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Kinetics and Antioxidative Sites of Capsaicin in Homogeneous Solution

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Abstract Reliable quantitative kinetic data on the antioxidant activity of capsaicin (CAP) is lacking, and the antioxidative mechanism of CAP is still unclear. Therefore, an investigation aimed at elucidating the antioxidative sites of CAP that react with chain-propagating peroxyl radicals was undertaken. First, the reaction of CAP with 2,2-diphenyl-1-picrylhydrazyl (DPPH) was investigated, and it was found that the stoichiometric factor of CAP is 2. Then, the rate constant for the reaction of CAP with peroxyl radicals derived from cumene was measured. CAP reacted with peroxyl radicals at a constant rate of $k_{inh} = 5.6 \times 10^3$ M^{-1} s^{-1}. Furthermore, the inhibitory effects of various related compounds against cumene oxidation were measured, showing that the phenolic OH group is the active portion of the molecule. In addition, the kinetic solvent effects of DPPH/CAP reactions were measured in methanol, acetonitrile, acetone and tetrahydrofuran. In particular, an enhancement in the reaction rate was observed in alkaline methanol, indicating that these results are due to the partial ionization of the phenol of CAP and very fast electron transfers from the phenolate anion to DPPH. We interpreted these results as indicating that the phenolic OH group of CAP is mainly associated with peroxyl radical scavenging.

Keywords Capsaicin · Rate constant · DPPH · Peroxyl radical · Phenolic OH group · Antioxidative site

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Introduction

Capsaicin (CAP) is a major pungent principle present in hot peppers and is widely consumed as a food additive throughout the world [1]. Due to degradation of foods containing lipids induced by free radicals, natural antioxidants present in plant foods have attracted considerable interest [2]. It is generally known that CAP shows antioxidant activity against lipid peroxidation in animal tissues, especially rodent lung [3, 4] and liver [5, 6]. We previously reported that CAP inhibits oxidation of phosphatidylcholine liposomal membranes as effectively as α-tocopherol (α-toc) [7]. We also showed that CAP is capable of scavenging hydroxyl radicals and its ability is characterized by the reaction against hydroxyl radicals with a rate constant of 3.5×10^{10} M^{-1} s^{-1} [8]. However, as far as we know, reliable quantitative kinetic data on its antioxidant activity is lacking. Furthermore, the antioxidative mechanisms of CAP have not been well documented. Kogure et al. [9] investigated the antioxidative mechanism of CAP and reported that the radical scavenging site of CAP was the C7-benzyl carbon rather than the phenolic OH group.

It is well known that the free radical scavenging ability of phenolic antioxidants depends on the reaction environment in which the reaction occurs [10–14]. That is, free radicals react with phenols (ArOH) via two different

mechanisms: (1) the direct abstraction of the hydrogen atom of ArOH by free radicals (HAT mechanism), and (2) the electron-transfer process from ArOH or its phenolate anion (ArO^-) to free radicals (ET mechanism) [15]. These reaction rates are strongly affected by the nature of the solvents. In general, the HAT reactions are predominant in apolar solvents and nonprotic polar solvents reduce the rate of many ArOH-free radical reactions. Therefore, if the rate of reaction of CAP with radicals is dependent on the solvents, this fact suggests that the antioxidant effect of CAP is conveyed by its phenolic hydroxyl group.

By discussing the structure–activity relationship of CAP (structures in Fig. 1) and the kinetic solvent effects of the radical reaction with CAP, we provide here convincing evidence that the phenolic OH group of CAP is the most important site as an antioxidant.

Materials and Methods

Materials

CAP, *N*-benzylacetamide, and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and used without purification. 2-Methoxy-4-methylphenol was obtained from Sigma-Aldrich (St. Louis). Cumene, 2,2′-azobis (isobutyronitrile) (AIBN), 2,2-diphenyl-1-picrylhydrazyl (DPPH), galvinoxyl and chlorobenzene were obtained from Wako Pure Chemical Industries (Osaka, Japan). AIBN was recrystallized from methanol, and cumene was purified on a silica-gel column before use.

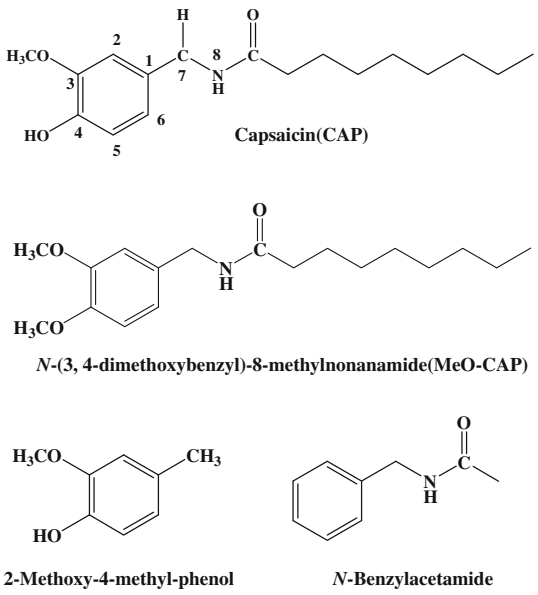

Fig. 1 Structures of CAP and its derivatives in this work

α-Toc was from the Kanto Chemical Co. (Tokyo, Japan) and used without purification. All other reagents of the highest grade were commercially available.

