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Antioxidative Activity of Sulfur-Containing Compounds in *Allium* Species for Human Low-Density Lipoprotein (LDL) Oxidation in Vitro

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Sulfur-containing compounds contributing to health promotion in *Allium* species are produced via enzymic and thermochemical reactions. Sulfur-containing amino acids and volatile organosulfur compounds were prepared for an antioxidative assay. The inhibitory activity of *S*-alk(en)yl-L-cysteines and their sulfoxides, volatile alk(en)yl disulfides and trisulfides, and vinyldithiins in *Allium* species against lipid hydroperoxide (LOOH) formation in human low-density lipoprotein (LDL) was examined. It was elucidated that the alk(en)yl substituents (methyl, propyl, and allyl) and the number of sulfur atoms in the compounds were important for the antioxidative activity. 3,4-Dihydro-3-vinyl-1,2-dithiin, which is produced by a thermochemical reaction of allyl 2-propenethiosulfinate, exhibited the highest antioxidative activity of human LDL among sulfur-containing compounds.

KEYWORDS: ***Allium*** **species; sulfur-containing compounds; antioxidative activity; life-style-related diseases; low-density lipoprotein**

INTRODUCTION

There is a growing interest in the relationship between food and life-style-related diseases such as cancer and heart disease among people all over the world. *Allium* species such as onion and garlic are fascinating to us, because large numbers of *Allium* vegetables are consumed in many cuisines such as American, European, Chinese, and Japanese (*1*). *Allium* species, onion (*A. cepa* L.), garlic (*A. sativum* L.), and caucas (*A. victorialis* L.) etc., have been cooked to season foods in many countries and used since ancient times as pharmaceuticals. Historically, these species have been utilized in folk medicine for the treatment of such varied physical disorders as burns, wounds, headaches, chest colds, and rheumatism (*2*).

Sulfur-containing components in *Allium* species consist of volatiles (disulfides and trisulfides, etc.) and nonvolatiles (sulfur-containing amino acids). The volatile sulfur compounds such as dialk(en)yl disulfides and dialk(en)yl trisulfides are produced by enzymic reaction of alk(en)yl-L-cysteine sulfoxides with C−S lyase (EC 4.4.1.4) and followed by thermochemical reactions when *Allium* vegetables are cut or ruptured (*3*−*7*). Consequently, the produced volatile sulfur-containing compounds are the main source of the distinctive odor of *Allium* species. We have previously indicated that the sulfur-containing flavor compounds were enzymically produced from caucas (*A. victorialis* L.) and thermochemically transformed to vinyldithiins, which have antithrombotic activities (*1*, *8*−*10*).

Various physiological functions of garlic and onion have been reported as well as antibacterial activities of allicin (2-propene-1-thiolsulfinate) and related thiosulfinates, so-called platelet aggregation inhibitory activities of garlic and onion (*10*−*14*), inhibitory effects of diallyl disulfide on the proliferation of human tumor cells (*15*, *16*), antioxidative activity of thiosulfinates and *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* (*17*), and apoptotic effect of diallyl disulfide in HL-60 cells (*18*).

Atherosclerosis is an inflammatory disease that is mediated by the progressive accumulation of cholesterol, more specifically, low-density lipoprotein (LDL), within the vascular walls. Accordingly, it is thought that high concentrations of LDL are strongly correlated with the development of atherosclerosis. In particular, oxidatively modified low-density lipoprotein (Ox-LDL) was recognized to be an important step in the initiation and progression of atherosclerotic lesions (*19*−*24*). When LDL is oxidized, it is modified in a variety of ways through the reaction with reactive oxygen species (ROS) (*22*, *25*) or in various cultured cells (*26*). Ox-LDL is recognized by receptor on monocyte-derived macrophages and excessively internalized to lead to foam cell formation (*27*), so it is important to develop functional foods that contain antioxidative substances in connection with the prevention of atherosclerosis. Lau et al. (*28*) demonstrated that the durability of the inhibitory effect for human LDL oxidation was increased by aged garlic extract (AGE) and compounds (*S*-allyl-L-cysteine, *N*-acetyl-*S*-allyl-L-cysteine, alliin, SAMC, allixin, and allicin) in garlic. Although it has been so far reported that some sulfur compounds in *Allium* species exhibit platelet aggregation inhibition (*10*, *11*, *13*, *29*), the antioxidative activity of the compounds against human LDL oxidation has not been investigated systematically. Rosen et al. (*30*) reported that allyl methyl sulfide as a major volatile

