ORIGINAL

Inhibitory effects of caraway (*Carum carvi* L.) and its component on *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced mutagenicity

Masanori Mazaki[1], Keiko Kataoka[1], Takemi Kinouchi[1], Usanee Vinitketkumnuen[2], Masami Yamada[3], Takehiko Nohmi[3], Tomomi Kuwahara[1], Shigeru Akimoto[1], and Yoshinari Ohnishi[1]

[1]*Department of Molecular Bacteriology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan ;* [2]*Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand ; and* [3]*Division of Genetics and Mutagenesis, Biologica 1 Safety Research Center, National Institute of Health Sciences, Tokyo, Japan*

Abstract : To elucidate the mechanism of antimutagenicity of caraway, we examined the effects of caraway seed extract on *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)-induced mutagenesis in DNA methyltransferase-deficient *Salmonella typhimurium* strains, O^6-methylguanine DNA adduct formation, and thiol content in *S. typhimurium* cells. MNNG was highly mutagenic for ogt^- strains YG7104 ($ogt^- ada^+$) and YG7108 ($ogt^- ada^-$), and it showed slightly higher mutagenicity in strain YG7100 ($ogt^+ ada^-$) than in strains TA100 and TA1535. Hot water extract of caraway seeds inhibited MNNG-induced mutation only in the ogt^+ strains. In the presence of caraway extract, O^6-methylguanine DNA adducts in strain YG7100 were decreased in proportion to the decrease of MNNG-induced mutagenesis. Although MNNG is known to degrade in the presence of thiols to produce methyl cation which can react with DNA, caraway had no effect on cellular concentrations of acid-soluble thiols. These results indicate that caraway does not directly inactivate MNNG and that Ogt-O^6-methylguanine-DNA methyltransferase may be involved in the antimutagenic activity of caraway. J. Med. Invest. 53:123-133, February, 2006

Keywords **:** *caraway ; MNNG ; antimutagenicity ; O^6-methylguanine*

INTRODUCTION

Methylating agents such as *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) induce mutation by modification of the O^6-position of guanine and cause tumor formation (1, 2). O^6-Methylguanine can mispair with thymine during DNA replication, resulting in G : C→A : T transition mutation, which has been implicated in activation of oncogenes such as *K-ras* and inactivation of tumor suppressor genes in methylating agent-induced tumorigenesis (3). The adduct is repaired by O^6-methylguanine-DNA methyltransferase(4). Alkylation of DNA is also induced by endogenous metabolites (5, 6), and the deficiency of repair of methylated bases is correlated with alkylating agent-induced brain tumor (1, 7). Therefore, screening of anti-alkylating agents and study on the antimutagenicity mechanisms are very important for cancer chemoprevention.

A wide spectrum of chemical compounds occurring

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Address correspondence and reprint requests to Keiko Kataoka, Department of Molecular Bacteriology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan. and Fax : +81-88-633-7453.

in natural dietary products has been observed to be associated with some protective effects against chemically induced toxicity and carcinogenesis (8-10). Most spices contain organosufides, phenols, aromatic isothiocyanates,flavones and terpenes, which have been found to be antimutagenic and anticarcinogenic (11, 12). A case control study in Italy (13) also indicates that low cancer risk is related to consumption of spices, olive oil and garlic, in addition to increased intake of raw vegetables, fresh fruits and citrus fruits. Many kinds of plant compounds are known to work as anti-initiators by various mechanisms. Some of the essential oils derived from spices influence carcinogen-metabolizing enzymes and hepatic levels of acid-soluble sulfhydryl (14). Black pepper also modulates the hepatic detoxication system (15). Some naturally occurring flavorings have been demonstrated to inhibit or enhance mutagenesis by modifying DNA replication and/or repair systems after cellular DNA is damaged by mutagens (16). Ellagic acid is known to inhibit tumorigenesis by enhancing the detoxication system, masking DNA from DNA-damaging agents and direct binding to ultimate mutagens (17).

Caraway seeds are used in rye bread, cookies and cheese as seasoning. We have reported that hot water extract of caraway seeds is antimutagenic against MNNG, nitrosodimethylamine and ICR-170 (18). In this study, we examined the effects of caraway seeds extracts on MNNG-induced mutagenesis in DNA methyltransferase-deficient strains, O^6-methylguanine DNA adduct formation, and thiol content in *Salmonella typhimurium* cells to elucidate the mechanism of antimutagenicity.

MATERIALS AND METHODS

Chemicals

MNNG, methyl methanesulfonate (MMS), and D-carbone were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis, USA. *N*-Ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG), methylazoxymethanol (MAM) acetate, 7-methylguanine, 3-methyladenine, Tris, and glutathione were obtained from Sigma Chemical Co., St. Louis, Mo, USA. *N*-Methyl-*N*-nitrosourea (MNU) and *N*-ethyl-*N*-nitrosourea (ENU) were obtained from Katayama Chemical Co., Osaka, Japan. Ethyl methanesulfonate (EMS) was from Nakalai Tesque Co., Ltd., Kyoto, Japan. Ribonucleases A and T1 were purchased from Worthington Biochemical Co., Freehold, NJ. Other chemicals were reagent grade or higher and were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. O^6-Methylguanine was kindly supplied by Drs. M. Ikenaga and K. Ishizaki, Kyoto University, Kyoto, Japan.

