Table 2. Mutagenicity of 11 chemicals in *S. typhimurium* strains with and without plasmid carrying the *dinB* gene encoding *E. coli* DNA pol IV in the presence and the absence of the *nfsB* and *oat* genes

Chemicals	Group	S9	TA1538	YG5161 +DNA pol IV	YG7158 ΔnfsBΔoat	YG5185 ΔnfsBΔoat +DNA pol IV	Dose
10-AzaB[a]P	A	+	2 (1.0)	21 (10.5)	2 (1.0)	28 (14.0)	25 μg/plate
B[a]P	A	+	8 (1.0)	103 (12.9)	9 (1.1)	126 (15.8)	10 μg/plate
3-MC	A	+	8 (1.0)	51 (6.4)	5 (0.6)	48 (6.0)	10 μg/plate
2-AA	A	+	531 (1.0)	1,111 (2.1)	432 (0.8)	1,680 (3.2)	2 μg/plate
DMBA	A	+	5 (1.0)	15 (3.0)	4 (0.8)	9 (1.8)	5 μg/plate
ENNG	A	−	2 (1.0)	11 (5.5)	2 (1.0)	12 (6.0)	10 μg/plate
1-AA	B	+	18 (1.0)	53 (2.9)	5 (0.3)	14 (0.8)	10 μg/plate
1-NB[a]P	B	−	192 (1.0)	2,042 (10.6)	72 (0.4)	326 (1.7)	0.5 μg/plate
1-NP	C	−	145 (1.0)	230 (1.6)	23 (0.2)	29 (0.2)	1 μg/plate
1,8-DNP	C	−	130,560 (1.0)	218,040 (1.7)	2,040 (0.02)	3,120 (0.02)	25 ng/plate
Glu-P-1	C	+	41,240 (1.0)	45,550 (1.1)	1,710 (0.04)	1,670 (0.04)	100 ng/plate

Each chemical was assayed with 4–7 doses on triplicate plates with four strains in parallel. The numbers of His^+ revertants per plate per μg are calculated at the doses indicated with arrows in Fig. 2. The numbers in parenthesis represent the values relative to the numbers of His^+ revertants per μg in TA1538. Differences of relative mutability more than two fold or less than half were regarded as significant effects of the introduction of plasmid pYG768 carrying *dinB* encoding DNA pol IV or the deletion of the *oat* and *nfsB* genes on the mutability. Group A: chemicals whose mutagenicity was enhanced by DNA pol IV but was not reduced by *ΔnfsΔoat*; Group B: chemicals whose mutagenicity was enhanced by DNA pol IV and was reduced by *ΔnfsΔoat*; Group C: chemicals whose mutagenicity was not enhanced by DNA pol IV but was reduced by *ΔnfsΔoat*.

DNA surrounding the *nfsB* gene was amplified from the colonies by PCR using primers flanking the *nfsB* gene, and the products were analyzed by agarose gel electrophoresis (Fig. 1B). If the chromosomal *nfsB* gene is correctly replaced with the introduced DNA fragments carrying the Km^r gene by recombination using the flanking homologous sequences, the Km^r colonies will exhibit single DNA bands of 1.8 kb. If no such true replacement occurs and the introduced DNA fragments are only integrated into the vicinity of the chromosomal *nfsB* gene, they will exhibit double bands of 2.4 kb (the intact *nfsB* gene) and 1.8 kb (the Km^r gene). As expected, some Km^r colonies exhibited single 1.8-kb bands while others exhibited double bands of 2.4 kb and 1.8 kb. We suggested that the Km^r colonies exhibited single 1.8-kb bands were deficient in the *nfsB* gene as well as the *oat* genes, and named the resulting strain YG7158. Plasmid pYG768 carrying the *dinB* gene encoding *E. coli* DNA polymerase IV was introduced and the resulting strain was referred to as YG5185, which was used for the subsequent mutation assays.

Specificity and sensitivity of strain YG5185 to genotoxic PAHs: We compared the sensitivity of strain YG5185 to 11 mutagens with those of three reference strains: the parent strain, i.e., TA1538, the derivative of strain TA1538 harboring plasmid pYG768, i.e., YG5161, and the *ΔnfsBΔoat* derivative of strain TA1538, i.e., YG7158 (Table 2). Introduction of plasmid pYG768 did not affect the spontaneous mutation frequencies in strains TA1538 and YG7158. Based on the order of sensitivity of the strains, we classified the chemicals into Group A to C as follows. For Group A compounds, i.e., 10-AzaB[a]P, B[a]P, 3-MC, 2-AA, DMBA and ENNG, deletion of the *nfsB* and *oat* genes did not reduce the sensitivity, and introduction of plasmid pYG768 substantially enhanced it (Fig. 2A). Thus, YG5185 exhibited similar or comparable sensitivity to Group A compounds as did strain YG5161, and the order of the sensitivity was YG5185=YG5161>TA1538=YG7158. For Group B