Methods

Synthesis of *N*-(3,4-dimethoxybenzyl)-8-methylnonanamide (MeO-CAP)

MeO-CAP was synthesized from CAP according to the method of Hashimoto et al. [16]. After the reaction was over, MeO-CAP was applied on an RP-18 preparative TLC plate and developed with methanol/water (3:2, v/v) up to 3 cm from the upper edge. Then, MeO-CAP was extracted by methanol, and confirmed using mass spectrometry (direct insertion). The mass spectra of MeO-CAP was measured with a JEOL JMS-AM II series spectrometer (inlet temperature 200 °C, 70 eV); MS *m/z* (relative intensity): 307 (M^+, 20%), 151 (100, C_6H_5 $(CH_3O)_2CH_2^+$).

Inhibitory Effect of CAP on Cumene Oxidation in a Chlorobenzene Solution

Cumene (5.35 M) in chlorobenzene in the presence of an appropriate amount of CAP was incubated at 30 °C in air. A solution of AIBN (23 mM) in chlorobenzene was added to this reaction mixture. The rate of the cumene oxidation was followed by measurement of the cumene hydroperoxides (CHP) generated from the cumene using a reverse phase HPLC (0.3 mL/min, methanol/water (85/15), Shiseido CAPCELLPAK C_{18} column 3.0 mm × 150 mm × 5 μm) and the peaks were detected at 260 nm [17, 18]. The consumption of CAP was analyzed at the same time using a C_{18} HPLC with a UV detector at 279 nm.

Reactivity of CAP Toward DPPH, Galvinoxyl, and ABTS Cationic Radical ($ABTS^{\cdot+}$)

DPPH (50 μM) and CAP were dissolved at 30 °C in chlorobenzene, methanol, acetonitrile, acetone, and tetrahydrofuran (THF), respectively. On the other hand, galvinoxyl (5.0 μM) and CAP were dissolved at 30 °C in chlorobenzene and methanol, respectively. The decays of DPPH or galvinoxyl by CAP were followed using a spectrophotometer (BECKMAN DU7000) at 517 or 429 nm, respectively [17, 18]. At the same time, the consumption of CAP by reaction with DPPH was analyzed using a reverse phase HPLC (0.3 mL/min, methanol/water (85/15), Shiseido CAPCELLPAK C_{18} column 3.0 mm × 150 mm × 5 μm) and the peaks were detected at 279 nm. In the same way, the rates of the DPPH or galvinoxyl-scavenging reactions were monitored at 517 or 429 nm in methanol

containing 10 mM acetic acid [12] or 50 μM potassium hydroxide, respectively [15]. $ABTS^{\cdot+}$ was prepared according to the method of Feng et al. [19]. The absorbance at 734 nm of the mixture became stable and was monitored by a spectrophotometer after CAP was added to the $ABTS^{\cdot+}$ solution at room temperature.

Reactivity of CAP and MeO-CAP toward Peroxyl Radicals

The reactivity of CAP and MeO-CAP toward the peroxyl radicals was estimated using a radical initiator, AIBN. The consumption of CAP (150 μM) and MeO-CAP (150 μM) with AIBN (15 mM) in acetonitrile at 50 °C was analyzed using an HPLC equipped with an RP-C18 column (3.0 mm × 150 mm × 5 μm, Shiseido) and methanol/water (7:3, v/v) as an eluent at a constant flow rate of 0.3 mL/min. CAP and MeO-CAP were detected by UV absorption at 279 nm using an HPLC detector (Shiseido Nanospace SI-2). At the same time, the absorption spectrum (200–350 nm) of CAP and CAP were measured every 1.0 nm using a BECKMAN spectrophotometer DU7000.

Calculation of Bond Dissociation Enthalpy Values

The calculation of the C7-benzyl carbon–hydrogen bond dissociation enthalpy (BDE)'s values of CAP and MeO-CAP was performed for the gas-phase by the Gaussian 03 program (Gaussian, Inc., Carnegie, PA, USA) [20] using the density functional theory (DFT) as follows: geometrical optimization and determination of the vibrational frequencies were performed using HF/6-31+G (2d, p). The single-point electronic energies were obtained using B3LYP/6-31+G (2d, p).

