DNA Repair Effect of Traditional Sweet Pepper Fushimi-togarashi: Seen in Suppression of UV-induced Cyclobutane Pyrimidine Dimer in Human Fibroblast

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The aqueous fraction of Fushimi sweet pepper increased the repair effect of the solvent control against UV-induced cyclobutane pyrimidine dimers in human fibroblast to 150%, but ordinary sweet pepper did not have a statistically significant effect. When Fushimi sweet pepper was boiled, the activity of the aqueous fraction was elevated to 209% of the control (p < 0.05), while that of the grilled state was decreased to 125% of the control. The repair activity of a dialyze (MW <12,000) of the aqueous fraction from Fushimi sweet pepper showed 191% of the control (p<0.05). The dialyze was contained 1.9% in the weight of the fresh fruit body of Fushimi sweet pepper, and the activity can be stable in its boiling state, and it might be therefore considered to be the worthy source for expecting the DNA repair activity in human diet.

Key words: sweet pepper; vegetable; antimutagen; pyrimidine dimer; excision repair

Carcinogenesis begins with genetic mutation and progresses as such mutations accumulate.1) Factors that can enhance DNA repair, called bioantimutagens, can help to prevent cancer.2) Certain traditional vegetables in Kyoto, called “Kyo-yasai” in Japanese, have greater bioantimutagenicity in tests that use Escherichia coli irradiated with ultraviolet (UV) light than the common cultivars.3) One reason may be that consumer preference has tended to be for milder tasting vegetables, and that selective breeding has resulted in the vegetables without strong flavors and also with decreased amounts of some components that may promote health and help prevent disease. Growers of traditional vegetables in Kyoto, however, have tended to preserve the cultivars without much change some 300 years or more; shapes and flavors are highly characteristic, and certain components with bioantimutagenic activity may have been preserved in abundant in them at the same time.3)

Here, we examined whether four traditional vegetables (Fushimi-togarashi sweet pepper, Kamo-nasu eggplant, Katsura-uri oriental pickling melon, and Shishigatani-kabocha pumpkin), which contained antimutagens in studies done with E. coli also enhance repairing UV damage in human cells. Ultraviolet light produces two major photoproducets, cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct (6-4PP). From 3 to 6.7 times as much CPD forms as 6-4PP, and CPD is removed from the genes much more slowly than 6-4PP by the nucleotide excision repair (NER) system.4,5) CPD therefore is probably more important factor in mutation caused by UV light than 6-4PP, and we concentrated mostly on CPD when examining repair activity in human fibroblasts.

First, we surveyed the repair activity of each four fractions (n-hexane, chloroform, ethyl acetate, and aqueous) of four traditional vegetables in Kyoto. Next, we compared the activity in active fractions from the traditional vegetables and their more common counterparts; we also found whether the activity was in fractions of low or high molecular weight. Last, we investigated whether the activity was affected by cooking of the vegetables.

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Abbreviations: CPD, cyclobutane pyrimidine dimer; 6-4PP, (6-4)photoproducet
Materials and Methods

Traditional vegetables in Kyoto used. Fushimitoragashiki sweet pepper (Capsicum annuum var. grossum), Kamo-nasu eggplant (Solanum melongena), Katsura-uri oriental pickling melon (Cucumis melo var. conomon), and Shishigatani-kabocha pumpkin (Cucurbita moschata) were harvested at the Kyoto Prefectural Agricultural Research Institute between June and October 1999. Common sweet pepper (Capsicum annuum var. grossum) was purchased from a supermarket in Kyoto City.

Extraction and fractionation of vegetables. A 500-g portion of a vegetable was homogenized with 500 ml of water and the homogenate was filtered. The residue was extracted three times with 500 ml of methanol each time. Aqueous and methanol filtrates were combined and evaporated to 300 ml of crude extract. The extract was adjusted to pH 2 with 1 N HCl and partitioned three times with n-hexane, chloroform (CHCl₃), and ethyl acetate (EtOAc), in that order, with 250 ml each time. The n-hexane, and EtOAc layers were evaporated to dryness at less than 40°C, and the aqueous layer was lyophilized.

Dialysis of aqueous fraction of Fushimi sweet pepper. The residue of the aqueous fraction (1.36 g) partitioned from 50 g of Fushimi sweet pepper was dissolved in 20 ml of distilled water and dialyzed at 4°C with a dialysis membrane (size 36; Mₛ excluded, < 12,000; Wako, Osaka, Japan) three times with 400 ml of distilled water each time.