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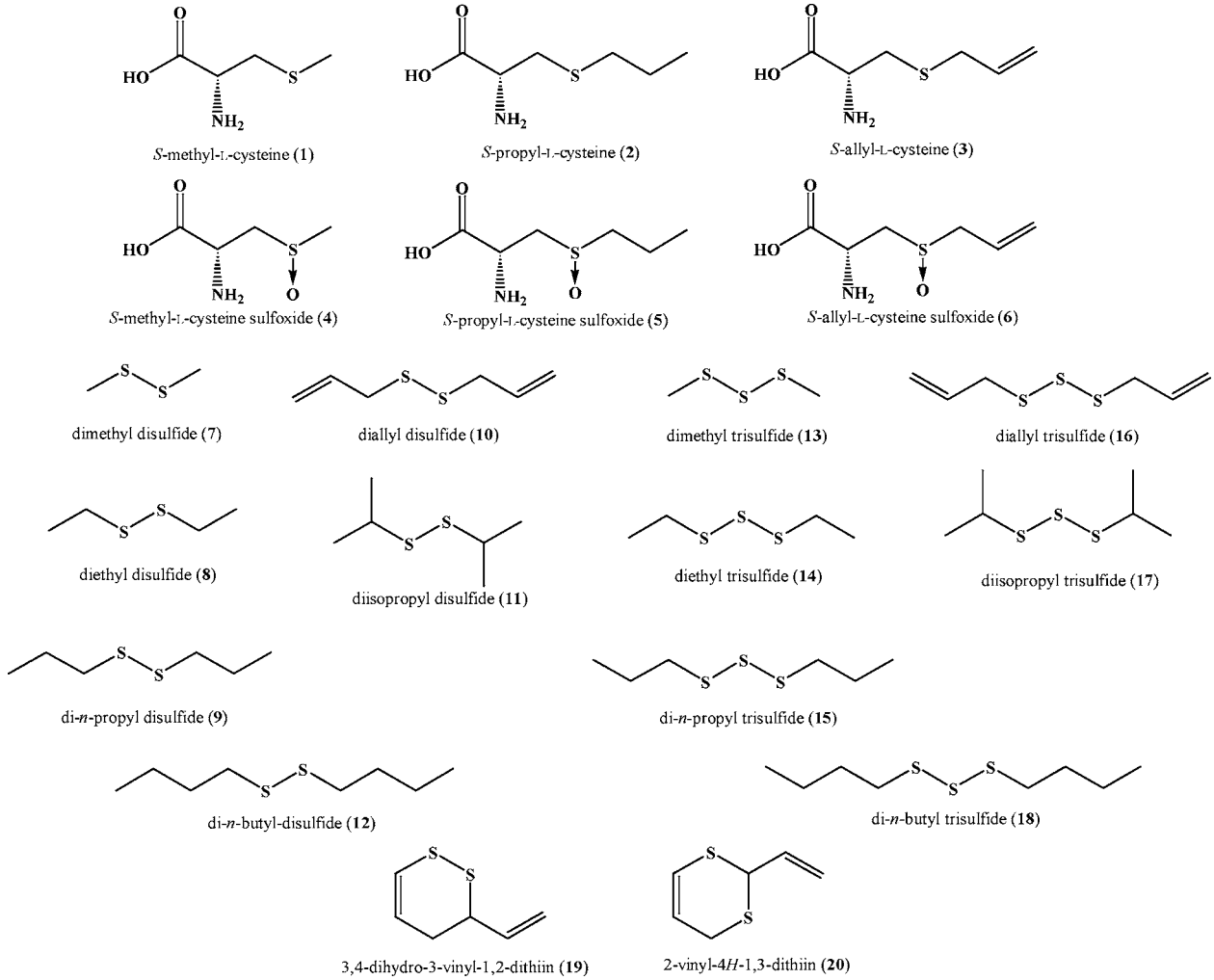

Figure 1. Chemical structures of synthesized sulfur-containing amino acids, disulfides, trisulfides, and vinyldithiins.

compound was detected in human breath 5 h after the consumption of garlic. This result indicates that garlic or its sulfur-containing compounds may be absorbed and metabolized in vivo.

The present paper deals with the effect of sulfur-containing compounds in *Allium* species on copper ion-induced human LDL oxidation, which is evaluated by measuring lipid hydroperoxide (LOOH), and with the structure−antioxidative activity relationships.

MATERIALS AND METHODS

Chemicals. Cupric sulfate and butylated hydroxytoluene (BHT) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Tween 20 [poly(oxyethylene) sorbitan monolaurate] was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Ascorbic acid, acetonitrile, and methanol (HPLC grade) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). The water used in this experiment was purified with Milli-Q Labo equipment (Millipore Japan, Tokyo, Japan).

Syntheses. *S*-Methyl-L-cysteine (**1**), *S*-propyl-L-cysteine (**2**), *S*-allyl-L-cysteine (**3**), *S*-methyl-L-cysteine sulfoxide (**4**), *S*-propyl-L-cysteine sulfoxide (**5**), and *S*-allyl-L-cysteine sulfoxide (**6**) were synthesized according to a modified method of du Vigneaud et al. (*31*) and purified by recrystallization. Structures are shown in **Figure 1**.

Dimethyl disulfide (**7**), diethyl disulfide (**8**), di-*n*-propyl disulfide (**9**), diallyl disulfide (**10**), diisopropyl disulfide (**11**), di-*n*-butyl disulfide (**12**), dimethyl trisulfide (**13**), diethyl trisulfide (**14**), di-*n*-propyl trisulfide (**15**), diallyl trisulfide (**16**), diisopropyl trisulfide (**17**), and di-*n*-butyl trisulfide (**18**) (**Figure 1**) were synthesized according to a modified method of Kirner and Richter (*32*). Vinyldithiins, 3,4-dihydro-3-vinyl-1,2-dithiin (**19**), and 2-vinyl-4*H*-1,3-dithiin (**20**) (**Figure 1**) were synthesized and purified according to the method of Beslin (*33*).