Preparation of caraway extract

Caraway (*Carum carvi L.*) seeds were purchased in Chiang Mai in Thailand. Hot water extract was prepared as described previously (18). After grinding caraway seeds with a mixer, 2.5 volume of boiling water was added and the suspension was centrifuged at 6000×g for 20 min (TOMY No.9 N rotor). The residue was extracted twice with 1.3 volume of boiling water, and combined extracts were centrifuged at 10^5×g for l hr. The supernatant (S-100) was filtered through a 0.45 μm filter and was used for experiments as hot water extract.

To examine which components in hot water extract of caraway involve in antimutagenic activity, the extract was further fractionated into ether-soluble basic, acidic, neutral fractions and remained aqueous layer as follows. Hot water extract was mixed with 2N sulfuric acid and was extracted with diethyl ether 3 times. Remained aqueous layer was added 12 N sodium hydroxide (NaOH) to adjust pH to 11.0 and was extracted with diethyl ether 3 times to get ether-soluble basic fraction. The first ether extract was combined and mixed with equal volume of 2N NaOH and ether layer was removed as ether-soluble neutral fraction. After two more extraction with ether, aqueous layer was mixed with 10 N hydrochloric acid (HCl) to adjust pH to 1.0 and extracted with ether 3 times (ether-soluble acidic fraction). These ether-soluble fractions were evaporated and dissolved in dimethyl sulfoxide (DMSO).

If necessary, ground caraway seeds were extracted sequentially with *n*-hexan, methanol, and boiling water like as the preparation of hot water extract. The extracts were evaporated and dissolved in DMSO.

Mutagenicity test

Mutagenicity of MNNG and other alkylating agents were assayed in duplicate by the procedure of Maron and Ames (19) with the modification of preincubation (20) under yellow lamps. *S. typhimurium* strains TA 100 (*hisG46, rfa, uvrB*, pKM101), TA1535 (*hisG46, rfa, uvrB*), G46 (*hisG46*), and O^6-methylguanine-DNA methyltransferase-deficient strains YG7100 (*ada*$^-$ *ogt*$^+$), YG7104 (*ada*$^+$ *ogt*$^-$), YG7108 (*ada*$^-$ *ogt*$^-$) were used as tester strains. Strains YG7100, YG7104 and YG7108 were constructed from strain TA1535 by M. Yamada *et al.* (21), and was used to investigate

the mechanisms of inhibitory effect of caraway on mutagenicity of MNNG. The genes *ogt* and *ada* encode constitutive and inducible O^6-methylguanine-DNA methyltransferase, respectively.

In standard mutagenicity test, mutagen dissolved in 100 μl of DMSO was mixed with 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4), 0.1 ml of bacterial culture, and 50 μl of caraway extract or water. After incubation at 37°C for 20 min, the mixtures were added 2ml of 0.6% agar- 0.5% sodium chloride (top agar), and poured onto minimal plate. After 48 hr incubation at 37°C, number of His^+ revertants was counted. The number of spontaneous revertants was determined separately and was subtracted. The numbers of spontaneous revertants were 21±18 in YG7108, 23±21 in YG7104, 6±3 in YG7100, 108± 22 in TA 100, 9±3 in TA1535 and 3±2 in *hisG46*.

If necessary, *S.typhimurium* strain YG7100 cells were incubated with caraway extract before MNNG treatment (pre-treatment), during MNNG treatment (co-treatment), or after MNNG treatment (post-treatment). In the post-treatment, 0.1 ml of overnight culture in Nutrient broth was incubated with MNNG (0.5 μg, dissolved in 0.1 ml of DMSO) in 0.5 ml of sodium phosphate buffer (pH7.4) at 37°C for 20 min, washed with saline, and resuspended in 0.1 ml of Nutrient broth. Then 0.5 ml of sodium phosphate buffer (pH 7.4) and caraway extract were added. After incubation at 37°C for 20 min, 2 ml of top agar was added and the mixture was poured onto a minimal plate. In the pre-treatment, YG7100 cells were first incubated with caraway extract in sodium phosphate buffer, and washed cells were incubated with MNNG as described above. In the co-treatment, YG7100 cells were treated with MNNG in the presence of caraway extract, and after washing with saline the cells were suspended in 0.1 ml of Nutrient broth and incubated for 20 min in sodium phosphate buffer (pH 7.4).

Quantification of O^6-methylguanine DNA adducts in S. typhimurium strain YG7100.

O^6-Methylguanine was analyzed by HPLC as described by Herron and Shank (22). Overnight culture of *S. typhimurium* strain YG7100 (70 ml) was mixed with 70 ml of MNNG (10 μg /ml) and 350 ml of 0.1 M sodium phosphate buffer (pH 7.4) in the presence or absence of 10 ml of hot water extract of caraway. Final concentrations of MNNG and caraway were 1.40 mg/ml and 5.7 mg of original weight of caraway seed/ml, respectively. After 60-min incubation at 37°C, 0.7 ml of the reaction mixture was mixed with 2 ml of top agar and poured onto a minima1 plate to measure MNNG-induced mutation. Bacterial cells were collected by centrifugation at 4,000×g for 10 min at 4°C and washed with saline. Cells were suspended in 6 ml of 0.15 M NaCl-0.1 M EDTA (pH 8.0) containing 2 mg of lysozyme per ml and incubated at 37°C for 20 min. After addition of 6 ml of 0.1 M Tris-1.0% SDS-0.1 M NaCl (pH 9.0), DNA was extracted with phenol as described previously (23). DNA was then hydrolyzed in 50 μl of HCl at 70°C for 30 min. Hydrolysates were centrifuged at 13,000 x g for 10 min at 4°C and analyzed by HPLC with using a strong cation-exchange column, Chemcosorb 7-SCX(6A)(4.6×250 mm, Chemco Scientific Co., Ltd., Osaka, Japan), and 0.05 M diammonium phosphate buffer (pH 3.0) at a flow rate of 1.0 m1/ min. Elution of fluorescent bases was monitored using a 286-nm excitation wavelength with a 366-nm emission interference filter. Standard solutions of methylated bases or normal bases were prepared in 0.01 N HCl and analyzed under the same conditions.

