2,4-Nonadienal and Benzaldehyde Bioantimutagens in Fushimi Sweet Pepper (*Fushimi-Togarashi*)

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Fushimi sweet pepper, "*Fushimi-togarashi*", is one of the "*Kyo-yasai*", traditional vegetables, in Kyoto, Japan. The chloroform fraction of Fushimi sweet pepper showed bioantimutagenicity on UV induced mutation in *Escherichia coli* B/r WP2. The bioantimutagen was purified with silica gel chromatography and identified as 2,4-nonadienal (ID_{50} = 20 μg/plate) on the basis of GC retention time and EI-MS spectrum of authentic 2,4-nonadienal. The sweet pepper also contained a known bioantimutagen, benzaldehyde (ID_{50} = 2 mg/plate). Additive bioantimutagenicity was also observed by 2,4-nonadienal with benzaldehyde. 2,4-Nonadienal did not show bioantimutagenicity in an UV excision repair deficient strain, *E. coli* B/r WP2s *uvrA*$^-$. Furthermore no delay of the first cell division after UV irradiation was observed in *E. coli* B/r WP2. These results indicate that the bioantimutagenic activity of 2,4-nonadienal on UV mutagenesis might depend on the excision repair system in *E. coli* B/r WP2.

Keywords: *2,4-Nonadienal; benzaldehyde; antimutagen; vegetable; sweet pepper; additive effect*

INTRODUCTION

Antimutagens suppress a genetic mutation and its accumulation in the development of cancer. Thereby finding the antimutagen from daily food could be of importance for cancer prevention. Against the DNA lesion formed by mutagens, bioantimutagens play a role to enhance to repair the lesion, followed by reducing the mutagenic frequency.

Antimutagenic activity has been classified into two main groups on the basis of their mode of action: as desmutagen is an inactivating factor toward a chemical mutagen, and a bioantimutagen activates the DNA damage repair system until the mutation is fixed (Kada et al., 1981). Fewer bioantimutagens than desmutagens have been found from food so far. It has reported that some food ingredients such as cinnamaldehyde (Ohta et al., 1983a), coumarin (Ohta et al., 1983b), tannic acid (Shimoi et al., 1985), catechins (Shimoi et al., 1986; Matsuo et al., 1993), vitamin B_6 (Shimoi et al., 1992), and *S*-methyl methanethiosulfonate (MMTS) (Nakamura et al., 1996) show bioantimutagenicity in *Escherichia coli* B/r WP2. Besides researchers have tended to identify only the strongest or a stronger bioantimutagen in food, bioantimutagenicity was tested by each substance alone. When we discuss whole bioantimutagenicity of food, we should also consider the additive and synergistic activity of plural substances related to the bioantimutagenicity rather than only the strongest bioantimutagen.

For more than 15 years regarding vegetables one of the pending problems has been that most of the vegetable crops lost valuable components and tastes compared with former vegetables owing mainly to selective breeding for higher yield, fewer days to harvest, and disease resistance. In contrast, agriculturists in Kyoto have been carefully preserving species of traditional vegetables in Kyoto, which are known as "*Kyo-yasai*" in Japan, for more than 300 years, thus retaining their original shapes and taste. Traditional vegetables in Kyoto might therefore contain useful ingredients more than common vegetables. For these reasons we have studied traditional vegetables in Kyoto.

Fushimi sweet pepper, which is called "*Fushimi-togarashi*" in Japan, is one of the traditional vegetables in Kyoto. Fushimi sweet pepper, which is 10−15 cm in fruit length and is not pungent, has recently been introduced as a source of bioantimutagens (Nakamura et al., 1998). However, so far the identification of principal antimutagen from Fushimi sweet pepper has not been reported. In the present paper, we reported the isolation of a new bioantimutagen 2,4-nonadienal and the detection of a known bioantimutagen, benzaldehyde, from Fushimi sweet pepper. We also described the bioantimutagenicity of 2,4-nonadienal and its additive activity with benzaldehyde against the UV-induced mutation of *E. coli* B/r WP2.