Purification. The synthesized disulfides, trisulfides, and vinyldithiins were purified by chromatography using a Lobar LiChroprep RP-18 column (310 × 25 mm, Merck Rahway, NJ). The high-performance liquid chromatography (HPLC) equipment consisted of a JASCO PU-980 pump and a UV-970 Intelligent UV/VIS detector set at 254 nm, with a mobile phase of acetonitrile/water [70:30, v/v (for vinyldithiins), 80:20, v/v (for disulfides and trisulfides)] at a flow rate of 3.0 mL/min. The purified compounds were characterized by the interpretation of ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), mass, and IR spectral data.

S-Methyl-L-cysteine (**1**): ^{1}H NMR (400 MHz, D_2O) δ 2.07 (3H, s, CH_3), 2.88−3.03 (2H, m, SCH_2 at C-β), 3.85 (1H, dd, J = 7.8 and 4.4 Hz, CH at C-α); ^{13}C NMR (100 MHz, D_2O) δ 14.9, 34.8, 53.5, 173.5; IR (KBr, cm^{-1}) 2963−2860, 2580, 2120 (NH_3^+), and 1580 (COO^-); MS *m*/*z* (rel intensity) 135 (M^+,100), 118 (17.0), 90 (75.7), 87 (38.1), 74 (87.9), 61 (94.3), 43 (37.4).

S-Propyl-L-cysteine (**2**): ^{1}H NMR (400 MHz, D_2O) δ 0.87 (3H, t, J = 8.0 Hz, CH_3), 1.52 (2H, m, CH_2), 2.50 (2H, t, J = 7.2 Hz, SCH_2 at *n*-propyl), 2.89−3.05 (2H, m, SCH_2 at C-β), and 3.82 (1H, dd, J = 7.6 and 4.4 Hz, CH at C-α); ^{13}C NMR (100 MHz, D_2O) δ 12.9, 22.5, 32.3, 33.8, 54.0, 173.4; mp 210−212 °C (dec); IR (KBr, cm^{-1}) 2965−2860, 2580, 2120 (NH_3^+), and 1580 (COO^-); MS *m*/*z* (rel intensity) 163 (M^+, 12.4), 118 (21.7), 90 (40.3), 89 (100), 74 (31.0), 61 (46.5), 47 (65.1), and 43(89.9).

S-Allyl-L-cysteine (**3**): ^{1}H NMR (400 MHz, D_2O) δ 2.80−2.95 (2H, m, SCH_2 at C-β), 3.10 (2H, dq, J = 7.3 and 0.8 Hz, SCH_2 at allyl), 3.73 (1H, dd, J = 7.2 and 4.4 Hz, CH at C-α), 5.08−5.14 (2H, m,

vinylic CH_2), and 5.72 (1H, m, vinylic CH); ^{13}C NMR (100 MHz, D_2O) δ 31.8, 34.2, 54.1, 118.9, 133.9, 174.6; mp 208−210 °C (dec); IR (KBr, cm^{-1}) 3020−2870, 2590, 2120 (NH_3^+), 1580 (COO^-), 99.0, and 918; MS *m*/*z* (rel intensity) 161 (M^+, 14.2), 116 (8.4), 88 (55.8), 87 (100), 74 (90.0), 45 (46.8), 41(87.6), and 39(32.4).

S-Methyl-L-cysteine sulfoxide (**4**): mp 195−198 °C (dec); IR (KBr, cm^{-1}) 1012 (sulfoxide).

S-*n*-Propyl-L-cysteine sulfoxide (**5**): mp 195−198 °C (dec); IR (KBr, cm^{-1}) 1012 (sulfoxide).

S-Allyl-L-cysteine sulfoxide (**6**): mp 165 °C (dec); IR (KBr, cm^{-1}) 1020 (sulfoxide), 990 and 915 cm^{-1}(allyl double bond).

Dimethyl disulfide (**7**): ^1H NMR (400 MHz, $CDCl_3$) δ 2.43(3H, *s*, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.1; bp 116−118 °C; IR (cm^{-1}) 3000−2840; MS *m*/*z* (rel intensity) 94 (M^+, 100), 79 (95.9), 64 (15.6), 61 (29.1), 46 (43.7), 45 (56.0).

Diethyl disulfide (**8**): ^1H NMR (400 MHz, $CDCl_3$) δ 1.33 (3H, t, J = 7.3 Hz, CH_3), 2.70 (2H, q, J = 7.3 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.9, 33.3; bp 152−154 °C; IR (cm^{-1}) 2975−2875; MS *m*/*z* (rel intensity) 122 (M^+, 100), 94 (66.9), 66 (61.6).

Di-*n*-propyl disulfide (**9**): ^1H NMR (400 MHz, $CDCl_3$) δ 1.00 (3H, t, J = 7.3 Hz, CH_3), 1.71 (2H, m, J = 7.3 Hz, CH_2), 2.67 (2H, *t*, J = 7.3 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.1, 22.5, 41.1; bp 195 °C; IR (cm^{-1}) 2980−2890; MS *m*/*z* (rel intensity) 150 (M^+, 100), 108 (83.7), 66 (18.0), 43 (89.5), 41 (32.5).

Diisopropyl disulfide (**10**): ^1H NMR (400 MHz, $CDCl_3$) δ 1.30 (6H, d, J = 6.8 Hz, CH_3), 2.97 (1H, m, J = 6.7 Hz, CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.6, 41.5; bp 174−176 °C; IR (cm^{-1}) 2960−2875; MS *m*/*z* (rel intensity) 150 (M^+, 99.8), 108 (89.0), 66 (21.0), 43 (100).