Measurement of total acid soluble-thiols

The amounts of acid-soluble thiols in cells were determined according to the method of Lawley and Thatcher (24). Overnight cultures of *S. typhimurium* strains (0.7 ml) were incubated with 0.1 M sodium phosphate buffer (pH 7.4, 4.3 m1) and caraway S-100 (99 μl ; final concentration, 5.7 mg/ml) or distilled water for 20 or 60 min at 37°C. Cells were harvested by centrifugation at 3,900×g for 10 min at 4°C, washed twice with saline, and resuspended in 0.5 ml of ice-cold 5% trichloroacetic acid. After centrifugation at 13,000×g for 10 min at 4°C, 0.2 ml of the clear supernatant was mixed with 1.4 ml of a solution containing 200 μg of 5, 5'-dithiobis (2-nitrobenzoic Acid) per ml, and absorbance at 410 nm was read immediately. Concentration of thiols was determined spectrophotometrically by the value of e max at 410 nm, 1.36×10^4.

RESULTS

Mutagenicity of MNNG for various strains of *S. typhimurium* is shown in Fig. 1. The number of MNNG-induced revertants increased dose-dependently in ogt^- strains and MNNG was highly mutagenic in O^6-methylguanine-DNA methyltransferase-deficient strains, especially in YG7108 ($ada^- ogt^-$) and YG7104 ($ada^+ ogt^-$). In ogt^+ strains YG7100 ($ada^- ogt^+$), TA100 ($ada^+ ogt^+$) and TA1535 ($ada^+ ogt^+$), MNNG-induced mutation was not observed below a dose of 0.3 mg/ plate.

Inhibitory effects of hot water extract of caraway

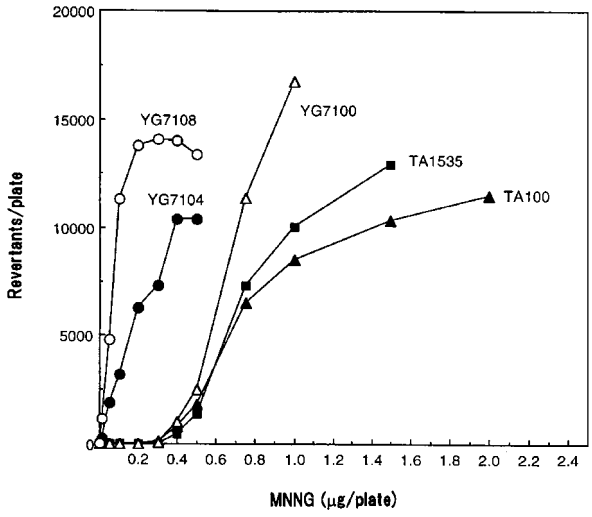

Fig. 1. Mutagenicity of MNNG for various strains.

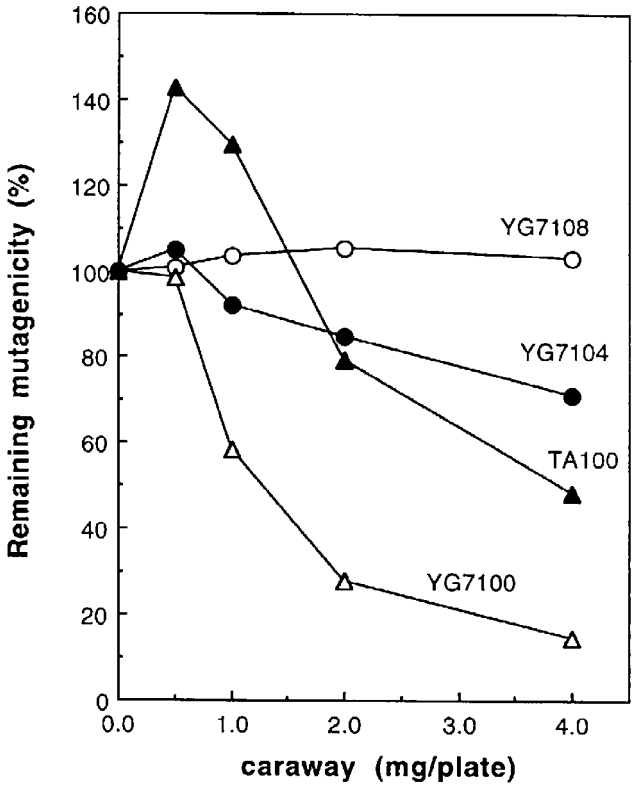

Fig. 2. Effect of caraway extract on the mutagenicity of MNNG for various strains. One hundred percent is the number of His+ revertants in the absence of caraway extract : 1,611 revertants/plate in YG7108 ; 1,144 in YG7104 ; 2,812 in YG7100 ; 2,223 in TA100 ; and 332 in TA1535. Doses of MNNG were 0.02 μg/plate in YG7108, 0.05 μg/plate in YG7104 and 0.5 μg/plate in the other strains.

on the mutagenicity of MNNG are shown in Fig. 2 and Table l. The dose of MNNG for each strain was determined from the dose-response data shown in Fig. 1. Caraway extract dose-dependently decreased the number of MNNG-induced revertants in strains TA100 and YG7100 but did not in the *ogt*$^-$ strains YG7104 and YG7108, even when the same ratio of caraway extract to MNNG was used (Table 1). In strain TA1535, the number of revertants increased to 4.9-fold at 0.5 mg of original weight of caraway/plate and decreased to the same level as that of the control

Table 1. Effects of caraway extract on the mutagenicity of MNNG for various strains.