MATERIALS AND METHODS

Materials. Fushimi sweet pepper (*Capsicum annum* var. *grossum*) is sown in January to February, planted in April to May, and harvested from June to October in an open field culture in Kyoto, Japan. We used what was harvested in Kyoto Prefectural Agricultural Research Institute on June 1996 and September 1997. 2,4-Nonadienal (>90% GC, extra pure grade) and methyl salicylate (>98% GC, extra pure grade) were purchased from Wako Pure Chemical Industries Ltd. (Osaka,

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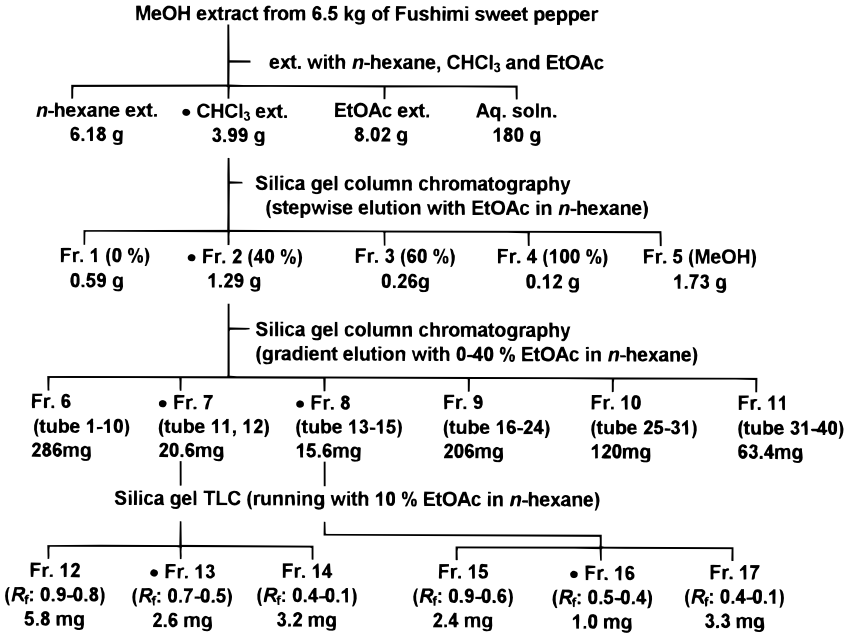

Figure 1. Purification scheme for bioantimutagen from Fushimi sweet pepper. •: bioantimutagenic fraction in UV irradiated *E. coli* B/r WP2.

Japan). Benzaldehyde (>98% GC, guaranteed reagent) was purchased from Nacalai Tesque (Kyoto, Japan). 2-Pentylfuran (>98% GC, extra pure grade) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO, spectra grade) was purchased from Dojin Lab (Kumamoto, Japan).

Gas Chromatography−Mass Spectrometry. All analyses were performed on a JEOL JMS-SX102A mass spectrometer, coupled on a Hewlett-Packard 5890 series II gas chromatography. The capillary column was used a DB-5 (25 m × 0.2 mm, 0.33 μm film thickness; J&W Scientific, Folsom, CA). The column oven temperature was held at 60 °C for 2 min and then was increased to 240 °C at 10 °C/min.