Di-*n*-butyl disulfide (**11**): ^1H NMR (400 MHz, $CDCl_3$) δ 0.93 (3H, t, J = 7.4 Hz, CH_3), 1.41 (2H, m, J = 7.3 Hz, CH_2), 1.66 (2H, m, J = 7.4 Hz, CH_2), 2.69 (2H, t, J = 7.4 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) 13.7, 21.7, 31.3, 38.9; bp 226 °C; IR (cm^{-1}) 2960−2880; MS *m*/*z* (rel intensity) 178 (M^+, 80.8), 122 (70.5), 57(100), 41(27.5).

Diallyl disulfide (**12**): ^1H NMR (400 MHz, $CDCl_3$) δ 3.54 (2H, d, J = 7.4 Hz, CH_2), 5.18 (2H, m, CH_2), 5.84 (1H, m, CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 42.7, 118.8, 133.9; bp 174 °C; IR (cm^{-1}) 3080, 2975−2920, 1670, 918; MS *m*/*z* (rel intensity) 146 (M^+, 74.5), 113 (17.9), 105 (24.5), 81(51.3), 41 (100), 39 (39.9).

Dimethyl trisulfide (**13**): ^1H NMR (400 MHz, $CDCl_3$) δ 2.57 (3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.0; bp 165−170 °C; IR (cm^{-1}) 2980−2920; MS *m*/*z* (rel intensity) 126 (M^+, 100), 111 (54.0), 79 (88.9), 64 (27.4), 45 (56.0).

Diethyl trisulfide (**14**): ^1H NMR (400 MHz, $CDCl_3$) δ 1.39 (3H, t, J = 7.3 Hz, CH_3), 2.89 (2H, q, J = 7.3 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.6, 33.0; IR (cm^{-1}) 2975−2880; MS *m*/*z* (rel intensity) 154 (M^+, 100), 125 (11.4), 93 (27.0), 61 (82.5), 59 (14.6).

Di-*n*-propyl trisulfide (**15**): ^1H NMR (400 MHz, $CDCl_3$) δ 1.02 (3H, t, J = 7.4 Hz, CH_3), 1.78 (2H, m, J = 7.3 Hz, CH_2), 2.86 (2H, t, J = 7.3 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.1, 22.1, 40.8; IR (cm^{-1}) 2960−2880; MS *m*/*z* (rel intensity) 182 (M^+, 100), 140 (8.5), 117 (5.1), 98 (11.4), 75 (81.2), 43 (59.7).

Diisopropyl trisulfide (**16**): ^1H NMR (400 MHz, $CDCl_3$) δ 1.36 (6H, d, J = 6.7 Hz, CH_3), 3.21 (1H, m, J = 6.7 Hz, CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.9, 42.2; IR (cm^{-1}) 2975−2880; MS *m*/*z* (rel intensity) 182 (M^+, 100), 140 (16.6), 117 (2.2), 98 (45.5), 75 (57.9), 43 (74.9).

Di-*n*-butyl trisulfide (**17**): ^1H NMR (400 MHz, $CDCl_3$) δ 0.94 (3H, t, J = 7.3 Hz, CH_3), 1.43 (2H, m, J = 7.3 Hz, CH_2), 1.73 (2H, m, J = 7.4 Hz, CH_2), 2.88 (2H, t, J = 7.3 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.6, 21.7, 30.9, 38.6; IR (cm^{-1}) 2960−2880; MS *m*/*z* (rel intensity) 210 (M^+, 100), 154 (12.6), 89 (78.8), 57 (53.1), 41(23.1).

Diallyl trisulfide (**18**): ^1H NMR (400 MHz, $CDCl_3$) δ 3.50 (2H, d, J = 7.3 Hz, CH_2), 5.22 (2H, m, CH_2), 5.89 (1H, m, CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 41.7, 119.1, 132.7; IR (cm^{-1}) 3080, 2975−2920, 1635, 990, and 920; MS *m*/*z* (rel intensity) 178 (M^+, 24.0), 113 (100), 105 (24.5), 73 (91.9), 41 (55.4), 39 (24.3).

3,4-Dihydro-3-vinyl-1,2-dithiin (**19**): ^1H NMR (400 MHz, $CDCl_3$) δ 2.41−2.68 (2H, m, allylic methylene), 3.68−3.76 (1H, m, methine proton), 5.23−5.32 (2H, m, CH_2=C), 5.94 (1H, dd, exo olefinic = CH), 6.04 (1H, m, endo olefinic, SC=CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.3 (C4), 44.1 (C3), 117.5 (C8), 120.6 (C7), 126.0 (C5), 136.4 (C6); mp °C (dec); IR (KBr, cm^{-1}) 1630 (C=C), 985, and 915 (vinyl double bond); MS *m*/*z* (rel intensity) 144 (M^+, 78), 111 (100), 103 (51), 97 (70), 85 (17), 79 (43), 77 (48), 72 (57), 71 (55), 49 (59), 39 (40).

