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Effect of Garlic (*Allium sativum*) on Glutathione S-Transferase Activity and the Level of Glutathione in the Mouse Liver

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Abstract

The effect of garlic on glutathione S-transferase activity and the level of glutathione in the mouse liver was studied. The intraperitoneal injection of the methanol extract of garlic and allyl sulfide which is one of possible active compounds in garlic to ICR mouse before the injection of aflatoxin B_1 (AFB_1) increased the levels of glutathione and nonprotein-SH in microsomal fraction of the livers. The injection of the chloroform fraction from the methanol extract of garlic and further fractionated ASC (*Allium sativum* chromatography) fraction 2 which revealed the highest antimutagenic activity in our previous research resulted in the increase of the activity of glutathione S-transferase and the levels of glutathione and nonprotein-SH. The glutathione itself also had the antimutagenic effect on AFB_1 and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *Salmonella typhimurium* TA98 and TA100 *in vitro*.

Key words : garlic, glutathione S-transferase, glutathione, antimutagenic effect

INTRODUCTION

Garlic (*Allium sativum*) has been used in the oriental food as a favorite spice and is considered to have medicinal effects. The therapeutic effect of garlic on diseases such as haemorrhoids, dematitis rheumatism, cough, loss of appetite was already written in the ancient Indian records[1].

With the expectation about curing effect of garlic, a number of the investigations have been continued. It was observed that the organic sulfur compounds from garlic showed antibacterial and antifungal activities[2~4]. By *in vitro* tests in several microorganisms, especially, allicin was equally effective aganist gram positive and gram negative microoganisms and inhibited both germination of spores and growth of hyphae[3,4]. Several investigators suggested that raw and boiled garlic prevented the increase in serum cholesterol in butter fat lipaemia[1].

Recently, garlic was also studied in the negative relation with the mutagenicity and cancer. Attempts have been made to prove the preventive effect of garlic and it's active compound on the growth of tumor and cancer cells. The results showed that garlic extract and it's active principle have tumor-inhibiting and anticancer effects[5~8]. Weisberger and Pensky[5] concluded that interference with sulfhydryl carrying components like garlic's could block necessary step for division and growth of cells. It was also reported that allicin of garlic oxidized -SH compounds which might stimulate cell growth and division and inhibited tumor growth. Son and Hwang[8] observed the cytotoxic activities of ethanol extract of garlic against various cancer cells.

In our previous investigation, the inhibitory effects of garlic extracts on the mutagenicity of various mutagens in *Salmonella* assay system and on the growth of the cancer cells were observed[9,10]. The chloroform fraction from the methanol extract of garlic revealed stronger inhibitory activities than the aqueous fraction. Eighteen compounds including methyl linoleate were identified from ASC (*Allium sativum* chromatography) fraction 2 which was separated from the chloroform fraction and exhibited the highest anti-

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mutagenic activity. But these findings were for the antimutagenic and anticarcinogenic effect of garlic *in vitro*.

Glutathione S-transferase was known as an important enzyme it has the capable effect for the detoxification and because it may be a possible mechanism of the antimutagenicity of chemical carcinogens *in vivo*[11].

In the this study, the effect of active extracts from garlic on glutathione S-trasferase activity and glutathione level in the mouse liver was investigated.

MATERIALS AND METHODS

Garlic extracts and sample preparation

Garlic produced in Eui-Sung, Kyeong-Nam was extracted and fractionated by the same methods as shown in our previous study[10]. It was extracted with methanol and then fractionated into chloroform and aqeous soluble fraction. The chloroform fraction was further fractionated by silica gel column and thin layer chromotographres. As the highest active fraction of garlic, ASC (*Allium sativum* chromatography) fraction 2 was obtained. Allyl sulfide and glutathione were purchased from Sigma Chemical Co. (USA). The sample used for the test were sterilized through millipore membrane (0.45μm) filtration or were autoclaved. And samples were dissolved in dimethyl sulfoxide (DMSO).

Carcinogens / mutagens

Aflatoxin B_1 (AFB_1) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) were purchased from Sigma Chemical Co. (USA). AFB_1 and MNNG were dissolved in DMSO and distilled water, respectively.

Antimutagenicity test

Salmonella typhimurium TA98 and TA100 were kindly presented by Dr. B. N. Ames, University of Califonia, Berkery, CA, USA. The genotypes of tester strains were confirmed routinely for their histidine mutation, deep rough (*rfa*) mutation, *uvrB* mutation and the presence of the R factor by the methods of Maron and Ames[12].