Assay for Bioantimutagenicity. The present assay was carried out by the method described by Shimoi et al. (1985) and Nakamura et al. (1996) with a slight modification. *E.coli* B/r WP2 *trpE65* or WP2s *uvrA155 trpE65* was cultured overnight (13.5 h) in nutrient broth (0.375 g/15 mL), washed twice with ice-cold 1/15 M phosphate buffer (PBS, pH 7.4), and then resuspended in 5 mL of the PBS. The cell suspension in a Petri dish (90 mm in diameter) was UV (254 nm)-irradiated with a 6 W germicidal lamp fixed at 40 cm height in a handmade wooden box. The intensity was measured with a UV-radiometer (UVX digital radiometer; UVP, CA). Ultra violet (254 nm; 21.0 J/m^2 for WP2 or 1.5 J/m^2 for WP2s)-irradiated cell suspensions were diluted to 1×10^{-1} for revertant detection and 1×10^{-6} for survivors with PBS. All samples were dissolved and sterilized with DMSO and diluted to 50 μL of DMSO solution. Bioantimutagenicity can be detected by that sample when it is treated with UV-irradiated cells. Fifty microliters of sample solution, 0.2 mL of UV-irradiated and diluted cells, and 2 mL of molten top agar (0.7% Bacto-agar and 0.6% NaCl) were mixed well in 0.5 mL of PBS and poured onto the semienriched minimal agar medium (SEM) plate (0.1% $(NH_4)_2SO_4$, 1% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% trisodiumcitrate$\cdot 2H_2O$, approximately 0.3% KOH for neutralization, 0.4% glucose, 0.016% nutrient broth, and 1.5% Bacto-agar). The numbers of revertants and survivors were counted as colony-forming units on the same organized SEM plates after incubation at 37 °C for 2 days. The antimutagenicity was expressed as relative mutagenic activities (RMA, % of control). RMA was obtained from the calculation $[(m/M)/(s/S)] \times 100$ where m is the number of revertant colonies in the presence of the test sample; M is the number of revertant colonies in the absence of the test sample; s is the number of surviving colonies in the presence of the test sample; and S is the number of surviving colonies in the absence of the test sample. ID_{50} (the inhibitory dose for 50% RMA) and its 95% confidence interval were calculated from a linear regression line derived from points taken from over five concentrations. An inactive fraction is defined as one whose RMA did not decrease less than 50% at 10 mg/plate or the dose of that observed in 50% of survival.

Purification and Identification of the Bioantimutagen. Fushimi sweet pepper (6.5 kg) was stored at 4 °C until used. Each 0.5 kg of the pepper was homogenized in water (0.7 L) once for 15 s with a family type mixer (MX-320, Matsushita, Osaka, Japan). All homogenate was filtered, and the residue was extracted with methanol (8 L, three times). Aqueous and methanol extracts were mixed and evaporated under 40 °C to about 6 L of an aqueous crude extract solution with a rotary evaporator. Crude extract adjusted to pH 2 with 1 M HCl was partitioned three times by 2.5 L each of *n*-hexane, $CHCl_3$, and ethyl acetate (EtOAc) successively. Each fraction was evaporated under 40 °C to give dryness of *n*-hexane, $CHCl_3$, and EtOAc, respectively. Aqueous layer was neutralized with 1 M NaOH, lyophilized, and then weighed. All fractions were tested for bioantimutagenic activity. The fractionation procedure for each fraction is summarized in Figure 1. $CHCl_3$ fraction, showed bioantimutagenicity was divided to fractions 1−5 by 180 g of silica gel column chromatography (Nacalai; silica gel 60, 35−70 mesh; 110 × 2.5 cm) using 2.5 L of 0%, 40%, 60%, and 100% EtOAc in *n*-hexane and methanol as the eluents in a stepwise manner. Fraction 2 (1.29 g), showed bioantimutagenicity was then divided by 60 g of silica gel column chromatography (Merck; silica gel 60, 70−230 mesh; 45 × 2.0 cm), using 0−40% EtOAc in *n*-hexane as the gradient eluent (400 mL), and each 10 mL was collected for tubes 1−40. Each tube spotted for TLC plate (Merck; silica gel 60 F_{254}), developed in 5% EtOAc in *n*-hexane. Tubes 1−40 were combined to fractions 6−11. Fraction 7 and 8 were spotted on TLC plates (Merck; silica gel 60 F_{254}, 20 × 20 cm), developed in 10% EtOAc in *n*-hexane. A spot of the compound was detected visually by UV (254 nm)-irradiation. The broad band of R_f value 0.5−0.7 spots in fraction 7 and spots of R_f value 0.4−0.5 in fraction 8 were collected.