2-Vinyl-4*H*-1,3-dithiin (**20**): ^1H NMR (400 MHz, $CDCl_3$) δ3.23 and 3.40 (2H, two ddd, J = 17.5, and 2 Hz, allylic methylene), 4.73 (1H, *d*, J = 7 Hz, methine proton), 5.30 and 5.40 (2H, two *d*, J = 17 and 10 Hz, CH_2=C), 5.92−6.05 (2H, m, *exo* olefinic =CH and *endo* olefinic SC = CH), 6.31 (1H, dt, *endo* olefinic, SCH = C); ^{13}C NMR (100 MHz, $CDCl_3$) δ25.1 (C4), 45.1 (C2), 117.2 (C8), 118.3 (C7), 122.2 (C5), 134.3 (C6); mp °C (dec); IR (KBr, cm^{-1}) 1630 (C=C), 982, and 918 (vinyl double bond); MS *m*/*z* (rel intensity) 144 (M^+, 45), 111 (35), 103 (10), 97 (16), 85 (7), 79 (11), 72 (100), 71 (78), 45 (41), 39 (23).

Isolation of Human LDL. Human LDL was isolated from the heparinized plasma of healthy volunteers by ultracentrifugation (*34*). In this experiment, healthy volunteers gave written informed consent in accordance with the Helsinki Declaration.

The heparinized whole blood was centrifuged at 4 °C and 3000 rpm for 10 min, and 2.0 mL of plasma was obtained and pooled. Density (d = 1.24 g/mL) was adjusted by the addition of solid potassium bromide (KBr) of 0.3816 g/mL of plasma. The adjusted plasma solution (2 mL) was charged into the ultraclear tube (13.5 mL, Beckman, Palo Alto, CA), and 5 mM phosphate buffer solution (pH 7.4, 0.1 mM EDTA as antioxidant) was added to it. The sealed tube was prepared in a 70.1-Ti rotor on an L7 ultracentrifuge (Beckman) at 10 °C and 450000*g* for 4 h. The collected LDL fraction (upper layer) was stored at 4 °C under a nitrogen atmosphere until use for 1 week maximum. Before the assay was begun, EDTA and other smaller molecular weight substances were removed from the LDL fraction by ultrafiltration (Centricon Plus-20, Millipore Japan). The protein concentration of LDL was measured by using the modified Lowry protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as standard.

LDL Oxidation. The antioxidative activity of sulfur-containing compounds was assessed by measuring of lipid hydroperoxide (LOOH) in human LDL. The assay mixture (1 mL of total volume) consists of 150 μL of 0.1 mg/mL LDL solution (final concentration of 0.015 mg/mL), 100 μL of test sample, 710 μL of phosphate-buffered saline (PBS, pH 7.4), and 40 μL of cupric sulfate (final concentration of 2 μM). The LDL solution was prepared by dilution with PBS. The hydrophobic compounds (sulfides and vinyldithiins) were dissolved in PBS (supplemented with 1% Tween 20). In this case there was no effect of Tween 20 for LDL oxidation. The cupric sulfate, which is an inducer of the oxidation reaction, was dissolved in PBS. The reaction mixture was incubated at 37 °C under the condition of free air for 1, 2, and 3 h, respectively.

Measurement of Lipid Hydroperoxides in LDL. The total lipids in LDL were prepared in accordance with the Folch method (*35*). An ice-cold chloroform/methanol (0.5 mL, 2:1, v/v, containing 0.002% BHT as antioxidant) reagent was added to the reaction mixture (1 mL) and stirred with a vortex at 4 °C for 1 min under a nitrogen atmosphere and centrifuged at 4 °C and 3000*g* for 30 min. Fifty microliters of the organic layer was applied for the Lipid Hydroperoxide Assay Kit (Cayman Chemical).

Statistical Analyses. The data were expressed as the mean and standard deviation (SD). Statistical comparisons were made with Student's *t* test.

RESULTS AND DISCUSSION

Antioxidative activities of sulfur-containing amino acids, dialk(en)yl disulfides, dialk(en)yl trisulfides, and vinyldithiins were evaluated by the inhibition of LOOH formation on human LDL.

Antioxidative activity on human LDL of sulfur-containing compounds is shown in **Figure 2**. The final concentration of sulfur-containing compounds was 50 μM, and human LDL was oxidized at 37 °C for 2 h under the condition of free air. It was found that LOOH formation on human LDL was inhibited by the addition of *S*-methyl-L-cysteine, *S*-propyl-L-cysteine, *S*-allyl-

