Kinetic and mechanistic studies of allicin as an antioxidant

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We have undertaken a detailed study of the antioxidant activity of allicin, one of the main thiosulfinates in garlic, in order to obtain quantitative information on it as a chain-breaking antioxidant. The antioxidant actions of allicin against the oxidation of cumene and methyl linoleate (ML) in chlorobenzene were studied in detail using HPLC. The hydroperoxides formed during the course of the inhibited oxidation of ML were analyzed as their corresponding alcohols by HPLC, and it is apparent that an allylic hydrogen atom of the allicin is responsible for the antioxidant activity. Furthermore, it is clear that the radical-scavenging reactions of allicin proceed *via* a one-step hydrogen atom transfer based on the results of the reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) in the presence of Mg^{2+} and calculation of the ionization potential value. In addition, we determined the stoichiometric factor (n), the number of peroxyl radicals trapped by one antioxidant molecule, of allicin by measuring the reactivity toward DPPH in chlorobenzene, and the value of n for allicin was about 1.0. Therefore, we measured the rate constants, k_{inh}, for the reaction of allicin with peroxyl radicals during the induction period of the cumene and the ML oxidation. As a result, we found that allicin reacts with peroxyl radicals derived from cumene and ML with the rate constants $k_{inh} = 2.6 \times 10^3$ $M^{-1}s^{-1}$ and 1.6×10^5 $M^{-1}s^{-1}$ in chlorobenzene, respectively. Our results demonstrate for the first time reliable quantitative kinetic data and the antioxidative mechanism of allicin as an antioxidant.

Introduction

Garlic has been used for centuries as a medicinal herb.[1] It has been cultivated in the Middle East for more than 5000 years and has been an important part of traditional Chinese medicine.[2–4] In addition, it has been traditionally used around the world to treat many conditions, including parasites, respiratory problems, poor digestion, and low energy.[5–8]

It is generally accepted that the oxidative modification of low density lipoproteins (LDL) is a key initial event in the progression of atherosclerosis which eventually causes coronary heart disease and cerebral hemorrhage. Animal studies have shown that an antioxidant suppresses atherosclerosis, and epidemiological studies suggest that a high intake of vitamin E reduces the risk of coronary heart disease.[9] The role of vitamin E as an antioxidant is well known and this property is due to scavenging of the chain-carrying peroxyl radicals of the substrate by transfer of the hydroxylic hydrogen atom to produce the corresponding hydroperoxides.[10]

It has also been shown that garlic extracts can scavenge some radicals and prevent lipid peroxidation.[11–20] In particular, allicin, one of the main thiosulfinates in garlic, prevents lipid peroxidation and was found to be an effective antioxidant.[21] Furthermore, thiosulfinates are reported to be extremely reactive toward protein thiols and inhibit the oxidation of many essential proteins *in vitro*.[22] Thus, the role of garlic as an antioxidant has received much attention.

We recently reported that garlic extracts are capable of inhibiting methyl linoleate (ML) oxidation induced by a radical initiator in acetonitrile solution, and its antioxidant property is mostly due to the presence of allicin. Moreover, it was found that the combination of the allyl group ($–CH_2CH=CH_2$) and the –S(O)S– group is necessary for the antioxidant action of allicin.[23] Based on these described results, it is therefore important to evaluate the antioxidant activity of allicin in relation to the type of antioxidant and mechanism. The mechanisms of lipid peroxidation have been well documented. The peroxyl radicals, particularly, are some of the most important radicals to act as a chain-carrying species during lipid peroxidation because they are the target for the antioxidant that effectively inhibits the oxidation.[24] However, the mechanisms of allicin as an antioxidant have not yet been recognized, and no kinetic data have been reported regarding the reactivity of allicin toward peroxyl radicals. Therefore, we have undertaken the study of the antioxidative mechanism of allicin and determination of the rate constants for the reaction of allicin with peroxyl radicals on the oxidation of cumene and ML in chlorobenzene, respectively.