Preparation of cooked Fushimi sweet pepper. Fushimi sweet pepper (50 g) was boiled in water for 4 min or grilled on a gas range (3,100 kcal/h) for 3.5 min in the usual way for cooking. Cooked sweet pepper was homogenized with 50 ml of distilled water and the homogenate was filtered. The residue was extracted with 50 ml of methanol three times. Aqueous and methanol filtrates were combined and evaporated to 30 ml of crude extract. The extract was adjusted to pH 2 with 1 N HCl and washed with 25 ml of EtOAc three times. The aqueous layer remaining was lyophilized.

Assay of repair of UV-induced damage. WI-38 VA13 cells (2 × 10⁶) were plated in a 100-mm plastic dish with 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (10% FBS-DMEM) and cultured for 4 h. To cells, a sample mixed with 50 µl of dimethyl sulfoxide was added, and the dish incubated for 21 h. After the incubation, cells were washed with Dulbecco's phosphate buffered saline and irradiated with UV light (254 nm; 10.0 J/m² from a germicidal lamp) in an aseptic plastic box. The intensity was measured with a UV-radiometer (UVR-1; Topcon, Tokyo, Japan). Irradiated cells were incubated in 10 ml of 10% FBS-DMEM with 50 µl of a sample solution, and further incubated for 3 h. After incubation, cells were harvested with a cell scraper and stored at −80°C until their DNA was extracted. DNA was purified with a QIAMP blood kit (Qiagen, Hilden, Germany). The DNA photoproducts CPD and 6-4PP were assayed by an enzyme-linked immunosorbent assay with monoclonal antibodies TDM-2 and 64M-2, respectively. The method was as described by Mori et al.

The amount of photoproduct was calculated by use of a standard equation for the photoproduct derived from the cells UV-irradiated (0, 2.5, 5.0, 7.5, and 10.0 J/m²). The amount of photoproducts repaired in the presence of the sample as a percentage of total photoproducts without the sample added (the control), was used to express repair activity.

Results

Effects of traditional vegetables in Kyoto on DNA Repair activity greater than 100% by fractions from these of the traditional vegetables of UV-induced CPD when used to treat fibroblasts for 3 h are shown in Table 1. With CHCl₃, EtOAc, and aqueous fractions of 1 mg of Fushimi sweet pepper, aqueous fraction had 150% of the activity of the control (p < 0.01, paired t-test). With Kamo eggplant, the EtOAc and aqueous fractions increased repair activi-

<table>
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<tr>
<th>Vegetable</th>
<th>Repair for CPD (%) by fractions</th>
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<tr>
<td></td>
<td>n-Hexane</td>
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<tr>
<td>Fushimi sweet pepper</td>
<td>—</td>
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<tr>
<td>Kamo eggplant</td>
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<tr>
<td>Shishigatani pumpkin</td>
<td>118.9 ± 4.6</td>
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Cells were incubated with a sample (1 mg/dish) for 21 h, washed, irradiated with UV light (10 J/m²), and incubated with same sample for 3 h to allow repair. The values are the mean amount of repaired CPD with the sample added as a percentage of the control amount without a sample. Values are the means and SE of three to seven experiments with triplicate or quadruplicate dishes.

* Significantly different from the control (100%); p < 0.05 (paired t-test).
DNA Repair by Fushimi Sweet Pepper

Changes with time in repair of UV-induced CPD and 6-4PP by aqueous fraction of Fushimi sweet pepper

WI-38 VA13 cells themselves removed 27% of CPD and 88% of 6-4PP within 3 h after the end of UV irradiation, and removed 52% of CPD and 94% of 6-4PP within 6 h (Fig. 1). When an aqueous fraction of 1 mg of Fushimi sweet pepper was used, removal of CPD was 40% at 3 h and 66% at 6 h after the end of irradiation. However, the fraction affected the amount of 6-4PP little.

Comparison of effects of common and traditional sweet peppers

Common and Fushimi sweet peppers have the same scientific name, Capsicum annum var. grossum. The fruit bodies of both kinds of sweet peppers are not pungent. The kinds can be easily distinguished by their shapes (Fig. 2). Fushimi sweet pepper was further partitioned to Keihoku strain, and Takii strain which is mainly shipped to the vegetable market. Both fruit bodies cannot be usually distinguish by their shapes (Fig. 2). Aqueous fraction of Keihoku strain of Fushimi sweet pepper increased CPD repairing to 150% at the dose of 1 mg/dish against the control without additive (Fig. 3). The aqueous fraction of common sweet pepper and Takii

strain of Fushimi sweet pepper also increased the repair activity to 114% and 107% at the dose of 1 mg/dish. However, significant difference from the activity of the control was shown in the activity of the aqueous fraction from Keihoku strain of Fushimi sweet pepper, but was not shown in that of the aqueous fraction from the common sweet pepper and Takii strain of Fushimi sweet pepper (p<0.05; ANOVA and multiple comparison test of Fisher’s PLSD).