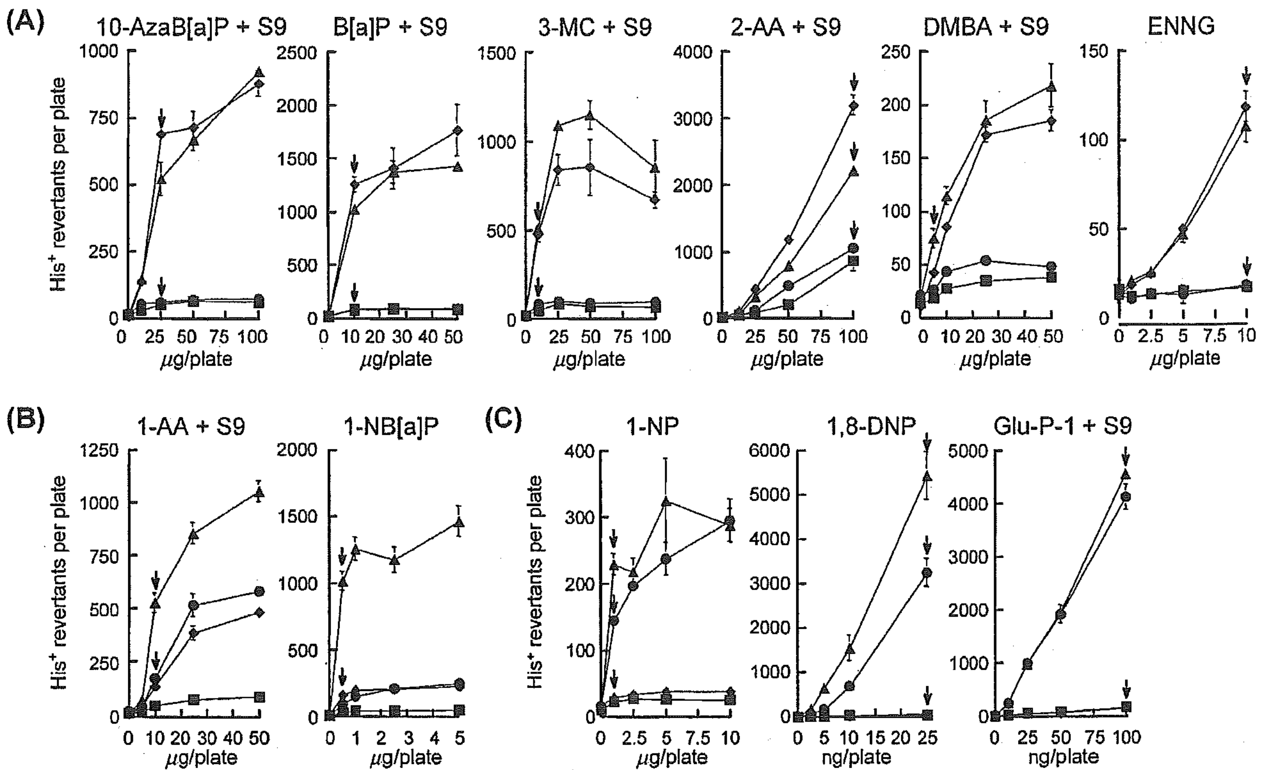

Fig. 2. Mutagenic responses of *S. typhimurium* newly constructed strains in the Ames test. The test chemicals are indicated on each panel. The chemicals are categorized into three Groups A, B and C, which are mentioned in the text and Table 2. Symbols are: ●, TA1538; ▲, YG5161; ■, YG7158; ◆, YG5185. The arrow indicates the dose that was used for the calculation of His$^+$ revertants per μg per plate in Table 2.

compounds, i.e., 1-AA and 1-NB[a]P, deletion of the *nfsB* and *oat* genes substantially reduced the sensitivity, and introduction of plasmid pYG768 enhanced it (Fig. 2B). Thus, the order of the sensitivity was YG5161 > TA1538 > YG5185 > YG7158. For Group C compounds, i.e., 1-NP, 1,8-DNP and Glu-P-1, deletion of the *nfsB* and *oat* genes severely reduced the sensitivity, and introduction of plasmid pYG768 did not substantially enhance it (Fig. 2C). The order of the sensitivity was YG5161 = TA1538 > YG5185 = YG7158.

Discussion

Genetically engineered Ames tester strains have been proven to be useful in environmental genotoxicology due to their extreme sensitivity and the mechanistic information they can provide (17). For example, *S. typhimurium* strain YG1021, 1024, 1026 and 1029 have been widely used for environmental research for their hypersensitivity to the genotoxic action of nitroarenes and aromatic amines (21). The strains harbor plasmids carrying *nfsB* or *oat*, conferring high enzymatic activities of nitroreductase or *O*-acetyltransferase in strain TA98 or TA100 (15,16,22,23). Strains YG7104 and YG7108, whose repair systems for the damage by alkylating agents are disrupted, exhibit hyper sensitivity to alkylating agents, such as methyl methanesulfonate and dimethylnitrosamine (24,25), and they are used for mechanistic analyses for chemical mutagenesis and carcinogenesis (26–28). Strain YG3001, YG3002 and YG3003 are highly sensitive to oxidative mutagens due to the lack of the *mutM* gene encoding 8-hydroxyguanine DNA glycosylase (29), and are shown to be useful for the studies on oxidative DNA damage (30,31).

To expand this line of research, we have previously established *S. typhimurium* strain YG5161 by introduction of plasmid carrying *dinB* encoding *E. coli* DNA polymerase IV into strain TA1538 to increase the sensitivity to genotoxic PAHs (10). Because of the error-prone nature of DNA polymerase IV, strain YG5161 exhibited higher sensitivity to PAHs such as B[a]P, 10-AzaB[a]P and 3-NB[a]P than did standard Ames strain TA1538 or TA98. Nevertheless, strain YG5161 has a potential defect as a bio-detector of genotoxic PAHs in complex mixtures, which is the cross sensitivity to other classes of genotoxic compounds, i.e., nitroaromatics and aromatic amines. Since these compounds are ubiquitously present in the environment and their genotoxicity in *S. typhimurium* is extremely amplified by the presence of intracellular metabolic activation enzymes, i.e., nitroreductase and *O*-acetyltran-

sferase, they can veil the potential genotoxicity of PAHs in the complex mixtures (17). For example, 1,8-DNP could be dominantly detected as a principle genotoxic compound in complex mixtures if 1,8-DNP and B[a]P were present at a weight ratio of 1:1,000. This is because the genotoxicity of 1,8-DNP, i.e., the numbers of His^+ revertants per plate per μg, is more than 15,000 times higher than B[a]P in strain TA1538 (Table 2). Despite the potent genotoxicity in *S. typhimurium*, 1,8-DNP is categorized into Group 2B (possible human carcinogens) by International Agency for Research on Cancer (IARC) while B[a]P is classified into Group 2A (probable human carcinogens) (32). Hence, we found it important to increase the specificity of tester strains to genotoxic PAHs.