Experimental

Materials

ML was obtained from the Sigma Chemical Co. (St. Louis, MO, USA) and purified on a silica-gel column before use. α-Tocopherol (α-toc) was from the Kanto Chemical Co. (Tokyo, Japan) and used without purification. 2,2′-Azobis(isobutyronitrile)

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(AIBN), chlorobenzene, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and cumene were purchased from Wako Pure Chemical Industries (Osaka, Japan). AIBN was recrystallized from methanol, and cumene was purified on a silica-gel column before use. All other reagents were of the highest grade commercially available.

Preparation of allicin from garlic slices

Allicin was extracted from garlic slices using supercritical fluid extraction with CO_2 according to the method of Rybak *et al.*[25] and purified by preparative HPLC using a UV detector at 254 nm with C_{18} column (10 mL min^{-1} methanol + 80% H_2O to methanol + 45% H_2O, Waters, μBondapak™ 10 μm 300 × 19 mm column). Allicin was identified by comparing its mass spectrum with that reported by Calvey *et al.*[26]; MS *m/z* 163 (MH^+, 11%), 73 (CH_2=CH–CH_2–S^+, 100) and 41 (25). A Micromass ZQ mass spectrometer (Waters, MA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source was used in the positive ionization mode. The mass ionization conditions were as follows: desolvation temperature, 400 °C; source temperature, 120 °C; cone voltage, 9 V; and desolvation gas flow, 160 L h^{-1}.

Inhibitory effect of allicin on cumene and ML oxidation in chlorobenzene solution

In a typical experiment, cumene (5.35 M) or ML (91 mM) in chlorobenzene in the presence of an appropriate amount of allicin was incubated at 30 °C in air. A solution of AIBN (23 mM for cumene and 40 mM for ML) in chlorobenzene was added to this reaction mixture. The rates of the substrates oxidation were followed by measuring the peroxides generated from each substrate using reverse phase HPLC (0.3 mL min^{-1} methanol + 15% water, Shiseido CAPCELLPAK C_{18} 5 μm 3.0 × 150 mm column) and the peaks were detected at 260 nm for cumene hydroperoxide (CHP) and at 234 nm for ML hydroperoxide (MLOOH). The consumption of allicin, at the same time, was analyzed using C_{18} HPLC with a UV detector at 254 nm.

Reactivity of allicin toward DPPH

DPPH (50 μM) and allicin (50 μM) were dissolved in chlorobenzene at 30 °C. The rates of the DPPH-scavenging reactions of allicin was monitored at 517 nm by a spectrophotometer. In the same way DPPH (48 μM) and allicin (48 μM) were dissolved in methanol in the presence of $Mg(ClO_4)_2$ (0.2 M) and monitored at 516 nm by a spectrophotometer.

Reactivity of allicin toward ML hydroperoxide

MLOOH was prepared by the autoxidation of ML at room temperature and purified on a silica-gel column. MLOOH (50 μM) was dissolved in chlorobenzene in the presence of ML (91 mM) and allicin (50 μM) at 30 °C. The amount of MLOOH was determined by HPLC using a C_{18} column (0.4 mL min^{-1} methanol + 10% H_2O, Shiseido CAPCELLPAK 5 μm 150 × 3.0 mm column) with a UV detector (234 nm).

Analysis of MLOOH isomers

The oxidation of ML (91 mM) was performed in chlorobenzene with AIBN (40 mM) in the presence of allicin (50 μM) at 30 °C in air. Samples (0.5 mL) were withdrawn from the mixture at known intervals during the induction period and were immediately reduced to ML hydroxides (MLOH) by adding 1.0 mL of 2 mM triphenylphosphine in chlorobenzene to this sample. The analysis was performed using silica-gel HPLC (1.0 mL min^{-1} *n*-hexane + 1.0% 2-propanol, Supelco SUPELCOSIL LC–Si 5 μm 250 × 4.6 mm column) and the peaks were detected at 234 nm.

Calculation of ionization potential (IP) values

The calculation of the IP value of allicin and α-toc were performed by the Gaussian 03 program (Gaussian, Inc., Carnegie, PA, USA)[27] as follows: the geometry optimization and determination of vibrational frequencies were performed using the HF/6-31G(d).[28] The single-point electronic energies were obtained by B3LYP/6-31G(d).

