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Antimutagenic Compounds Identified from the Chloroform Fraction of Garlic (*Allium sativum*)

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Abstract

Methanol extract of garlic was fractionated to chloroform and aqueous fractions. The chloroform fraction possessed the highest antimutagenic activity in *Salmonella typhimurium* TA100 and TA98, and was further fractionated into four *Allium sativum* chromatography fractions (ASC F1, 2, 3 and 4) by column and thin layer chromatographies. The ASC F2 exhibited the highest antimutagenic activity and contained 18 chemical compounds tentatively identifed by GC-MS, NMR and FT-IR. Among the 18 compounds, methyl linoleate was a major compound to exhibit the antimutagenicity.

Key words : antimutagenic compounds, garlic, methyl linoleate

INTRODUCTION

Garlic (*Allium sativum*) has been a folk medicine for a thousand years. The Codex Ebers (an Egyptian medical papyrus dating back to about 1550 B. C.) gives more than 800 therapeutic formulas, of which 22 mention garlic as an effective remedy for a variety of aliments including heart problems, headaches, bites, worms and tumors[1].

Garlic has been used as a spice in various oriental foods such as Korean-style pickled Chinese cabbage (Kimchi). Recently, studies have investigated the pharmathuetical effect of garlic. Attempts have been made to fractionate and isolate biologically active compounds from garlic. Investigations also performed more than a century ago established that cutting garlic bulbs releases a number of low molecular weight organic molecules incorporating sulfur atoms in bonding forms, which are rarely encountered in nature. The sulfur containing molecules are highly reactive. They spontaneously transform into other organic sulfur compounds, which display a remarkable range of biological effects such as antibacterial, antifungal and antithrombotic activities[1-5].

In the area of garlic cancer research, the most interesting finding is its antitumorigenic effect. Certain sulfur-containing compounds such as allicin is reported as an antitumorigenic compound[6]. The mechanism of the anticarcinogenic effect has been suggested that the allicin inactivates sulfhydryl enzyme, resulting in the inhibition of cell growth and division by sulfurhydryl poison, thus providing protection from the incidence of cancer. Sulfur compounds including allicin were most effectively extracted into ethanol/aqueous fraction[7] indicating that they are water soluble. However, recent studies indicate that garlic compounds extracted into more nonpolar solvents such as

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petroleum ether or chloroform exhibited more antimutagenic and/or anticarcinogenic activities than those into ethanol/aqueous solvent. Son and Hwang[8] reported that fat-soluble compounds of garlic had anticarcinogenic activities on murine leukemic lymphocyte cells of mice, and rectal and colon cancer cells of humans. Kim[9] also reported that the chloroform fraction of garlic had an antimutagenic effect. However, they did not chemically identify the antimutagenic compounds in the chloroform fraction.

In the present study, we investigated antimutagenic activities of chloroform fractions from garlic and identified some chemical compounds in the fraction.

MATERIALS AND METHODS

Fractionation and isolation of antimutagenic compounds

Solvent extraction and fractionation

Garlic produced in Eui-Sung, Kyeong-Buk was purchased from a local grocery and minced in a blender. The minced garlic (1kg) was extracted with glass distilled methanol (10 L) by shaking for 12hrs according to the method of Takahashi et al[10]. After decanting the supernatant of the methanol extract, additional 10 L glass-distilled methanol was added to the garlic residue and shaken for 12hrs, followed by separating supernatant again. The combined methanol extracts (20L) were concentrated to 200ml under a vacuum rotary evaporator (Hedolph Co. model W 2000) at 60℃. The concentrated extract was further fractionated into chloroform and aqueous soluble fractions using 400ml of chloroform : water (1 : 1, v/v).

Silca gel column chromatography

The chloroform fraction was further fractionated using silica gel column chromatography. The sample was mixed with silica gel (10g) and then placed on the column (100cm × 5cm i.d.) packed with silica gel (245g). ASC (*Allium sativum* chromatography) fractions were eluted with $CHCl_3$-MeOH-H_2O (65 : 35 : 10) and concentrated to 2ml under vacuum.

