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Antigenotoxic effect of *Pleurotus cornucopiae* extracts on the mutagenesis of *Salmonella typhimurium* TA98 elicited by benzo[a]pyrene and oxidative DNA lesions in V79 hamster lung cells

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Abstract

Pleurotus cornucopiae (PC) mushroom with a brilliant yellow pileus is found in the field and known in Japan as Tamogi dake mushroom. The purpose of this paper is to investigate the mechanism of the antimutagenic effect of PC mushroom using both the Ames test and Comet assay. We have found a strong inhibitory effect of both aqueous and organic PC extracts on the mutagenicity elicited by benzo[a]pyrene (B[a]P). This inhibition was dose-dependent in reaction mixtures containing cytosolic and microsomal fractions (S-9) from untreated rat liver as well as in those containing S-9 from aryl hydrocarbon receptor (Ah) ligand of Sudan III-treated rats. Sudan III was a potent inducer of cytochrome P450 1A (CYP1A) activity. We treated rats with Sudan III to enhance the metabolic activation of B[a]P by the S-9 fraction. To explain whether this antimutagenicity was due to the inhibition of CYP1A activity that metabolically activates B[a]P, we tested the effects of the extracts on activities of CYP1A1 and CYP1A2, represented by ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD), respectively. Both aqueous and organic extracts inhibited EROD activity at all dose levels, while the inhibitory effect was only observed at high doses with regard to MROD activity. Furthermore, pre-treatment of Chinese hamster V79 cells with PC extracts significantly reduced H₂O₂-induced-DNA damage, indicating that PC extracts provide a protective effect against oxidative DNA damage. These results indicate that whole-mushroom extracts contain compounds that

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may inhibit the metabolic activation of B[a]P by CYP1A1 as well as prevent oxidative DNA damage.

Key words : Antimutagenesis, Comet assay, CYP1A1, Mushroom, *Pleurotus cornucopiae*

Introduction

Since it is not always possible to reduce human exposure to mutagens, attempts have been directed at identifying potential antimutagens and anticarcinogens for use in protecting the population against environmental disease. Several dietary constituents may be important factors capable of increasing or decreasing cancer incidence. Thus, the specific and predetermined manipulation of diet may turn out to be a promising non-invasive approach to minimizing cancer¹⁶.

Edible mushrooms are nutritionally functional foods and a source of physiologically beneficial and nontoxic medicines. They have been used in folk medicine throughout the world since ancient times¹³. *Agaricus blazei* Murill (Basidiomycetes) mushroom is consumed as a food and for possible medicinal value. Infusion of the dried fruiting bodies has been used as a stimulant and as an auxiliary treatment of various diseases, including cancer⁹. Many isolated polysaccharides and protein-bound polysaccharides from *A. blazei* have been shown to have direct anti-tumor activity¹². Menoli et al. observed a tea preparation of *A. blazei* extracts to be antimutagenic in Chinese hamster lung fibroblast V79 cell lines²⁰. Similarly to *A. blazei*, the mushroom *Letinus edodes* also was observed to be effective in protecting cells from DNA damage, which can be responsible for the initiation of carcinogenesis⁶.

Pleurotus species are commonly called Oyster mushrooms. There are about 40 species of this mushroom distributed worldwide

in both temperate and tropical areas. Oyster mushrooms now rank as the second most important cultivated mushroom in the world²⁶. In Japan and far eastern Russia, a form of *Pleurotus cornucopiae* (PC) known also as *P. citrinopileatus* Singer with a brilliant yellow pileus is commonly sold in markets. Filipic et al. reported a strong antimutagenic effect (more than 50%) of PC mushroom on the gene expression of umuC⁸. However, the mechanism of antimutagenicity has not been investigated. The aim of the present study was to evaluate the antimutagenic effect of different extracts of PC mushroom in the Ames test using TA98, and protection against oxidative DNA damage from hydrogen peroxide in the Comet assay using V79 hamster lung cells.

Materials and methods

Chemicals :

Benzo[a]pyrene (B[a]P), ethoxyresorufin, methoxyresorufin and resorufin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan). All other chemicals and solvents used were of the highest quality commercially available.

