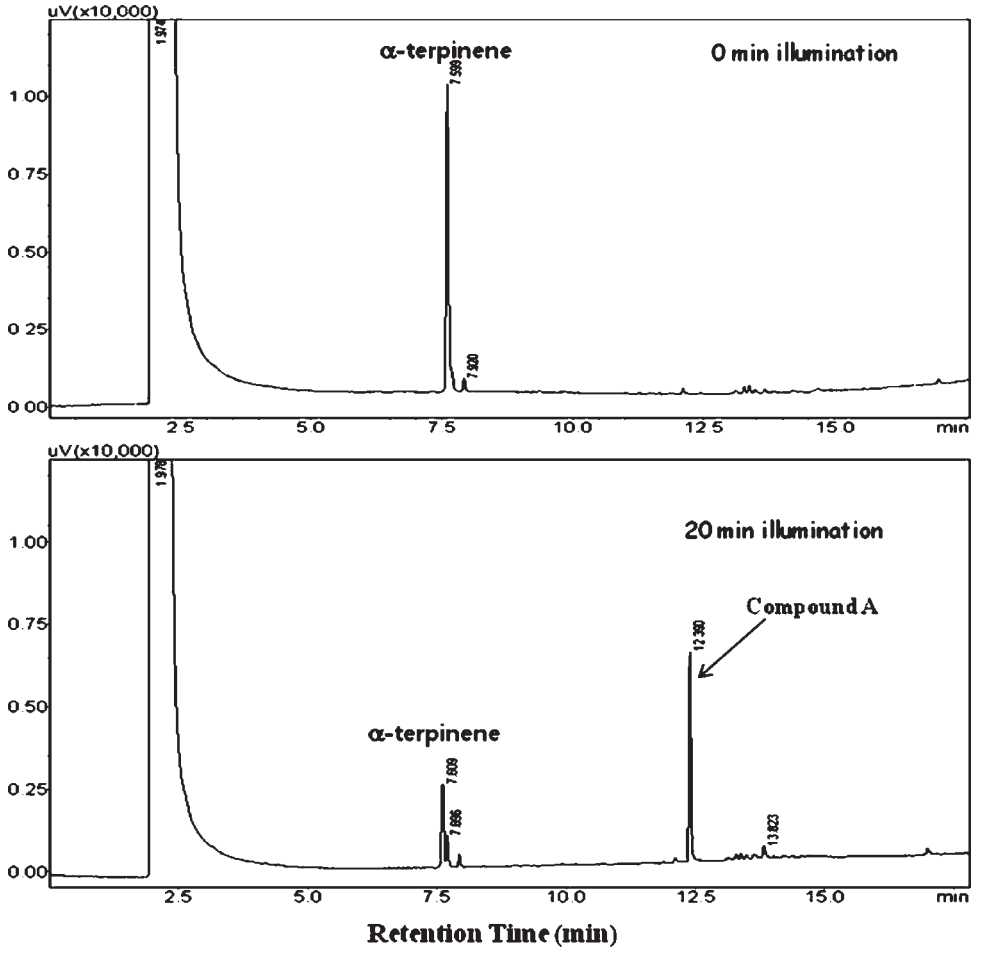

Figure 5. Gas chromatograms of α-terpinene and its oxidation products in methanol containing methylene blue before and after light illumination.

of 1268 nm. The higher the resveratrol concentration, the lower the emission signal height at 1268 nm was. **Figure 4** shows that the light excitation at 665 nm in methanol containing methylene blue also induced light emission with maximum height at the wavelength of 1268 nm, showing the production of $^1O_2$ by methylene blue photosensitization. Resveratrol also decreased light emission at the wavelength of 1268 nm induced by methylene blue photosensitiztion, in a dose-dependent manner (**Figure 4**). It was easy to set the bottom baseline in the emission spectra obtained using rose bengal, but, as shown in **Figure 4**, it was very difficult to set the true bottom baseline in the emission spectra obtained using methylene blue. Thus, it was impossible to quantitatively compare the singlet oxygen quenching ability of resveratrol with the emission spectrum obtained using different photosensitizers of methylene blue and rose bengal. Nevertheless, our present results are unambiguous and direct evidence of singlet oxygen quenching by resveratrol.