Strain	MNNG (μg/plate)	caraway (mg/plate)	Ratio of conc. of caraway and MNNG (×10^3)	Revertants/plate	Survivor[a] (%)
YG 7108	0.02	0	—	1672	100
(*ada*$^-$*ogt*$^-$)		2	100	1771	70.8
YG 7104	0.05	0	—	1489	100
(*ada*$^+$*ogt*$^-$)		2	40	1820	100.5
		5	100	2106	89.7
YG 7100	0.5	0	—	3039	100
(*ada*$^-$*ogt*$^+$)		2	4	974	92.7
		50	100	348	101.7
TA 100	0.5	0	—	2880	100
(*ada*$^+$*ogt*$^+$)		2	4	1100	102.4
		50	100	1690	109.9
TA 1535	0.5	0	—	388	100
(*ada*$^+$*ogt*$^+$)		0.5	1	1904	96.2
		1	2	1112	99.8
		2	4	822	95.5
		4	8	342	87.4
		50	100	466	84.9

[a] Survivor was assayed on the nutrient broth agar after diluting a portion of the preincubation solution in the Ames test.

(no caraway) at 4 mg/plate. Caraway extract was not toxic below 50 mg/plate because survivors of tester strains were over 70% after treatment with caraway (Table l). Caraway had no effect on the growth of *S. typhimurium* strains in Nutrient broth at 5.7 mg/ml (data not shown).

The number of MNNG-induced revertants decreased only in the case of co-treatment of bacterial cells with MNNG and caraway (Table 2). The number of revertants did not decrease in post-treatment with caraway. Pretreatment of cells with the extract increased the number of MNNG-induced revertants.

O^6-Methylguanine in DNA of *S. typhimurium* strain YG7100 treated with MNNG in the presence or absence of caraway was quantified by HPLC analysis (Fig. 3 and Table 3). From 70 ml of overnight culture of strain YG7100, 664 μg (MNNG-treated), 752 μg (MNNG+caraway) and 1650 μg (non-treated, 90 ml of overnight culture) of DNA were yielded. After acid hydrolysis of each DNA sample (non-treated, 302 μg; MNNG-treated, 332 μg; MNNG+caraway, 376 μg), methylated bases were separated by HPLC and quantified from fluorescent peak areas (Fig. 3). The amount of *O*^{6}-methylguanine was 59.0 pmol/100 μg of DNA in MNNG-treated cells and decreased to 17.4 pmol/100 μg (29.5%) in the presence of caraway. The number of revertants was also decreased to 41.4% by the addition of 5.7 mg/ml of caraway extract in consistent with the decrease of *O*^{6}-methylguanine.

Concentrations of acid-soluble thiols in *S. typhimurium* YG7100 cells were determined after incubation with

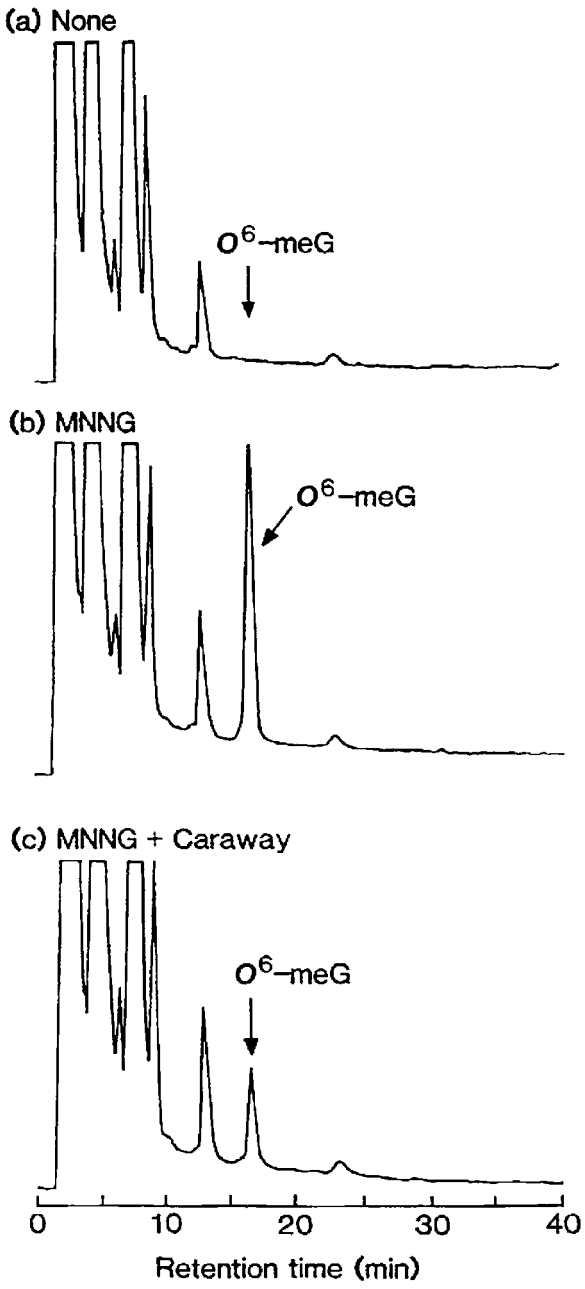

Fig. 3. Effect of caraway extract on *in vivo* formation of *O*^{6}-methylguanine in *Salmonella typhimurium* strain YG7100. Overnight culture of strain YG7100 was incubated with MNNG in the absence or presence of hot water extract of caraway. Purified DNA was hydrolyzed and analyzed by HPLC as described in Materials and Methods. Standards were eluted as follows : thymine, 2.9 min ; guanine, 4.0 min ; cytosine, 6.4 min ; adenine, 7.2 min ; 7-methylguanine, 7.6 min; *O*^{6}-methylguanine, 6.8 min; 3-methyladenine, 19.5 min.