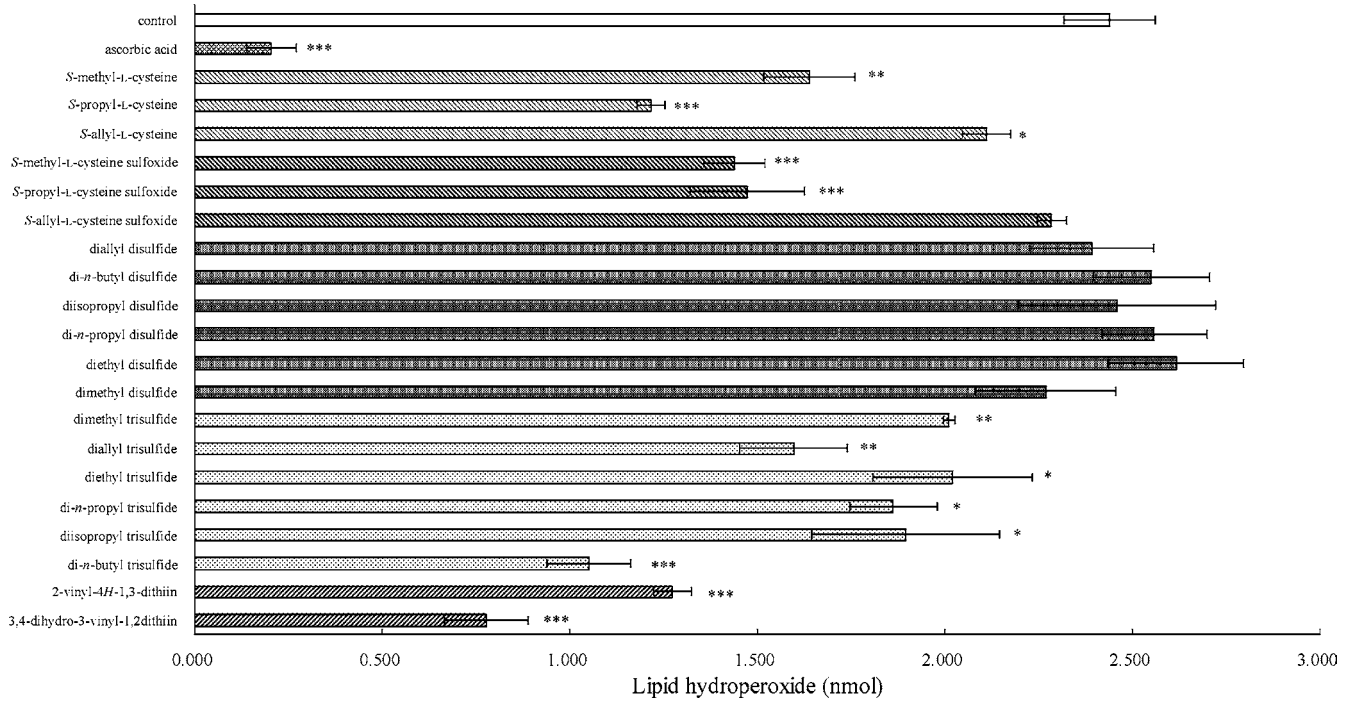

Figure 2. Effect of sulfur-containing amino acids, disulfides, trisulfides, and vinyldithiins on human LDL oxidation. The amount of lipid hydroperoxide shows mean ± standard deviation of three replicates. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, by Student's *t* test.