Here, we disrupted the *nfsB* and *oat* genes of strain TA1538, introduced plasmid pYG768 carrying *dinB* into the *ΔnfsBΔoat* strain and established novel *S. typhimurium* strain YG5185 (Table 1). When compared the sensitivity of strain YG5185 and YG5161, they exhibited comparative sensitivity to Group A compounds where four out of six compounds, i.e., 10-azaB[a]P, B[a]P, 3-MC and DMBA, were PAHs (Table 2, Fig. 2A). Thus, strain YG5185 appears to be able to detect the genotoxic PAHs with similar high sensitivity as does strain YG5161. The remaining two compounds in Group A are 2-AA and ENNG. It cautions that the compounds that are more sensitively detected by strain YG5161 or YG5185 compared with strain TA1538 are not necessarily PAHs. They can be aromatic amines or alkylating agents.

For Group B compounds, i.e., 1-AA and 1-NB[a]P, the genotoxicity was significantly reduced by the deletion of *nfsB* and *oat*, as in the case of Group C compounds (Fig. 2B and C). Unlike Group C compounds, however, the genotoxicity of 1-AA and 1-NB[a]P was three to 10 times enhanced by the introduction of plasmid carrying *dinB* (Fig. 2B). Actually, the genotoxicity of two compounds was more sensitively detected with strain YG5161 compared with standard strain TA98 (10). Thus, it seems that DNA lesions induced by 1-AA and 1-NB[a]P are more efficiently bypassed by DNA polymerase IV in an error-prone manner than by DNA polymerase RI encoded by *mucAB* carried by plasmid pKM101 in strain TA98. Interestingly, the genotoxicity of 1-AA was reduced by more than 70% by the deletion of *nfsB* and *oat* while the genotoxicity of 2-AA was not (Table 2, Fig. 2A and B). These results suggest that the intracellular metabolic activation mechanisms are markedly different between two aromatic amino compounds despite the structural similarity.

In Group C compounds, the deletion of *nfsB* and *oat* reduced the genotoxicity of 1-NP by more than 85% and those of 1,8-DNP and Glu-P-1 by more than 95% (Table 2). Strain YG5185 exhibited much lower sensitivity to the genotoxicity of nitroaromatics and aromatic amine than did strain YG5161 (Fig. 2C). Thus, we concluded that strain YG5185 more specifically detects the genotoxicity of PAHs than does strain YG5161. Strain YG5185 could help and facilitate the successful isolation of genotoxic PAHs in complex mixtures. We have to point out, however, that the genotoxicity of 1,8-DNP and Glu-P-1 in strain YG5185 is still more than 25 times and 10 times higher than that of B[a]P. Hence, there is a possibility that genotoxic nitroaromatics or aromatic amines can be detected as principle mutagens if the complex mixtures are heavily contaminated with nitroaromatics or aromatic amines.

During the strain construction, we noticed that *S. typhimurium* TA1538/1,8-DNP could have mutations in the genes other than the *oat* gene. This is because introduction of plasmid pYG768 carrying *dinB* did not enhance the sensitivity of the strain to B[a]P while introduction of the same plasmid into YG7158 (*ΔnfsBΔoat* strain) or YG7129 (*Δoat* strain) enhanced it more than 10 times. It may not be surprising that strain TA1538/1,8-DNP has unexpected mutations because it was generated by random mutagenesis with 1,8-DNP (33). To avoid such confusion by extra mutations, we specifically disrupted the *oat* and *nfsB* genes by a targeting method, i.e., the pre-ligation method, which has been developed in this laboratory (20).

In summary, we established novel *S. typhimurium* strain YG5185 by introduction of plasmid carrying *dinB* encoding *E. coli* DNA polymerase IV into *ΔnfsBΔoat* derivative of standard Ames tester strain TA1538. The newly constructed strain exhibited higher sensitivity to the genotoxic compounds including PAHs but reduced sensitivity to nitroaromatics and aromatic amines. We propose that strain YG5185 is useful to detect the genotoxic PAHs in complex mixtures extracted from various polluted environmental sources.

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ORIGINAL

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

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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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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 1 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℃ 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℃, 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℃ 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℃ 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℃, 0.7 ml of the reaction mixture was mixed with 2 ml of top agar and poured onto a minimal plate to measure MNNG-induced mutation. Bacterial cells were collected by centrifugation at 4,000×g for 10 min at 4℃ 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℃ 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℃ for 30 min. Hydrolysates were centrifuged at 13,000 x g for 10 min at 4℃ 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 ml/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 ml) and caraway S-100 (99 µl ; final concentration, 5.7 mg/ml) or distilled water for 20 or 60 min at 37℃. Cells were harvested by centrifugation at 3,900×g for 10 min at 4℃, 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℃, 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

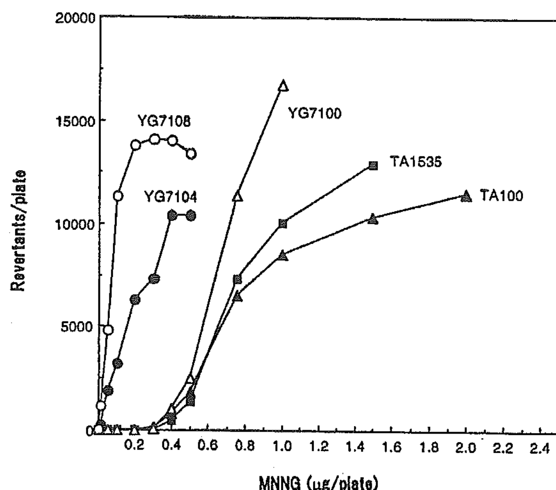

Fig. 1. Mutagenicity of MNNG for various strains.