Table 2. Effects of pre-, co-, and post-treatments of *Salmonella typhimurium* strain YG7100 cells with caraway extract on MNNG-induced mutagenesis in strain YG7100.

Treatment		Caraway (mg/plate)	Revertants/plate
Mutagenicity test (normal condition)	YG7100+MNNG +Caraway 7℃, 20 min	0	3730
		2	914
Pre-treatment	YG7100+Caraway, 37℃, 20 min →wash→YG 7100+MNNG, 37℃, 20 min	0	718
		2	1630
Co-treatment	YG7100+MNNG+ Caraway, 37℃, 20 min →wash→YG7100, 37℃, 20 min	0	7140
		2	526
Post-treatment	YG7100 + MNNG , 37℃, 20 min →wash→YG7100+ Caraway, 37℃,20 min	0	6190
		2	6920

Table 3. Effects of caraway extract on MNNG-induced mutagenicity and *in vivo* formation of *O*^{6}-methylguanine in *Salmonella typhimurium* strain YG7100.

MNNG (μg/plate)	Caraway (mg/plate)	Revertants/plate	*O*^{6}-methylguanine (pmol/100 μg DNA)(%)	
0	0	0	0.0031	(0.005)
1.0	0	21100	59.0	(100)
1.0	4	8730	17.4	(29.5)

caraway extract at 37°C for 20 or 60 min. Caraway had no effect on thiol concentrations within this incubation period (Table 4).

Effects of caraway extract on the mutagenicity of other kinds of alkylating agents are shown in Table 5. Caraway dose-dependently decreased the number of revertants induced by dimethylnitrosamine and methylazoxymethanol acetate (MAM acetate) in ogt^+ strains but had no effect in ogt^- strains. However, the number of revertants induced by the other alkylating agents, ENNG, MNU, ENU, MMS and EMS, was not inhibited or was rather enhanced by the extract.

Table 4. Acid-soluble thiols in *Salmonella typhimurium* cells with or without incubation with caraway extract.

	Amount of acid-soluble thiols (μmol/10^{12} cells)			
	Water		Caraway	
Strain	20 min	60 min	20 min	60 min
YG7108	2.0	2.4	2.7	2.9
YG7104	3.2	2.9	3.2	3.2
YG7100	1.9	2.4	2.6	2.6
TA100	3.3	3.9	4.1	4.3
TA1535	2.6	3.2	3.1	3.6

D-Carvone, a main constituent in caraway seed oil, also decreased the number of revertants induced by MNNG in strain YG7100 but not in strain YG7108 as well as the hot water extract of caraway seeds (Fig. 4a). D-Carvone did not decrease the number of ENNG- and MNU-induced revertants in either strain YG7108 or YG7100 (Fig. 4 b and c). To determine whether the antimutagenicity of the hot water extract of caraway is derived from D-carvone or not, the extract was further fractionated into diethylether-soluble and aqueous fractions. The ether-soluble fraction did not show antimutagenicity even at 40 mg of original weight/plate, while the antimutagenic activity remained in the aqueous fraction (Table 6). When caraway seeds were extracted sequentially with *n*-hexan, methanol and boiling water, hexan extract most strongly decreased the number of MNNG-induced revertants in YG7100 (Fig. 5a), but the inhibition was not observed in strain YG7108 (Fig. 5b). However, the last hot water extract still showed ogt^+- dependent antimutagenicity for MNNG (Fig. 5 a and c).