Determination of Singlet Oxygen Quenching Rate Constant ($k_r + k_q$). The singlet oxygen quenching rate constant of antioxidants was determined by using a steady state kinetic equation. In our reaction system, α-terpinene was oxidized in methanol with photochemically induced singlet oxygen in the presence of methylene blue. When a solution of α-terpinene is exposed to singlet oxygen, the following reactions take place:

$$MB + {}^3O_2 \xrightarrow{\text{light}} {}^1O_2 \quad (1)$$

$${}^1O_2 \xrightarrow{k_d} {}^3O_2 \quad (2)$$

$${}^1O_2 + \alpha\text{-terpinene} \xrightarrow{k_{ox}} \text{oxidized } \alpha\text{-terpinene} \quad (3)$$

$${}^1O_2 + Q \xrightarrow{k_r} QO_2 \quad (4)$$

$${}^1O_2 + Q \xrightarrow{k_q} {}^3O_2 + Q \quad (5)$$

In the above reactions, k_d is 1/the singlet oxygen lifetime in the solvent, k_{ox} is the chemical reaction rate of α-terpinene with singlet oxygen, k_r is the singlet oxygen chemical quenching rate constant, k_q is the singlet oxygen physical quenching rate constant, Q is the quencher, and QO_2 is the oxidation product of the quencher.

If a quencher is present, $^1O_2$ disappears by four routes: nonradiative decay (reaction 2), reaction with α-terpinene (reaction 3), singlet oxygen chemical quenching (reaction 4), and singlet oxygen physical quenching (reaction 5). In the absence of a quencher, $^1O_2$ disappears by only two routes: nonradiative decay (reaction 2) and reaction with α-terpinene (reaction 3). If there are two solutions of equal volume, one containing quencher and one without a quencher and each having the same initial concentration of α-terpinene, and they are each exposed to the same amount of $^1O_2$, the following equation can be derived from steady state kinetics (*23*−*25*):

$$S_0/S_s = 1 + [(k_r + k_q)/k_d][Q] \quad (6)$$

S_0 and S_s are slopes of the first-order plots of disappearance of singlet oxygen acceptor (α-terpinene) in the absence and presence of antioxidant, respectively.

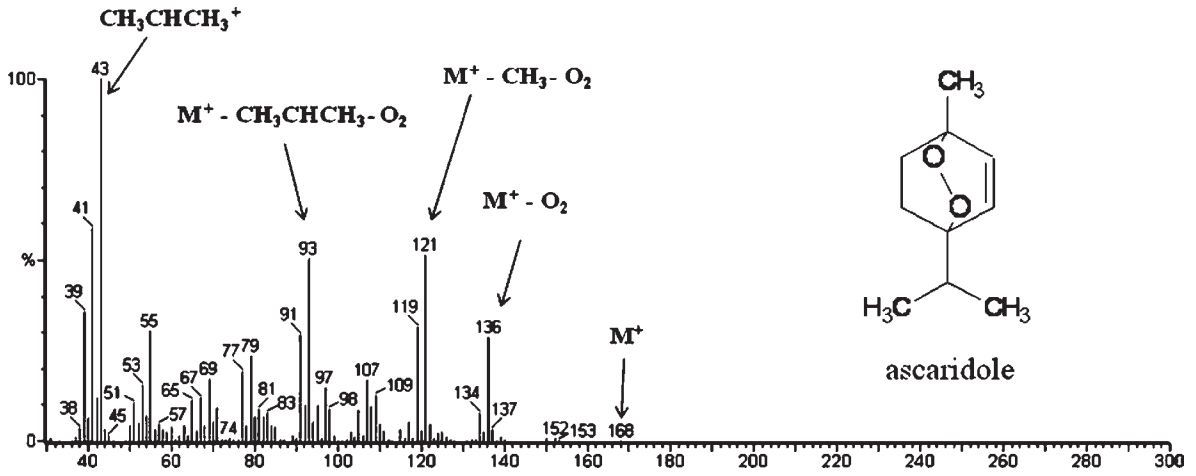

Figure 6. GC-MS spectrum of a major oxidation product of α-terpinene with a retention time of 12.39 min.