Thin layer chromatography

The ASC fractions were further fractionated on precoated TLC silica gel plates (Kiesel gel 60 F_{254} plate, Art No. 5735, Merck). The plates were developed with hexane - ethyl acetate (10 : 1, v/v), and then 50% H_2SO_4 solution was sprayed on carbonate spots.

Antimutagenicity test

Bacterial strains

Salmonella typhimurium strains TA100 and TA98, histidine requiring mutants, were provided by Dr. B. N. Ames, University of California, Berkley, CA, USA and were maintained as descriged by Maron and Ames[11].

The genotypes of tester strains were checked routinely for their histidine requirements, deep rough(*rfa*) character, UV sensitivity(*uvr*B mutation) and for the presence of R factor.

Chemicals

AFB_1 (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in spectrophotometric grade dimethyl sulfoxide (DMSO) obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was also obtained from Aldrich Chemical Co., These chemicals were sterilized through millipore membrane filtration or were autoclaved.

Mutagenicity test

A modified plate incorporation test (liquid preincubation of the organisms with the test com-pound) was employed[12]. 0.5ml of S9 mix prepared by the method of Maron and Ames[11] was distributed in sterile capped tubes in an ice bath and then 0.1ml of testers from overnight culture($1 \sim 2 \times 10^9$cells /ml) and 0.1ml of test compounds were added. The tubes were vortexed gently and preincubated at 37 ℃ for 30min. 2ml of the top agar in each tube kept at 45℃ were added and vortexed for 3 seconds. The resulting entire mixture was over-laid on the minimal agar plate. The plates were incubated at 37℃ for 48hrs and then the revertant bacterial colonies on each plate were counted.

GC-MS (Gas chromatography-mass spectroscopic) analysis

GC-MS analysis of ASC F2, which showed antimutagenic activity, was carried out with a HP 5970 Mass spectrometer connected with HP 5890 Gas chromatograph using a bonded polyethylene glycol-fused silica capillary column (HP-5 fused silica WCOT capillary column). The mass spectra were

recorded at an electron energy of 70eV and the ion source temperature was 280℃. The column was operated with temperature program from100℃ to 280℃ at 15℃ /min, and then hold for 15min at 280 ℃. Helium was used as carrier gas (1ml/min, split ratio 1/25). Each peak was identified based on Chamstation (HP 91153C, NBS-REVEL) mass spectral data base and/or mass spectrum of authentic compound.

FT-IR and NMR spectral analysis

IR spectrum of ASC F2 was obtained using KBr disk method by Bomen MB-100 FT-IR spectrophotometer. NMR spectrum was obtained by Brucker-AM spectrophotometer using TMS as an internal standard.

Table 1. Effects of chloroform and aqueous fraction (5%) from methanol extract of garlic on the mutagenicity of aflatoxin B_1 (AFB_1, 1μ g/plate) in *Salmonella typhimurium* TA98 and TA100

	Revertants/plate	
	TA 98	TA100
Spontaneous	26±4	103±7
AFB_1	1428±54	1118±28
AFB_1 + Methanol extracts	755±20	626±18
AFB_1 + Chloroform fraction	30±6	135±7
AFB_1 + Aqueous fraction	1453±24	1101±24

RESULTS AND DISCUSSION

Methanol extract of garlic was fractionated into chloroform and aqueous fractions and tested for the antimutagenic activity against AFB_1(Table 1).

The results indicate that the 5% chloroform fraction possessed higher antimutagenic activity than the methanol and aqueous fractions did. This result agrees with a previous report[8] that the fat soluble fraction of garlic extracted with petroleum ether exhibited anticarcinogenic activity. However, most of the study concluded that ethanol/aqueous solvent extractable fractions of garlic had anti-carcinogenic/antimutagenic effects[7, 13], and allicin and allin were considered as major compounds in antimut-agenic/anticarcinogenic fractions[13]. Since then, allicin has been believed to be the miracle biologi-cal compound in garlic and many studies have been carried out on this single compound. In the present study, we found that chloroform-extractable fract-ion rather than the aqueous phase exhibited anti-mutagenic activity.

