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Nonenzymatic Antioxidant Activity of Four Organosulfur Compounds Derived from Garlic

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The nonenzymatic antioxidant activity of diallyl sulfide (DAS), diallyl disulfide (DADS), *S*-ethyl cysteine (SEC), and *N*-acetyl cysteine (NAC) in the liposome system was examined. The antioxidant protection from these organosulfur agents was concentration dependent ($p < 0.05$). SEC and NAC showed significantly lower lipophilicity and greater reducing power than DAS and DADS ($p < 0.05$). Greater antioxidant protection was presented in the combinations of α-tocopherol with four organosulfur agents than α-tocopherol treatment alone ($p < 0.05$), and SEC and NAC showed greater sparing effects on α-tocopherol ($p < 0.05$). Four organosulfur agents lost antioxidant activity when the temperature was 65 °C ($p < 0.05$). At pH 2.5 and 10, DAS and DADS still showed antioxidant activity ($p < 0.05$). On the basis of the observed nonenzymatic antioxidant protection, these organosulfur compounds are potent agents for enhancing lipid stability.

KEYWORDS: Organosulfur compounds; nonenzymatic antioxidant activity; iron-chelating effect; reducing power; partition coefficient

INTRODUCTION

Diallyl sulfide (DAS), diallyl disulfide (DADS), *S*-ethyl cysteine (SEC), and *N*-acetyl cysteine (NAC) are four organosulfur compounds derived from *Allium* plants such as garlic and onion. Several in vivo studies have observed that these organosulfur compounds exerted their antioxidant protection via modulating antioxidant related enzymes such as 3-hydroxy-3-methylglutaryl-CoA reductase, glutathione-*s*-transferase, and catalase (*1*−*3*). The enzymatic antioxidant activity of these compounds supported their application in human and animals for healthy consideration; however, these findings may not support their application in food systems for antioxidant protection.

So far, less attention has been paid to the nonenzymatic antioxidant capabilities of these compounds. The possible nonenzymatic antioxidant actions include reducing power (*4*−*6*), metal ions chelating effect (*7*−*9*), and interacting with biomembranes and/or with other antioxidants (*10*−*12*). It is known that the interaction of an antioxidant with biomembranes or with other antioxidant agents is strongly related to its lipophilicity (*12*−*14*). To evaluate the nonenzymatic antioxidant activity of these organosulfur agents, it would be helpful to determinate their partition coefficients. On the other hand, the information regarding the influence of temperature and pH upon the antioxidant activity of these organosulfur compounds was lacking. If these agents are able to show antioxidant protection in lower pH and/or higher temperature, they will be useful in a wide variety of food systems.

In this study, a liposome system without any enzymes was used to examine the nonenzymatic antioxidant property of these organosulfur compounds, and their antioxidant interactions with α-tocopherol. The lipophilicity, reducing power, and iron chelating capability of these organosulfur compounds, as well as the influence of temperature and pH upon the antioxidant activity of these agents, were evaluated.

MATERIALS AND METHODS

Chemicals. *S*-Ethyl cysteine (99.5%), *N*-acetyl cysteine (99.5%), diallyl sulfide (purity 97%), and crude diallyl disulfide (purity 80%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Diallyl disulfide was further purified by fractional distillation and its final purity was analyzed by HPLC to be 98% (*15*). The structures of four test organosulfur compounds are shown in **Figure 1**. In this study, the antioxidant activities of these organosulfur compounds were compared with α-tocopherol purchased from Sigma Chemical Co. (St. Louis, MO). Control groups contained no organosulfur compound. 2,2′-Azobis-(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), trichloroacetic acid (TCA), thiobarbituric acid (TBA), and chemicals used for liposome preparation were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All chemicals used in these measurements were of the highest purity commercially available.