The preincubation test was employed for the antimutagenicity test as described by Lee *et al.*[13]. To activate the indirect mutagen, the S9 mixes were prepared from the S9 fraction (microsomal fraction of rat liver) according to the method by Maron and Ames[11]. The S9 mix were administered with 8mM $MgCl_2$, 33 mM KCl, 5mM glucose-6-phosphate, 4mM NADP, 100mM sodium phosphate, pH 7.4 and the S9 fraction (10%).

With or without 0.5ml of the S9 mix, 0.1ml of a test strain from an overnight culture ($1\sim2\times10^9$cells/ml) and 0.1ml (50μl of mutagen+50μl of test sample) of the test compound were preincubated at 37° C for 30 minutes. And then 2ml of the top agar, kept at 45° C, were added and vortexed for 3 seconds. The entire mixture was poured on the minimal agar plate. After incubating for 48 hours at 37° C, the revertant bacterial colonies on each plate were counted.

The toxicity tests[12] for the samples were also carried out before the antimutagenicity tests.

In vivo test of active compounds of garlic

ICR mice (male) weighing 22 ± 2g were prepared with five per each group. First, the methanol extract of garlic and allyl sulfide were administered with 60 mg per kg of the mouse by the intraperitoneal injection once a day. On the third day, AFB_1 was injected with 7mg per kg of the mouse causing acute poisoning[14]. And then after 24 hours, their livers were removed for the test. Second, the further extracted chloroform fraction (40mg/kg) and the ASC fraction 2 (10 mg/kg) were administered (i.p.) twice with the interval of 24 hours. After 3 hours the livers of the mice were removed. The removed livers were homogenized with 0.15M KCl (3ml/g liver) and separated in the centrifuge with $9000\times g$ for 10 minutes, and then the supernatant was used for the test.

Measurement for the activity of glutathione S-transferase

The activities of glutathione S-transferase in the treated mouse livers were measured by the method of Habig *et al.*[15]. The reaction system composed with 1 ml of 2.5mM 1-chloro-2,4-dinitrobenzene, 0.5ml of 5mM glutathione (GSH) and 0.25mM phosphate buffer (pH 6.5), and the mixture was preincubated for 5

minutes at 25° C. After the addition of the enzyme source in the system, the change of absorbance at 340 nm for 5 minutes was measured.

Determination of nonprotein-SH, glutathione (GSH), protein-SH and protein in the mouse liver

Nonprotein-SH

By the method of Sedlak and Lindsay[16], 1ml of 10% TCA was added in 1ml of 10% liver homogenate. 0.2ml of the supernatant was reacted with 0.5ml of the solution administered with 0.01M of $NaNO_2$ and 0.2N H_2SO_4(1 : 9) for 5 minutes. After 0.2ml of 0.5% sulfumic acid ammonium solution was added, the system was mixed strongly for 2 minutes. Again, sulfanilamine and N-1-napthylenediamine were added. After 5 minutes, the absorbance at 540nm was measured. 125mM GSH solution was used as the standard solution.

Glutathione (GSH)

The level of glutathione was determined by subtracting the levels of cysteine-SH from that of nonprotein-SH. The level of cysteine-SH was measured by the methods of Higashi[17]. In order to measure the level of cysteine, 1ml of 10% TCA was added to 1ml of 10% liver homogenate and centrifuged. 0.5ml of acetic acid and 0.5ml of ninhydrine were added to 0.5ml of the above centrifuged solution. That combined solution was boiled for 10 minutes and then cooled immediately. After adding 3ml of ethanol, the absorbance of that solution was measured at 560nm.

Protein-SH

After 0.2ml of 5% liver homogenate was added in 1ml of 0.2M tris buffer, 0.1ml of 0.01M DTNB (5,5'dithiobis-2-nitrobenzoic acid) and 4ml of methanol, those solutions were reacted at 24° C for 15 minutes. After centrifuging at 4000rpm for 30 minutes, the absorbance of the supernatant was measured at 412 nm[16].

Protein

0.5ml of the centrifuged microsomal fraction of the liver was reacted with 2.5ml of alkaline solution and then 0.5ml of Folin-Ciocalteau was added. After the reaction for 30 minutes, the absorbance was measured at 750nm[18].

Statistical analysis

The statistical analysis of the test data was performed by the analysis of variance. Significant differences between treatment means were determined by using Student's t test[19].