Results and discussion

Cumene and ML oxidation in chlorobenzene solution

In our previous paper,[23] we reported that allicin is capable of scavenging peroxyl radicals and inhibiting the ML oxidation induced by radical initiator in an acetonitrile solution. Therefore, we have undertaken a detailed study of the antioxidant activity of allicin in order to obtain quantitative information as a chain-breaking antioxidant.

Burton *et al.*[29] and Chepelev *et al.*[30] reported that the autoxidation of a substrate, such as ML, cumene, and styrene, initiated by AIBN in chlorobenzene, has certain advantages for determining the rate constants for peroxyl radical trapping by antioxidants. For example, the slow thermal decomposition of AIBN, followed by the very rapid reactions of the formed peroxyl radicals, ensures a controlled rate of chain initiation (R_i) throughout the experiments. In fact, this is a very important requirement for such quantitative studies. We consequently decided to investigate the antioxidant activity of allicin against the oxidation of cumene and ML in chlorobenzene using HPLC.

Fig. 1 shows a plot of the CHP during the initiated oxidation of cumene in chlorobenzene in the presence of allicin. Allicin produced a well-defined induction period in the cumene oxidation. Moreover, the rate of oxidation after the induction period was almost the same as that in the absence of allicin. Therefore, we

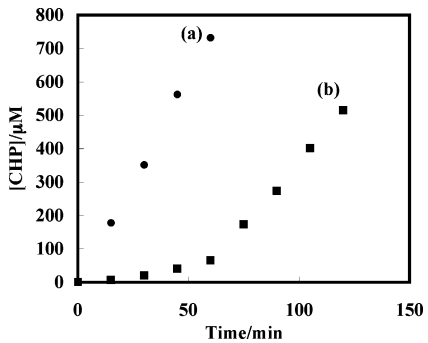


Fig. 1 Inhibitory effect of allicin on the oxidation of cumene induced by AIBN in chlorobenzene. Cumene (5.35 M) was oxidized at 30 °C in chlorobenzene under air with AIBN (23 mM) in the absence (a) and presence (b) of 30 μM allicin. CHP was measured by HPLC.

measured the amount of unreacted allicin during this induction period. Due to quantitative studies in homogeneous solution systems, it is necessary to determine whether the allicin is completely consumed by the end of the induction period of the cumene oxidation. As a result, it was found that allicin was completely consumed by the end of the induction period of the cumene oxidation (data not shown).

In the same way we also checked the inhibitory effect of allicin on the ML oxidation in chlorobenzene and the same results were also obtained in this system (Fig. 2). In addition, we studied the effect of allicin as a hydroperoxide decomposer in chlorobenzene at 30 °C. From the results, allicin had no significant effect on decomposing the MLOOH during the induction period (data not shown).

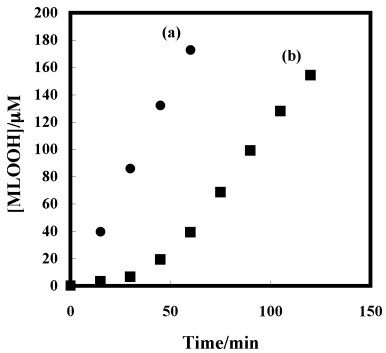


Fig. 2 Inhibitory effect of allicin on the oxidation of ML induced by AIBN in chlorobenzene. ML (91 mM) was oxidized at 30 °C in chlorobenzene under air with AIBN (40 mM) in the absence (a) and presence (b) of 50 μM allicin. MLOOH was measured by HPLC.

Taken together, it may be concluded that quantitative kinetic studies on cumene and the ML oxidation in chlorobenzene are quite applicable.