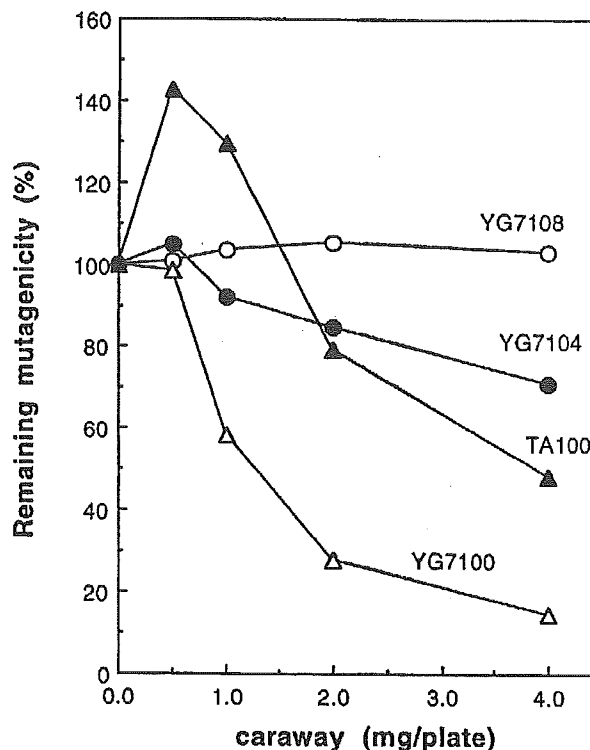

Fig. 2. Effect of caraway extract on the mutagenicity of MNNG for various strains. One hundred percent is the number of His^+ revertants in the absence of caraway extract: 1,611 revertants/plate in YG7108; 1,144 in YG7104; 2,812 in YG7100; 2,223 in TA100; and 332 in TA1535. Doses of MNNG were 0.02 μg/plate 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 1. 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 ($\times 10^3$)	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 1). 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

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

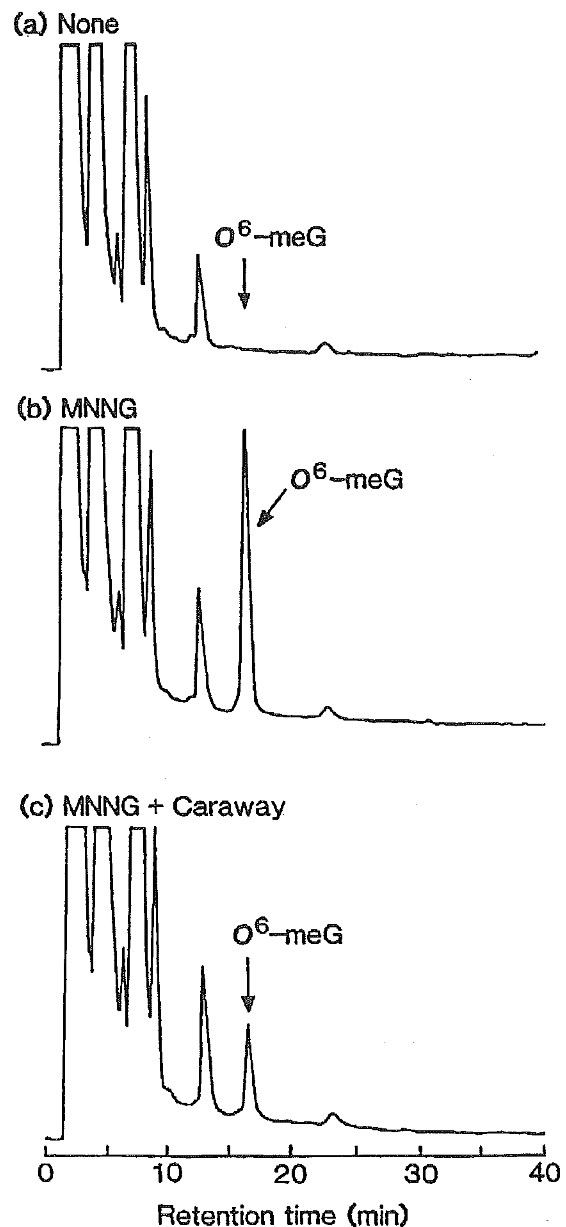

Fig. 3. Effect of caraway extract on *in vivo* formation of O^6-methylguanine in *Salmonella typhimurium* strain YG7100. Overnight culture of strain YG7100 was incubated with MNNG in the absence or presence of hot water extract of caraway. Purified DNA was hydrolyzed and analyzed by HPLC as described 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 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℃ 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).