Assay for Cell Growth after UV Irradiation. Five milliliters of *E. coli* B/r WP2 were UV (21.0 J/m^2 of 254 nm)-irradiated and then diluted to 1×10^{-1} with PBS. A portion (0.8 mL) of the irradiated cells was mixed with 10.2 mL of nutrient broth. 2,4-Nonadienal was dissolved, sterilized with DMSO, and diluted to 20 μg/50 μL of DMSO solution.

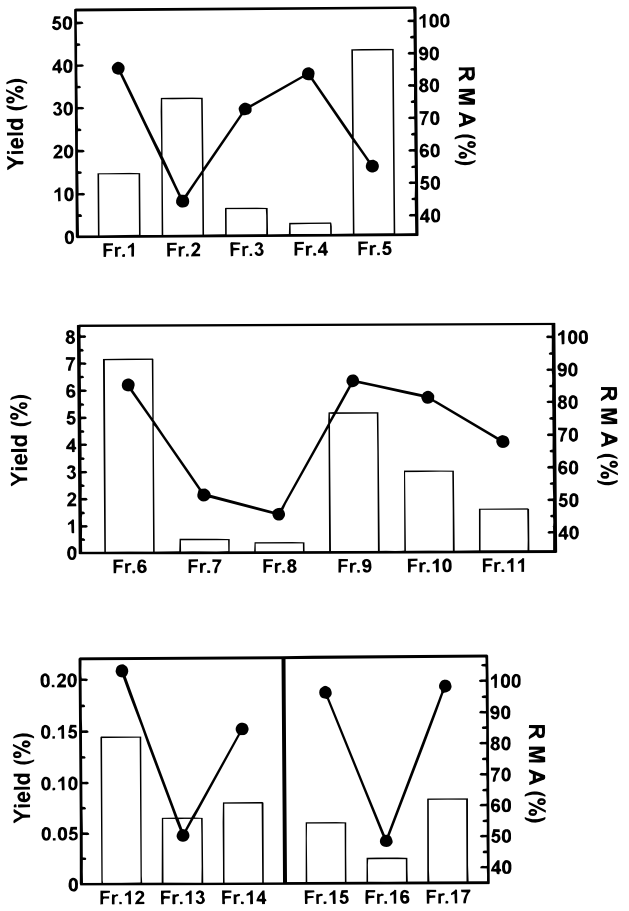

Figure 2. Yield and bioantimutagenicity of fractions 1−17. Bar represents a percentage of yield against the $CHCl_3$ fraction. Closed circle represents RMA at the dose of 3 mg/plate in fractions 1−5, 1 mg/plate in fractions 6−11, 0.6 mg/plate in fractions 12−14, and 0.2 mg/plate in fractions 15−17, respectively.

Fifty microliters of DMSO solution with or without 2,4-nonadienal was added to each 2.7 mL of NB solutions containing irradiated cells and incubated at 37 °C. This concentration reproduced the condition where 2,4-nonadienal shows 50% of RMA without toxicity in the assay for bioantimutagenicity. After 60, 90, 120, and 150 min of incubation, a portion (25 μL) of the cell solution was withdrawn and diluted to 1×10^{-6} with PBS. Two hundred microliters of diluted cells and 2 mL of molten top agar were mixed well in 0.5 mL of PBS and poured onto the SEM plate. The number of survivors was counted as colony-forming units on SEM plates after incubation at 37 °C for 2 days.

