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Isolation and Identification of an Antioxidant Substance from Heated Garlic (*Allium sativum* L.)

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Abstract The objectives of this study were to identify antioxidant substance in heated garlic juice (HGJ). We evaluated the antioxidant activities of heated garlic juice exposed to 120, 130, and 140°C for 2 hr. The HGJ was partitioned using the solvents of hexane, chloroform, ethyl acetate, butanol, and water. The ethyl acetate fraction of HGJ treated at 130°C for 2 hr showed strong antioxidant activity; this extract was isolated and purified using silica gel column chromatography and semi-preparative high-performance liquid chromatography. The structure of the purified compound was determined using spectroscopic methods, i.e., ultraviolet, mass spectrometry, infrared, ^{1}H NMR, ^{13}C NMR, DEPT, HMBC, and HMQC. The isolated compound was identified as thiacremonone (2,4-dihydroxy-2,5-dimethyl-thiophene-3-one). Thiacremonone showed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, with a 50% inhibition concentration (IC_{50}) of 22.25±0.44 μg/mL, which is much higher than that of the antioxidants ascorbic acid (30.06±0.42 μg/mL), α-tocopherol (71.30±0.97 μg/mL), and butylated hydroxyanisole (50.54±0.94 μg/mL).

Keywords: garlic (*Allium sativum* L.), antioxidant activity, heat treatment, thiacremonone (2,4-dihydroxy-2,5-dimethyl-thiophene-3-one)

Introduction

Garlic, *Allium sativum* L., which is a member of the *Liliaceae*, is grown in many areas and has been used by many civilizations, including Greek, Egyptian, Asian, and Indian, since antiquity (1). Many *Allium* species have antioxidant properties (2, 3). Garlic and garlic extracts have strong antioxidant activity in various *in vitro* models. The antioxidant activity of *Allium* plants has mainly been attributed to a variety of sulfur-containing compounds such as diallyl sulfide, diallyl trisulfide, allyl-cysteine, and selenium compounds (4-7). In addition to its antioxidant activity, it has antimicrobial, antibacterial, antiviral, antifungal, and antiprotozoal properties, as well as beneficial effects for the cardiovascular and immune systems (8). An antioxidant is a substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate (9). However, the safety and continued use of artificial antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food is being questioned (10, 11). Therefore, a search for antioxidants of natural origin has attracted increasing attention.

Compared to fresh foods, thermally processed foods, especially fruits and vegetables, have increased biological activity caused by chemical changes during heat treatment (12). The polyphenol and flavonoid contents and antioxidant activity increase with high temperature and pressure in plants such as ginseng (13), garlic (14), pear (15), *shiitake* mushroom (16), sweet corn (17), citrus peel (18), and tomato (12).

Our aim was to isolate the active compounds from heated garlic. The isolation of active compounds was performed in three steps: multiple extractions, fractionation using column chromatography, and purification using semi-preparative high-performance liquid chromatography (HPLC). We then determined both the structure of the compounds using spectroscopy and antioxidant activity.

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Materials and Methods

Sample preparation Garlic, *Allium sativum* L., was purchased from the Chungbuk Agriculture and Marine Products Market in June 2006 and stored at −20°C. Heat treatment was performed using a temperature- and pressure-controlling apparatus (Jisico, Seoul, Korea). The samples were heated at temperatures of 120, 130, and 140°C for 2 hr. The heated samples were juiced and then filtered (Whatman filter paper No. 2, Maidstone, England) on a Büchner funnel under a vacuum. The garlic juice was kept at −20°C until analysis.

Selection of the solvent layer Heated garlic juice (HGJ) was partitioned consecutively in a separating funnel using solvents of increasing polarity: *n*-hexane, chloroform, ethyl acetate, butanol, and water. The solvent was evaporated using a rotary evaporator (N-1000; Eyela, Tokyo, Japan) at 40°C. The dried residues of the 5 extracts were measured for 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA) radical scavenging activity and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) radical cation scavenging activity.