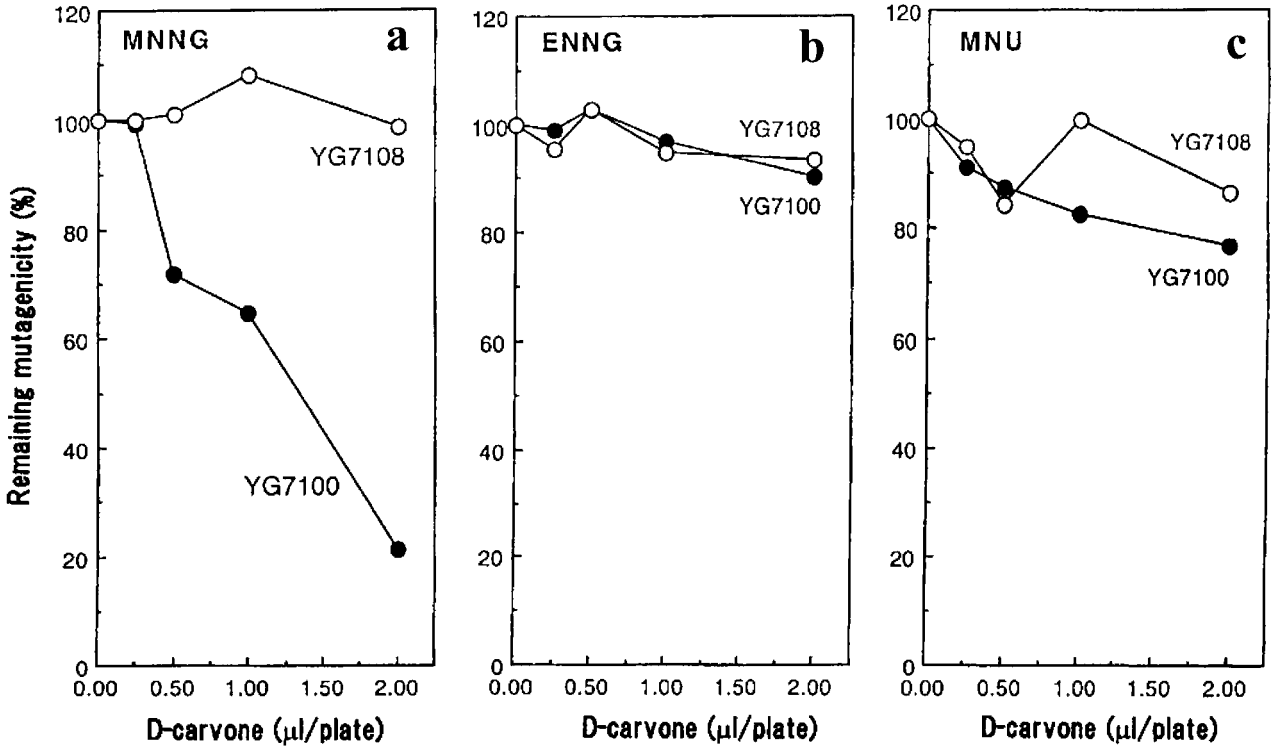

Fig. 4. Effects of D-carvone on the mutagenicity of (a) MNNG, (b) ENNG and (c) MNU. One hundred percent of remaining mutagenicity corresponded to 1,119 revertants/plate in YG7100 at 0.6 μg MNNG/plate, 1,909 in YG7108 at 0.02 μg MNNG/plate, 196 in YG7100 at 2.5 μg ENNG/plate, 1,347 in YG7108 at 0.025 μg ENNG/plate, 10,869 in YG7100 at 50 μg MNU/plate, and 5,169 in YG 7108 at 5 μg MNU/plate.

Table 5. Effects of caraway extract on the mutagenicity of alkylating agents.

Mutagen	Strain	Dose of mutagen	Mutagenicity (revertants/plate) Caraway (mg/plate) 0	0.5	1.0	2.0	4.0
		μg/plate					
N-methyl-	YG7108	5	2852	3953	3863	3424	4202
N-nitrosourea	YG7104	10	1873	2232	2311	2050	2585
	YG7100	50	547	2707	2160	1874	2293
	TA100	40	87	212	232	233	252
	TA1535	40	90	174	232	165	188
		μg/plate					
N-ethyl-	YG7108	5	579	768	670	680	704
N-nitrosourea	YG7104	10	635	680	743	634	639
	YG7100	300	162	349	374	339	349
	TA100	300	447	1967	2289	1391	1846
	TA1535	300	383	869	1047	1029	980
		μg/plate					
Methyl-	YG7108	0.03	1871	1883	1931	2011	1737
methane-	YG7104	0.25	1439	1275	1409	1384	1372
sulfonate	YG7100	1.0	50	52	49	56	31
	TA100	1.0	1358	1589	1535	1568	1571
	TA1535	2.0	76	107	113	107	99
		μg/plate					
Ethyl-	YG7108	0.05	2953	2868	2710	2630	2637
methane-	YG7104	0.05	2509	3147	2983	3123	2661
sulfonate	YG7100	7.0	2082	1967	1693	1578	1565
	TA100	5.0	735	820	913	887	814
	TA1535	5.0	880	818	850	830	964
		μg/plate					
N-Ethyl-*N'*-	YG7108	0.025	934	2330	2214	2232	2457
nitro-*N*-nitroso	YG7104	0.025	1070	2165	2299	1922	2463
guanidine	YG7100	2.5	107	161	204	173	207
	TA100	2.5	165	417	443	516	530
	TA1535	2.5	78	112	122	129	112
		μg/plate					
Methylazoxy-	YG7108	0.1	1311	1957	2460	2187	2266
methanol	YG7104	0.5	2199	1882	1573	1427	1463
acetate	YG7100	4.0	640	181	133	126	82
	TA100	4.0	787	359	287	204	154
	TA1535	4.0	850	177	160	128	128
	G46	3.0	1012	297	281	208	157
		μg/plate					
Dimethy-	YG7108	0.625	11871	10734	10643	8521	8004
nitrosamine*	YG7104	1.25	8716	8472	8442	7177	7050
	YG7100	100	3295	3007	2528	1447	732
	TA100	100	5179	5653	4826	4516	3203
	TA1535	100	6671	6574	6252	5048	2920

*Mutagenicity of dimethylnitrosamine was determined by preincubation in the presence of metabolic activation system which consisted of 9000×g supernatant of rat liver homogenate, $NADP^+$, glucose 6-phosphate and 0.1 M sodium phosphate buffer (pH 7.4). Dimethylnitrosamine dissolved in 0.1 ml of DMSO was mixed with 0.5 ml of metabolic activation sysytem and 0.1 ml of bacterial culture, and then incubated at 37℃ for 45 min.