Preparation of PC mushroom extracts :

Aqueous extract (tea-like preparation) : Fresh PC mushroom was minced and dried under vacuum at room temperature for 2 h. Distilled water (40ml/g) was added with agitation for one hour and then the homogenate

was placed in a water bath at 60°C for 15min. The resultant homogenate was filtered with suction, and the filtrate was centrifuged at 10,000g for 20min to remove any mushroom debris. The supernatant was sterilized by filtration through two Millipore filters (0.22 µm) and then stored at 4 °C.

Organic extraction of PC mushroom : Fresh PC was minced and dried under vacuum at room temperature for 2 h. Equal volumes of methanol and chloroform were added at 8 ml/g. The homogenate was agitated for 2 h, filtrated with normal filter paper, refluxed for 3 h or until completely dry with a rotary evaporator at 50°C, and finally stored at 4°C.

Ames test :

Salmonella typhimurium TA 98 was kindly provided by Dr. Ames (University of California, Berkeley, CA, USA). S-9 was prepared from male Wistar rats (7 weeks old) purchased from Japan SLC Inc. (Shizuoka, Japan) and used for experiments after 1 week of acclimatization. All experiments on the animals were performed under the supervision and with the permission of the University's Institutional Animal Care and Use Committee. S-9 fractions were prepared from untreated and Sudan III (SIII, 40mg/kg/daily for 3 days, dissolved in corn oil) -treated Wistar rats. Since SIII is a potent inducer of CYP1A activity, treatment with SIII should enhance the metabolic activation of B[a]P by the S-9 fraction. The rats were sacrificed on the fourth day and their livers were excised and perfused with ice-cold KCl 1.15% (w/v)²¹. For the preparation of the S-9 fraction, livers were homogenized in 3 volumes of 1.15% KCl and centrifuged at 9,000 g for 20 min. Protein concentrations were determined by the method of Lowry et al¹⁸.

The B[a]P mutagenesis assay was performed according to the method of Ames et al¹,

with some modifications. An overnight culture of strain TA 98 of *S. typhimurium* (0.1ml) was pre-incubated for 20 min at 37°C with and without a S-9 incubation mixture containing a complete NAD(P)H generating system, B[a]P (5 µg) or DMSO, and either crude aqueous or organic extracts of 1, 5, 10 and 50 mg of PC mushroom. Plates without B[a]P or PC mushroom extracts were used as a negative control. Immediately after 20 min pre-incubation, 2 ml of soft agar containing 0.5 mM L-histidine and 0.5 mM biotin was added. The mixtures were poured onto a glucose agar plate. The numbers of revertant colonies were counted after 48 h of incubation at 37°C. Each sample was assayed using triplicate plates and the data were presented as the mean ± standard error of three independent assays. The mutagenicity of B[a]P (positive control) in the absence of PC mushroom extract is defined as 0 in the mutagenicity figures.

Assay for hepatic microsomal monooxygenase activities :

Ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) activities were assayed in the presence and absence of crude aqueous or organic extracts of 1, 5, 10 and 50 mg of PC mushroom. The fluorescence of resorufin was detected with excitation and emission wavelengths of 528 nm and 590 nm respectively using a JASCO FP-777 spectrofluorometer³.

The Comet assay :

V79 Chinese hamster lung fibroblasts were obtained from Riken Bio Resource Center, Tsukuba, Japan. Cells were grown in Eagle's Minimum essential medium (Eagle's MEM) obtained from Sigma-Aldrich, supplemented with 6 % fetal calf serum (Sigma-Aldrich) and antibiotics (penicillin 200 U/ml, streptomycin 100 mg/ml, and kanamycin 100

mg/ml). Cells were cultured on glass petri dishes at 37°C in a humidified atmosphere of 5% CO₂. The V79 cells were treated with aqueous extracts of PC mushroom for 2 h prior to administration of the standard DNA and peroxidative hydrogen peroxide (H₂O₂) (Wako Chemical, Osaka, Japan). The treatment doses were 1, 10 and 50 mg of crude extract of PC mushroom, diluted in PBS and filtered through Millipore filters (0.22 µm). The H₂O₂ stock solution (10M) was diluted immediately before use in PBS buffer (Ca²⁺ and Mg²⁺ free). Cells treated with H₂O₂ (100 mM) for 15 min were used as a positive control, while negative control cells were treated only with PBS.