DPPH radical scavenging activity The DPPH radical scavenging activity of the extracts, fractions, and isolated compound, based on the scavenging activity of the stable DPPH free radical, was measured according to the method of Bektas *et al.* (19), with some modifications. Aliquots of 0.8 mL of 0.2 mM DPPH methanolic solution were mixed with 0.2 mL of the extracts. The mixture was shaken vigorously and then left to stand for 30 min under low light. The absorbance was measured at 520 nm using a spectrophotometer (DU 650; Beckman, Fullerton, CA, USA). The percentage inhibition of activity was calculated as $(A_0-A_1)/A_0\times100$, where A_0 is the absorbance without the sample and A_1 is the absorbance with the sample. Sample concentrations providing the 50% inhibition concentration (IC_{50}) were calculated from a graph of inhibition percentage versus sample concentration. All samples were analyzed in triplicate.

ABTS radical scavenging activity The ABTS radical cation scavenging activity of the extracts and fractions was measured according to the method of Lee *et al.* (20) and Kim *et al.* (21), with some modifications. The ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate (Sigma-Aldrich) solution and leaving the mixture to stand overnight in the dark at room temperature. The ABTS radical cation solution was diluted with distilled water to obtain an absorbance of 1.4-1.5 at 735 nm. A 1 mL aliquot of diluted ABTS radical cation solution was added to 50 μL of the extract, fractions, ascorbic acid (Sigma-Aldrich) standard solution, or distilled water. The absorbance at 735 nm was determined using a spectrophotometer (DU 650; Beckman) after 60 min. The ascorbic acid equivalent antioxidant activity (AEAC) was calculated as $(A/A_{AA})\times C_{AA}$, where A is the change in absorbance after the addition of the sample, A_{AA} is the change in absorbance after the addition of ascorbic acid standard solution, and C_{AA} is the concentration of the ascorbic acid standard solution. The ABTS radical cation scavenging activity was expressed as the AEAC in mg of ascorbic acid equivalents. All samples were analyzed in triplicate.

Thin layer chromatography (TLC) Analytical TLC of extracts, fractions, and the isolated compound was performed using silica gel 60 F254 glass plates (0.25 mm thick, 20×20 cm; Merck, Darmstadt, Germany), which were developed using appropriate solvents for each sample, i.e., dichloromethane:methanol mixtures in different ratios. The resultant bands were located using ultraviolet (UV) light (254 and 365 nm) and 20% sulfuric acid solution in 10% vanillin/ethanol spray, followed by heating in an oven for about 10 min at 110°C. For the qualitative detection of radical scavenging compounds, the TLC plates were sprayed with 1 mM methanol solution of DPPH, which produced yellow spots on a purple background.

Open column chromatography Isolation of the active compound from the ethyl acetate layer of HGJ treated at 130°C for 2 hr was subjected to column chromatography on silica gel (Fig. 1). HGJ (2 kg) was partitioned consecutively using various solvents. The ethyl acetate extract (4.8 g) was subjected to open-column (500×35 mm, i.d.) chromatography using silica gel (Kiesel gel 60, 70-230 mesh; Merck); elution was carried out using a mixture of dichloromethane :methanol with an increasing amount of methanol (20:1, 10:1, 5:1, 1:1, 0:1, v/v). Five fractions were collected and assayed for antioxidant activity or used for TLC by pooling them into 1 major fraction, A1. The active fraction A1 (1.6 g) was subjected to further open-column (300×10 mm, i.d.) chromatography using silica gel; elution was carried out using a mixture of dichloromethane:methanol with increasing amounts of methanol (20:1, 10:1, 5:1, 1:1, 0:1, v/v). A total of 24 fractions was collected and assayed for antioxidant activity or used for TLC by pooling them into 4 major fractions, B4-B6 (0.26 g). The removal of solvents from fractions was performed using a rotary evaporator at 40°C.