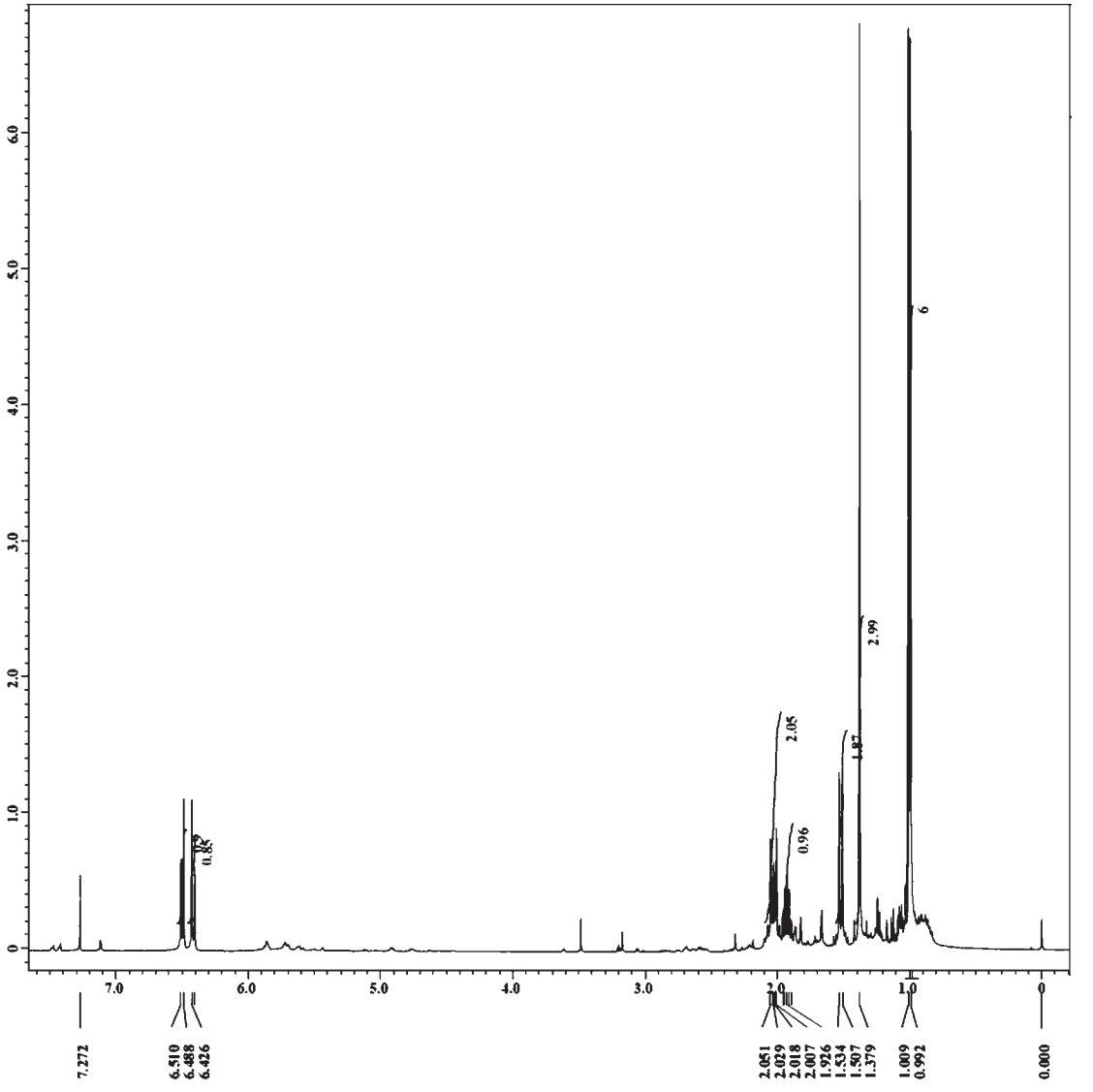

Figure 7. ^{1}H NMR spectrum of reaction product of α-terpinene after light illumination. ^{1}H NMR spectra were measured on a 400 MHz NMR spectometer in $CDCl_3$ with teramethylsilane, and chemical shifts are given as δ values.