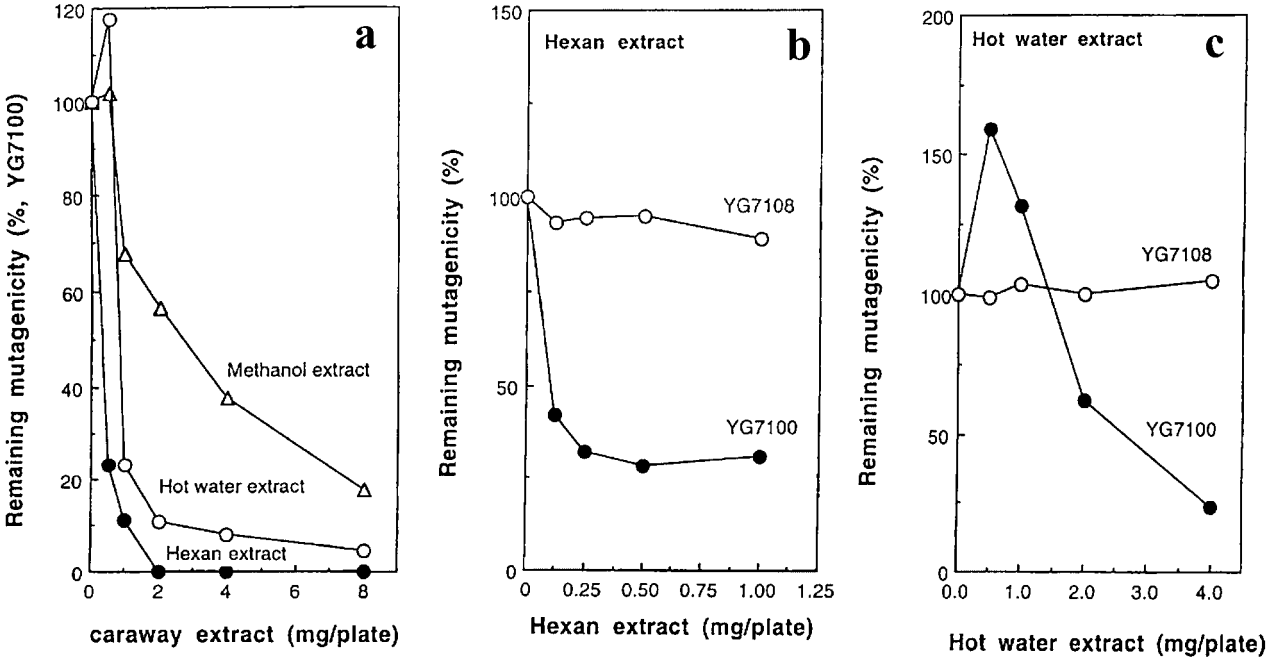

Fig. 5. Effects of caraway fractions prepared by sequential extraction with *n*-hexan, methanol and hot water on MNNG-induced mutagenesis. (a) Inhibition of MNNG-induced mutagenesis by each extract. (b) and (c) Strain difference in inhibition of MNNG by hexan or hot water extract. MNNG induced 2,822 revertants/plate at 0.6 μg/plate in YG7100 and 2,203 revertants/plate at 0.02 μg/plate in YG7108 in the absence of caraway extracts. These values correspond to 100% of remaining mutagenicity in each strain.

Table 6. Inhibition of MNNG-induced mutagenicity by fractions of caraway extract fractionated with diethylether.

Fraction	Dose (mg/plate)	Revertants/plate	Remaining mutagenicity(%)
None	—	8613	100
Caraway extract	4	1349	15.7
Ether-soluble neutral fraction	4	8538	99.1
	40	7980	92.7
Ether-soluble basic fraction	4	8277	96.1
	40	8210	95.3
Ether-soluble acidic fraction	4	8288	96.1
	40	6882	79.9
Remained aqueous fraction	4	5517	64.1
	40	5058	58.7

Hot water extract of caraway was extracted and fractionated into ether-soluble acidic, neutral, and basic fractions as described in Materials and Methods. Inhibitory effect of these fractions on the mutagenicity of MNNG (0.5 μg/plate) was examined in *S. typhimurium* strain YG7100. Dose of each fraction corresponded to 4 or 40 mg of caraway seed.

DISCUSSION

Many kinds of plant components have been demonstrated to be antimutagenic and antitumorigenic (8-10, 11, 12, 18, 25) and epidemiological studies suggested that consumption of spices is related to lowered risk of gastric cancer (13). Caraway is a fresh smelling spice, and we previously reported that hot water extract of caraway seeds inhibited the mutagenicity of MNNG (18). In addition, D-Carvone, a main component of caraway oil, has been shown to inhibit the development of diethylnitrosamine-induced stomach and pulmonary tumors (11, 26, 27). MNNG is a typical methylating agent and causes tumors at administered sites of animals (1, 2). O^6-Methylguanine in methylated DNA can mispair with thymine during DNA replication. Since methylation of DNA by endogenous metabolites *in vivo* has been demonstrated (6) and the difference in repair efficiency has been shown to correlate with organ specificity of MNU-induced DNA adducts and tumors (7), it is important to clarify the mechanism of inhibition of methylation by chemopreventive agents. In this study, to elucidate the mechanism of antimutagenicity of caraway, we examined the effects of caraway extract on MNNG-induced mutation, DNA methylation and thiol content in bacterial cells using O^6-methylguanine-DNA methyltransferase-deficient strains of *S. typhimurium*.