Determination of stoichiometric factor (*n*)

The stoichiometric factor, *n*, the number of peroxyl radicals trapped by one molecule of an antioxidant, can be determined by measurement of the reactivity toward DPPH (Fig. 3). DPPH has often been used in the estimation of the reactivity of antioxidants toward radicals and can also be used for estimating the antioxidant stoichiometries.[31] We then examined the reactivity of allicin toward DPPH in chlorobenzene at 30 °C. As shown in Fig. 4, allicin, despite its lower reactivity than that of α-toc ($n = 2$), reacted with equimolar quantities of DPPH. This result shows that the *n* for allicin is almost 1.0 in chlorobenzene at 30 °C.

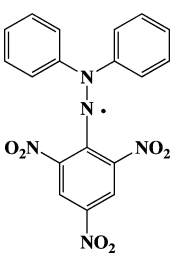


Fig. 3 Structure of DPPH.

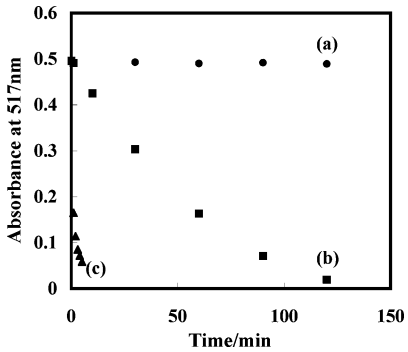


Fig. 4 Reactivity of allicin toward DPPH in chlorobenzene. DPPH (50 μM) was reduced at 30 °C in chlorobenzene under air in the absence (a) and presence (b) of 50 μM allicin, and (c) 25 μM α-toc.

Antioxidative mechanism of allicin

Recently, we showed that the –S(O)S–CH_2CH=CH_2 portion of allicin was found to contribute to the antioxidant action.[23] Because CH_3–S(O)S–CH_2CH=CH_2 exhibits a significantly higher antioxidant activity than CH_2–CH=CH_2–S(O)S–CH_3 and the bond strength of the allylic C–H bond is the weakest of all the C–H bond strengths in allicin, we speculate that the antioxidant property of allicin is due to scavenging the chain-carrying peroxyl radicals of the substrates by transfer of this allylic hydrogen to form hydroperoxides. Therefore, this assignment was confirmed by demonstrating that the allylic hydrogen of allicin contributes to its antioxidant activity.

The MLOOH formed during the controlled oxidation of ML provides very important information on the mechanism of lipid peroxidation and the action of antioxidants. Porter and Wujek[32] showed that the *cis*,*trans* to *trans*,*trans* (*c*,*t* : *t*,*t*) ratios of the MLOOH formed at the 9- and 13-positions of ML provide a simple linear relationship to the hydrogen atom donating ability of the antioxidants. This means that the amount of the *cis*,*trans*-MLOOH will increase and the *trans*,*trans*-MLOOH will decrease with the increasing concentrations of the substrates and hydrogen-donating antioxidants. We found it useful to use this ratio to monitor the effect of allicin. Therefore, we have undertaken an experimental study of the antioxidative mechanism of allicin as an antioxidant.

When adding the appropriate amounts of allicin to the ML oxidation induced by the AIBN in chlorobenzene, the MLOOH formed during the course of the inhibited oxidation was reduced by triphenylphosphine to their the corresponding alcohols, and these alcohols were analyzed by HPLC. These results are shown in Fig. 5. The *c*,*t* : *t*,*t* ratio formed during the uninhibited AIBN-initiated oxidation of ML in chlorobenzene remained constant at about 0.01 from 5 to 30 min. On the other hand, the ML oxidation, when inhibited by 50 μM allicin, showed a *c*,*t* : *t*,*t* ratio of about 0.5 during the course of the induction period. The result of this experiment suggests the contribution of the allylic hydrogen atom of the –S(O)S–CH_2CH=CH_2 transfer acting as an antioxidant (Scheme 1).