Liposome Preparation. Phosphatidylcholine (PC) with oleic acid (at the Sn-1 position) and linoleic acid (at Sn-2 position) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Liposomes (multilamellar vesicles) were prepared from PC, cholesterol, and dicetyl phosphate at 4 °C as described by Yin et al. (*16*). The buffer for liposomes suspension was sodium citrate buffer (0.05 M, pH 2.5, 4,

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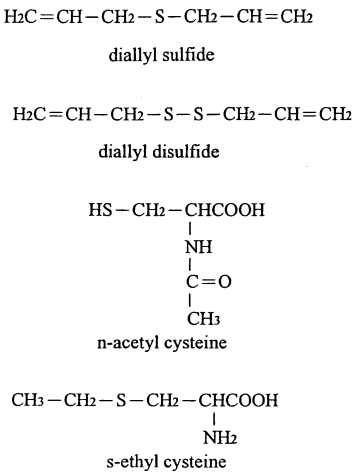

Figure 1. Structures of diallyl sulfide (DAS), diallyl disulfide (DADS), *N*-acetyl cysteine (NAC), and *S*-ethyl cysteine (SEC).

and 5.5) or sodium phosphate buffer (0.05 M, pH 7, 8.5, and 10). After preparation, all samples were incubated at 4, 25, 37, 45, 55, and 65 °C for oxidation measurements.

Antioxidant Treatments. The individual effectiveness of antioxidant action was measured with the concentrations of α-tocopherol, DAS, DADS, SEC, and NAC at 5 and 10 μM. The antioxidant interaction experiments were measured with 5 μM of α-tocopherol combined with 5 μM organosulfur agent. On the basisi of lipid solubility, α-tocopherol, DAS, and DADS were incorporated into multilamellar vesicles with phosphatidylcholine for liposome preparation. SEC or NAC was added to the buffer used for liposome preparation.

Lipid Oxidation Measurements. At the start, 10 μM $FeSO_4$, 2 mM AAPH, and 0.5 mM AMVN were added to liposomes to induce lipid oxidation. AMVN was first dissolved in 95% ethanol. The residue of ethanol in liposomes did not significantly affect oxidation level (data not shown). Lipid oxidation was measured by the thiobarbituric acid (TBA) assay as described by Yin et al. (*16*). One milliliter of sample was mixed with 0.5 mL of 30% TCA and the mixture was centrifuged at 1400*g* for 5 min at 4 °C, and then 1 mL of supernatant was mixed with 1 mL of 0.02 M TBA and the mixture was stored in the dark for 20 h at 25 °C. The absorbance of the final solution was measured by UV−vis spectrophotometry at 532 nm and recorded as the TBA number (TBA no.), which was directly used to express the lipid oxidation level. The lipid stability of purchased PC was examined, and the PC with TBA number ≤0.01 was used for liposome preparation. On the other hand, conjugated diene (CD) formation in liposomes was also determined for lipid oxidation measurement according to the method described by Medina et al. (*17*). The lipid oxidation of liposomes containing 5 or 10 μM of each organosulfur agent was initiated at 37 °C by 0.1 mM $CuCl_2$. Absorbance at 234 nm was continuously recorded for 60 min at 37 °C by a Hitachi U-2001 spectrometer with a constant temperature recirculator (Tokyo, Japan). The lag-phase, expressed as minutes, was defined as the period where no oxidation occurred. Longer lag-phase indicated less conjugated diene formation.

Chelating Effects on Ferrous Ions. The method of Shimada et al. (*18*) was used to determine the chelating effect of α-tocopherol, DAS, DADS, SEC, and NAC on ferrous ions. Each agent in methanol (2 mg/mL) was mixed thoroughly with 200 μL of 1 mM tetramethyl murexide and 2 mL of a solution consisting of 30 mM hexamine, 30 mM potassium chloride, and 9 mM ferrous sulfate. Control groups contained no test agent. Absorbance at 485 nm was measured after 3-min incubation at 25 °C. Lower absorbance indicates higher iron-chelating effect. In this study, the iron-chelating ability of test agent was compared with that of EDTA, and was expressed in percent.

