Research Note

Kinetic study on the singlet oxygen quenching activity of nordihydroguaiaretic acid (NDGA) using methylene blue sensitized photooxidation of α -terpinene

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Abstract Singlet oxygen is highly reactive and can therefore induce rapid oxidation of a range of biological molecules, causing cell damages. The effects of nordihydroguaiaretic acid (NDGA) on the photochemical singlet oxygen oxidation of α -terpinene in methanol were studied. NDGA showed strong protective activity on the singlet oxygen oxidation of α -terpinene in methanol in a dose dependent manner. The protective activity of NDGA was considerably higher than that of butylated hydroxytoluene and 1,4-diazabicyclo[2.2.2]octane. Total singlet oxygen quenching rate constant ($k_r + k_q$) of NDGA was determined by a steady state kinetic equation. The total singlet oxygen quenching rate constant of NDGA was $9.81 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. The result showed that NDGA possessed strong singlet oxygen quenching activity, indicating its potential for the protection of molecules, cells and nutrients from the highly reactive singlet oxygen. To the best of our knowledge, this is the first report on the singlet oxygen quenching rate constant of NDGA.

Keywords: NDGA, singlet oxygen, *Larrea tridentata*, α -terpinene, quenching rate constant

Introduction

Singlet oxygen ($^1\text{O}_2$), in the electronically excited state ($^1\Delta_g$), has no unpaired electrons and therefore no net electron spin with 22.4 kcal per mole higher energy than ordinary triplet oxygen (1,2). Singlet oxygen is highly reactive to important biological components (low density lipoprotein cholesterol, lipids, amino acids proteins, vitamins, sterols, DNA, and RNA), resulting in fast deleterious oxidative damages (3–7). Singlet oxygen can be produced in biological systems as well as food system through various routes (enzymatic, chemical and photochemical pathways) (1,2,8–10). Photochemical reaction is the main pathway for the production of singlet oxygen in biological systems (1,2). The antioxidants with high singlet oxygen quenching activities could protect a range of molecules and cells in biological systems as well as important nutrients in foods from singlet oxygen induced oxidation. Ascorbic acid, carotenoids, tocopherols, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), and catechins have been reported to have protective activity against the singlet oxygen induced oxidation of various nutrients (1,2,8,9,11–16). Nordihydroguaiaretic acid (NDGA) is a phenolic compound naturally present in the evergreen shrub *Larrea tridentata* (17,18). NDGA has been reported to have beneficiary functionality in the treatment of diverse diseases, including diabetes,

cancer, arthritis, kidney injury, and neurological disorders (18,19). Cell culture and animal studies have supported its medicinal properties (20–22). The evergreen shrub, *Larrea tridentata*, has been used as a nutritional supplement due to this functionality even though the safety and possible toxicity from its application must still be determined in clinical studies. NDGA has been also reported to significantly expand the life spans of mosquitos and male mice (23,24). The medicinal property of NDGA may be attributed to its scavenging ability of various reactive oxygen species. The scavenging activity of NDGA on the various reactive oxygen species has been extensively studied (25–27). However, there are only a few reports regarding the singlet oxygen quenching ability of NDGA (25,27). For the evaluation of antioxidant efficiency as a singlet oxygen quencher, it is essential to determine the total singlet oxygen quenching rate constants. To the best of our knowledge, however, there is no reported kinetic study on the singlet oxygen quenching rate constant of NDGA.

Herein, we report the protective activity of NDGA on the singlet oxygen induced α -terpinene oxidation and its singlet oxygen quenching rate constant ($k_r + k_q$). In this study, α -terpinene was selected as a substrate for singlet oxygen induced photooxidation since it is particularly susceptible to singlet oxygen oxidation to form a specific oxidation product (ascaridole).