The chloroform fraction was further separated on silica gel column and thin layer chromatogra-phies into 4 spots which showed Rf values of 1, 0.53, 0.28 and 0.14 and designated as ASC F1, 2, 3 and 4, respectively.

Antimutagenic activities of ASC fractions are shown in Table 2. ASC F2 and F3 showed strong antimutagenic effects among the ASC fractions. Since ASC F2 was a major fraction (67%), only the ASC F2 was used for the structural identification and antimutagenicity test.

Table 2. Effect of ASC(*Allium sativum* column) fractions which were separated from chloroform fraction of methanol extract of garlic on the mutagenicity of aflatoxin B_1 (AFB_1, 1μ g/plate) in the *Samonella typhimurium* TA100 and their relative amount

	Revertants / plate	Relative amount(%)
AFB_1	1417±32	
AFB_1 + ASC F1	777±32	13
AFB_1 + ASC F2	347±11	67
AFB_1 + ASC F3	349±25	15
AFB_1 + ASC F4	1042±35	5

ASC F2 collected from the silica gel column were concentrated with vacuum evaporator and then separated on HP-5 capillary column with GC.

The compounds in this fraction was identified by GC-MS (Fig. 1). Eighteen peaks were separated on HP-5 capillary GC columns and detected at the GC-MS detector sensitivity we used. The compounds tentatively identified include 5-methyl-1, 2, 3-thiadiazole, 1-methoxy-2-hexene, 5-ethenyl-3-oxazolidinethion, 2-oxazolidinethion, 4-methanol-2-pentadecyl-3-dioxolane, 7-quinolinol, 2, 2-dimethyl-dimethyl ester-pentanedioic acid, 1H-1, 2, 3 tri-azole, 3-allylthio-propionic acid, hexadecanoic acid methyl ester, 10, 13- octadecanoic acid methyl ester, 9, 12- octadecanoyl chloride, and 3-nitro-1, 2-benzen dicarboxylic acid (Table 3).

Among the peaks separated on HP-5 GC column, the peak no. 15 had the highest total ion counts and was considered as a major peak in ASC F2. This major peak had a typical ion fragments m/z 67, 81, 95, 82, 54, 68, 96, 109, 294 and 110 with relative abundances of 100, 97, 60, 57, 50, 52, 31, 29 and 25, respectively. This indicates that the