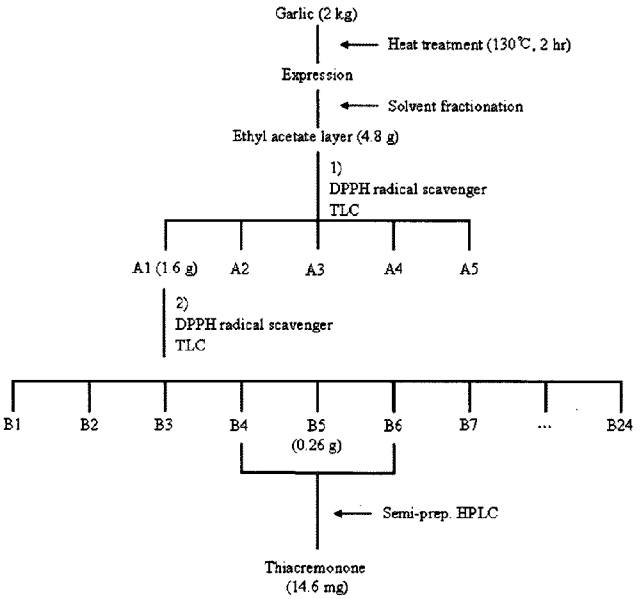

Fig. 1. Extraction scheme for the isolation of active compound from heated garlic (*Allium sativum* L.) at 130°C for 2 hr. Silica gel column chromatography: [1),2)]dichloromethane:methanol= 20/1, 10/1, 5/1, 1/1, 0/1, v/v

Semi-preparative HPLC The active fractions B4-B6 were purified by preparative RP-HPLC (Discovery® C18 column; 250×10 mm, i.d., 5 μm, Supelco, Bellefonte, PA, USA) on a Younglin SP930D Instrument (Anyang, Korea) equipped with a UV detector, operating at 365 nm, at room temperature and a flow rate of 3.5 mL/min. The elution gradient was a water: acetonitrile phase at 95:5 (v/v) for the first 11 min, followed by a continuous change from 95:5 to 50:50 over the next 29 min. The pure compound was obtained after evaporating the solvents using a rotary evaporator.

Structural identification of the isolated compound The structure of the purified compound was determined using several spectroscopic methods. The UV spectrum in methanol was recorded on a spectrophotometer (UV-1650; Shimadzu, Kyoto, Japan). The infrared (IR) spectrum was recorded on an FT-IR spectrometer (IFS-66/FRA106S; Brucker, Karlsruhe, Germany). Gas chromatography-mass spectrometry (GC-MS) was performed (Agilent 6890 gas

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of isolated compound[1)]

Position	δ_C	δ_H	HMBC (H to C)
2	85.7	-	
3	199.1	-	
4	148.3	-	
5	139.8	-	
2-CH_3	27.1	1.63 (3H, s)	C-2, C-3
3-CH_3	14.9	2.18 (3H, s)	C-4, C-5

[1)]Measured in CD_3OD, Assignments confirmed by DEPT and HMQC experiments.

chromatograph/5973N; Agilent, Palo Alto, CA, USA). The ^{1}H nuclear magnetic resonance (NMR, 500 MHz), ^{13}C NMR (125 MHz), Distortionless Enhancement by Polarization transfer (DEPT), Heteronuclear Multiple Bond Correlation (HMBC), and Heteronuclear Multiple Quantum Correlation (HMQC) spectra were recorded on a spectrometer (Avance 500; Bruker) using CD_3OD as a solvent. The compound was a colorless oil; $[\alpha]^{25}_{D}\pm0$ (c 0.1, methanol); UV λ_{max} (methanol) 365 nm; IR (KBr) ν_{max} 3350, 2981, 1679, 1601, 1395, 1360, 1252, 1188, 1104, 921, 845, and 563/cm; GC-MS *m/z* 160 $[M]^{+}$; ^{1}H-NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) : see Table 1.