The Comet assay was performed according to the method of Singh et al.²⁵⁾ with some modifications⁵⁾. In this study, we did not use any computer-applications to measure tail-moments. Since Collins et al.⁴⁾ showed the accuracy and reproducibility of visual scoring in the Comet assay using the data from computer analyses, we referred to their protocol. Briefly, slides were coated with a first layer of 1% normal agarose. Approximately 20,000 cells were suspended in 50 µl of 0.5% low melting agarose and layered onto the slides, which were then immediately covered with cover slips. After agarose solidification at 4 °C for 5 min, cover slips were removed and to remove cellular proteins, slides were immersed for 1 h at 4 °C in fresh lysis solution (2.5M NaCl, 100 mM Na₂EDTA, and 10 mM Tris, pH 10) containing 1% Triton X-100. The slides were equilibrated in alkaline solution (1 mM Na₂ EDTA, 300mM NaOH, pH>13) for 40 min. Electrophoresis was carried out for 30 min at 1 V/cm. Afterwards, slides were neutralized by washing them three times with 0.4 M Tris buffer (pH 7.5) every 5 min. Slides were stained with ethidium bromide (2 µg/ml). Nucleoids were examined and the images were

scored using a fluorescent microscope (Olympus BX 50 equipped with a 520-550 nm excitation filter). Based on the extent of strand breakage, cells were classified according to tail length into five categories, ranging from 0 (no visible tail) to 4 (head of the Comet still detectable but most of the DNA in the tail). The following formula¹⁷⁾ in which N is the number of cells in each category (e.g. N₃ is the number of cells in category 3) was used to calculate scores.

$$\text{Score} = \frac{(N_0 + N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)}{(N_0 + N_1 + N_2 + N_3 + N_4)}$$

Experiments were done in duplicate and repeated at least twice.

Statistical Analysis :

Differences in means were assessed with the analysis of variance (ANOVA), followed by Fisher's protected least significance difference test. The level of statistical difference was set at P<0.05.

Results

The antimutagenic effects of both aqueous and organic PC mushroom extracts were evaluated using the Ames test with TA98 as the test strain of bacteria. The numbers of revertant colonies decreased markedly on the addition of the aqueous extract to preincubation mixtures containing hepatic S-9 from SIII-treated or un-treated rats (Figure 1). The mutagenicity of B[a]P also decreased dose-dependently upon the addition of the organic extract of PC mushroom (Figure 2A). Organic PC mushroom extract also had a significant antimutagenic effect when hepatic S-9 from SIII-treated rats was used in the B[a]P mutagenic assay (Figure 2). The antimutagenic effect of the aqueous extract appeared to be somewhat stronger than that

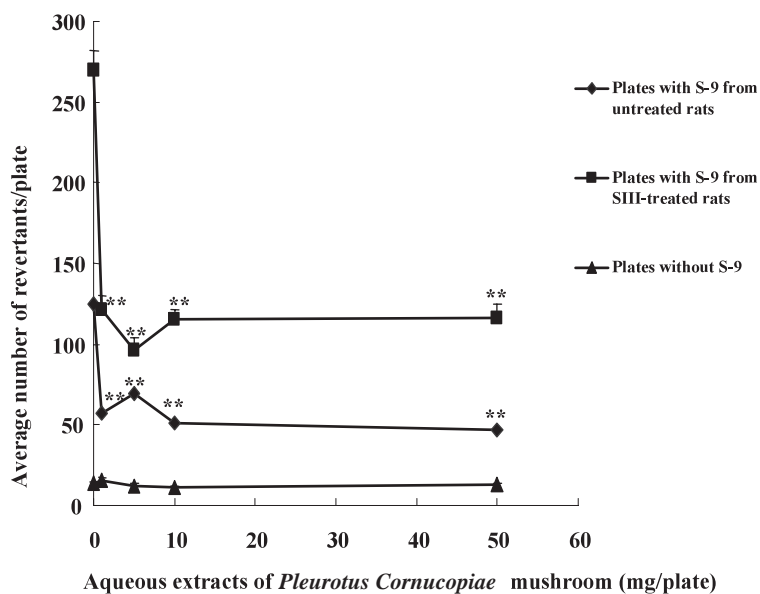


Figure 1 . Antimutagenic effect of aqueous extract of *Pleurotus Cornucopiae* mushroom against B[a]P. Effect of the addition of aqueous PC mushroom extracts on mutagenesis produced by B[a]P with *S. typhimurium* TA98. The Ames assay was performed with hepatic S-9 from SIII-treated and untreated rats. Each value is expressed as the mean number of revertant colonies/plate \pm SE (the mean for three plates from three independent assays). The number of spontaneous revertants was subtracted from each count. ** $p < 0.01$, significantly different from control.

of the organic extract.