Results and Discussion

Antioxidant Activity of CAP on Cumene Oxidation in Chlorobenzene Solution

In our previous paper [7, 8], we reported that the antioxidant activity of CAP on methyl linoleate oxidation is much less than that of α-toc in an acetonitrile solution. Similar results were reported for the reaction of CAP toward DPPH in a homogeneous solution. Therefore, at first we undertook a detailed study of the antioxidant activity of CAP in chlorobenzene solution in order to obtain quantitative information as a chain-breaking antioxidant. Chepelev et al. [21] also reported that the autoxidation of cumene initiated by AIBN in chlorobenzene has certain advantages for determining the rate constants for peroxyl radical trapping by antioxidants. Recently, we showed the antioxidant activities of allicin, one of the main thiosulfinates in garlic, and *S*-benzyl phenylmethane-thiosulfinate from *Petiveria alliacea* L. against the oxidation of cumene in a chlorobenzene solution [17, 18]. We decided to investigate the antioxidant activity of CAP under this same condition as had been used for the measurement of the rate constant, k_{inh}, for allicin.

Figure 2A shows a plot of the CHP during the initiated oxidation of cumene in chlorobenzene in the presence of CAP or α-toc. It is clear that both the CAP and α-toc were highly effective chain-breaking antioxidants that gave sharp, well-defined induction periods in this condition. For quantitative studies in a homogeneous solution, it was necessary to determine whether the CAP was consumed by the end of the inhibition period of the cumene oxidation. As shown in Fig. 2B, CAP was found to be almost completely consumed by the end of the inhibition period of the cumene oxidation. Moreover, the time of the induction period, τ, generated by CAP was the same as that of α-toc. This means that one molecule of CAP can scavenge two peroxyl radicals derived from cumene, namely, the number of peroxyl radicals trapped by one molecule of an antioxidant is 2 because α-toc is known to trap two peroxyl radicals per molecule under this condition [22]. In order to identify this fact, we measured the rate of reduction of DPPH by CAP and the amount of unreacted CAP remaining after the CAP was mixed with equimolar quantities of DPPH in chlorobenzene. As shown in Fig. 3, it was found that 50% of the CAP remained, despite the fact that DPPH was completely reduced by the CAP. This result was also confirmed by demonstrating that one molecule of CAP reacted with two molecules of DPPH [23].

In general, 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. We then determined the rate constant, k_{inh}, by measuring the R_i and R_{inh}, the rate of chain initiation and the rate of CHP formation, respectively, during the induction period of the cumene oxidation. The rate of the inhibited oxidation is given by Eq. 1 [24, 25],

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

where ROOH, RH, IH, n, and k_p are the substrate hydroperoxide (CHP), substrate (cumene), antioxidants (CAP), stoichiometric number (the number of peroxyl radicals trapped by one molecule of an antioxidant), and the rate constant for the chain propagation, respectively. R_i was determined by the inhibitor method using α-toc as a reference antioxidant: $R_i = 2[\alpha\text{-toc}]/\tau$. The k_p value was 0.18 M^{-1} s^{-1} under this condition [26]. As a result, CAP and α-toc reacted with peroxyl radicals derived from