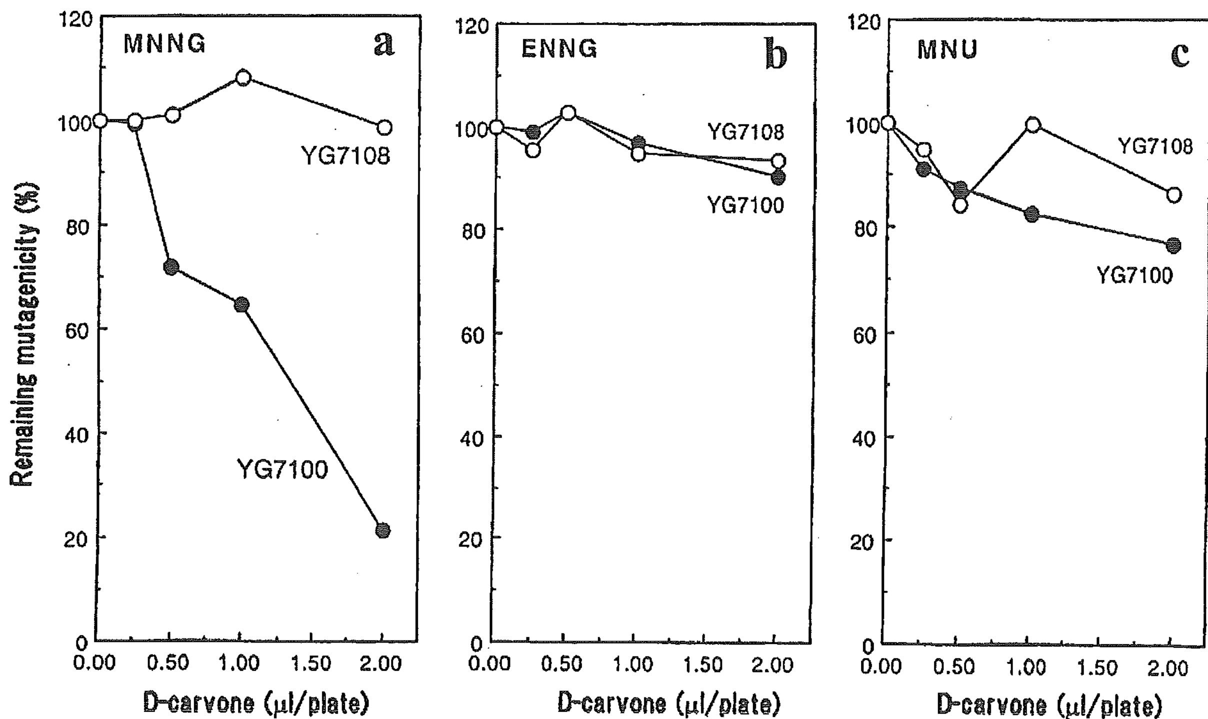

Fig. 4. Effects of D-carvone on the mutagenicity of (a) MNNG, (b) ENNG and (c) MNU. One hundred percent of remaining mutagenicity corresponded to 1,119 revertants/plate in YG7100 at 0.6 μg MNNG/plate, 1,909 in YG7108 at 0.02 μg MNNG/plate, 196 in YG7100 at 2.5 μg ENNG/plate, 1,347 in YG7108 at 0.025 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-*N*-nitrosourea	YG7108	5	2852	3953	3863	3424	4202
	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-*N*-nitrosourea	YG7108	5	579	768	670	680	704
	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-methane-sulfonate	YG7108	0.03	1871	1883	1931	2011	1737
	YG7104	0.25	1439	1275	1409	1384	1372
	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-methane-sulfonate	YG7108	0.05	2953	2868	2710	2630	2637
	YG7104	0.05	2509	3147	2983	3123	2661
	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*'-nitro-*N*-nitroso guanidine	YG7108	0.025	934	2330	2214	2232	2457
	YG7104	0.025	1070	2165	2299	1922	2463
	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-methanol acetate	YG7108	0.1	1311	1957	2460	2187	2266
	YG7104	0.5	2199	1882	1573	1427	1463
	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-nitrosamine*	YG7108	0.625	11871	10734	10643	8521	8004
	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.

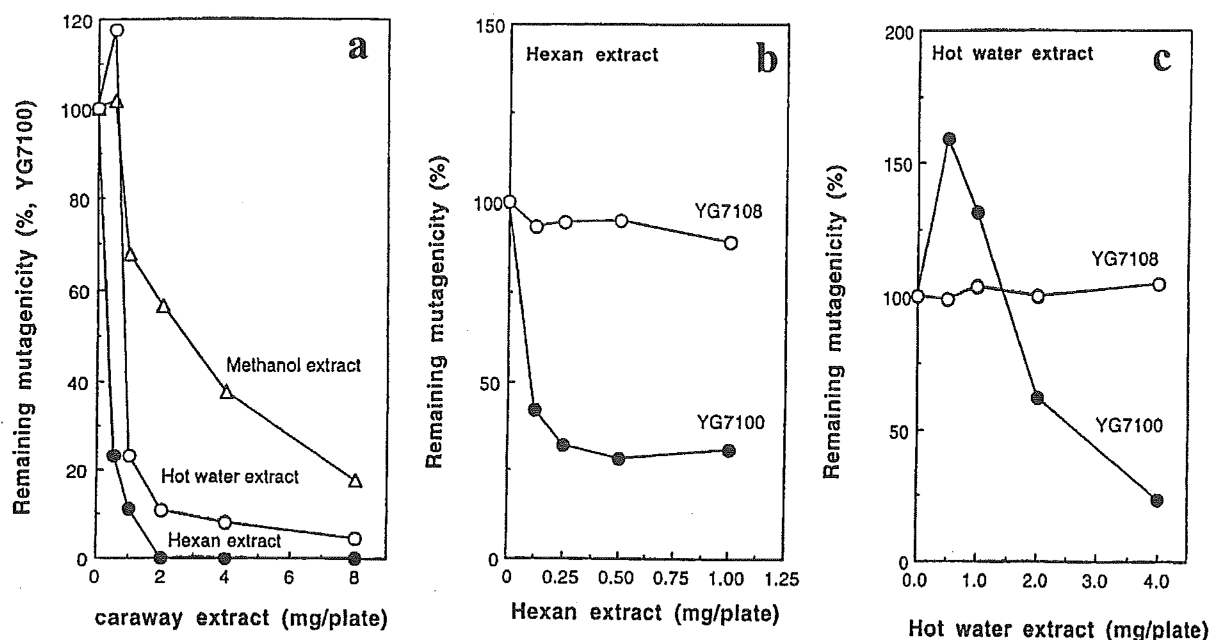

Fig. 5. Effects of caraway fractions prepared by sequential extraction with *n*-hexan, methanol and hot water on MNNG-induced mutagenesis. (a) Inhibition of MNNG-induced mutagenesis by each extract. (b) and (c) Strain difference in inhibition of MNNG by hexan or hot water extract. MNNG induced 2,822 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 pretreatment 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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Visualization of the interaction between archaeal DNA polymerase and uracil-containing DNA by atomic force microscopy

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Deamination of cytosine to uracil is a hydrolytic reaction that is greatly accelerated at high temperatures. The resulting uracil pairs with adenine during DNA replication, thereby inducing G:C to A:T transitions in the progeny. Interestingly, B-family DNA polymerases from hyperthermophilic Archaea recognize the presence of uracil in DNA and stall DNA synthesis. To better understand the recognition mechanism, the binding modes of DNA polymerase B1 of *Sulfolobus solfataricus* (Pol B1) to uracil-containing DNA were examined by gel mobility shift assays and atomic force microscopy. Although PolB1 per se specifically binds to uracil-containing single-stranded DNA, the binding efficiency was substantially enhanced by the initiation of DNA synthesis. Analysis by the atomic force microscopy showed a number of double-stranded DNA (dsDNA) in the products of DNA synthesis. The generation of ds DNA was significantly inhibited, however, by the presence of template uracil, and intermediates where monomeric forms of Pol B1 appeared to bind to uracil-containing DNA were observed. These results suggest that Pol B1 more efficiently recognizes uracil in DNA during DNA synthesis rather than during random diffusion in solution, and that single molecules of Pol B1 bind to template uracil and stall DNA synthesis.