RESULTS

Purification of the Bioantimutagen. Whole purification scheme for bioantimutagen from Fushimi sweet pepper is shown in Figure 1. The bioantimutagenic activity and weight of the fractions is shown in Figure 2 and Table 1. As shown in Figure 1 and Table 1, the $CHCl_3$ fraction (3.99 g) showed bioantimutagenic activity, while the *n*-hexane (6.18 g), EtOAc (8.02 g), and aqueous fractions (180 g) did not show the activity. Fraction 2 (40% EtOAc elution from the $CHCl_3$ fraction) showed the bioantimutagenic activity (RMA 44.9%, 3 mg/plate) and yielded 1.29 g. Fraction 2 was further divided resulting in one active fraction 8 (RMA 46.0%, 1 mg/plate) and close active fraction 7 (RMA 52.0%, 1 mg/plate). Fractions 7 and 8 respectively yielded 1.6% and 1.2% of fraction 2 (Figure 2). By the purification step, the bioantimutagen was concentrated effectively. Fractions 7 and 8 were purified with silica gel TLC. Fraction 13, showing the band of TLC R_f value 0.5−0.7 from fraction 7, showed close bioantimutagenic activity (RMA 50.8%, 0.6 mg/plate) and yielded 2.6 mg. Fraction 16, showed the band of TLC R_f value 0.4−0.5 from fraction 8 and bioantimutagenic activity (RMA 48.6%, 0.2 mg/plate) and yielded 1.0 mg.

Table 1. Bioantimutagenic Activities of the Fractions and Compounds Identified on UV-Induced Mutation in *E. coli* B/r WP2[a]

fraction or compound	dose (mg/plate)	RMA (%)
control	0	100
$CHCl_3$ fraction	10	40.8
fraction 2 (40% EtOAc elution)	3	44.9
fraction 7 (tube 11, 12)	1	52.0
fraction 13 (TLC R_f: 0.5−0.7)	0.6	50.8
fraction 8 (tube 13−15)	1	46.0
fraction 16 (TLC R_f: 0.4−0.5)	0.2	48.6
authentic 2-pentylfuran	0.01	103
	0.03	116
	0.1	toxic
authentic methyl salicylate	0.3	112
	0.45	116
	0.6	toxic

[a] Cell suspensions of *E. coli* in a Petri dish were irradiated with a germicidal lamp at a dose of 21 J/m^2. Two hundred microliters of the cell suspension diluted appropriately was poured on SEM plate with soft agar containing sample. Values represent the mean of one or two experiments with duplicate plates for the fractions or authentic compounds, respectively.

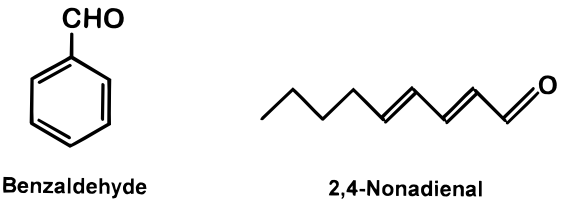

Figure 3. Chemical structures of 2,4-nonadienal and benzaldehyde.

Identification of Bioantimutagen in Fraction 13. Fraction 13 gave three compounds (**1**−**3**) on the total ion chromatogram from the GC-EI mass analysis. Compound **1**, appearing at t_R 6.65 min showed the ion peak at *m*/*z* 138 $(M)^+$ and prominent fragment ions with masses of 123, 109, 95, 81, 67, and 53. This was identified to be 2-pentylfuran by comparing the mass spectrum and retention time with those of authentic 2-pentylfuran but showed no bioantimutagenic activity (Table 1). Compound **2**, appearing at t_R 6.90 min, showed prominent ion peaks at *m*/*z* 106 $(M)^+$, 105 $(M - H)^+$, 77 $(M - CHO)^+$, and 51 $(M - C_2H_2CHO)^+$. The chemical structure of the compound was identified to be that of benzaldehyde by comparing the mass spectrum and retention time with those of authentic benzaldehyde. The structure of benzaldehyde is shown in Figure 3. Authentic benzaldehyde decreased UV mutagenesis dose-dependently at the range of 0.1−3 mg/plate, and its ID_{50} was 1.85 mg/plate. Benzaldehyde showed the lowest RMA (28.2%) at the dose of 3 mg/plate (Figure 4). Compound **3**, appearing at t_R 11.00 min, showed the ion peak at *m*/*z* 152 $(M)^+$ and prominent fragment ions with masses of 120, 92, 65, and 64. This was identified to be methyl salicylate by comparing the mass spectrum and retention time with those of authentic methyl salicylate but showed no bioantimutagenic activity (Table 1).