Repair activity of dialyzate and non-dialyzate from aqueous fraction of Fushimi sweet pepper

The aqueous fraction (1.36 g) was extracted from 50 g of Fushimi sweet pepper. Nine hundreds sixty
milligrams of a aqueous dialyzate (MW < 12,000) and 400 mg of a non-dialyzate (MW > 12,000) were obtained from the aqueous fraction. The dialyzate showed the 191% of repair activity for CPD against the control at the dose of 1 mg/dish (Fig. 4). On the contrary, the non-dialyzate did not increase the activity at the dose of 1 mg/dish. The activity of the dialyzate was significantly different from those of the control and the non-dialyzate (p<0.05; ANOVA and multiple comparison test of Fisher’s PLSD).

Repair activity of aqueous fraction of cooked Fushimi sweet pepper

The aqueous fraction of row Fushimi sweet pepper possessed 150% of the repair activity for CPD against the control at the dose of 1 mg/dish (Fig. 5). Boiled Fushimi sweet pepper showed 209% of the repair activity at the dose of 1 mg/dish, and the activity was significantly different from those of the control, row and grilled states (p<0.05; ANOVA and multiple comparison test of Fisher’s PLSD). However, 1 mg/dish of grilled Fushimi sweet pepper showed 125% of the repair activity of which the value was less than that of the row state, and the activity was not significantly different from that of the control and row (p<0.05; ANOVA and multiple comparison test of Fisher’s PLSD).

Discussion

This study has primarily demonstrated that DNA repair activity is increased with extract of vegetable in human fibroblast cells. The candidate to investigate the repair activity in this study were subjected to the previous result of that four traditional vegetables in Kyoto (Fushimi-togarashi sweet pepper, Kamo-nasu eggplant, Katsura-uri oriental pickling melon and Shishigatani-kabocha pumpkin) were superior to their counterpart of common vegetables on bioantimutagenicity in UV-irradiated E. coli.\textsuperscript{39} Recently it has been reported that thymidine dinucleotide up-regulate three DNA repair regarding proteins (G1 arrest and excision repair protein, p53; proliferating cell nuclear antigen, PCNA; and excision repair enzyme, XPA) in human keratinocyte.\textsuperscript{7} Further, human interferon-β has been appeared to enhance the mRNA level of excision repair enzyme XPG in human Cockayne syndrome cells.\textsuperscript{8} However, it has not been so far identified the extract or component of food possessed the repairing for UV-induced photosystem in human cells. Here only the aqueous fraction of Fushimi-togarashi traditional sweet pepper in Kyoto (Keihoku strain) was found as a significantly enhancing fraction of repair activity against UV-induced CPD in human fibroblast WI-38 VA13 cells (Table 1). In contrast, its chloroform extract and 2,4-nonadienal, which is a potent bioantimutagen in its chloroform extract in the assay of E. coli,\textsuperscript{9} did not show significantly repair activity in the present assay of WI-38 VA13 (data not shown). On the one hand, the inconsistent is sometimes inevitable due to the difference of fundamental cell systems between prokaryotic and eukaryotic cells, or induction pathway of DNA repair system.\textsuperscript{10,11} On the other hand, some bioantimutagens in the assay using E.
coli have been found the cancer preventive effect in the mammalian assay in vivo. The present result should also suggest another candidate for studying cancer chemoprevention in the future.

Human fibroblast WI-38 VA13 cells usually repair 90% of the initial (6-4) photoproduct (6-4PP) within 3 h and approximately 25% and 50% of initial cyclobutane pyrimidine dimer (CPD) at 3 h and 6 h after UV-irradiation (Fig. 2). Cells, being induced DNA-lesion, act to arrest in the G1 phase and induce various repair system to avoid the fixation of genetic mutation which occurs during the S phase. It is thus considered that predominantly facilitating of the repair against slower removing CPD than 6-4PP is important for lowering the frequency of genetic mutation. Although the aqueous fraction of Fushimi sweet pepper do not change the repair rate of 6-4PP within 6 h, it is interesting that the fraction possesses the enhancing repair activity against more genetically toxic lesion CPD.