Table 3. Compounds identified from ASC F2 and their mass spectral data

Peak no.	Compounds	Mass spectral data : m/z (% base peak)
1	1, 2, 3 - Thiadiazole, 5 - methyl	72 (100), 71 (81), 45 (80), 144 (42), 39 (41), 39 (41), 111 (40) 97 (22), 43 (20), 103 (19)
2	2 - Hexane, 1 - methoxy	71 (100), 41 (60), 45 (33), 39 (21), 28 (18), 43 (18), 27 (13), 29 (13), 32 (8), 58 (8)
3	Unknown	45 (100), 129 (73), 41 (65), 28 (62), 69 (57), 59 (51), 29 (47), 43 (45), 27 (35), 39 (30)
4	2 - Oxazolidinethion 5 - ethenyl	129 (100), 45 (96), 41 (91), 69 (69), 59 (50), 43 (42) 39 (39), 29 (26), 27 (24), 55 (23)
5	2 - Oxazolidinethion	45 (100), 129 (92), 41 (87), 69 (64), 39 (40), 43 (40), 29 (39), 27 (35), 59 (35), 28 (29)
6	Unknown	129 (100), 45 (95), 41 (91), 69 (78), 59 (45), 39 (41), 43 (41), 29 (32), 55 (26), 101 (26)
7	1, 3 - Dioxolane - 4 - methanol, 2 - pentadecyl	145 (100), 113 (61), 41 (45), 39 (30), 45 (27), 28 (26) 32 (20), 69 (18), 27 (16), 43 (15)
8	7 - Quinolinol	145 (100), 113 (61), 41 (36), 45 (28), 39 (25), 69 (23), 28 (17), 29 (13), 51 (13), 79 (12)
9	Pentanedioic acid 2, 2 - dimethyl, dimethyl ester	69 (100), 129 (63), 41 (46), 39 (29), 45 (29), 59 (18) 27 (17), 67 (12), 28 (11), 73 (9)
10	1H - 1, 2, 3 - Triazole	69 (100), 129 (60), 41 (41), 45 (36), 39 (29), 28 (17), 59 (14), 27 (14), 67 (12), 29 (12), 70 (9)
11	Propionic acid, 3 - (allylthio) -	41 (100), 103 (93), 45 (89), 39 (81), 73 (81), 145 (67) 71 (44), 111 (41), 99 (35), 67 (32)
12	Hexadecanoic acid methyl ester	74 (100), 87 (73), 75 (22), 270 (16), 143 (16), 97 (13) 59 (10), 129 (10), 98 (8), 101 (8)
13	Unknown	41 (100), 71 (73), 103 (71). 72 (63), 39 (58), 45 (53), 73 (47), 177 (43), 28 (40), 145 (39)
14	Unknown	149 (100), 41 (17), 29 (12), 28 (11), 39 (9), 150 (9), 27 (8), 32 (7), 76 (7), 223 (5)
15	10, 13 - Octadecanoic acid methyl ester	67 (100), 81 (97), 95 (60), 82 (57), 54 (52), 68 (50) 95 (50), 109 (31), 294 (29), 110 (25)
16	9, 12 - Octadecadienoyl chloride	41 (100), 55 (91), 67 (71), 81 (63), 79 (60), 44 (53), 69 (49), 29 (46), 83 (40), 95 (40)
17	Unknown	129 (100), 57 (55), 28 (47), 41 (41), 55 (38), 70 (36), 71 (30), 43 (27), 112 (27), 147 (27)
18	1, 2 - Benzenedicarboxylic acid, 3-nitro-	149 (100), 167 (41), 57 (28), 43 (18), 70 (18), 41 (15) 71 (15), 28 (14), 150 (12), 55 (11)

relative abundances of 100, 97, 60, 57, 50, 52, 31, 29 and 25, respectively. This indicates that the major compound in ASC F2 is methyl linoleate comparing with data (Chamstation ; NB-REVFL) and mass spectrum of authentic methyl linoleate. FT-IR and H-NMR spectral data of ASC F2 support that methyl linoleate is the major compound in the ASC F2. FT-IR spectra (Fig. 2) show that the typical IR spectrum data of methyl linoleate (-C=O;