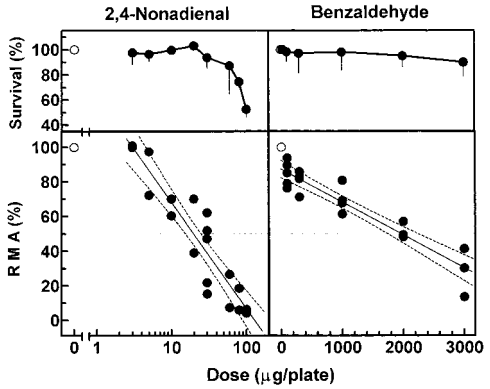

Figure 4. Bioantimutagenic effect of 2,4-nonadienal and benzaldehyde on UV induced mutation in *E. coli* B/r WP2. Cell suspensions of *E. coli* in a Petri dish were irradiated with a germicidal lamp at a dose of 21 J/m^2. Two hundred microliters of the cell suspension was poured on SEM plate with soft agar containing DMSO (○) and sample (●). Each point represents the mean of duplicate plates in one experiment. A linear regression line and its 95% confidence interval solid line were expressed in RMA graphs.

Table 2. Bioantimutagenic Activities of 2,4-Nonadienal on UV-Induced Mutation in *E. coli* B/r WP2s (*uvrA*$^-$)[a]

dose (μg/plate)	survival (%)	RMA (%)
0	100	100
10	97.3	99.2 ± 0
30	93.1	78.9 ± 0.1
45	103	76.3 ± 3.4
60	88.5	88.5 ± 6.3

[a] Cell suspensions of *E. coli* B/r WP2s *uvrA*$^-$ in a Petri dish were irradiated with a germicidal lamp at a dose of 1.5 J/m^2. Two hundred microliters of the cell suspension diluted appropriately was poured on SEM plate with soft agar containing 2,4-nonadienal. Values represent the mean ± range of two experiments with duplicate plates.

Identification of Bioantimutagen in Fraction 16. Fraction 16 gave one main compound on the total ion chromatogram from the GC-EI mass analysis. The compound, appearing at t_R 12.15 min, showed prominent ion peaks at *m/z* 138 $(M)^+$, 109 $(M - CHO)^+$, 95 $(M - CH_2CHO)^+$, 81 $(M - C_2H_4CHO)^+$, 67 $(M - C_3H_6CHO)^+$, and 53 $(M - C_4H_8CHO)^+$. The chemical structure of the compound was identified to be that of 2,4-nonadienal by comparing the mass spectrum and retention time with those of authentic 2,4-nonadienal. The structure of 2,4-nonadienal are shown in Figure 3. 2,4-Nonadienal was contained at a level of about 0.15 ppm in the fresh Fushimi sweet pepper during the purification process. Authentic 2,4-nonadienal decreased UV mutagenesis dose-dependently at the range of 3−100 μg/plate, and its ID_{50} was 20.0 μg/plate (Figure 4). 2,4-Nonadienal showed the lowest RMA (5.6%) at the dose of 100 μg/plate.