RESULTS AND DISCUSSION

The methanol extract of garlic and allyl sulfide which was considered as one of the possible active compounds in garlic were injected (i. p.) to ICR mouse once a day for two days. A single intraperitoneal injection of AFB_1 (7mg/kg) was administered to the each mouse on the third day. On the fourth day, the livers were removed from the mice and homogenized at 9000 × g. With the upper portion (microsomal fraction), the activity of glutathione S-transferase and the levels of glutathione, nonprotein-SH and protein-SH of the liver samples were measured.

As shown in Table 1, the levels of glutathione and nonprotein-SH increased in the group injected with the methanol extract of garlic or allyl sulfide by 73 to 123 percent. Glutathione was known to bind with electrophiles of various carcinogens and thus it blocked the binding with DNA[20]. Since almost all of ultimate carcinogens or mutagens are electrophilic, the conjugation of glutathione with them by itself or via glutathione S-transferase was important in a mechanism for the carcinogen detoxification[11]. Therefore the increase of the level of glutathione by garlic extract and allyl sulfide in this result seems to be very benefical in the prevention of cancer.

The activity of glutathione S-transferase which was believed to be one of the enzyme involved in the detoxification pathway for the carcinogens in the livers was not changed in this test system (Table 1). It was considered that because the livers were removed in a day after the injection of AFB_1, the activity of glutathione S-transferase increased just after the injection and then decreased gradually during 24 hours.

The revertant numbers of *Salmonella typhimurium* TA100 in the absence and presence of AFB_1 (1μg/plate) when variously treated S9s used are shown in

Table 1. The activity of hepatic glutathione S-transferase and the levels of glutathione, nonprotein-SH and protein-SH in the microsomal fractions (S9s)[1] of the livers from the mice treated with methanol extract from garlic or allyl sulfide plus aflatoxin B_1 (AFB_1)

Treatment	Glutathione S-transferase (μmol/min/mg protein)	Glutathione (nmol/mg protein)	Nonprotein-SH (nmol/mg protein)	Protein-SH (nmol/mg protein)
Control (DMSO + AFB_1)	6.51±0.25	104.8±25.1	113.2±25.9	1079.8±31.6
Methanol extract of garlic + AFB_1	6.59±0.35	186.8±49.0	197.5±50.1	1091.0±27.4
Allyl sulfide + AFB_1	6.20±0.41	233.3±38.4*	245.3±39.1**	953.7±50.2

[1] The mouse liver S9s were prepared as follows, methanol extract of garlic (60mg/kg), allylsulfide (6mg/kg) and DMSO (control) were injected (i.p.) to the mice twice and then aflatoxin B_1 (7mg/kg) was injected (i.p.) to those each mouse. The mice were killed after 24 hrs and the microsomal fractions (S9s) were collected from the homogenized livers

*The value is significantly different at the $p<0.05$ level

**The value is significantly different at the $p<0.01$ level

Table 2. The revertant numbers of *Salmonella typhimurium* TA100 in the absence or presence of aflatoxin B_1 (AFB_1, 1μg/plate) when variously treated S9s[1] used

Treatment	Revertants/plate	
	Absence of AFB_1	Presence of AFB_1
Control (DMSO + AFB_1)	125±2	1554±133
Methanol extract of garlic + AFB_1	100±6*	1564±115
Allyl sulfide + AFB_1	95±8*	1521±112

[1]See the detailed explanation in Table 1

*The value is significantly different at the $p<0.05$ level

Table 2. The revertants of *Salmonella typhimurium* TA100 decreased ($p<0.05$) in the group of methanol extract of garlic or allyl sulfide injected S9s prior to AFB_1 treatment compared to the control. However, the decreasing effect of the revertants by methanol extract of garlic or allyl sulfide was not shown when the S9s were used for the mutagenicity of AFB_1.

The chloroform fraction from methanol extracts of garlic and ASC fraction 2 which showed the highest antimutagenic effect in the previous study[9] were also administered by the intraperitoneal injection with twice in the interval of 24 hours. In 3 hours after the second injection, the livers of the mice were taken out. And then the activity of glutathione S-transferase and the levels of glutathione, nonprotein-SH, protein-SH in S9 fraction were determined. The injection of the chloroform fraction and ASC fraction 2 from the garlic resulted in the increase of the activity of glutathione S-transferase (Table 3). It was known that glutathione S-transferase played an important role as one of the mechanism for carcinogen detoxification[21]. Lotlikar *et al.*[22] had also reported that glutathione S-transferase in microsome induced the conjugation between AFB_1-epoxide and glutathione, and prevented the binding of AFB_1 to DNA. Therefore the increase of glutathione S-transferase activity in these results might indicate one possible antimutagenic effect aganist AFB_1.