Results and Discussion

Antioxidant activity of the solvent fraction and isolation of the active compound from heated garlic The optimum heating condition for garlic was previously determined as 130°C for 2 hr (14). We isolated the active compound from HGJ exposed to 120, 130, and 140°C for 2 hr and successively fractionated with hexane, chloroform, ethyl acetate, butanol, and water. We used chemical assays based on the ability of the compound to scavenge model free radicals, i.e., DPPH and ABTS radicals, because of their simplicity and worldwide acceptance for comparative purposes. The antioxidant activity of the 5 fractions from HGJ is shown (Fig. 2 and 3). The antioxidant activity of the ethyl acetate fraction was higher than that of the hexane, chloroform, butanol, and water fractions. The ethyl acetate fraction of HGJ treated at 130°C for 2 hr showed strong antioxidant activity compared with those from the other heating conditions. Therefore, we isolated and purified the active compound from the ethyl acetate fraction of HGJ treated at 130°C for 2 hr. The 4.8 g ethyl acetate fraction of 2 kg of heated garlic exposed to 130°C for 2 hr was subjected to activity-guided repeated fractionation on a silica gel column and eluted with an increasing concentration of methanol in dichloromethane. Silica gel chromatography resulted in one active fraction, A1 (1.6 g; data not shown). The active fraction A1 was repurified using silica gel chromatography (dichloromethane:methanol) to obtain 3 active fractions, B4-B6 (0.26 g; data not shown). We then attempted to isolate the active compound from the B4-B6 fractions using semi-preparative HPLC on a C18 column (Fig. 1). The yield of the purified active compound was about 14.6 mg. The chemical structure of the isolated compound was determined using spectroscopic methods.

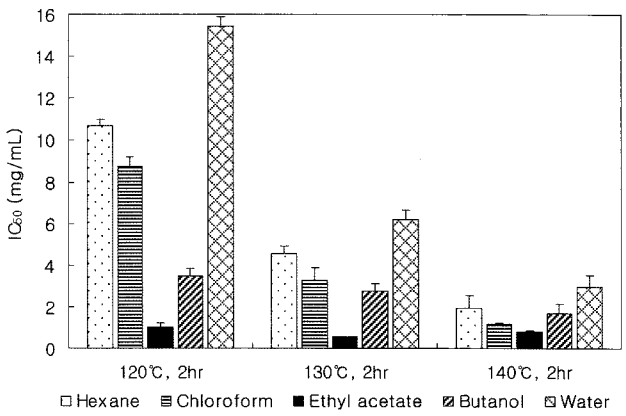

Fig. 2. IC_{50} values of electron donating ability (%) on the solvent fraction of heated garlic juice at 120, 130, and 140°C for 2 hr. DPPH radical scavenging activity (%) on solvent fraction of raw garlic juice (10 mg/mL): hexane (9.88±1.37%), chloroform (8.77±2.47%), ethyl acetate (6.46±1.36%), butanol (31.76±1.99%), water (5.74±2.11%).

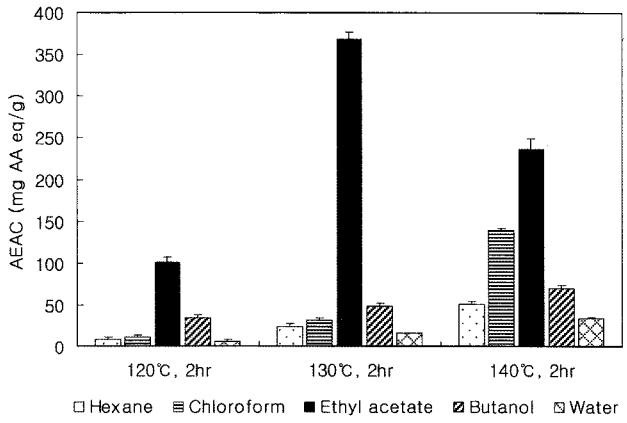

Fig. 3. Total antioxidant activities (AEAC) on the solvent fraction of heated garlic juice at 120, 130, and 140°C for 2 hr. AEAC (mg AA eq/g) on solvent fraction of raw garlic juice (10 mg/mL): hexane (3.5±0.2 mg AA eq/g), chloroform (3.4±0.2 mg AA eq/g), ethyl acetate (3.5±0.3 mg AA eq/g), butanol (5.4±0.3 mg AA eq/g), water (2.2±0.3 mg AA eq/g).