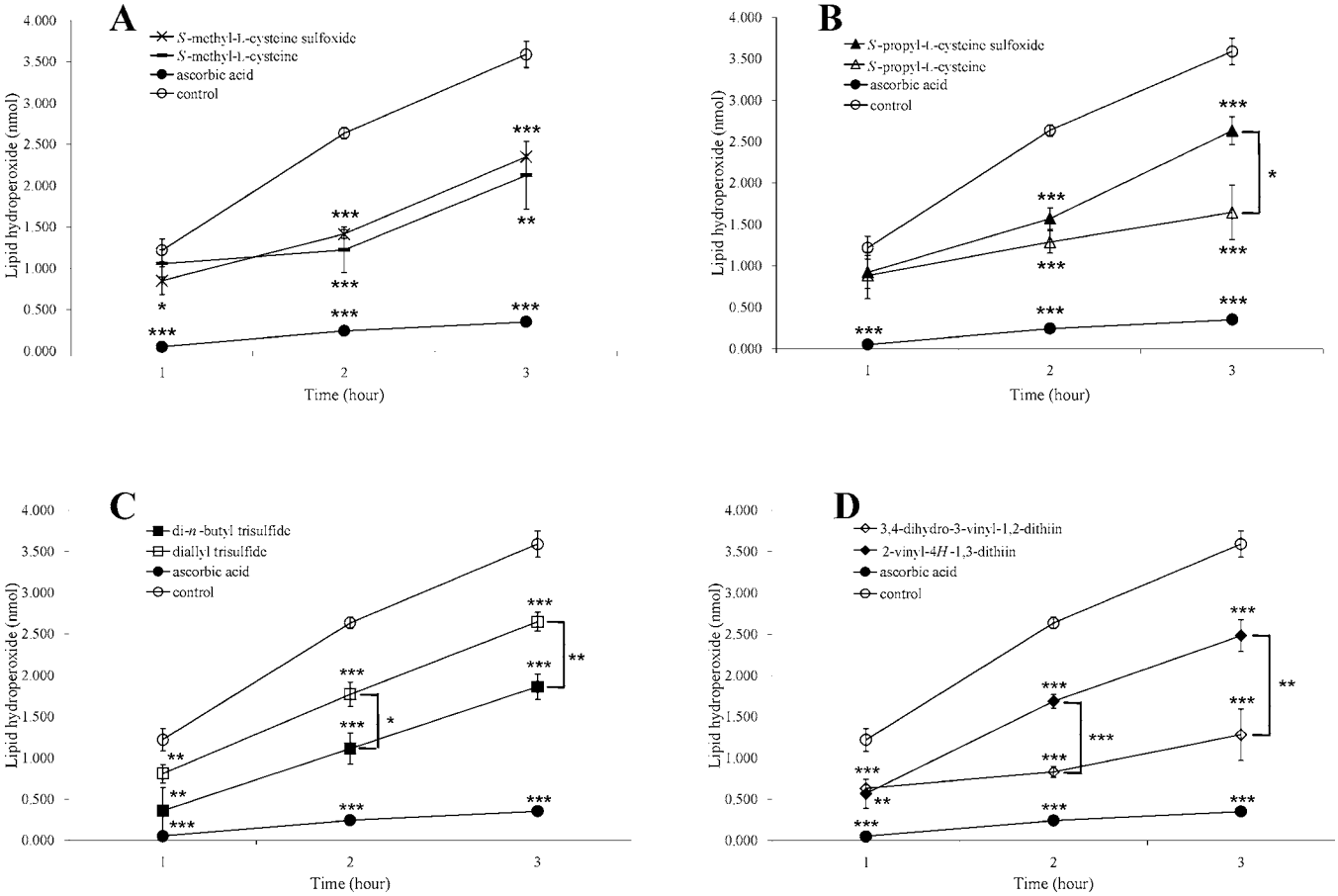

Figure 3. Time course relationships of *S*-methyl-L-cysteine and its sulfoxide (**A**), *S*-propyl-L-cysteine and its sulfoxide (**B**), diallyl trisulfide and di-*n*-butyl trisulfide (**C**), and 2-vinyl-4*H*-1,3-dithiin and 3,4-dihydro-3-vinyl-1,2-dithiin (**D**); final concentration = 50 μM. The amount of lipid hydroperoxide shows mean ± standard deviation of three replicates. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, by Student's *t* test.

L-cysteine, *S*-methyl-L-cysteine sulfoxide, *S*-propyl-L-cysteine-sulfoxide, dimethyl trisulfide, diallyl trisulfide, diethyl trisulfide, di-*n*-propyl trisulfide, diisopropyl trisulfide, di-*n*-butyl trisulfide, 2-vinyl-4*H*-1,3-dithiin, and 3,4-dihydro-3-vinyl-1,2-dithiin. On the other hand, *S*-allyl-L-cysteine sulfoxide, diallyl disulfide, di-*n*-butyl disulfide, diisopropyl disulfide, di-*n*-propyl disulfide,

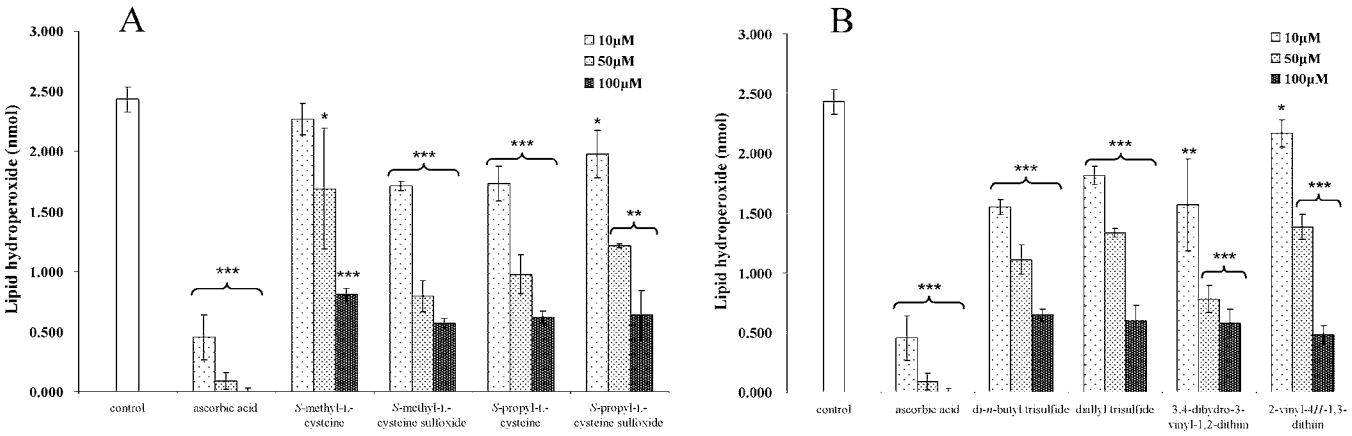

Figure 4. Dose-dependent effect of *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide (**A**) and diallyl trisulfide, di-*n*-butyl trisulfide, 2-vinyl-4*H*-1,3-dithiin, and 3,4-dihydro-3-vinyl-1,2-dithiin (**B**) on human LDL oxidation. The amount of lipid hydroperoxide shows mean ± standard deviation of three replicates. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, by Student's *t* test.

diethyl disulfide, and dimethyl disulfide exhibited no significantly activity.

A time course relationship of *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide are shown in **Figure 3A**,**B**. In **Figure 3A**,**B**, the final concentration of *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide was 50 μM, and the amount of LOOH on human LDL was measured at 1, 2, and 3 h, respectively. The antioxidative activities of sulfur-containing amino acids themselves and ascorbic acid (at positive control) were compared. It was found that LOOH formation on human LDL was nearly equally inhibited by *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide until 3 h. However, the antioxidative activity of ascorbic acid was higher than that of *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide. In **Figure 3A**, the antioxidative activity of *S*-methyl-L-cysteine sulfoxide was not different from that of *S*-methyl-L-cysteine. However, in **Figure 3B**, the antioxidative activity of *S*-propyl-L-cysteine sulfoxide was more effective than that of *S*-propyl-L-cysteine.