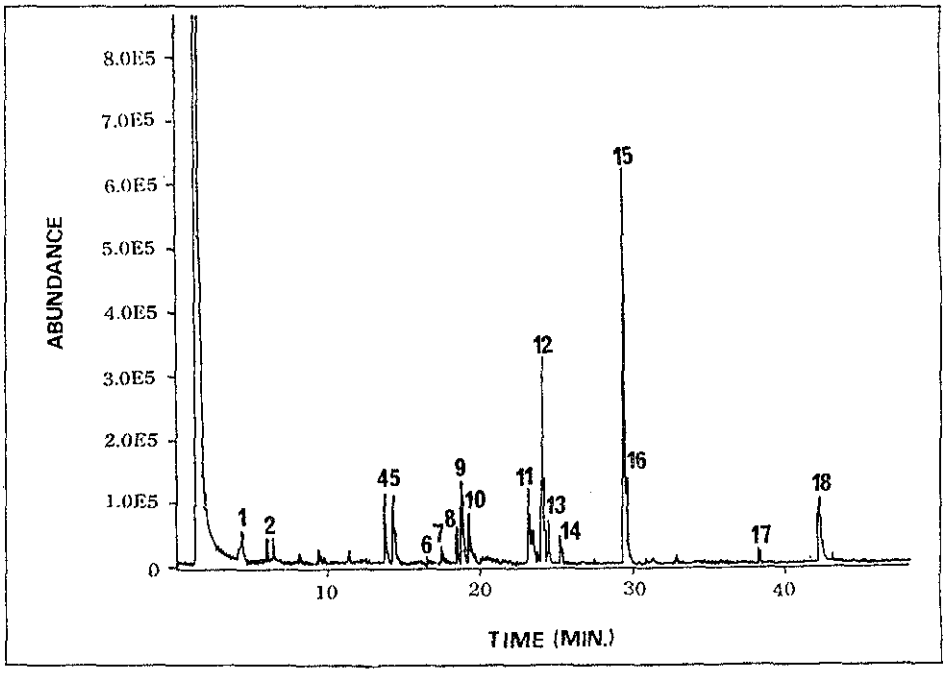

Fig. 1. Total ion chromatogram (TIC) of GC-MS of ASC F2 from garlic.

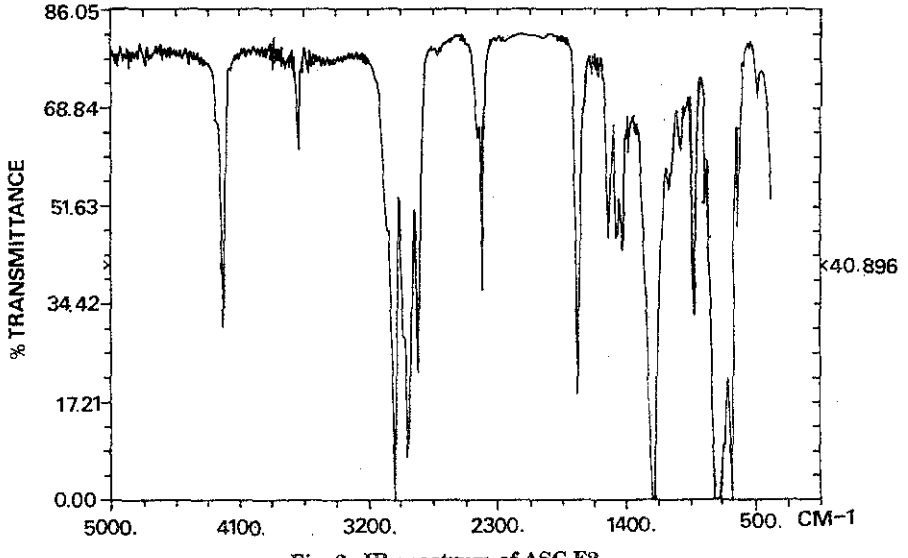

Fig. 2. IR spectrum of ASC F2.

linoleate (-C=O;1734 cm^{-1}, -C-O ; 1213 cm^{-1}, H-C=C-H ; 754 cm^{-1}). H-NMR data (Fig. 3) has -$(CH_2)_n$-CH_3 (d 0.8-0.9 ppm), R-CH_2-R (d 1.2-1.35ppm), -COO-CH_3 (d 3.6 ppm, s), -CH=CH- (d 5.30 ppm, m) supporting this compound as methyl linoleate.

We tested antimutagenic activity with the same amount of authentic methyl linoleate contained in the sample tested. Its antimutagenic activity against AFB_1 was similar to the total antimutagenic activity shown in the ASC F2, indicating that the methyl linoleate is a major contributor to the antimutagenic activity observed.