With regard to EROD activity, there was a remarkable and significant dose-dependent inhibition with the addition of the aqueous as well as organic PC mushroom extract to the reaction mixture. The effects of both extracts were highly significant at the high dose, but there was no significant difference between the low-dose (extract of 1 mg of crude PC mushroom) group and the control group treated with organic extract (Figure 3). MROD activity was significantly inhibited in groups that received aqueous extracts of 10 and 50 mg of crude PC mushroom, while in other groups there were no significant differences between control and aqueous PC mushroom extracts (Figure 4). MROD in organic PC mushroom extract had a significant effect

at the highest dose only (extract of 50 mg of crude PC mushroom) (Figure 4).

We evaluated the preventive action of PC mushroom extracts against direct DNA single stranded breaks (ssb) with the Comet assay, which could detect DNA ssb in H_2O_2 -treated cells immediately after treatment. A statistically significant reduction in the number of DNA ssb was evident with all the concentrations of aqueous PC mushroom extract tested and this reduction was dose-dependent (Figures 5 and 6).

Discussion

There have been few studies on the antimutagenicity of mushrooms. However, one study using the Ames test reported weak mutagenicity of 40 edible mushroom species¹⁰.

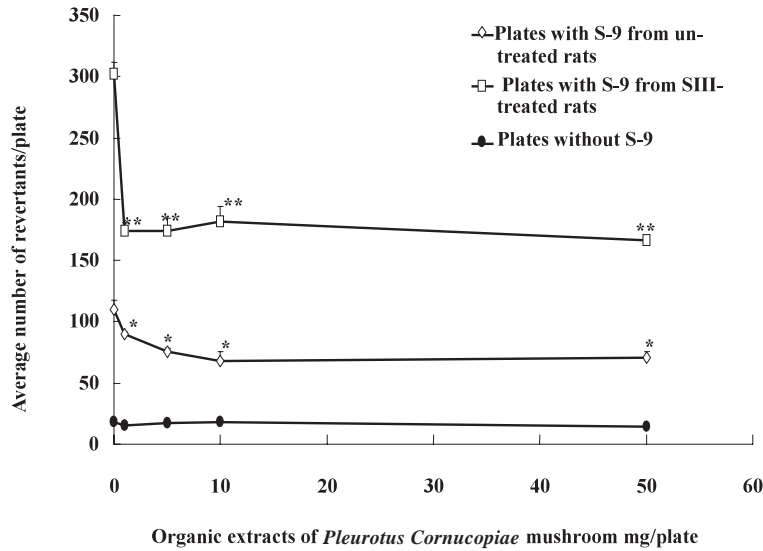


Figure 2 . Antimutagenic effect of organic extracts of *Pleurotus Cornucopiae* mushroom against B[a]P. Effect of the addition of organic PC mushroom extracts on mutagenesis produced by B[a]P with *S. typhimurium* TA98. The Ames assay was performed with hepatic S-9 from SIII-treated and untreated rats. Each value is expressed as the mean number of revertant colonies/plate \pm SE (the mean for three plates from three independent assays). The number the spontaneous revertants was subtracted from each count. * $p < 0.05$, ** $p < 0.01$, significantly different from control.

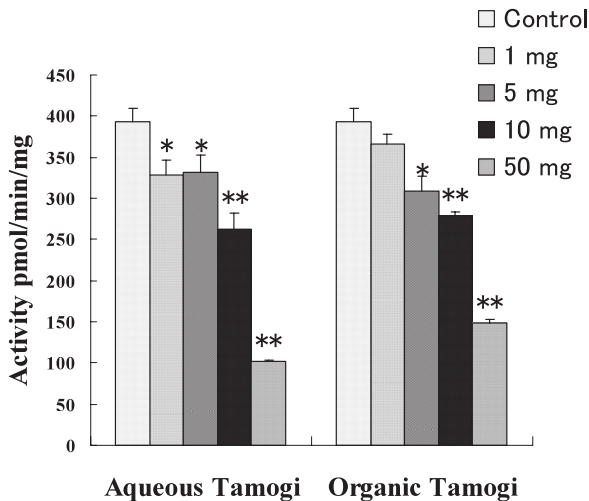


Figure 3 . Ethoxyresorufin O-deethylase (EROD) activity in different *Pleurotus Cornucopiae* extracts. Inhibition of hepatic microsomal EROD activity by both aqueous and organic PC mushroom extracts. * $p < 0.05$, ** $p < 0.01$, significantly different from control.