Identification of the isolated compound The compound obtained was colorless oil. The GC-MS spectrum showed a molecular ion peak at *m/z* 160, corresponding to a molecular formula of $C_6H_8O_3S$. The UV and IR spectra of the compound indicated the presence of an α, β-unsaturated ketone, substituted with an oxygen in the a position and a sulfur in the β position. The ^{1}H NMR spectrum of the compound showed signals assignable to 2 quaternary methyl groups at δ 1.63 and δ 2.18. The ^{13}C NMR and DEPT spectra confirmed that the molecule contained 6 carbon atoms consisting of 2 methyl groups at δ 14.9 and δ 27.1, 2 olefinic carbons at δ 139.8 and δ 148.3, a ketone signal at δ 199.1, and a quaternary oxygen-bearing carbon at δ 85.7. This indicates that the compound has a 3-thiphenone ring. The 2 methyl groups were located at C-2 and C-5, based on HMBC correlations between the methyl signals at δ 1.63 and δ 85.7 (C-2) and δ 199.1 (C-3), as

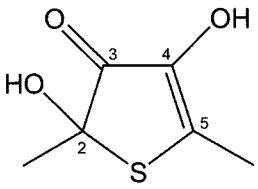

Fig. 4. Structure of active compound isolated from heated garlic juice at 130°C for 2 hr.

Table 2. DPPH radical-scavenging activity of isolated compound from heated garlic juice at 130°C for 2 hr

Active compound[1)]	IC_{50} value (μg/mL)
Ethyl acetate fraction	540.00±0.03[2)]
Thiacremonone	22.25±0.44
Vit. C	30.06±0.42
Vit. E	71.30±0.97
BHA	50.54±0.94

[1)]Ascorbic acid, α-tocopherol, and butylated hydroxyanisole were used as positive control compounds for vit. C, vit. E, and BHA, respectively.
[2)]Value are mean±SD of 3 replicate analyses.

well as between the other methyl signals at δ 2.18 and δ 148.3 (C-4) and δ 138.8 (C-5). Therefore, the isolated active compound from HGJ treated at 130°C for 2 hr was identified as thiacremonone (2,4-dihydroxy-2,5-dimethyl-thiophene-3-one; Fig. 4) by comparing its physicochemical and spectroscopic data with those from the literature (22). According to the HPLC analysis, the thiacremonone of raw garlic juice was not detected. We study results are consider that isolated the thiacremonone was synthesized with heat treatment. This is the first report of the isolation of thiacremonone from heated garlic, although it has been isolated from the fungus *Acremonium* sp. strain HA33-95, an inducer of differentiation in mammalian cells (22).

DPPH radical scavenging activity of isolated thiacremonone The ethyl acetate fraction of HGJ treated at 130°C for 2 hr and the isolated compound showed concentration-dependent antiradical activity by reducing the stable radical DPPH to a yellow-colored diphenylpicrylhydrazine derivative (Table 2). The known natural antioxidants ascorbic acid and α-tocopherol, and the synthetic antioxidant BHA, were used as positive controls. The IC_{50} of the thiacremonone was 22.25±0.44 μg/mL, which was higher than those of the antioxidants ascorbic acid (30.06 ±0.42 μg/mL), α-tocopherol (71.30±0.97 μg/mL), and BHA (50.54±0.94 μg/mL).