However, it is necessary to investigate the antimutagenic activity of the minor compounds

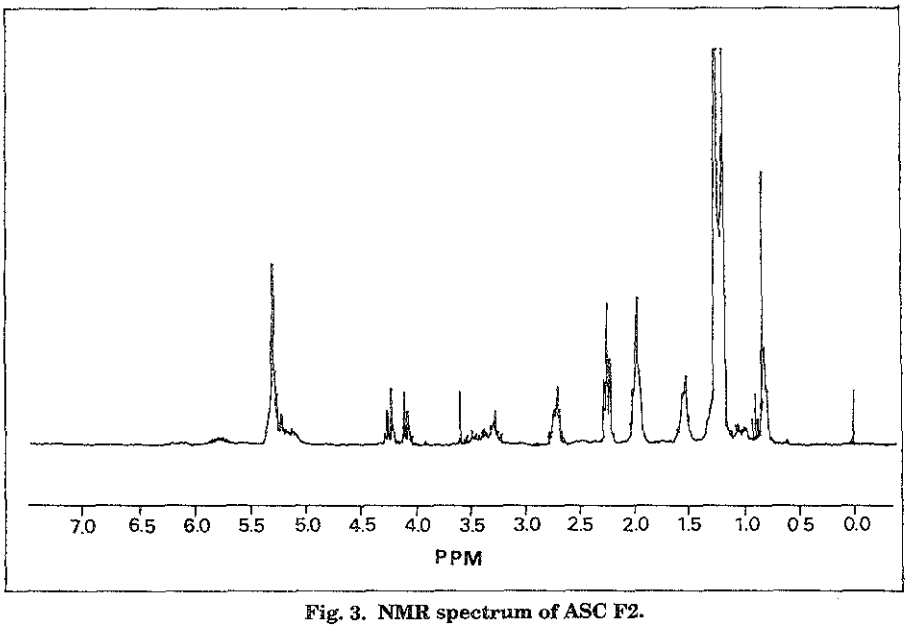

Fig. 3. NMR spectrum of ASC F2.

identified in the ASC fraction 2 currently being investigated in our laboratory. Recently new findings on the anticarcinogenic effect of fatty acids such as conjugated linoleic acid[14, 15] and linoleic acid[16-18] have been reported. Therefore, it is not a totally unexpected result that the fat soluble component, methyl ester of linoleic acid, has an antimutagenic activity.

The origin of methyl linoleate was not completely understood. However, it is possible that the ester of linoleic acid could be biologically synthesized in garlic. It is also possible that the methyl linoleate was formed during the experimental process from linoleic acid in garlic and methanol used for extraction. So the ester formation during the sol-vent extraction and separation of compounds, were tested but no methyl linoleate formation was observed. Therefore, it was concluded that methyl linoleate originated from biosynthetic processes in garlic.

In conclusion, the ASC F2 from the chloroform fraction of garlic showed antimutagenic activity and contained 18 chemical compounds tentatively identified. Among the 18 compounds, methyl linoleate was a major compound to exhibit the antimutagenicity for the *Salmonella typhimurium* TA100 and TA98.

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마늘의 클로로포름 분획에서 동정된 항돌연변이 물질

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요 약

마늘의 메타놀 추출물을 클로로포름층과 수층으로 분획하여 각 추출물의 aflatoxin B_1(AFB_1)에 대한 항돌연변이 효과를 시험한 결과, *Salmonella typhimurium* TA98과 TA100에서 클로로포름층이 가장 효과가 크게 나타났다. 클로로포름층을 다시 silica gel column 및 thin layer 크로마토그라피를 사용하여 분리한 결과, 4개의 분획(ASC F1, 2, 3, 4)를 얻었는데, 이중 ASC F2가 AFB_1에 대해 항돌연변이 효과가 가장 컸었다. ASC F2로부터 18가지의 화합물이 GC-MS, NMR, FT-IR을 이용하여 잠정적으로 분리, 동정되었으며, 이중 methyl linoleate는 가장 다량으로 함유되어 있는 화합물이었다. Methyl linoleate 표준품의 항돌연변이 효과를 조사한 결과 효과가 매우 큰 것으로 나타났다.