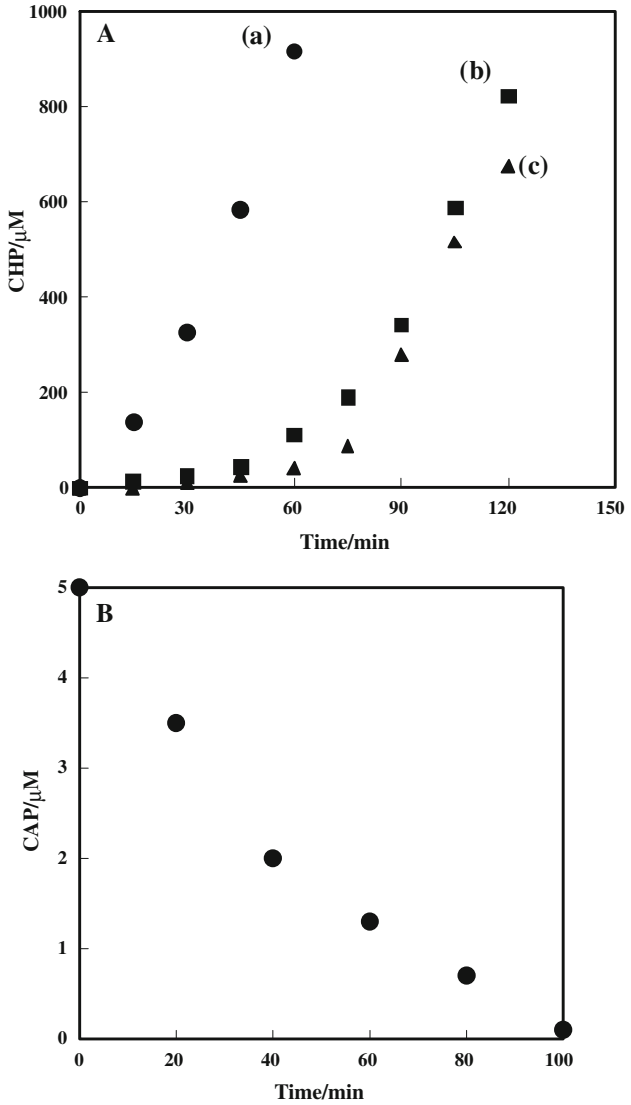

Fig. 2 Inhibitory effect of CAP on the oxidation of cumene induced by AIBN in chlorobenzene. Cumene (5.35 M) was oxidized at 30 °C in chlorobenzene under air with AIBN (23 mM). **A** A plot of CHP during cumene oxidation in the absence *(a)* and presence *(b)* of 5.0 μM CAP, and *(c)* 5.0 μM α-toc. **B** Consumption of CAP during cumene oxidation. The decay of CAP was measured by HPLC

cumene with a rate constant of $k_{inh} = 5.6 \times 10^3$ and $6.3 \times 10^4 M^{-1}\ s^{-1}$, respectively. This means that the antioxidant activity of CAP was about one-eleventh that of α-toc in this system. From the above results, it was found that the stoichiometric numbers, *n*, for CAP and α-toc are the same, but the k_{inh} for CAP is smaller than that of α-toc. This is because the R_{inh} for α-toc is smaller than that of CAP. α-Toc and 2,6-di-*tert*-butyl-4-methylphenol are examples of such antioxidants [27]. Thus, there are many other cases where the stoichiometric numbers are the same, but the antioxidant activities are different. However, we should notice the importance of scavenging two peroxyl radicals per one molecule of CAP.

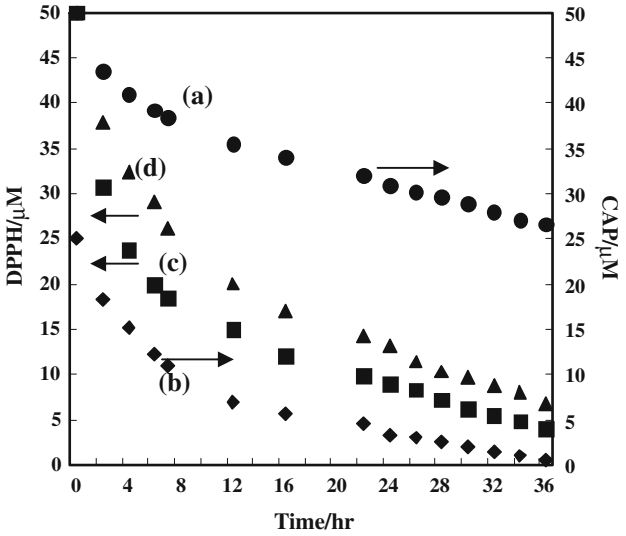

Fig. 3 Consumption of CAP and DPPH by reaction of CAP with DPPH in chlorobenzene. DPPH (50 μM) was reduced at 30 °C in chlorobenzene under air in the presence of *(a)* and *(c)* 50 μM, and *(b)* and *(d)* 25 μM CAP. The decay of CAP was measured by HPLC

Antioxidative Site of CAP

Structure–Activity Relationship Studies of CAP

Kogure et al. [9] reported that the phenolic OH group of CAP is not associated with the radical scavenging reaction and the radical scavenging site of CAP is the C7-benzyl carbon (Fig. 1). From the results of quantum chemical calculations of various radical intermediates derived from the related CAP compound and identification of reaction products of CAP with DPPH, they proposed that the presence of acetamide moiety should be important for hydrogen abstraction from C7-benzyl carbon of CAP by free radicals. Therefore, we investigated the antioxidative site of CAP by measuring the inhibitory effects of its related compounds against cumene oxidation in chlorobenzene. Hussain et al. [28] also reported that the various related compounds used provided a useful way of targeting reactive sites of antioxidant activities in compounds. Recently, Feng et al. [19] clarified the antioxidant ability of the phenolic group in curcumin by comparison with that of the activity of curcumin derivatives.

First of all, we determined the inhibitory effect of MeO-CAP, phenolic O-methylation of CAP, for whether phenolic hydrogen of CAP contributes to its activity. MeO-CAP must show the inhibitory effect against cumene oxidation if the radical scavenging site of CAP is the C7-benzyl carbon because CAP and MeO-CAP have almost identical C7-benzyl carbon–hydrogen BDE's (86.0–86.6 kcal/mol). As shown in Fig. 4, MeO-CAP had no significant inhibitory effect against cumene oxidation.