Reducing Power. The method of Oyaizu (*19*) was used to determine the reducing power of α-tocopherol, DAS, DADS, SEC, and NAC. Each agent was dissolved in methanol (2 mg/mL), and then was mixed with a solution containing 2.5 mL of sodium phosphate buffer (pH 6.6, 200 mM) and 2.5 mL of 1% potassium ferricyanide. After the mixture was incubated at 50 °C for 20 min, 2.5 mL of 10% trichloroacetic acid was added. Then, the resulting suspension was centrifuged at 650 rpm for 10 min. The supernatant was mixed thoroughly with 5 mL of deionized water and 1 mL of 0.1% ferric chloride. Absorbance at 700 nm was measured and directly used to express reducing power. Higher absorbance indicates higher reducing power.

Partition Coefficient Measurement. A method similar to that of Foti et al. (*20*) was used to measure the partition coefficient of α-tocopherol, DAS, DADS, SEC, and NAC. Each agent at 1 mM *n*-octanol/water (1:1) was mixed thoroughly by vortexing for 10 min at the highest speed and then incubated at 37 °C for 2 h. A UV spectrum was run and the value of the absorbance at the maximum was measured: 210 nm for DAS; 240 nm for DADS; 195 nm for NAC; and 180 nm for SEC. The UV spectrum of the *n*-octanol layer was run every 30 min until the absorbance value was constant. A solution of *n*-octanol saturated with water was used as a blank. The concentration of agent in the *n*-octanol layer was calculated from the absorbance value. The partition coefficient of an agent was (its concentration in *n*-octanol)/(its concentration in water).

Statistical Analysis. The effect of each treatment was analyzed on liposomes from five different preparations ($n = 5$). Data were subjected to analysis of variance (ANOVA) and computed using the SAS General Model (GLM) procedure (*21*). Difference among means was determined by the least significance difference test with significance defined at $p < 0.05$.

RESULTS

The antioxidant activities of four organosulfur compounds and α-tocopherol against lipid oxidation determined by TBA assay and CD formation are presented in **Table 1**. All organosulfur compounds and α-tocopherol significantly delayed lipid oxidation when compared with control groups ($p < 0.05$). The antioxidant activity of each test agent increased significantly with increasing the concentration from 5 μM to 10 μM ($p < 0.05$). When Fe^{2+} or AAPH was used to initiate lipid oxidation, SEC and NAC showed greater antioxidant protection than DAS, DADS, and α-tocopherol ($p < 0.05$); however, the latter three agents showed greater antioxidant protection when AMVN was used to initiate lipid oxidation ($p < 0.05$).

The partition coefficient, chelating effect, and reducing power of these five agents are shown in **Table 2**. α-Tocopherol was more lipid-soluble than DAS and DADS, and SEC and NAC were more hydrophilic ($p < 0.05$). SEC and NAC showed less reducing power than α-tocopherol ($p < 0.05$), but showed greater reducing power than DAS and DADS ($p < 0.05$). The iron-chelating effect of NAC or SEC was mild, and DAS or DADS did not show detectable iron-chelating effect. The antioxidant interactions of four organosulfur compounds with α-tocopherol are shown in **Figure 2**. When AMVN was used to initiate lipid oxidation, 10 μM α-tocopherol showed less antioxidant activity than 5 μM α-tocopherol plus 5 μM DAS, DADS, NAC, or SEC (**Figure 2A**, $p < 0.05$). NAC and SEC showed greater sparing effects upon α-tocopherol than DAS or DADS (**Figure 2B**, $p < 0.05$).

The influence of temperature and pH upon antioxidant activities of four organosulfur compounds is shown in **Tables 3** and **4**, respectively. Although SEC and NAC showed greater antioxidant activities than other agents at 4, 25, and 37 °C, both showed less antioxidant activities than other agents at 45 °C and high temperatures ($p < 0.05$) (**Table 3**). Both SEC and NAC showed greater antioxidant protection than the other three agents at pH 5.5, 7, and 8.5 ($p < 0.05$); however, SEC and NAC lost their antioxidant activity when the pH was down to 2.5 and up to 10 ($p < 0.05$). On the contrary, DAS and DADS still showed antioxidant activity at pH 2.5 or 10 ($p < 0.05$).