Materials and Methods

Materials NDGA, methylene blue, BHT, DABCO, and α -terpinene were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Effects of NDGA on singlet oxygen oxidation of α -terpinene To study the effects of NDGA on singlet oxygen oxidation of α -terpinene, the sample solutions containing α -terpinene (0.2 mM), NDGA (0, 0.5, 1.0, 1.5, and 2.0 mM) or BHT, DABCO (2.0 mM), and methylene blue (13.2 μ g/mL) in oxygen-saturated methanol were prepared in duplicate. For the preparation of the oxygen saturated methanol, pure oxygen was purged through methanol for 10 min. Then, an aliquot (10 mL) of the sample solutions was added into 20-mL capacity glass vials with screwcaps. The sample vials were placed in a light storage box and illuminated under the condition of fluorescence light (1,500 lux, 3×15 W, 30 cm distance) at 25±1°C for 20 min. The oxidation of α -terpinene was determined by previously described gas chromatography (8,16).

Determination of total singlet oxygen quenching rate (k_r+k_q) To determine the total quenching rate constant of NDGA, the sample solutions containing α -terpinene (0.2 mM), NDGA (0 and 3.0 mM), and methylene blue (13.2 μ g/mL) in oxygen-saturated methanol were prepared. The prepared samples were stored in the light box for 25 min. Samples were taken every 5 min, and the effects of NDGA on the oxidation of α -terpinene were determined by GC analysis. A steady-state kinetic equation (11-13,16) was applied for the calculation of singlet oxygen quenching rate constants. The total quenching rate constant of NDGA was determined in triplicate.

Gas chromatography for the determination of α -terpinene contents

The content of α -terpinene was determined using a gas chromatograph (GC-2120; Shimadzu, Kyoto, Japan) as previously reported (8,16). The column used was a nonpolar capillary column (RTX-5MS, 30 m×0.25 mm ID, 0.25 mm film thickness, Restek Co., Bellefonte, PA, USA). The temperatures of injector and detector used were 140 and 250°C, respectively. Injections were made in the split mode (20:1). The oven temperature was programmed from initially 70°C (held for 3 min) to 150°C at an increase rate of 7°C/min (held for 10 min at 150°C). The inlet head pressure of the carrier gas (helium) was 100 kPa. The ratio of the gas chromatography peak area to that of known amounts of its authentic compound was calculated for the quantification of α -terpinene (8).

Statistical analysis The statistical analysis was conducted by the Analysis of variance (ANOVA) and Duncan's multiple range test to ascertain the statistical differences of the data at $\alpha=0.05$ using a SPSS statistical analysis program (SPSS version 19.0; SPSS Inc., Chicago, IL, USA).

Results and Discussion

Effects of NDGA on the methylene blue sensitized photooxidation of α -terpinene

After 20 min light illumination, there was a considerable decrease in α -terpinene content and considerable increase in the formation of the oxidized compound (ascaridole), which was elucidated by our previous report (8). Herein the identity of ascaridole was doubly verified by conducting the GC-MS with the authentic ascaridole. Ascaridole has been known to be produced by the 1,4-cycloaddition of singlet oxygen to α -terpinene through Type II reaction mechanism (8,16,28). In this experiment, the ascaridole was the only major compound formed during the light illumination (figure not shown). The result suggested that this experiment used in the reaction system efficiently induced the singlet oxygen oxidation of α -terpinene. The effects of NDGA on the photochemically induced singlet oxygen oxidation of α -terpinene are shown in Fig. 1. The addition of 0.5, 1.0, 1.5, and 2.0 mM NDGA in the system showed the 14.8, 26.5, 36.8 and 47.1% inhibition of α -terpinene oxidation. BHT and DABCO at the same concentration of 2 mM showed 8.9% and 7.8% inhibition of α -terpinene, respectively (Fig. 1). The statistical result showed that NDGA had significantly higher protective activity than BHT and DABCO for the methylene blue sensitized photooxidation of α -terpinene. The result also suggested that NDGA is an efficient antioxidant for the protection of biological molecules from the singlet oxygen induced oxidation.

Singlet oxygen quenching rate constant (k_r+k_q)

A steady state kinetic equation was used for the calculation of singlet oxygen quenching rate constant of NDGA. When α -terpinene is exposed to singlet oxygen produced by methylene blue under a light illumination condition, the following reactions take place (9,11,13,16):

where, k_d is a singlet oxygen decaying rate, k_{ox} is α -terpiene reaction rate with singlet oxygen, k_r is rate constant of singlet oxygen chemical quenching, k_q is rate constant of singlet oxygen physical quenching, Q is a quencher, and QO₂ is the reaction product of the quencher with singlet oxygen.