Moreover, *N*-benzylacetamide, a related compound which does not have the phenol moiety of CAP but has a C7-benzyl carbon, did not inhibit the oxidation of cumene in chlorobenzene (Fig. 4). These results show that the phenolic hydrogen of CAP is the site for radical scavenging and the antioxidant activity of CAP is independent on the C7-benzyl hydrogen and acetamide moiety. In practice, 2-methoxy-4-methyl-phenol inhibits the oxidation of cumene in chlorobenzene as effectively as CAP (Fig. 4). This result in turn has important implications for the antioxidant activity of CAP, indicating that only a 2-methoxy-4-methyl-phenol moiety can suffice for the antioxidant activity of CAP.

Absorption Spectrum Studies of CAP

Kogure et al. [9] monitored a peak at 280 nm due to the phenolic OH group of CAP in order to elucidate the antioxidative site of CAP. This peak at 280 nm must change if the phenolic OH group is associated with the radical scavenging. However, since this absorption spectrum of CAP did not change after incubation with DPPH, they concluded that the phenolic OH group of CAP is not associated with the radical scavenging reaction.

Therefore, we monitored the absorption spectrum (200–350 nm) of CAP in the presence of AIBN in acetonitrile at 50 °C, and we measured, at the same time, the consumption of CAP using an HPLC. As shown in Fig. 5A, B, the spectrum shape at around 280 nm gradually changed with

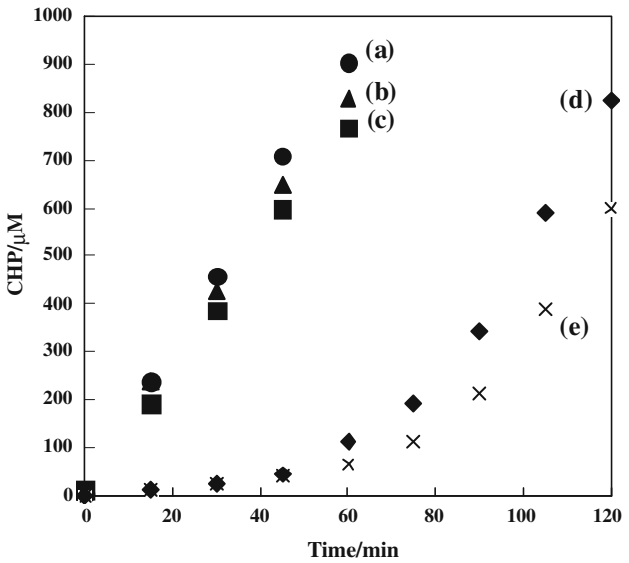

Fig. 4 Inhibitory effect of MeO-CAP, *N*-benzylacetamide, 2-methoxy-4-methyl-phenol, and CAP on the oxidation of cumene induced by AIBN in chlorobenzene. Cumene (5.35 M) was oxidized at 30 °C in chlorobenzene under air with AIBN (23 mM) in the absence *(a)* and presence *(b)* of 50 μM MeO-CAP, *(c)* 50 μM *N*-benzylacetamide, *(d)* 50 μM 2-methoxy-4-methyl-phenol, and *(e)* 50 μM CAP