Furthermore, a dose-dependent relationship is shown in **Figure 4A**. The final concentration of *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide was adjusted to 10, 50, and 100 μM to evaluate the dose-dependent effect of those amino acids. The amount of LOOH was measured at 2 h after the oxidation reaction. The antioxidative activities of *S*-methyl-L-cysteine and its sulfoxide and *S*-propyl-L-cysteine and its sulfoxide and of ascorbic acid (a positive control) were compared. It was found that each sulfur-containing amino acid had dose-dependently inhibited the oxidation of human LDL.

As shown in **Figure 2**, the antioxidative activities of *S*-methyl-L-cysteine, *S*-propyl-L-cysteine, and their sulfoxides in onion were higher than those of *S*-allyl-L-cysteine and its sulfoxide in garlic. This result suggests that sulfur and alk(en)yl groups (methyl, propyl, and allyl) in *S*-alk(en)yl-L-cysteine and its sulfoxide are very significant in terms of the antioxidative activity for human LDL. This agreed well with the high reaction rate of sulfides and sulfoxides with reactive oxygen species such as $^{\bullet}$OH or $^{\bullet}$OOH radicals (*1*).

Furthermore, the antioxidative properties of volatile sulfur compounds that are produced by both enzymic and thermochemical reactions of sulfur-containing amino acids in cooked *Allium* vegetables were examined. A time course relationship of trisulfides (diallyl trisulfide and di-*n*-butyl trisulfide) and vinyldithiins (3,4-dihydro-3-vinyl-1,2-dithiin and 2-vinyl-4*H*-1,3-dithiin) is shown in **Figure 3C**,**D**, respectively. The final concentrations of trisulfides and vinyldithiins were 50 μM. Human LDL was oxidized in the same manner as mentioned above. It was found that LOOH formation on human LDL was significantly inhibited by diallyl trisulfide, di-*n*-butyl trisulfide, 3,4-dihydro-3-vinyl-1,2-dithiin, and 2-vinyl-4*H*-1,3-dithiin until 3 h. It was also indicated that the antioxidative activity of di-*n*-butyl trisulfide was higher than that of diallyl trisulfide and that the activity of 3,4-dihydro-3-vinyl-1,2-dithiin was higher than that of 2-vinyl-4*H*-1,3-dithiin.

Furthermore, a dose-dependent effect of dialk(en)yl trisulfide and vinyldithiins on human LDL is demonstrated in **Figure 4B**. Final concentrations of trisulfides and vinyldithiins were 10, 50, and 100 μM, and human LDL was oxidized at 37 °C under the condition of free air for 2 h. Ascorbic acid as a positive control was used to compare with trisulfides and vinyldithiins on the antioxidative activity. It was elucidated that diallyl trisulfide, di-*n*-butyl trisulfide, 3,4-dihydro-3-vinyl-1,2-dithiin, and 2-vinyl-4*H*-1,3-dithiin had dose-dependent antioxidative effects, although ascorbic acid showed a higher antioxidative activity than all sulfur-containing compounds in all concentrations. In the case of trisulfides, it seems that an allyl double bond and a length of the *S*-substituted alk(en)yl group are important to reveal higher antioxidative activity, because the diallyl trisulfide and di-*n*-butyl trisulfide showed higher antioxidative activities than other trisulfides as shown in **Figure 2**. However, the role of the hydrophobic alk(en)yl group in trisulfides has not been elucidated yet. The double bond associated with nonbonding electron on sulfur may enhance the antioxidative activity. Furthermore, the antioxidative activity depends on the number of sulfur atoms in molecules because trisulfides generally exhibit higher activity than disulfides as shown in **Figure 2**.

3,4-Dihydro-3-vinyl-1,2-dithiin belonging to vinyldithiins with exo and endo double bonds in a ring system indicated higher activity than aliphatic dialk(en)yl disulfides and trisulfides. It seems that the conjugated double bond to a nonbonding electron on sulfur in a ring system plays an important role in enhancement of the antioxidative activity for human LDL.

In conclusion, we demonstrated that sulfur-containing compounds in *Allium* species revealed a high antioxidative activity at low or physiological concentration for human LDL oxidation in vitro. Especially, it was indicated that the antioxidative activity of 3,4-dihydro-3-vinyl-1,2-dithiin was higher than that of other sulfur-containing compounds, and the substituent and the number of sulfur atoms were important. Our attention has been focused on the different oxidation mechanisms by reactive

oxygen species between organic sulfur compounds and polyphenols, which are proton donor types of antioxidants such as caffeic acid, tocopherol, and quercetin. It is considered that organic sulfides and sulfoxides in *Allium* species scavenge •OH or •OOH radicals to produce sulfoxides and sulfones in vivo, respectively (*1*), because a human oral administration of 100 g of *A. victorialis* vegetable every day for 8 days gave rise to decreased phosphatidylcholine hydroperoxide (LOOH) in plasma (*36*). To increase the amount of volatile sulfur-containing compounds, which have a preventive effect against life-style-related diseases, it is necessary that the C−S lyase activity in *Allium* species and processing temperature are controlled. Therefore, it is very important to determine the cooking or processing temperature and duration of *Allium* vegetables. Our findings may be helpful for the prevention of arteriosclerosis because the sulfur-containing compounds in *Allium* species inhibit LOOH formation on human LDL.

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