Fig. 1. Protective effects of NDGA on α -terpinene in methanol containing methylene blue after 20 min light illumination at different concentration and the comparison with other antioxidants (BHT and DABCO).

With a quencher (NDGA), singlet oxygen is converted to triplet oxygen (reaction 2), reacts with α -terpinene (reaction 3), reacts chemically with the quencher (reaction 4), and is physically quenched by the quencher (reaction 5). However, without the quencher (NDGA), singlet oxygen can be removed only through reactions 2 and 3. The following steady state kinetics can be obtained from the reaction of singlet oxygen the α -terpinene samples containing various concentration of quencher (9,11,13,16).

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

where, S_0 and S_s are the slopes of the α -terpinene oxidation plot with and without NDGA, respectively. The slope of the plot of S_0/S_s vs concentration of a quencher (NDGA) would be $(k_r + k_q)/k_d$. Then, with the known k_d value, singlet oxygen quenching rate can be calculated. The adopted singlet oxygen decaying rate (k_d , 1.0×10^5) in methanol from the previous reports was used (16,29).

Figure 2A shows the effects of different concentrations of NDGA (0, 0.5, 1.5, and 2.0 mM) on the α -terpinene oxidation in methanol containing methylene blue during a time course of light illumination. α -Terpinene oxidation follows the 1st order kinetic equation. As the NDGA concentration increased, the slope of the α -terpinene oxidation decreased. The slopes (k), the oxidation rates of α -terpinene, obtained from the 1st order kinetic equation ($\ln[\alpha\text{-terpinene}] = -kt + \ln[\alpha\text{-terpinene}]_0$) for the samples treated with 0, 0.5, 1.0, 1.5, and 2.0 mM NDGA were 0.08278, 0.05857, 0.045284, 0.035056, and 0.022253 mM/min, respectively, where k is the slope and t is the time in min. Thus, the calculated S_0/S_s values for the 0, 0.5, 1.0, 1.5, and 2.0 mM NDGA were 1.0, 1.412, 1.182, 2.360, and 2.981, respectively. The plot of S_0/S_s vs [NDGA] was drawn as shown in Fig. 2B. From the slope of the plot (Fig. 2B) and the known k_d value, the $k_r + k_q$ could be calculated using Eq. 6. The slope of this plot (Fig. 2B) was 981.94. The determined singlet oxygen quenching rate constants ($k_r + k_q$) of NDGA in methanol was $981.94 \times k_d = 9.81 \times 10^7$. Our $k_r + k_q$

Fig. 2. Effect of different concentration of NDGA on the α -terpinene oxidation in methanol during light illumination (A) and the plot of S_0/S_s vs concentration of NDGA (B).

value for NDGA was higher than that for BHT (9,30,31). Criado *et al.* (30) determined that the total singlet oxygen quenching rate constant for BHT in methanol was $3.3 \times 10^6 M^{-1} s^{-1}$. Thomas and Foote (31) determined that the total singlet oxygen quenching rate constant for BHT in methanol was $5.55 \times 10^6 M^{-1} s^{-1}$. Our previous reported total singlet oxygen quenching rate constant of BHT in methanol was $3.41 \times 10^6 M^{-1} s^{-1}$ (9). The results clearly showed that NDGA had approximately 30 fold higher singlet oxygen quenching ability than BHT. This result was consistent with our previous results for the protection of singlet oxygen oxidation of α -terpinene. NDGA showed significantly higher protective activity on the singlet oxygen induced α -terpinene oxidation than BHT at the same concentration. NDGA, a phenolic compound present in the evergreen shrub *Larrea tridentate*, has been reported to have various medicinal applications (18,19,23, 24). Our present results demonstrated that the medicinal properties of NDGA may be, at least to some extent, attributed to its efficient quenching ability of singlet oxygen which is a highly aggressive reactive oxygen species.

Disclosure The authors declare no conflict of interest.

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