If a plot of S_0/S_s versus [Q] is drawn, the slope would be $(k_r + k_q)/k_d$. The total singlet oxygen quenching rate constant $(k_r + k_q)$ can be calculated from eq 6. The lifetime of $^1O_2$ $(1/k_d)$ in methanol can be obtained from a previous paper. The singlet oxygen decaying rate (k_d, 1.0×10^5) in methanol was adopted from a previous paper (*28*).

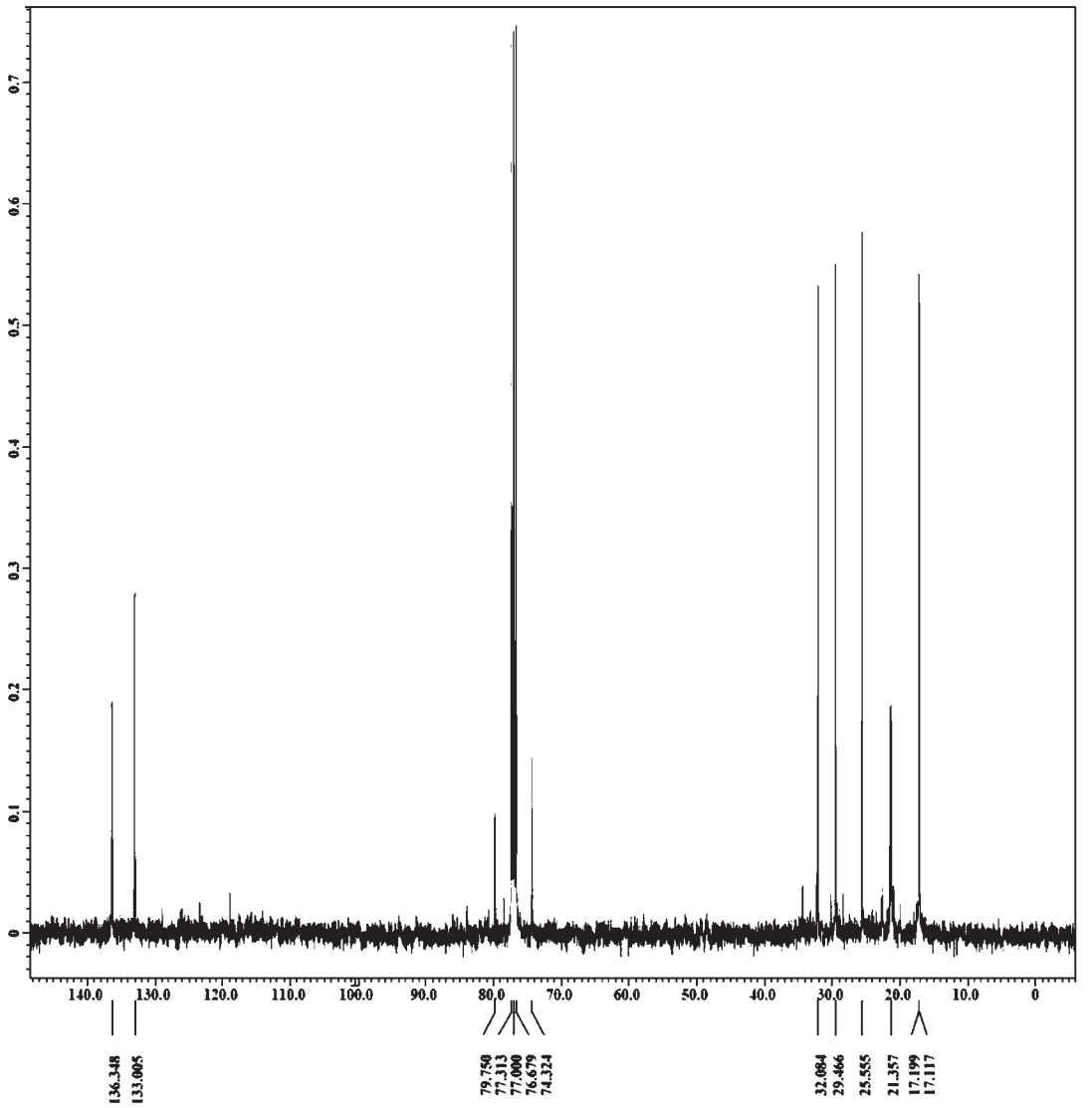

Figure 8. ^{13}C NMR spectrum of reaction product of α-terpinene after light illumination. ^{13}C NMR spectra were measured on a 400 MHz NMR spectometer in $CDCl_3$ with teramethylsilane, and chemical shifts are given as δ values.