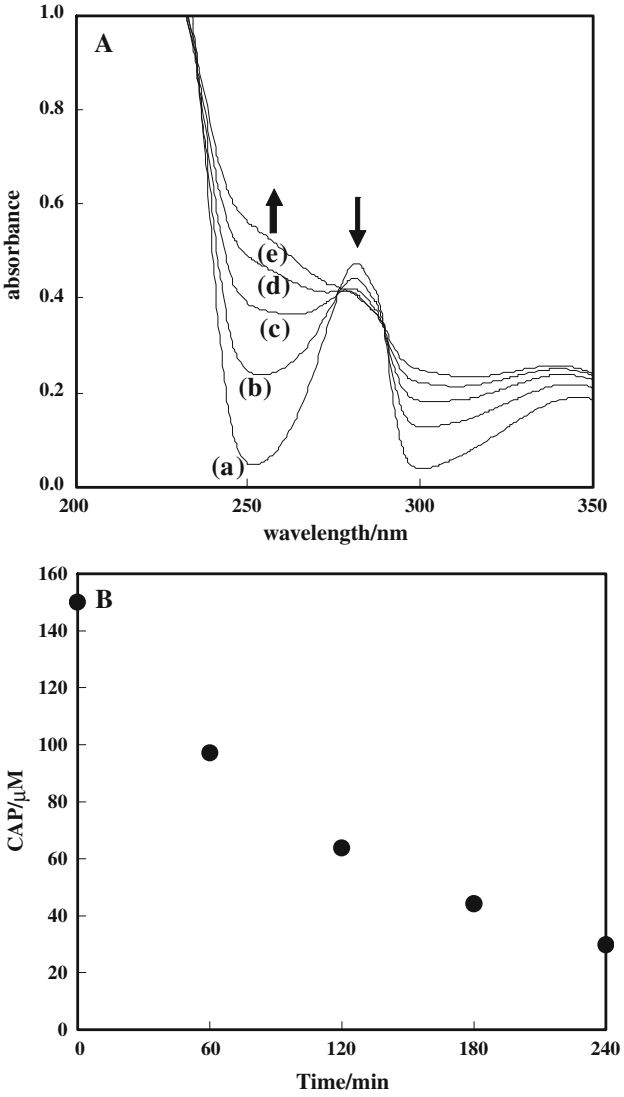

Fig. 5 Reactivity of CAP with AIBN in acetonitrile. **A** Absorption spectra of CAP (150 μM) with AIBN (15 mM) in acetonitrile at 50 °C under air for *(a)* 0 time, *(b)* 1.0 h, *(c)* 2.0 h, *(d)* 3.0 h, and (e) 4.0 h. **B** Consumption of CAP (150 μM) with AIBN (15 mM) in acetonitrile at 50 °C under air. The decay of CAP was measured by HPLC

the consumption of the CAP. This result clearly shows that the phenolic OH group of CAP is associated with peroxyl radical scavenging.

We also monitored the absorption spectrum of MeO-CAP in acetonitrile. As shown in Fig. 6, the absorption spectra (200–350 nm) of CAP and MeO-CAP are almost identical. This indicates that a peak at 280 nm is not due to the phenolic OH group of CAP. Moreover, as shown in Fig. 7, the absorption spectrum of CAP at around 280 nm is derived from the guaiacol structure, not from the phenol structure. On the other hand, the spectrum shape of *N*-benzylacetamide is almost distinct from that of CAP. In addition, we monitored the absorption spectrum

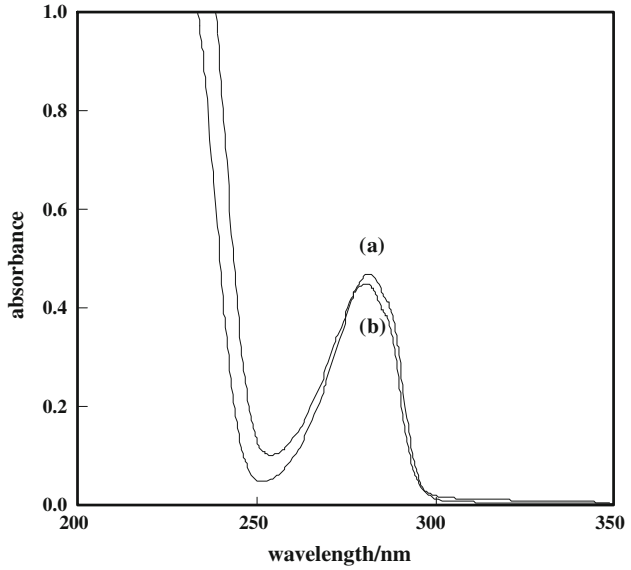

Fig. 6 Absorption spectra of CAP and MeO-CAP in acetonitrile. *(a)* CAP (150 μM) or *(b)* MeO-CAP (150 μM) was dissolved in acetonitrile at room temperature under air

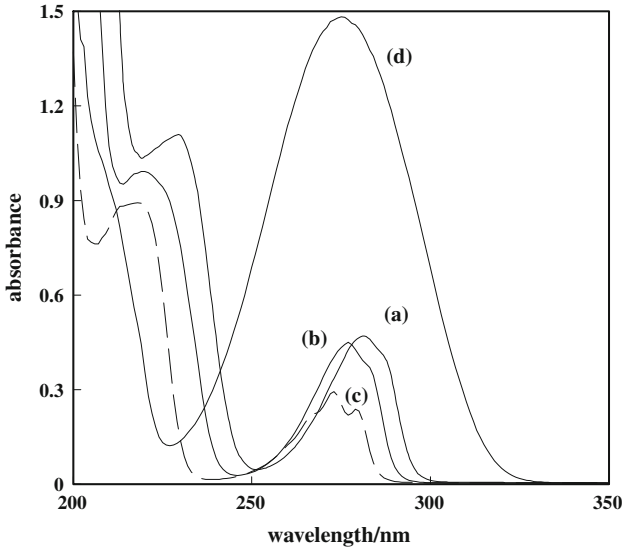

Fig. 7 Absorption spectra of CAP, guaiacol, phenol, and *N*-benzylacetamide in acetonitrile. *(a)* CAP (150 μM), *(b)* guaiacol (150 μM), *(c)* phenol (150 μM), and *(d)* *N*-benzylacetamide (50 μM) were dissolved in acetonitrile at room temperature under air

(200–350 nm) of MeO-CAP in the presence of AIBN in acetonitrile at 50 °C. As a result, the spectrum shape at around 280 nm did not change at all although the amount of MeO-CAP was reduced to about 80% after 4.0 h of incubation. Consequently, we could not obtain any available data from this absorption spectrum of CAP.