Table 1. The Individual Antioxidant Activity of α-Tocopherol (α-Toc), Diallyl Sulfide (DAS), Diallyl Disulfide (DADS), *S*-Ethyl Cysteine (SEC), and *N*-Acetyl Cysteine (NAC) against Fe^{2+}, AAPH, or AMVN Induced TBARS Formation after a 72-h Incubation at 37 °C, and against Cu^{2+}-Induced Conjugated Diene (CD) Formation at 37 °C

		TBA no. [a]			CD[a]
treatment	concn (μM)	Fe^{2+}	AAPH	AMVN	lag-phase (min)
control[b]		0.576 ± 0.042d	0.558 ± 0.037f	0.582 ± 0.048f	15 ± 1.2a
α-Toc	5	0.327 ± 0.024c	0.381 ± 0.019e	0.259 ± 0.015b	28 ± 2.5d
	10	0.285 ± 0.025b	0.330 ± 0.023d	0.174 ± 0.018a	44 ± 2.0f
DAS	5	0.342 ± 0.027c	0.347 ± 0.025d	0.307 ± 0.020c,d	25 ± 1.5c
	10	0.273 ± 0.019b	0.292 ± 0.021c	0.236 ± 0.023b	42 ± 1.0f
DADS	5	0.334 ± 0.024c	0.342 ± 0.014d	0.293 ± 0.023c	32 ± 2.1e
	10	0.284 ± 0.027b	0.290 ± 0.023c	0.228 ± 0.019b	49 ± 2.5g
SEC	5	0.251 ± 0.015b	0.263 ± 0.017b	0.409 ± 0.032e	21 ± 1.7b
	10	0.142 ± 0.018a	0.156 ± 0.012a	0.335 ± 0.029d	34 ± 2.3e
NAC	5	0.256 ± 0.016b	0.270 ± 0.013b	0.395 ± 0.035e	23 ± 2.0b,c
	10	0.153 ± 0.022a	0.164 ± 0.021a	0.327 ± 0.028d	34 ± 2.7e

[a] Least-squares means with a common superscript within a column are not different at the 5% level. [b] Controls contained no antioxidant agent.

Table 2. Partition Coefficient, Chelating Effect, and Reducing Power of α-Tocopherol, Diallyl Sulfide, Diallyl Disulfide, *S*-Ethyl Cysteine, and *N*-Acetyl Cysteine

		chelating effect (%)[b]		reducing power[a,c]	
agent	partition coeff[a]	5 μM	10 μM	5 μM	10 μM
α-tocopherol	551 ± 9.4c	21.6 ± 1.3	38.1 ± 2.4	0.426 ± 0.018c	0.635 ± 0.015c
diallyl sulfide	22 ± 1.3b	*d*		0.187 ± 0.011a	0.322 ± 0.008a
diallyl disulfide	25 ± 2.7b			0.156 ± 0.014a	0.308 ± 0.010a
s-ethyl cysteine	0.3 ± 0.2a	2.6 ± 0.6	3.3 ± 0.8	0.347 ± 0.009b	0.528 ± 0.014b
n-acetyl cysteine	0.4 ± 0.2a	2.8 ± 0.5	4.1 ± 0.9	0.361 ± 0.013b	0.540 ± 0.016b

[a] Least-squares means with a common superscript within a column are not different at the 5% level. [c] The absorbance of the control group at 700 nm was 0.022 ± 0.007. [b] EDTA was used for comparison, its chelating effect was 100%. [d] The chelating effect was too low to be detected.

Table 3. The Influence of Temperature on the Antioxidant Activity of 10 μM α-Tocopherol, Diallyl Sulfide, Diallyl Disulfide, *S*-Ethyl Cysteine, and *N*-Acetyl Cysteine against Fe^{2+}-Induced Lipid Oxidation after a 72-h Incubation at pH 7.0