Introduction

Deamination of exocyclic amino groups of DNA bases such as deamination of the amino group of cytosine poses a common genotoxic risk in all organisms (Lindahl 1993; Pearl 2000). The hydrolytic deamination of cytosine leads to the formation of uracil in DNA, and G:U base pairs result in G:C to A:T transitions in a half of the progeny if not repaired before replication. Since the rate of the hydrolytic reaction is greatly accelerated at high temperatures (Lindahl & Nyberg 1974), hyperthermophilic organisms, which live in habitats at more than 80 °C, are supposed to be exposed to massive DNA damages by the deamination. However, the spontaneous mutation rate in hyperthermophilic archaea *Sulfolobus acidocaldarius* is reported to be similar to that of *Escherichia coli* (Grogan *et al.* 2001). Thus, hyperthermophilic archaea appear to possess mechanisms to protect a stability of the genomic DNA from the mutagenic threat of deaminated bases generated at high temperatures.

Interestingly, B-family DNA polymerases from hyperthermophilic archaea such as *Sulfolobus solfataricus* DNA polymerase B1 (Pol B1) or *Pyrococcus furiosus* DNA polymerase (Pfu) recognize the presence of uracil in DNA and tightly bind to uracil-containing oligonucleotides (Lasken *et al.* 1996; Greagg *et al.* 1999). Pol B1 is likely to play an important role in DNA replication in *S. solfataricus* because the activity is highly stimulated by PCNA-like and RFC-like factors in *in vitro* (De Felice *et al.* 1999). Pol B1 and Pfu stall DNA polymerization

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three to four base pairs (bps) before template uracil. The recognition and stalling mechanisms by the polymerases appear to contribute to the genome integrity of hyperthermophilic archaea because they may prevent the misincorporation of adenine opposite the template uracil. More surprisingly, a recent study suggests that the enzymes also bind to the deamination product of adenine, i.e. hypoxanthine, in a template DNA strand and stall DNA synthesis upstream of the lesion (Gruz *et al.* 2003). Hypoxanthine can pair with cytosine, thereby inducing A:T to G:C transitions if not repaired (Lindahl 1993). Thus, the recognition mechanisms of deaminated bases by archaeal B family DNA polymerases may play more important roles in maintaining the genome stability than previously thought. The recognition mechanism seems unique to archaeal B family DNA polymerases because viral B family DNA polymerases such as T4 DNA polymerase or DNA polymerases from hyperthermophilic eubacteria such as *Thermus aquaticus* (Taq) do not stall DNA synthesis when the template DNA has uracil or hypoxanthine (Greagg *et al.* 1999; Gruz *et al.* 2003). There are no specific reports on the recognition of uracil in DNA by eukaryotic B-family DNA polymerases as far as we know. Structural analysis for uracil recognition by archaeal B family DNA polymerases indicates a pocket in the N-terminal domains interacting with a template strand is responsible for the discrimination of uracil from normal DNA bases (Fogg *et al.* 2002).

Since Pol B1 is abundantly expressed in the cell, i.e. 1500 molecules per *S. solfataricus* cell, it may bind to the deaminated bases in the chromosome DNA even without DNA synthesis. In fact, it binds to uracil- or hypoxanthine-containing oligonucleotides without primers or dNTPs necessary for DNA synthesis (Gruz *et al.* 2003). If such a binding occurs *in vivo*, it may interfere with normal repair of the deaminated bases by DNA glycosylases (Sartori *et al.* 2002). Thus, there appear to be mechanisms that prevent non-productive binding of Pol B1 to the deaminated bases in non-replicating chromosomes. For further insights into the binding mechanisms of archaeal B family DNA polymerases to the deaminated bases in DNA, we compared the binding efficiencies of Pol B1 to uracil in DNA with or without DNA synthesis. For this purpose, we employed atomic force microscopy, which is suitable for the analysis of the behavior of individual molecules, as well as gel mobility shift assays (Engel & Muller 2000; Murakami *et al.* 2000). The results indicated the binding of Pol B1 to uracil in DNA is greatly accelerated by DNA synthesis and suggested that Pol B1 is targeted to the deaminated bases in replicating DNA. In addition, analyses with atomic force microscope (AFM) suggested that Pol B1 binds to uracil-containing DNA as a monomer, which directly supports the "read-ahead" mechanism where single molecules of DNA polymerase bind to template uracil and halt DNA replication (Greagg *et al.* 1999).

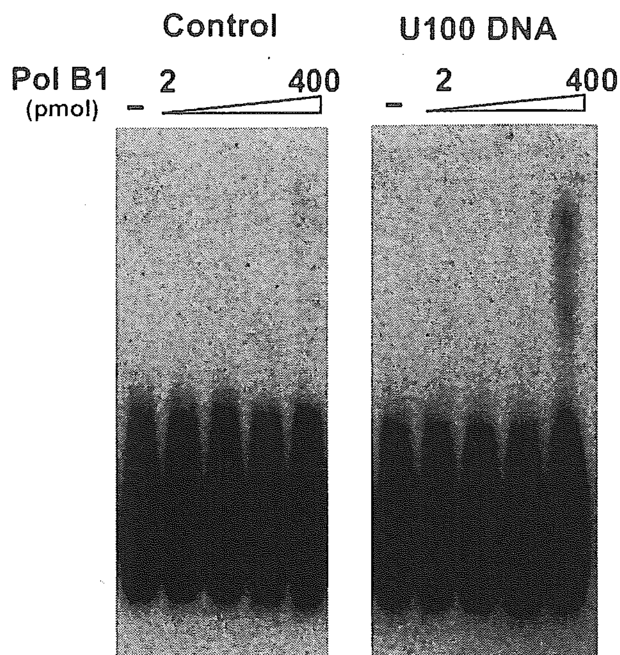

Figure 1 Binding of Pol B1 with template uracil without DNA synthesis. The reaction mixture (20 μL) contained Tris/KCl/ $MgCl_2$, ssDNA (control or U100 DNA, 2 pmol) and Pol B1 (2, 20, 200 or 400 pmol), and the mixture was incubated for 15 min at 55 °C. The products were analyzed by 1% agarose gel electrophoresis, followed by Southern hybridization. The bands were visualized with ChemiDoc.—no Pol B1 was added in the reaction mixture.