It was already reported that the compounds of benzyl isothiocyanate, β-naphthoflavone, coumarin, α-angelicalactone, disulfiram, indole-3-carbinol, indole-3-acetonitrile and the diets containing dried powdered preparations of brussels, sprouts, cabbage, coffee beans, or tea leaves increased the activity of glutathione S-transferase in the liver and small intestine of mouse[11].

The levels of glutathione and nonprotein-SH also increased in this test system (Table 3). But the increase of the levels was not the same as shown in Table 1. Glutathione S-transferase was known as an enzyme that was involved in the detoxification of a large group of hydrophobic compounds bearing an electrophilic center by the conjugation with the thiol group of glutathione[21]. Therefore, it could be considered that some glutathione was used as substrate in the metabolism with the increased glutathione S-transferase.

The revertants of *Salmonella typhimurium* TA98 and TA100 was determined in the test system which AFB_1 (1μg/plate) was added in the activating mixture containing S9 prepared as in Table 3. As shown in Table 4, it seemed that the S9s treated with garlic extracts had no effect on decreasing the mutagenicity of AFB_1.

Since the level of glutathione increased by garlic extracts *in vivo*, the antimutagenicity of glutathione was studied *in vitro*. The glutathione also suppressed the mutagenicity of AFB_1 in propotional with the co-

Table 3. The activity of hepatic glutathione S-transferase and the levels of glutathione, nonprotein-SH and protein-SH in the liver S9s[1] from the mice treated chloroform fraction from methanol extract of garlic and ASC fraction 2 from the chloroform fraction

Treatment	Glutathione S-transferase (μmol/min/mg protein)	Glutathione (nmol/mg protein)	Nonprotein-SH (nmol/mg protein)	Protein-SH (nmol/mg protein)
Control	1.49±0.10	22.74±1.26	26.56±1.06	244.8±7.7
Chloroform fraction[2]	1.56±0.10	27.22±1.61	30.42±1.51	281.5±20.9
ASC fraction 2[3]	1.66±0.04	26.28±2.16	29.37±1.91	285.4±7.8

[1] The mouse liver S9s were prepared as follows, chloroform fraction of methanol extract from garlic (40mg/kg), ASC fraction 2 (10mg/kg) and DMSO (control) were injected (i.p.) to the mice twice and then killed after 3 hrs and the homogenized each mouse liver used as the S9s

[2] Chloroform fraction was fractionated from the methanol extract of garlic

[3] ASC fraction was separated from the chloroform fraction

Table 4. The revertant numbers of *Salmonella typhimurium* TA98 and TA100 in the presence of aflatoxin B_1 (AFB_1, 1μg/plate) when variously treated S9s[1] used

Treatment	Revertants/plate	
	TA98	TA100
Control	725±55	664±99
Chloroform fraction[2]	684±74	629±97
ASC fraction[3]	664±40	675±81

[1~3] See the detailed explanation in Table 3

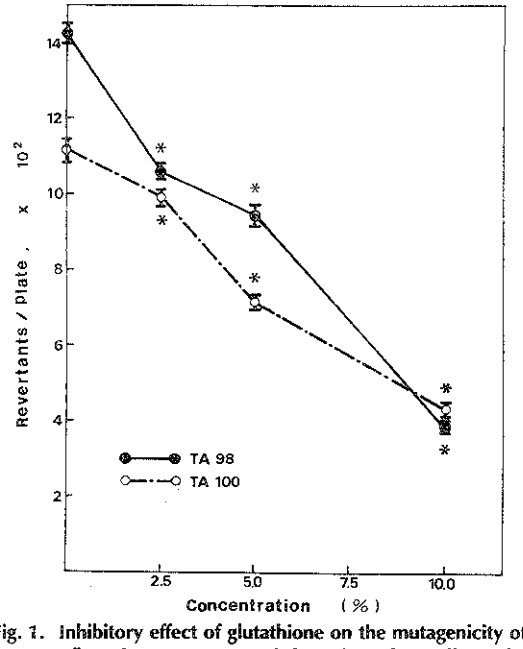

Fig. 1. Inhibitory effect of glutathione on the mutagenicity of aflatoxin B_1 (AFB_1, 1μg/plate) in *Salmonella typhimurium* TA98 and TA100.
*Significantly different from the control value ($p<0.05$)

ncentration in *Salmonella typhimurium* TA98 and TA100 (Fig. 1). The revertants numbers of *Salmonella*