Kinetic Solvent Effect Against DPPH, Galvinoxyl, and ABTS Cationic Radical ($ABTS^{\cdot+}$)

Next, we examined the solvent effects on the radical scavenging ability of CAP. Thavasi et al. [29] demonstrated the radical scavenging ability of phenols against DPPH in different solvent media, and indicated that the radical scavenging ability of phenols was the fastest in methanol and the slowest in THF. According to this report, methanol can potentially form hydrogen bonds with surrounding methanol molecules, therefore, the hydrogen atom devoting behavior of phenols to scavenge the free radicals is least affected in methanol. On the other hand, the higher electronegative nature of the oxygen atom in THF exerts a stronger interaction with the OH of the phenols, therefore, the rate of free radical scavenging of phenols is reduced in THF. That is to say, the rate of the DPPH scavenging reaction of phenols is affected by the nature of the solvent, the strength of the phenolic OH···solvent interaction; thus, phenols release their hydrogen atom at a considerably faster rate of decrease in the order methanol > acetonitrile > acetone > THF. Though this point will be examined again later, similar phenomena in the reactivity of phenols against DPPH were observed by Saito et al. [10] and Litwinienko et al. [12].

Therefore, we consequently decided to investigate the solvent effects on the DPPH scavenging ability of CAP in methanol, acetonitrile, acetone, and THF. As shown in Fig. 8, the rate of reactivity of CAP against DPPH in the decreasing order of methanol > acetonitrile > acetone > THF. This result was quite similar to that described by Thavasi et al. [29], which means that phenolic OH group of CAP is mainly associated with radical scavenging.

Litwinienko et al. [12] reported the solvent effects on hydrogen atom abstraction from phenol by the DPPH in a number of alcoholic and nonalcoholic solvents. Litwinienko et al. [12] also found that phenols react with DPPH at a higher rate in methanol due to the presence of traces of phenolate anions (ArO^-). That is, phenolate anions (ArO^-) would be expected to react very rapidly in methanol with the DPPH. According to Litwinienko et al. [12], since electron transfer from unionized phenols to electrophilic radicals is occasionally the rate-controlling step, a very fast electron transfer occurs from the electron-rich phenolate anion (ArO^-) to the highly electron-deficient DPPH. For example, in the reaction between DPPH and α-toc, the sequential proton loss electron transfer (SPLET) mechanism will be expressed as follows [12–14].

$$\mathrm{DPPH}\bullet + \alpha\text{-tocH} \underset{H^+}{\overset{-H^+}{\rightleftharpoons}} \mathrm{DPPH}\bullet + \alpha\text{-toc}:^- \xrightarrow[\text{fast}]{} \mathrm{DPPH}:^- + \alpha\text{-toc}\bullet$$
$$\xrightarrow{H^+} \mathrm{DPPHH} + \alpha\text{-toc}\bullet$$

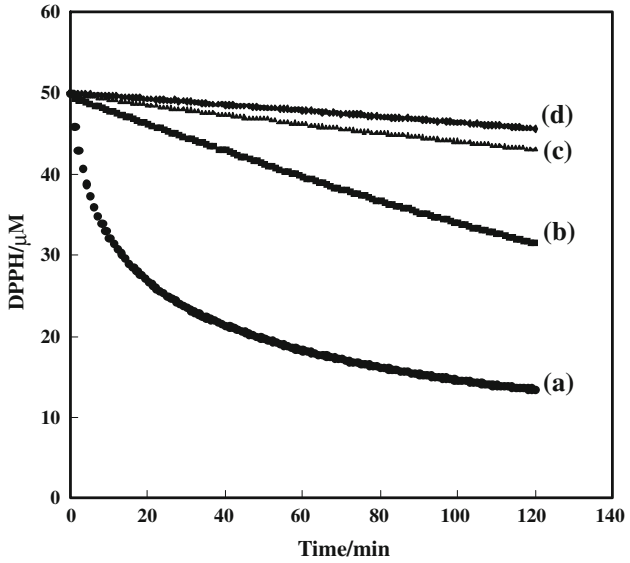

Fig. 8 Reactivity of CAP toward DPPH in solvents. DPPH (50 μM) was reduced at 30 °C in *(a)* methanol, *(b)* acetonitrile, *(c)* acetone, and *(d)* THF under air in the presence of 50 μM CAP