Results

Binding of Pol B1 to uracil-containing DNA is accelerated by DNA synthesis

To examine the binding of Pol B1 to uracil in single-stranded DNA (ssDNA), control or U100 DNA, which contains uracil instead of thymine in DNA, was incubated with various amounts of Pol B1, and the migration of DNA bands was analyzed by gel mobility shift assays (Fig. 1). A clear band shift was observed when U100 DNA was incubated with Pol B1. A smear band appeared in the upper part of the gel while the original DNA bands remained in the lower part. In these experiments, primers and dNTPs were omitted from the reaction mixtures. Thus, it seems that Pol B1 can bind uracil-containing ssDNA even without DNA synthesis. This is consistent

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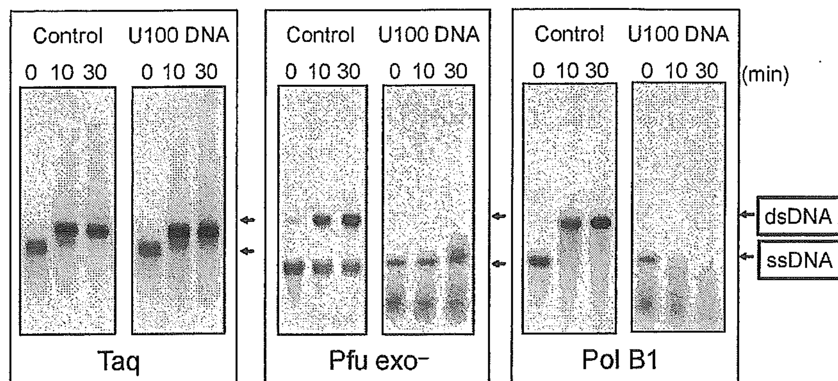

Figure 2 Template uracil inhibits DNA synthesis by Pol B1 and Pfu exo⁻ but not by Taq DNA polymerase. The reaction mixture (20 μL) contained Tris/KCl/$MgCl_2$, F1 primer (10 pmol), four dNTPs (4 nmol each), ssDNA (control or U100 DNA, 0.1 pmol) and Pol B1 (20 pmol). Taq DNA polymerase (1 unit) and Pfu exo⁻ DNA polymerase (2.5 units) were used as controls. The reaction was carried out for 10 or 30 min at 55 °C and was terminated by the addition of EDTA. The products were analyzed by Southern hybridization and visualized with ChemiDoc. Taq, Taq DNA polymerase; Pfu exo⁻, Pfu DNA polymerase exo⁻; Pol B1, DNA polymerase B1.

with our previous results that the K_d values of Pol B1 to primed DNA, ssDNA without uracil and ssDNA with uracil are 81, 55 and 4 nM, respectively (Gruz *et al.* 2003). It should be noted, however, that the binding was not efficient: it needed a large amount of Pol B1, i.e. 400 pmol, and no band shift was observed with lower amounts of the protein. The binding appears specific to uracil-containing DNA because no clear band shift was observed in control DNA.

Next, we examined the inhibitory effects of template uracil on DNA synthesis with U100 DNA (Fig. 2). DNA synthesis by Pol B1 was severely inhibited by the presence of uracil in template DNA. ssDNA containing uracil (U100 DNA) was hardly converted to double-stranded DNA (dsDNA), while control ssDNA was almost completely converted to dsDNA within 10 min. Most of ssDNA bands of U100 diminished during 10- or 30-min incubation without formation of dsDNA. Since Pol B1 possesses an exonuclease proofreading activity (Pisani & Rossi 1994), uracil-containing ssDNA might be partially digested by the exonuclease during the incubation. Like DNA synthesis by Pol B1, DNA synthesis by another archaeal B-family DNA polymerase, i.e. Pfu exo⁻, was severely inhibited by the presence of uracil in template DNA. In this case, however, ssDNA bands were observable even after 30-min incubation. The lack of exonuclease activity in Pfu exo⁻ DNA polymerase might account for the persistence of the ssDNA bands. In contrast to the archaeal enzymes, DNA synthesis by hyperthermophilic eubacterial DNA polymerase, i.e. Taq, was not inhibited by the presence of template uracil.

Since template uracil strongly inhibited DNA synthesis by Pol B1, the binding of Pol B1 during DNA synthesis might be stronger than that of Pol B1 without DNA synthesis. To examine the possibility, we analyzed the binding during DNA synthesis by the gel shift assay (Fig. 3). When Pol B1 and U100 DNA were incubated

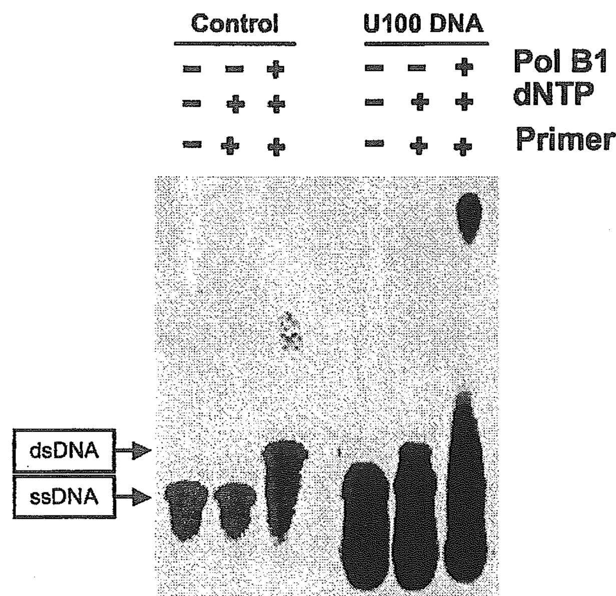

Figure 3 Specific binding of Pol B1 with template uracil during DNA synthesis. The reaction mixture (20 μL) contained Tris/KCl/$MgCl_2$, F1 primer (10 pmol), four dNTPs (4 nmol), ssDNA (control or U100 DNA, 2 pmol) and Pol B1 (20 pmol). The mixtures were incubated for 10 min at 55 °C, and the reactions were terminated by the addition of EDTA. The products were analyzed by Southern hybridization and visualized with ChemiDoc.