Acknowledgments

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References

1. Ingrid A, Jacques A. Seleno-compounds in garlic and onion. J. Chromatogr. A 1112: 23-30 (2006)
2. Anna MN, Riitta PP, Marjukka A, Kirsi-Marja OC. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. Food Chem. 81: 485-493 (2003)
3. Lee EJ, Kim KS, Jung HY, Kim DH, Jang HD. Antioxidant activities of garlic (*Allum sativum* L.) with growing districts. Food Sci. Biotechnol. 14: 123-130 (2005)
4. Kim SM, Kubota K, Kobayashi A. Antioxidative activity of sulfur-containing flavor compounds in garlic. Biosci. Biotech. Bioch. 61: 1482-1485 (1997)
5. Lampe JW. Health effects of vegetables and fruit: Assessing mechanisms of action in human experimental studies. Am. J. Clin. Nutr. 70: 475S-490S (1999)
6. Kodera Y, Suzuki A, Imada O, Kasuga S, Sumioka I, Kazenawa A. Physical, chemical, and biological properties of S-allylcysteine, an amino acid derived from garlic. J. Agr. Food Chem. 50: 622-632 (2002)
7. Yin M, Hwang S, Chan K. Nonenzymatic antioxidant activity of four organosulfur compounds derived from garlic. J. Agr. Food Chem. 50: 6143-6147 (2002)
8. Harris JC, Cottrell SL, Plummer S, Lloyd D. Antimicrobial properties of *Allium sativum* (garlic). Appl. Microbiol. Biot. 57: 282-286 (2001)
9. Anand PK, Somaradhya MA, Soundar D. Isolation and identification of a radical scavenging antioxidant-punicalagin from pith and carpellary membrane of pomegranate fruit. Food Chem. 87: 551-557 (2004)
10. Wee JH, Moon JH, Eun JB, Chung JH, Kim YG, Park KH. Isolation and identification of antioxidants from peanut shells and the relationship between structure and antioxidant activity. Food Sci. Biotechnol. 16: 116-122 (2007)
11. Boo HO, Chon SU, Kim SM, Pyo BS. Antioxidant activities of colored sweet potato cultivars by plant parts. Food Sci. Biotechnol. 14: 177-180 (2005)
12. Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agr. Food Chem. 50: 3010-3014 (2002)
13. Yang SJ, Woo KS, Yoo JS, Kang TS, Noh YH, Lee JS, Jenog HS. Change of Korean ginseng components with high temperature and pressure treatment. Korean J. Food Sci. Technol. 38: 521-525 (2006)
14. Kwon OC, Woo KS, Kim TM, Kim DJ, Hong JT, Jeong HS. Physicochemical characteristics of garlic (*Allium sativum* L.) on the high temperature and pressure treatment. Korean J. Food Sci. Technol. 38: 331-336 (2006)
15. Hwang IG, Woo KS, Kim TM, Kim DJ, Yang MH, Jeong HS. Change of physicochemical characteristics of Korean pear (*Pyrus pyrifolia* Nakai) juice with heat treatment conditions. Korean J. Food Sci. Technol. 38: 342-347 (2006)
16. Choi Y, Lee SM, Chun J, Lee HB, Lee J. Influence of heat treatment on the antioxidant activities and polyphenolic compounds of *shiitake* (*Lentinus edodes*) mushroom. Food Chem. 99: 381-387 (2006)
17. Dewanto V, Wu X, Liu RH. Processed sweet corn has higher antioxidant activity. J. Agr. Food Chem. 50: 4959-4964 (2002)
18. Jeong SM, Kim SY, Kim DR, Jo SC, Nam KC, Ahn DU, Lee SC. Effect of heat treatment on the antioxidant activity of extracts from citrus peels. J. Agr. Food Chem. 52: 3389-3393 (2004)
19. Bektas T, Munevver S, Akpulat HA, Atalay S. Screening of the antioxidant potentials of six Salvia species from Turkey. Food Chem. 95: 200-204 (2006)
20. Lee JH, Baek IY, Kang NS, Ko JM, Han WY, Kim HT, Oh KW, Suh DY, Ha TJ, Park KH. Isolation and characterization of phytochemical consitituents from soybean (*Glycine max* L. Merr.). Food Sci. Biotechnol. 15: 392-398 (2006)
21. Kim DO, Lee KW, Lee HJ, Lee CY. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. J. Agr. Food Chem. 50: 3713-3717 (2002)
22. Gehrt A, Erkel G, Anke T, Sterner O. Thiacremonone, a new inducer of differentiation of mammalian cells from an *Acremonium* sp. Nat. Prod. Lett. 14: 281-284 (2000)