Bioantimutagenicity of 2,4-Nonadienal. 2,4-Nonadienal showed bioantimutagenicity on UV-induced mutation in a repair competent strain, *E. coli* B/r WP2 (Figure 4). However no bioantimutagenicity of 2,4-nonadienal was shown in an UV excision repair deficient strain, WP2s *uvrA*$^-$ (Table 2). Further no delay of the first cell division after UV irradiation was observed under the condition where 2,4-nonadienal shows 50% of RMA in *E. coli* B/r WP2. The first cell division was observed at 90−120 min in both the absence and presence of 2,4-nonadienal (Figure 5).

Additive Bioantimutagenicity of 2,4-Nonadienal with Benzaldehyde. 2,4-Nonadienal and benzaldehyde were also shown a cobioantimutagenicity on UV induced mutation in *E. coli* B/r WP2. The effect was additive without a synergistic effect because each RMA line of 2,4-nonadienal was nearly parallel regardless of the benzaldehyde dose (Figure 6).

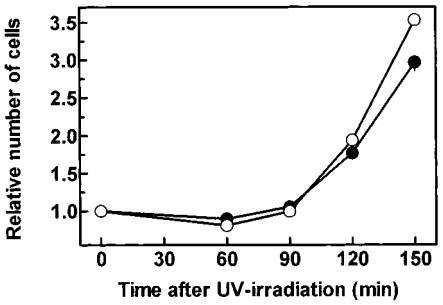

Figure 5. Effects of 2,4-nonadienal on cell growth after UV irradiation in *E. coli* B/r WP2. The UV (21.0 J/m^2)-irradiated cell suspensions of *E. coli* B/r WP2 were cultured in nutrient broth with (●) or without (○) 2,4-nonadienal (20 μg/2.75 mL) and incubated at 37 °C. After incubation, a portion of the cell was diluted and then poured onto the SEM plate. The number of viable cells were determined by colony formation on SEM plates. Each point represents the mean ± SD of one experiment with triplicate plates.

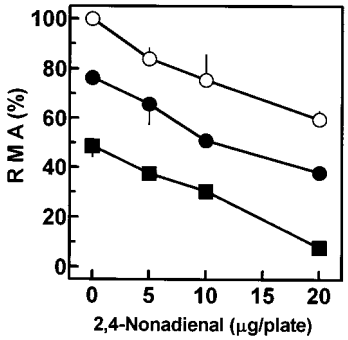

Figure 6. Additive bioantimutagenicity of 2,4-nonadienal with benzaldehyde on UV-induced mutation in *E. coli* B/r WP2. Cell suspensions of *E. coli* in a Petri dish were irradiated with a germicidal lamp at a dose of 21 J/m^2. Two hundred microliters of the cell suspension was poured on SEM plate with soft agar containing 2,4-nonadienal at the indicated dose and benzaldehyde (○: 0 mg/plate; ●: 1 mg/plate; ■: 2 mg/plate). Each sample was prepared to 25 μL of DMSO. Each point represents the mean of duplicate plates in two experiments.

DISCUSSION

We isolated a new bioantimutagen from the methanol extract of Fushimi sweet pepper and identified its chemical structure as that of 2,4-nonadienal. Furthermore we detected a known bioantimutagen, benzaldehyde (Watanabe et al., 1988).

2,4-Nonadienal suppresses UV-induced mutation at a dose of 3−100 μg/plate and showed a 20 μg/plate of ID_{50} in *E. coli* B/r WP2. In this assay system, most of the active compounds have shown 0.1−2 mg/plate of ID_{50}, and *S*-methyl methanethiosulfonate (MMTS), which is one of the most potent bioantimutagens in Cruciferae and Liliaceae vegetables, is shown a 2 μg/plate of ID_{50} (Nakamura et al., 1996). 2,4-Nonadienal is hence considered a bioantimutagen to show a lower ID_{50} among known bioantimutagens. 2,4-Nonadienal reduced RMA slightly but did not show ID_{50} in an UV excision repair deficient strain, WP2s *uvrA*$^-$. Thereby the antimutagenesis of 2,4-nonadienal may depend on