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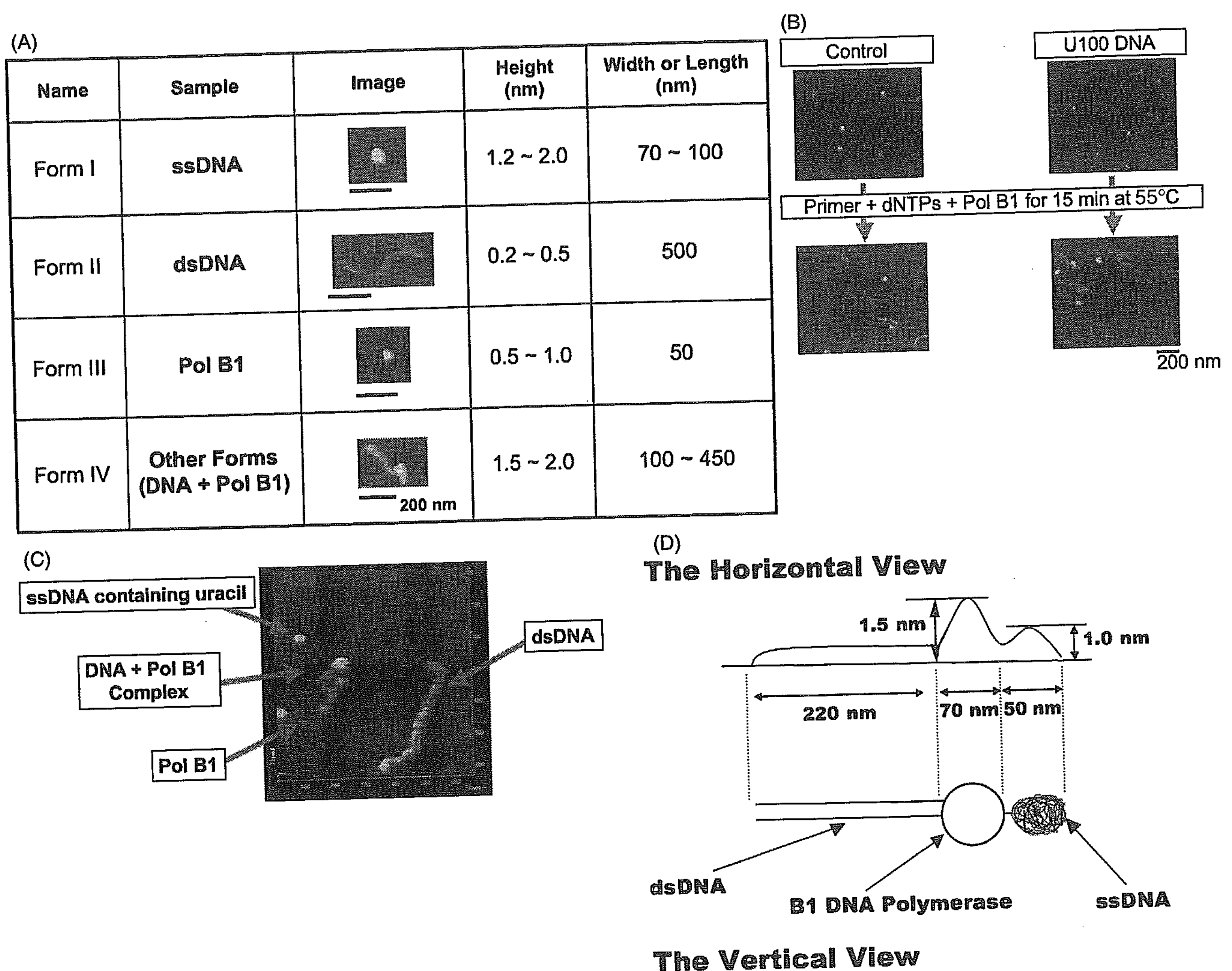

Name	Sample	Image	Height (nm)	Width or Length (nm)
Form I	ssDNA		1.2 ~ 2.0	70 ~ 100
Form II	dsDNA		0.2 ~ 0.5	500
Form III	Pol B1		0.5 ~ 1.0	50
Form IV	Other Forms (DNA + Pol B1)	200 nm	1.5 ~ 2.0	100 ~ 450

Figure 4 AFM images of DNA, Pol B1 and intermediates where Pol B1 binds with uracil-containing DNA. (A) Classification of the images of ssDNA (Form I), dsDNA (Form II), Pol B1 (Form III) and other forms including the intermediates (Form IV). (B) AFM images of the products of DNA synthesis with Pol B1 plus control DNA or U100 DNA. The reaction mixtures before and after incubation were analyzed by AFM. (C) Typical images of ssDNA, dsDNA, Pol B1 and the intermediates. (D) Possible explanation for the AFM image of the intermediate Form IV.

with primer and dNTPs, a smear band appeared on the gel. The band intensity was much stronger with U100 DNA than with control DNA. When we exposed the gel for shorter period of time, it became evident that substantial amounts of U100 DNA disappeared during the incubation (data not shown). It should be emphasized that the amount of Pol B1 (20 pmol, Fig. 3) required for binding with U100 DNA was 1/20 of that required for the binding to U100 DNA without primer and dNTPs in the reaction mixture (400 pmol, Fig. 1). These results suggest that the binding efficiency of Pol B1 with template uracil is substantially enhanced by DNA synthesis.

Pol B1 binds to uracil-containing DNA as a monomer

Atomic force microscopy is a powerful and convenient tool to visually analyze the behavior of individual DNA and protein molecules (Argaman *et al.* 1997; Murakami *et al.* 2001). The AFM method was employed to directly characterize the features of ssDNA, dsDNA and Pol B1 at the single molecular level (Fig. 4A). In the image analysis, ssDNA and dsDNA appeared as spherical and linear forms, respectively (Forms I and II). Pol B1 appeared as smaller spherical forms (Form III). The observed length of dsDNA, i.e. 500 nm, was consistent with the calculated

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