For this study, α-terpinene was oxidized in methanol with photochemically induced singlet oxygen in the presence of methylene blue. **Figure 5** shows the gas chromatograms of α-terpinene and its oxidized products in methanol containing methylene blue before and after light illumination. As the gas chromatograms in **Figure 5** showed, there was one major oxidized product (compound **A**) with several minor products after illumination. **Figure 6** showed the GC-MS spectrum of compound **A**. The spectrum showed the molecular ion at m/z 168 with major fragment ions at m/z 136, 121, 99, and 43. The fragment ion at m/z 136 was due to the neutral loss of two oxygen atoms (O_2) from the molecular ion (168 − 32). The fragment ion at m/z 121 was due to the neutral losses of O_2 and CH_3 (168 − 32− 15) from the molecular ion. The fragment ion at m/z 99 was due to the neutral losses of O_2 and $CH_3{-}CH{-}CH_3$ (168 − 32 − 43) from the molecular ion. The fragment ion at m/z 43 was due to the fragmentation of $[CH_3{-}CH{-}CH_3]^+$. On the basis of interpretation of the mass spectral data, compound **A** was tentatively identified as ascaridole. Compound **A** was extracted from the solution obtained after light illumination after 20 min by a liquid−liquid partition with hexane. The hexane layer was removed by nitrogen flushing to get compound **A**. Then the compound was dissolved in $CDCl_3$ containing tetramethylsilane to obtain the ^1H and ^{13}C NMR spectra. ^1H NMR data showed δ 1.00 (3H, s), 1.01 (3H, s), 1.38 (3H, s), 1.52 (2H, m), 1.92 (1H, m), 2.03 (2H, m), 6.42 (1H, d, J = 8.8 Hz), and 6.50 (1H, d, J = 8.8 Hz) (**Figure 7**). ^{13}C NMR data showed δ 17.1, 17.2, 21.4, 25.6, 29.5, 32.1, 74.3, 79.8, 133.0, and 136.3 (**Figure 8**). ^1H and ^{13}C NMR spectral data positively confirmed that compound **A** was ascaridole. It has been previously reported that α-terpinene selectively reacts with singlet oxygen to produce ascaridole (*29*). Our result indicated that singlet oxygen was efficiently produced in this reaction system by light illumination.

Figure 9 shows the effects of resveratrol on the α-terpinene oxidation in methanol containing methylene blue during light illumination. α-Terpinene was oxidized rapidly in methanol under light illumination. Resveratrol decreased the α-terpinene oxidation, and its protective activity was concentration dependent. α-Terpinene oxidation followed first-order kinetics. The slope in **Figure 9** decreased as the resveratrol increased. **Figure 10** shows the plot of S_0/S_s (obtained from **Figure 9**) versus [resveratrol]. The plot of S_0/S_s versus [resveratrol] showed that as the resveratrol concentration in the system increased, the S_0/S_s increased. The calculated slope of the plot in **Figure 10** was 2.55×10^2. From the slope of the plot and the known k_d value, $k_r + k_q$ was calculated using eq 6. The calculated singlet oxygen

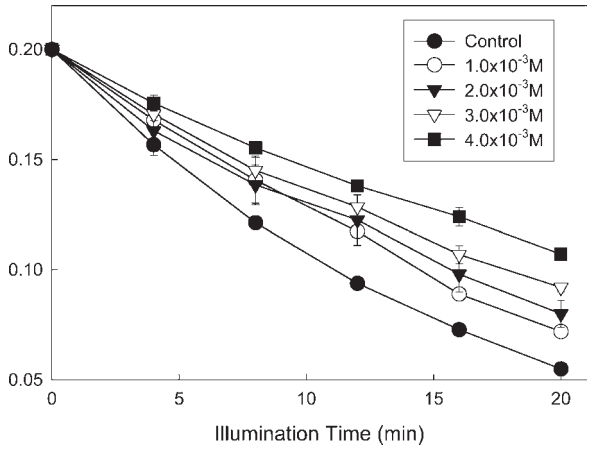

Figure 9. Effect of resveratrol on α-terpinene oxidation in methanol during light illumination (3000 lx) at 20 ± 1 °C.