It is well known that there are two mechanisms for the radical-scavenging reactions of antioxidants: a one-step hydrogen atom transfer; and an electron-transfer followed by a proton transfer.[33] In addition, the electron-transfer mechanism is known to be

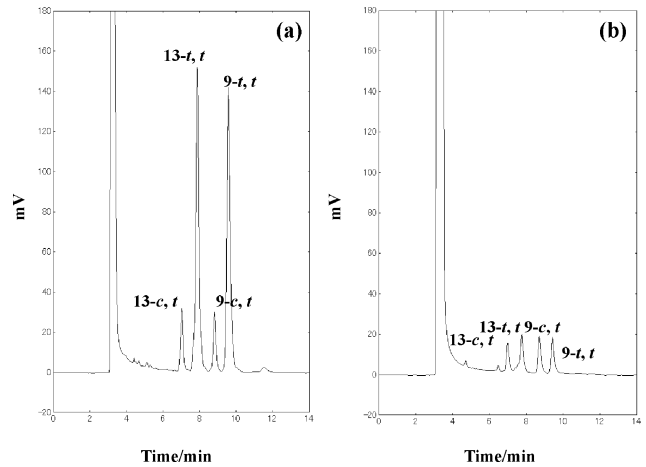
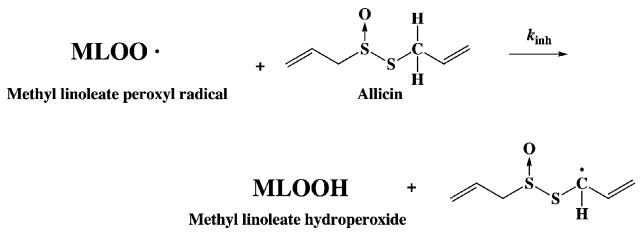


Fig. 5 Distribution of geometric isomers of the MLOH. ML (91 mM) was oxidized at 30 °C in chlorobenzene under air with AIBN (40 mM) in the absence (a) and presence (b) of 50 μM allicin. Samples (0.5 mL) were withdrawn from the mixture after 30 min and were reduced to MLOH by adding triphenylphosphine to the samples. MLOH was analyzed by HPLC.



Scheme 1 Radical-scavenging reaction by allicin.

significantly accelerated in the presence of Mg^{2+}.[34] This was investigated by examining the effect of Mg^{2+} on the radical-scavenging rates by allicin in methanol according to the method of Nakanishi *et al.*[34] As shown in Fig. 6, the rate of the DPPH scavenging reaction by allicin was not affected upon the addition of Mg^{2+} to the allicin–DPPH system in methanol. Thus, the radical scavenging reaction in methanol may not proceed *via* an electron transfer from allicin to DPPH. This result shows that the radical scavenging reaction of allicin with the peroxyl radical proceeded

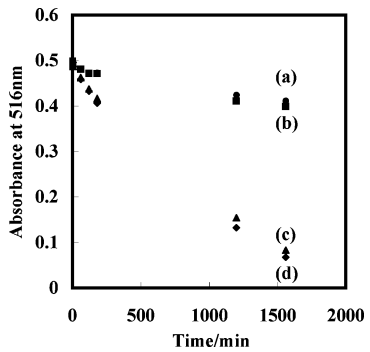


Fig. 6 Reactivity of allicin toward DPPH in the presence of Mg^{2+} in chlorobenzene. DPPH (50 μM) was reduced at 30 °C in chlorobenzene under air in the absence (a) and presence (b) of 0.2 M $Mg(ClO_4)_2$, (c) 50 μM allicin, and (d) 0.2 M $Mg(ClO_4)_2$ and 50 μM allicin.

via a one-step hydrogen atom transfer rather than *via* an electron transfer.

It is clear that the bond dissociation enthalpy (BDE) in a radical-scavenging type antioxidant will be an important factor in determining the efficacy of an antioxidant.[35] A one-step hydrogen atom transfer mechanism is governed by BDE to a large extent. The BDE value of the allylic C–H bond of allicin, in this connection, was calculated to be 85.8 kcal mol^{-1}. On the other hand, an electron-transfer mechanism is mainly determined by the ionization potential (IP).[33] The lower this value, the faster the electron-transfer mechanism. We then calculated the IP value of allicin using GAUSSIAN 03, which suggested that the IP value for allicin was 189.5 kcal mol^{-1}. The IP value was also determined using α-toc, which scavenges the peroxyl radical *via* an electron transfer in methanol, as a reference antioxidant. The result of the calculation provides the IP value for α-toc of 149.5 kcal mol^{-1}. A recent study by Wright *et al.*[35] has determined that the antioxidant mechanism is predominantly a one-step hydrogen atom transfer if the IP value is about 45 kcal mol^{-1} greater than that of an electron transfer type antioxidants. The IP value for allicin was about 40 kcal mol^{-1} higher than that of α-toc, suggesting that allicin can scavenge the peroxyl radical *via* a one-step hydrogen atom transfer.