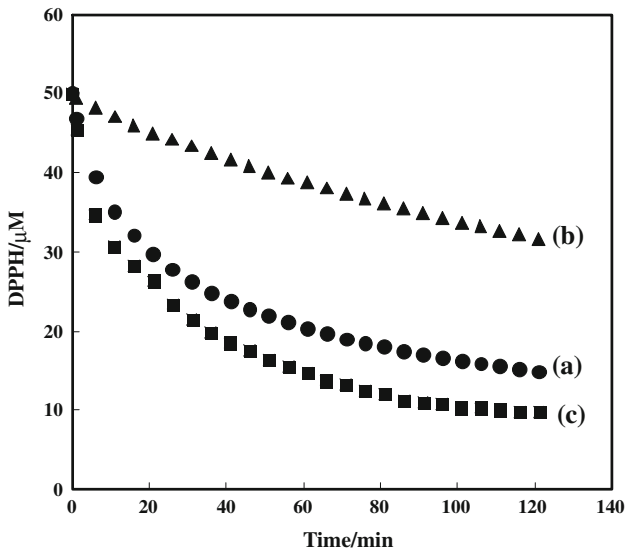

Fig. 9 Reactivity of CAP toward DPPH in acidic methanol and alkaline methanol. DPPH (50 μM) was reduced at 30 °C in *(a)* methanol, *(b)* 10 mM acetic acid in methanol, and *(c)* 50 μM potassium hydroxide under air in the presence of 50 μM CAP

Moreover, they found that the reaction rate of phenols with DPPH is sufficiently reduced in methanol containing 10 mM acetic acid because the phenols exist only in the molecular forms (ArOH) under the acidic conditions [12]. In practice, as shown in Fig. 9, the reaction of CAP with DPPH was slower in acidic methanol than that in methanol only. Also, Foti et al. [15] reported that the reaction of phenols with DPPH take place more rapidly in methanol containing 30 μM KOH because the phenols exist only in the phenolate anion (ArO^-) forms in the alkaline condition. Figure 9 shows that the CAP was allowed to react more rapidly with DPPH in alkaline methanol than that in methanol only.

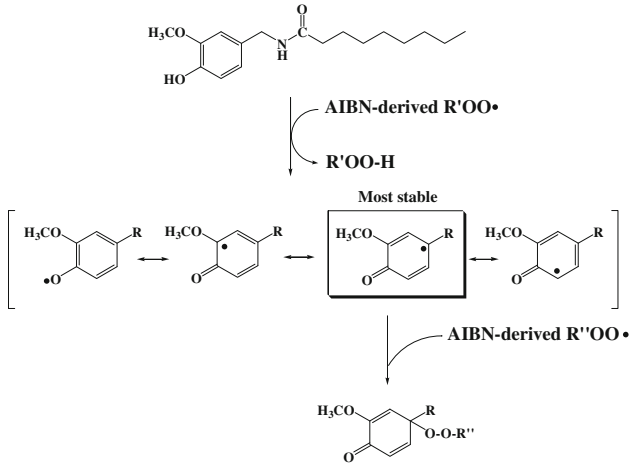

Fig. 10 Antioxidative mechanism of CAP

On the other hand, we investigated the reactivity of CAP with galvinoxyl and $ABTS^{\cdot+}$. Although the galvinoxyl was consumed less rapidly under air in chlorobenzene and methanol, the rate of reduction of galvinoxyl was much faster after the addition of CAP. In addition, CAP can also reduce the $ABTS^{\cdot+}$ in a concentration-dependent manner. In this case, nevertheless, $ABTS^{\cdot+}$ was also consumed slowly in ethanol without the addition of CAP. It is thus readily apparent that CAP can scavenge the O-centered radicals and the cationic radicals. However, it is difficult to account for the stoichiometric of CAP and the SPLET and HAT mechanism by CAP in galvinoxyl and $ABTS^{\cdot+}$ radical trapping test.

These experimental results obtained above mean that the reaction between CAP and DPPH in methanol may be proceeded by the mechanism of SPLET, which may occur parallel to the hydrogen atom transfer mechanism.

From above the results, we can say with fair certainly that the phenol OH group of CAP mainly contributes to determining the antioxidant activity.

Conclusions

In conclusion, CAP is an active antioxidant in cumene oxidation in chlorobenzene, having an antioxidant activity one-eleventh that of α-toc. However, most notably, we also clarified that the phenolic OH group of CAP is mainly associated with radical scavenging, and CAP is capable of scavenging two radicals per one molecule of CAP.

Saito et al. [11] and López-Giraldo et al. [30] reported that the methanol can act as a strong nucleophilic agent and

regenerate the oxidized phenols. Moreover, they suggested that the presence of methanol influences the DPPH scavenging activity.

We obtained our results concerning the antioxidant activity of CAP in chlorobenzene, therefore, we will now develop the hypothesis of antioxidative mechanism of CAP a little further. As shown in Fig. 10, our suggestion is as follows; the phenolic group of CAP scavenges peroxyl radicals to give a stable CAP radical (C1 carbon position) in its first stage, followed by the addition of another peroxyl radical to its CAP radical.

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