Common sweet pepper and traditional Fushimi sweet pepper are belonged to same species and not pungent, and therefore both sweet peppers have been applied to cuisines by using similar cooking manner. Both sweet peppers are thus considered to be appropriate samples to compare their DNA repair activities. We can easily distinguish between common and Fushimi sweet peppers in the vegetable market. However, it is difficult to recognize between Takii and Keihoku strains of Fushimi sweet pepper owing to their morphologically similar fruit bodies (Fig. 2). Keihoku strain has been performed less selective breeding due to using at limited area. In contrast Takii strain is produced with selective breeding and sold at widespread area commercially. In the present study, the repair activity against CPD of aqueous fraction of Keihoku strain is superior to that of common sweet pepper and Takii strain (Fig. 3). It might be reflected the previous hypothesis that the vegetable being closer to wild have been superior to its counterpart of improved strain on bioantimutagenicity in the assay of E. coli. In fact, some of more ingredient in Fushimi sweet pepper have been reported, for instance potassium and calcium contents of Fushimi are 2 and 1.6 times higher than those of common sweet pepper.

In order to further condense the active fraction, the aqueous fraction of Keihoku strain of Fushimi sweet pepper was partitioned to both fractions containing lower and higher than 12,000 of molecular weight substance. The lower molecular weight fraction (MW < 12,000) shows higher repair activity than that of higher molecular weight fraction (Fig. 4). The lower molecular weight fraction shares 70.6% weight of the aqueous fraction, thus the quantitative condensation of active ingredient is hardly performed, but the qualitative condensation can be well performed as shown in that its repair activity is increased to 191% of the activity from 150% of that of original aqueous fraction. In addition, no significantly repair activity was shown in the three partitions with further purification of the lower molecular weight fraction by using reverse-phase chromatography, and therefore the activity of the lower molecular weight fraction might be sustained with plural substances (data not shown). The appropriate purification to further condense the active fraction is under investigation.

It is also important for expecting the physiological ability on food to remain that in the edible state. We examined the repair activity of aqueous fractions from different cooking conditions of Fushimi sweet pepper. Fushimi sweet pepper has been well eaten as boiled and grilled states, or as row state. The repair activity of boiled Fushimi sweet pepper (Keihoku strain) is significantly higher than that of row state at the same dose, and the result suggests that the capable ingredient can be remained in the fruit body with boiling and is stable at 100°C for 4 min, and further some of inhibitor or interfered substance for repair activity might be conveniently leaked to boiling water from the fruit body, or rate of extraction of the capable ingredient might increase with boiling (Fig. 5). On the one hand, Rhee et al. have been recently reported that the boiling can facilitate to increase the content of some monosaccharide in immature black soybean "Murasakizukin", which is also traditional vegetables in Kyoto, owing to the activation of α-D-galactosidase to hydrolyze raffinose and stachyose.

On the other hand, we also have hypothetical speculation that the amount of some capable components increases in Fushimi sweet pepper during its boiling for some reason. The repair activity of the grilled Fushimi sweet pepper is not significantly different from that of the control and row state, and the result indicated that the capable ingredient should be unstable or broken at over 100°C. The traditional dish has been also applied Fushimi sweet pepper such as its grilled body with soy source and dried bonito. The additive seasonings can slightly (but not significantly) recover the diminished ability by grilling (data not shown). On the basis of the result, we hypothesize the combination between food material and appropriate seasonings might be of important for modifying the physiological activity, and the combination effect of food components is our interesting study that is now under investigation.

In the present study, Keihoku strain of Fushimi-togarashi traditional sweet pepper in Kyoto appears to higher DNA repair activity than a common sweet pepper and improved Takii strain of Fushimi sweet pepper in human cell on the basis of the quantification of UV-induced photoproduct, CPD. CPD shows higher genetic toxicity than 6-4PP due to its slower repairing rate, and therefore have been considered to the significant risk factor of skin cancer. Further CPD is one of the bulky DNA-lesions as same cate-
gory of DNA adducted aflatoxin, benz[a]pyrene and heterocyclic amines etc., and they can be removed from whole genome by the NER system, and therefore the higher repair activity of CPD is considered to the higher preventive ability for not only skin cancer but liver, lung and gastrointestinal cancers.\textsuperscript{17,18,19} The aqueous lower molecular fraction (MW <12,000) is abundantly contained as 1.9% of the fresh weight of Fushimi sweet pepper, and its repair activity can be stable in the boiling state, and thus it is considered the worthy source for expecting the repair capability. The study for the tolerance against digestion, efficiency of absorption and distribution \textit{in vivo} of the repair capable fraction should be important in order to consider to the application of the present data to cancer chemoprevention in human diet.

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\textbf{References}