Determination of antioxidant activity (k_{inh}) of allicin

As described above, it is evident that allicin was consumed by the end of the induction period of the cumene and ML oxidation in chlorobenzene, and the allylic hydrogen atom of allicin contributes to determining the antioxidant activity. We then determined the antioxidant activity, k_{inh}, by measuring the τ and R_{inh}, the time of the induction period and the rate of hydroperoxide formation, respectively, during the course of the induction periods of the cumene and ML oxidation.

The activity of a radical-scavenging antioxidant in a homogeneous solution is primarily determined by the rate constant of the scavenging peroxyl radicals by the antioxidant during the induction period of the substrate oxidation. The rate of the inhibited oxidation is given by eqn 1,[36,37]

$$R_{inh} = -\frac{d[ROOH]}{dt} = \frac{k_p[RH]R_i}{nk_{inh}[IH]} \quad (1)$$

where ROOH, RH and IH are the substrate hydroperoxide, substrate and antioxidant, and k_p and k_{inh} are the rate constants for the chain propagation and scavenging of the chain-carrying peroxyl radical by an antioxidant, respectively. This classical equation applies to the peroxidation of many organic substrates in a homogeneous solution. For example, the k_{inh} values for bilirubin and glutathione, not phenolic antioxidants, have also been determined using this equation.[38,39] The k_p values used for cumene and ML in chlorobenzene at 30 °C are 0.18 M^{-1} s^{-1} and 62 M^{-1} s^{-1},[40,41] respectively. The antioxidant activity and stoichiometric factor of allicin in chlorobenzene at 30 °C are summarized in Table 1 for comparison with that of α-toc. As a result, the k_{inh} values give the inhibition rate constants for allicin with the peroxyl radical derived from cumene and ML at 2.6×10^3 M^{-1} s^{-1} and 1.6×10^5 M^{-1} s^{-1}, respectively. This is about one-fiftieth that of α-toc in the cumene oxidation system and about one-seventh that of α-toc in the ML oxidation system. However,

Table 1 Summary of antioxidant activities of allicin and α-toc

	n	$k_p/M^{-1}\ s^{-1}$	R_i/s^{-1}	$R_{inh}/M\ s^{-1}$	τ/s	$k_{inh}/M^{-1}\ s^{-1}$
Cumene (5.35 M) + AIBN (23 mM) in chlorobenzene						
Allicin (25 μM)	1.0	0.18	2.46×10^{-9}	3.66×10^{-8}	2787.6	2.6×10^{3}
α-Tocopherol (25 μM)	2.0			3.87×10^{-10}	20286	1.2×10^{5}
ML (91 mM) + AIBN (40 mM) in chlorobenzene						
Allicin (50 μM)	1.0	62	5.28×10^{-9}	3.64×10^{-9}	2659.2	1.6×10^{5}
α-Tocopherol (50 μM)	2.0			2.73×10^{-10}	18948	1.1×10^{6}

our results provide an answer to the simple, but important subjects concerning the antioxidant activity of allicin and the discussion of these results suggests potential applications of allicin as an antioxidant. Furthermore, to the best of our knowledge, these results represent the first reported of the quantitative kinetic data for allicin as an antioxidant.

It is conceivable that allicin is not stable. However, it has been reported that half-life of allicin is 30–40 d at 23 °C in water at concentrations ranging from 0.6 to 6.0 mM.[25] On this basis, we speculate that allicin will play a more effective role in controlling lipid oxidation in micelles and bilayers. Therefore, we are currently extending this study to micellar and liposome systems.

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