MNNG was highly mutagenic for *ogt*$^-$ strains YG 7104 and YG7108, and it showed slightly higher mutagenicity in strain YG7100 than in strains TA 100 and TA 1535 as described by Yamada *et al*. (19). Hot water extract of caraway seeds inhibited MNNG-

induced mutation in *ogt*$^+$ strains but not in *ogt*$^-$ strains. O^6-Methylguanine DNA adducts in *S. typhimurium* YG7100 cells were decreased in the presence of caraway extract accompanied with the decrease in MNNG-induced mutagenesis. These results suggest the importance of O^6-methylation in mutagenicity of MNNG, and that O^6-methylguanine-DNA methyltransferase may be involved in the antimutagenic activity of caraway. Possible mechanisms of antimutagenicity of natural products in the initiation step in mutagenesis are 1) direct inactivation of mutagens, 2) inhibition of metabolic or chemical activation of mutagens, 3) modulation of the hepatic detoxication system, 4) protection of DNA from ultimate mutagens, and 5) modification of DNA replication and/or DNA repair. Strain difference in the inhibitory effect of caraway suggests that the extract may modify DNA repair. More than 75% of MNNG remained after incubation with caraway in a phosphate-buffer solution (pH 7.4) (data not shown), indicating that the caraway component might not degrade MNNG directly.

Teel *et al*. (17) reported that one of the mechanisms by which ellagic acid inhibits mutagenesis and carcinogenesis is by forming adducts with DNA, thus masking binding sites to be occupied by the mutagen or carcinogen. However, in the case of caraway, masking of DNA may not occur because its antimutagenicity appeared only in *ogt*$^+$ strains.

Nitrosoguanidines are easily degraded to produce active methyl cation in the presence of thiols, and the mutagenicity is highly dependent on where their reaction with thiols takes place (30). A mutant of *S. typhimurium* strain TA1535 with a decreased level of glutathione was reported to exhibit increased resistance to these alkylnitrosoguanidines (31). However, caraway had no effect on the cellular concentrations of acid-soluble thiols. Moreover, the extract did not inhibit the mutagenicity of ENNG, and pre-treatment of bacterial cells with caraway also had no inhibitory effect on the mutagenicity of MNNG. These results indicate that the action of caraway is not correlated with thiol-dependent activation of MNNG.

Caraway inhibited the mutagenicity of MNNG only in the case of co-treatment. Methylating agents such as MNNG and MNU produce a greater concentration of mutations near replication forks in *E. coli* than in non-replicating regions of the genome, probably due to the weak action of Ada methyltransferase protein on single-stranded DNA containing O^6-alkylguanine moieties (4, 28). Thus, O^6-alkylguanine and O^4-alkylthymine present in parental DNA strands at replication forks may be refractory to repair until replication restores the duplex structure by misincorporation in the daughter strand opposite the alkylated nucleotides (4, 29). Caraway components might help Ogt methyltransferase to act more easily on single-stranded DNA before methylated sites are replicated.

However, caraway was not effective in post-treatment and did not inhibit the mutagenicity of other methylating agents such as MNU and MMS, suggesting that the components of this spice might not simply enhance the Ogt methyltransferase activity. Although MNU produces methyldiazonium ion as an ultimate form to bind DNA as well as MNNG, its mutagenicity was not affected by caraway,suggesting that caraway might not trap this activated form to inhibit mutagenesis. Since SOS-dependent mutagens such as MMS and ENU were not inhibited by the extract, caraway may not modify error-prone SOS repair.

In Table 2, in the absence of caraway extract, co- and post-treatment increased the number of MNNG-induced revertants compared with the result of standard mutagenicity test, while very low number of revertants were observed in pre-treatment. These results can be explained as follows : in co- and post-treatment, MNNG-treated cells were further incubated at 37℃ for 20 min, resulting in efficient mispair of O^6-methylguanine, but incubation of *S. typhimurium* cells in sodium phosphate buffer before MNNG treatment may probably reduce DNA replication, resulting in decrease of methylation of DNA and mispair of O^6-methylguanine.

D-Carvone is a main constituent in caraway seed oil (about 50%) and was found to inhibit the development of forestomach tumors induced by diethylnitrosamine in A/J mice (26, 27). To determine the contribution of D-carvone to inhibition of MNNG mutagenesis by the hot water extract, we examined the effect of D-carvone on MNNG-induced mutation. D-Carvone was antimutagenic for MNNG in YG 7100 (*ada*$^-$ *ogt*$^+$) but not in YG7108 (*ada*$^-$ *ogt*$^+$) and did not affect ENNG and MNU, as well as hot water extract. However, D-carvone is practically insoluble in water, and the antimutagenic activity of hot water extract remained in the water fraction after extraction with diethyl ether. The *ogt*$^+$- dependent antimutagenicity remained in the hot water extract of caraway seeds after sequential extraction with *n*-hexan and methanol. Therefore, the antimutagenic component in the hot water extract may be water-soluble derivative(s) of D-carvone.

Hot water extract of caraway sometimes increased the number of MNNG-induced revertants especially

at lower dose. Enhancing effect was also observed in pre-treatment of YG7100 cells with hot water extract of caraway. When caraway seeds were sequentially extracted with *n*-hexan, methanol and boiling water, enhancing effect was observed only in the hot water extract. These results suggest the presence of water-soluble component(s) which enhance the mutagenicity of MNNG.

How caraway components interact with Ogt methyltransferase and decreases O^6-methylguanine DNA adducts is still unclear. We have been purifying the active component from hot water extract of caraway to study the inhibitory mechanism.

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