	TBA no.[a]					
agent	4 °C	25 °C	37 °C	45 °C	55 °C	65 °C
control	0.125 ± 0.014b	0.357 ± 0.034b	0.567 ± 0.034c	0.708 ± 0.035c	1.087 ± 0.045c	1.338 ± 0.035b
α-Toc	0.063 ± 0.005a	0.106 ± 0.012a	0.291 ± 0.022b	0.425 ± 0.032a	0.727 ± 0.025a	1.047 ± 0.043a
DAS	0.071 ± 0.008a	0.121 ± 0.008a	0.275 ± 0.021b	0.438 ± 0.029a	0.785 ± 0.031b	1.364 ± 0.041b
DADS	0.084 ± 0.012a	0.113 ± 0.011a	0.267 ± 0.018b	0.431 ± 0.025a	0.794 ± 0.034b	1.378 ± 0.032b
SEC	0.042 ± 0.008a	0.095 ± 0.011a	0.147 ± 0.015a	0.483 ± 0.022b	1.090 ± 0.041c	1.327 ± 0.037b
NAC	0.053 ± 0.007a	0.103 ± 0.012a	0.150 ± 0.023a	0.492 ± 0.017b	1.058 ± 0.042c	1.365 ± 0.040b

[a] Least-squares means with a common superscript within a column are not different at the 5% level.

Table 4. The Influence of pH on the Antioxidant Activity of 10 μM α-Tocopherol, Diallyl Sulfide, Diallyl Disulfide, *S*-Ethyl Cysteine, and *N*-Acetyl Cysteine against Fe^{2+}-Induced Lipid Oxidation after a 72-h Incubation at 37 °C

	TBA no.[a]					
agent	pH 2.5	pH 4	pH 5.5	pH 7	pH 8.5	pH 10
control	1.067 ± 0.044b	0.844 ± 0.037c	0.607 ± 0.030d	0.567 ± 0.027c	0.591 ± 0.029c	0.813 ± 0.023b
α-Toc	0.731 ± 0.034a	0.426 ± 0.023a	0.341 ± 0.022c	0.293 ± 0.020b	0.348 ± 0.022b	0.581 ± 0.034a
DAS	0.708 ± 0.032a	0.415 ± 0.032a	0.246 ± 0.018b	0.267 ± 0.019b	0.335 ± 0.024b	0.605 ± 0.026a
DADS	0.717 ± 0.026a	0.403 ± 0.027a	0.250 ± 0.016b	0.256 ± 0.020b	0.340 ± 0.028b	0.622 ± 0.029a
SEC	1.085 ± 0.035b	0.484 ± 0.025b	0.152 ± 0.011a	0.141 ± 0.015a	0.271 ± 0.019a	0.821 ± 0.025b
NAC	1.071 ± 0.023b	0.496 ± 0.020b	0.147 ± 0.015a	0.154 ± 0.020a	0.265 ± 0.021a	0.835 ± 0.027b

[a] Least-squares means with a common superscript within a column are not different at the 5% level.

DISCUSSION

Several studies have observed that these organosulfur compounds could modulate antioxidant enzymes (*1*−*3*); however, the results of the present study further found that these organosulfur compounds could exert their antioxidant protection via nonenzymatic actions. The antioxidant activity of garlic extract has been studied in our laboratory (*22*). The results of our present study suggested that these four organosulfur compounds might contribute to the antioxidant activity of garlic extract. So far, most studies regarding garlic and/or its components in food protection mainly focused on antimicrobial and/or flavor consideration (*23*−*25*). The results of this present study supported the application of garlic and its compounds in food systems for antioxidant protection.