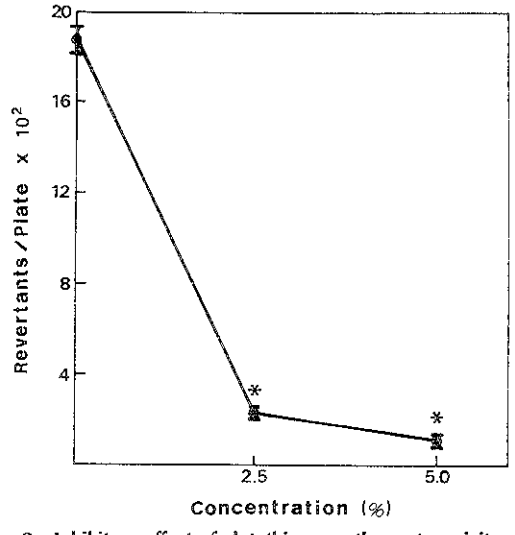

Fig. 2. Inhibitory effect of glutathione on the mutagenicity of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 1g/plate) in *Salmonella typhimurium* TA100.
*Significantly different from the control value ($p<0.05$)

typhimurium TA98 and TA100 decreased in the addition of 5% glutathione by about 40% and 39%, respectively. However, on the mutagenicity of MNNG, the addition of 5% of glutathione decreased the revertants of *Salmonella typhimurium* TA100 by 94 percent (Fig. 2). Therefore, it seemed that the glutathione had more antimutagenic effect on the direct mutagen than the indirect mutagen. It was already known that the glutathione protected the cells from the electrophiles of mutagen or carcinogen via glutathione S-transferase[11,20]. However the present result revealed that glutathione itself had the antimutagenic effect on AFB_1. The glutathione conjugation of AFB_1 had also been reported in the rat *in vivo* and *in vitro* [21~23]. As reactive metabolite of AFB_1 involved in conj-

ugation, AFB_1-2,3-epoxide, was suggested in the tentative characterization of the glutathione conjugate[23]. In addition, glutathione was known as an antioxidant which was possible inhibitor of chemical carcinogen[24]. Considering the reports and the present result, It seems that the glutathione plays an important role in the prevention of cancer by itself as well as via glutathione S-transferase.

Based on our studies of garlic, we suggest that garlic has antimutagenic effect *in vivo* as well as *in vitro*. But the question about how garlic enhanced glutathione S-transferase activity and the level of glutathione in the mouse liver remained and would be investigated continuously. More research regarding the antimutagenic or anticarcinogenic effects of garlic *in vivo* with a wide range of cancer will have to be continued. And we expect that the active compounds of garlic in the protective effects for health will be identified in further investigations.

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마늘이 생쥐간에서 Glutathione S-transferase 활성과 Glutathione 생성에 미치는 영향

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요 약

마늘의 메탄올 추출물과 이로 부터 분리한 클로르포름 분획물, 칼럼 크로마토그라피로 더욱 분리한 분획물들

에 투여하여, 간의 microsomal 분획물에서의 glutathione S-transferase 활성과 glutathione 생성에 대한 이들의 효과를 검토하였다. 마늘의 메탄올 추출물(60mg/kg)과 마늘의 주요 활성물질의 하나로 알려진 allyl sulfide(6mg/kg)를 1일 간격으로 2번 복강주사하고 3일째에 aflatoxin B_1 (AFB_1, 7mg/ kg)을 복강주사하고 다시 24시간 후에 간을 적출하여 조사한 결과, 간의 microsomal분획물의 glutathione과 이를 포함한 nonprotein-SH양은 마늘의 메탄올 추출물과 allylsulfide 투여군에서 73~123%까지 증가하였다. 또한 마늘의 메탄올 추출물에서 분획한 클로르포름 분획물과 여기서 더욱 분리된 ASC 분획 2의 투여는 발암물질의 해독에 효과적인 것으로 알려진 간의 glutathione S-transferase의 활성을 증가시켰으며, glutathione과 nonprotein-SH의 양도 증가시켰다. 또한 마늘 분획물들의 첨가로 증가를 볼 수 있었던 glutathione은 *Salmonella typhimurium* TA98 과 TA100에서 aflatoxin B_1 (1μg/plate)에 대해 그리고 TA100에서 N-methyl-N′-nitro-N-nitrosoguanidine (1μg/plate)에 대해 강한 항돌연변이 효과를 나타내었다.