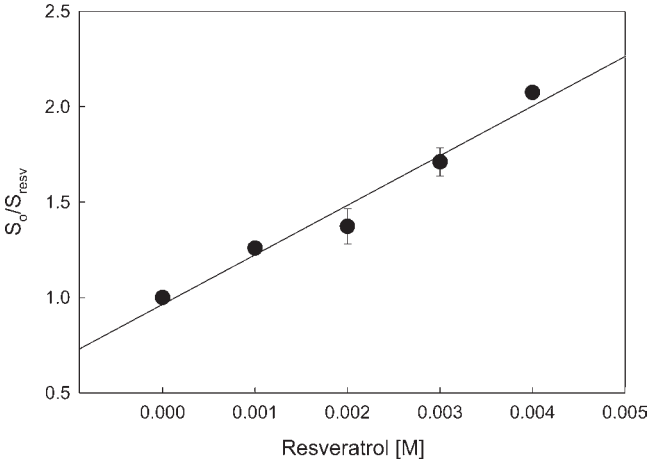

Figure 10. Plot of S_0/S_Q versus concentration of resveratrol obtained from α-terpinene oxidation in methanol containing various concentrations of resveratrol during light illumination (3000 lx) at 20 ± 1 °C.

quenching rate constant ($k_r + k_q$) of resveratrol in methanol was $(2.55 \pm 0.17) \times 10^7$ M^{-1} s^{-1}. The result showed that the $k_r + k_q$ value for resveratrol was close to that of BHA (*24*, *30*). Criado et al. (*30*) reported that the $k_r + k_q$ value for BHA in methanol was 2.97×10^7 M^{-1} s^{-1}. Kim et al. (*24*) reported that the $k_{ox\text{-}Q} + k_q$ value for BHA in methanol was 3.37×10^7 M^{-1} s^{-1}. The total singlet oxygen quenching rate constant of a similarly structured compound, pallidol, in water has been previously reported (*31*). To our knowledge, however, the total singlet oxygen quenching rate constant of resveratrol in methanol has never been previously reported.

Determination of Chemical Singlet Oxygen Quechning Rate Constant (k_r) of Resveratrol. The singlet oxygen chemical quenching rate (k_r) of resveratrol could be determined by measuring the relative oxidation rate of resveratrol as compared to the oxidation rate of α-terpinene, the k_r value of which is known, in the same solution during illumination with the equation (*23*)

$$k_r^R / k_r^{\alpha\text{-}T} = \{-\mathrm{d}[\text{resveratrol}]/\mathrm{d}t\}/\{-\mathrm{d}[\alpha\text{-terpinene}]/\mathrm{d}t\} \quad (7)$$

where $k_r^{\alpha\text{-}T}$ = chemical singlet oxygen quenching rate constant of α-terpinene, k_r^R = chemical singlet oxygen quenching rate constant of resveratrol, $-\mathrm{d}[\alpha\text{-terpinene}]/\mathrm{d}t$ = apparent rate of α-terpinene disappearance, and $-\mathrm{d}[\text{resveratrol}]/\mathrm{d}t$ = apparent rate of resveratrol disappearance.

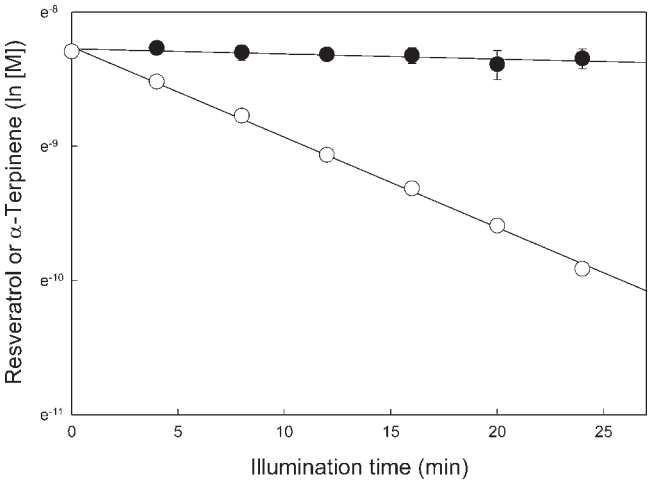

Figure 11. Relative singlet oxygen reaction rate of resveratrol in methanol solution as compared with α-terpinene.