the UV excision repair system. One hypothesis that has been described is that the bioantimutagens act by extending G_1 allowing DNA repair to go on longer to remove potential mutagenic lesions, hence lower mutation frequency (Shimoi et al., 1992). However, no delay on the cell growth was observed in the presence of 2,4-nonadienal in *E. coli* B/r WP2. Therefore antimutagenesis of 2,4-nonadienal does not increase the opportunity of the DNA repair. No mutagenicity of 2,4-nonadienal was observed in *E. coli* B/r WP2 (data not shown). It is suggested that antimutagenesis of 2,4-nonadienal has close relation to the UV excision repair system. α,β-Unsaturated unaromatic aldehydes show bioantimutagenicity of UV-irradiated *E. coli* B/r WP2, and its mechanism could be related to the enhancement of the DNA repair system by the modification of the enzyme-SH group (Aikawa et al., 1988, 1989). Accordingly the 2,4-nonadienal activity could be shown through the same path of other α,β-unsaturated unaromatic aldehydes. 2,4-Nonadienal was found in the volatiles of bitter melon (Binder et al., 1989) and endive (Götz-Schmidt et al., 1986) or in some processed animal products of pork (Ramarathnam et al., 1991) and chicken (Moerck et al., 1979). 2,4-Nonadienal is also formed from linoleic acid methyl and ethyl esters by moderate oxidative degradation (Nishiya et al., 1978). Furthermore 2,4-dienal derivatives have been added to meat and margarine for supplementing lesser aroma during their storage (Keppler et al., 1971). Thus we have daily been taking 2,4-nonadienal from some vegetables and food.

Benzaldehyde, which is a well-known ingredient in seeds of bitter almonds or pits of apricots and peaches, has been widely used in flavors such as almond and cherry. The bioantimutagenicity of benzaldehyde was reported on 4-nitroquinoline 1-oxide (4NQO) induced mutation in excision repair deficient strain, *E. coli* B/r WP2s (Watanabe et al., 1988). Benzaldehyde showed a 49% decreasing effect to the UV mimic, 4NQO-mutagenesis at the dose of 3.18 mg/plate. It is believed that *E. coli* possesses two main protective systems against UV, an excision or a recombination repair system. The antimutagenesis of benzaldehyde was thereby considered to be dependent on the recombination repair system. Benzaldehyde inhibited 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and induced lung tumorigenesis by reducing the activation in vivo, too (Morse et al., 1995). Benzaldehyde is in one unit of amygdalin (laetrile, or incorrectly called vitamin B_{17}). The hypothesis was that amygdalin would be broken down by an enzyme in cancerous tissue to liberate cyanide which would kill the cancer. However, no curative properties have been demonstrated for amygdalin administration in tumor-bearing rodents (Greenberg et al., 1980). The negative results of amygdalin against cancer (Jukes et al., 1990) were thus reported. Although such a background was known to cancerous cells, another liberated unit of amygdalin, benzaldehyde, could play a role of bioantimutagen to a cell having UV type mutagenic lesion, hence preventing cancer.

We conclude that most researchers have tested a physiological activity of one ingredient alone despite the fact that the whole activity of food was not able to be explained by the identification of one substance alone. One example is that MMTS is one of the strongest bioantimutagens in Chinese chives, but it would be necessary to take the MMTS from 24−41 kg of fresh Chinese chives daily for preventing colon carcinogenesis (Nakamura et al., 1996). However, along time might be necessary and also a little perception to clarify the additive or synergistic activity by combination with known plural active substances. In the present paper, it is interesting that two different types bioantimutagens, 2,4-nonadienal and benzaldehyde, are detected in Fushimi sweet pepper, and they also show additive bioantimutagenicity. To estimate the whole bioantimutagenicity of Fushimi sweet pepper by determining other active ingredients or their synergist should be important. It is also necessary to investigate the bioantimutagenic activity in mammalian cells or the cancer chemopreventive effect in vivo.

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