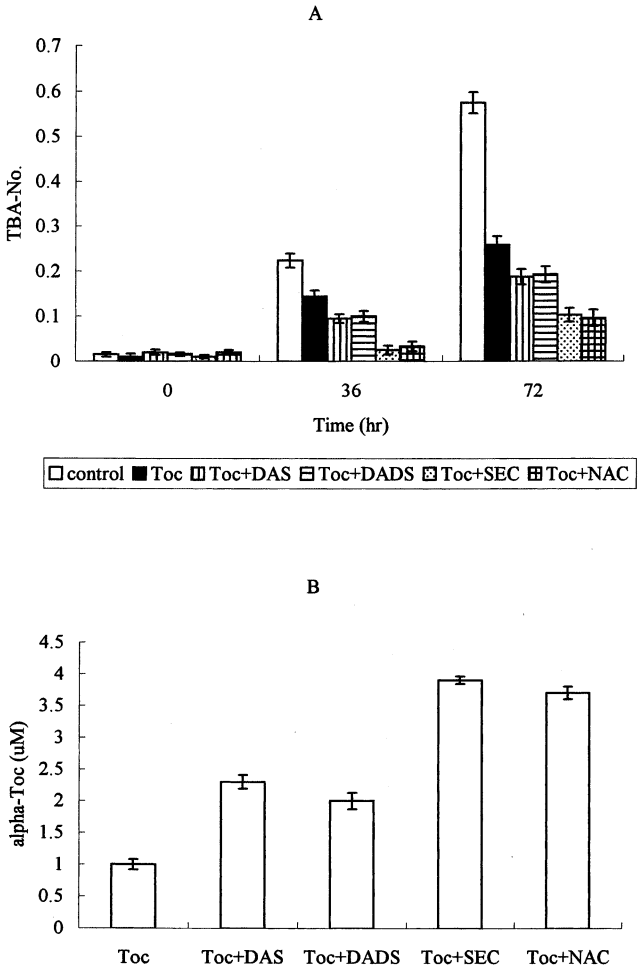

Figure 2. Antioxidant effect of 10 μM α-tocopherol (Toc) alone or 5 μM α-tocopherol plus 5 μM DAS, DADS, SEC, and NAC against AMVN-induced lipid oxidation in liposomes (A) at 0, 36, and 72 h at 37 °C. The content of α-tocopherol (α-Toc) was analyzed for every combination at 36 h (B). Each point represents the mean ± standard deviation from five experiments.

The lower partition coefficients of NAC and SEC indicated that these two agents were water-soluble and might more effectively scavenge the free radicals induced by Fe^{2+} or AAPH in the aqueous phase. This might partially explain why NAC and SEC with less iron-chelating effect and reducing power than α-tocopherol showed greater antioxidant effectiveness in retarding Fe^{2+}- or AAPH-induced lipid oxidation (**Table 1**). On the contrary, α-tocopherol with greater lipid solubility could interact more deeply with biomembranes and might be more effective in scavenging AMVN-induced radicals in the lipid phase. On the other hand, DAS or DADS with lower lipid solubility than α-tocopherol may have relatively higher affinity toward the radicals induced by Fe^{2+} in the aqueous phase, which resulted in the observed greater antioxidant protection from DAS and DADS than from α-tocopherol in retarding Fe^{2+}-induced lipid oxidation. These results strongly suggested that the interaction of an agent with biomembranes and/or other antioxidant agent was important in determining its antioxidant performance.

The greater antioxidant protection from α-tocopherol plus other agents than α-tocopherol treatment alone has been observed (*13*, *26*−*28*). The results of the present study further extended the greater antioxidant protection from α-tocopherol combined with four organosulfur compounds (**Figure 2A**). NAC or SEC provided a more sparing effect than DAS or DADS for α-tocopherol (**Figure 2B**), and this sparing effect might also contribute to the observed greater antioxidant protection presented in α-tocopherol plus SEC or NAC. Our previous study found that two antioxidants with a greater difference in their partition coefficients provided greater protection for membrane lipid stability when these two antioxidants were combined (*13*). In this study, a greater difference in partition coefficients was also presented between α-tocopherol and NAC (or SEC). The results of our present study agreed with our previous finding and suggested that the combination of two agents with greater difference in their partition coefficients might be more beneficial for lipid stability.

Both NAC and SEC were more sensitive to temperature and pH (**Tables 3** and **4**). This may be due to their protein-based property. Thus, using these two compounds in an acid food system as antioxidant agents may not be appropriate. On the contrary, DAS and DADS were more stable in lower or higher pH environments. This advantage suggested that these two agents were more useful in acidic or basic food systems for antioxidant protection. The antioxidant protection from these organosulfur compounds was not marked at 55 °C or higher. This finding suggested using these agents only after the target foods had finished heating process.

In conclusion, the nonenzymatic antioxidant activities of four organosulfur compounds were mainly from their reducing power and interactions with biomembranes and/or other antioxidant agent. The application of these agents in food systems via exogenous addition may be feasible and benefit for antioxidant protection.

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