Figure 11 shows the oxidation rates of α-terpinene and resveratrol in the same solution of methanol containing methylene blue during illumination. The results showed that the disappearances of resveratrol and α-terpinene followed the first-order kinetic equation and that the relative reaction rate of resveratrol as compared with that of α-terpinene were 0.0563. The k_r value of α-terpinene ($k_r^{\alpha\text{-}T} = 2.05 \times 10^7$) was adopted from our previous result (*32*). Thus, with eq 7, the singlet oxygen chemical quenching rate constant for resveratrol could be calculated: $k_r^R = (0.0562 \times 2.05) \times 10^7 = 1.15 \times 10^6$ M^{-1} s^{-1}. The percent ratio of chemical quenching over total singlet oxygen quenching ($k_r \times 100$) / ($k_r + k_q$) was 5.11% for resveratrol. The result showed that only a tiny portion of singlet oxygen was quenched through a chemical quenching mechanism. That is, resveratrol quenched singlet oxygen almost exclusively by a physical quenching mechanism. About 95% of singlet oxygen quenching by resveratrol was through its physical quenching mechanism. For the evaluation of antioxidant efficiency as a singlet oxygen quencher, it is essential to determine the chemical quenching rate constant of singlet oxygen along with the total quenching rate constant. If the chemical quenching rate of an antioxidant is high, the antioxidant will be rapidly consumed along with the protection of food components from the singlet oxygen, and thus its antioxidant activity cannot last long during storage. The result clearly suggested that these antioxidants could protect biological components from singlet oxygen induced oxidations without their significant losses during storage. The total and chemical singlet oxygen quenching rate constants of resveratrol in methanol have never been previously reported. Furthermore, the singlet oxygen quenching mechanism exerted by resveratrol has not been reported previously.

Effects of Resveratrol and BHA on the Methylene Blue-Sensitized Photooxidation of α-Terpinene in Methanol. Because it has been found that the singlet oxygen quenching activity of resveratrol was similar to that of BHA, we compared the effect of resveratrol with that of BHA on the α-terpinene photooxidation in methanol containing methylene blue. The α-terpinene content in the sample solution containing no added antioxidant in methanol decreased from 0.2×10^{-3} to 0.086×10^{-3} M after 15 min of illumination. The samples protected from light with aluminum foil did not induce any decrease in the α-terpinene content during the 15 min illumination under the same conditions (data not shown). Resveratrol and BHA at the concentration of 2.0×10^{-4} M greatly reduced the MB-sensitized photooxidation of α-terpinene during illumination (data not shown). After 15 min of light illumination, the contents of α-terpinene in samples

containing resveratrol and BHA were 0.134×10^{-3} and 0.147×10^{-3} M, respectively. That is, resveratrol exhibited a slightly lower protective activity than BHA. However, the protective effect of resveratrol on the MB-sensitized photooxidation of α-terpinene was not significantly different from that of BHA ($p > 0.05$). It is interesting to note that resveratrol and BHA, which have similar singlet oxygen quenching rate constants, also showed similar protective activities against α-terpinene oxidation with singlet oxygen induced in a photochemical way.

Singlet oxygen ($^1O_2$) has been reported to be extremely reactive to electron-rich biomolecules, resulting in the oxidative damage of biomolecules in biological systems and nutrient loss in foods (*1*−*5*). Singlet oxygen plays an important role in the induction of gene expression (*8*−*10*). Resveratrol reportedly protects biomolecules from photosensitized oxidation in biological systems (*18*, *19*). Our present result will unambiguously explain the mechanism of how resveratrol protects tissues and cells from damage in biological systems. This result also suggests that resveratrol could be involved in the regulation of gene expression activated by singlet oxygen in biological systems.

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