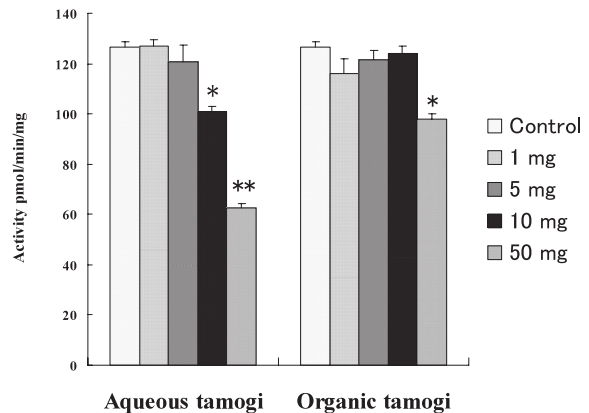


Figure 4 . Methoxyresorufin O-demethylase (MROD) activity in different *Pleurotus Cornucopiae* extracts. Inhibition of hepatic microsomal MROD activity by both aqueous and organic PC mushroom extracts. * $p < 0.05$, ** $p < 0.01$, significantly different from control..

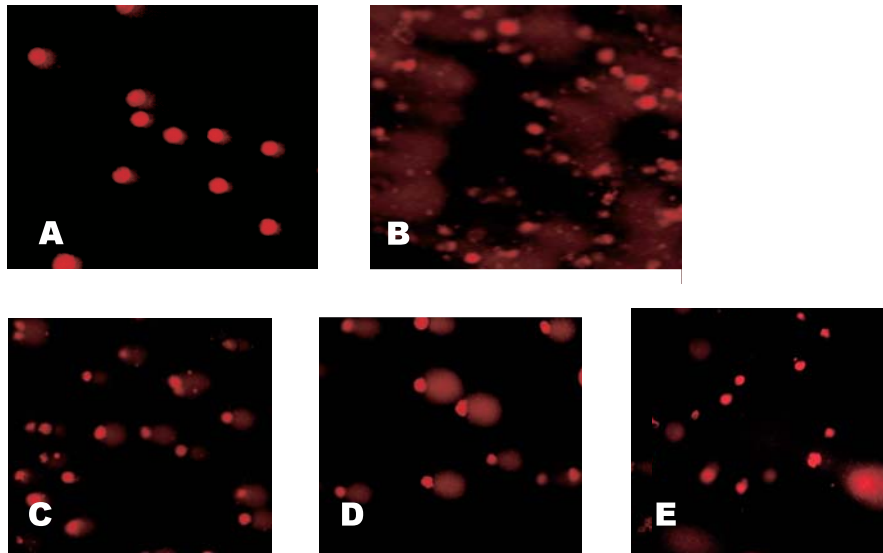


Figure 5 . Comet images of V79 cells treated with ; A : PBS. B : H₂O₂ for 15 min. C: pretreatment with 1 mg of aqueous PC mushroom extract 2 h prior to treatment. D: 10mg of aqueous PC mushroom extract 2 h prior to treatment. E: 50mg of aqueous PC mushroom extract 2 h prior to treatment.

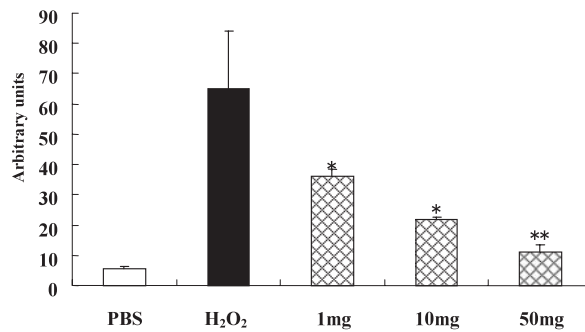


Figure 6 . DNA peroxidative damage using the aqueous extract of PC mushroom. The level of DNA strand breaks in PBS (white column) and H₂O₂-treated V79 cells in samples without (black columns) and with (hatched columns) pre-incubation with 1, 10, and 50 mg of aqueous PC mushroom extract for 2 h. -ve control : cells treated with PBS. +ve control : H₂O₂-treated cells. *(p<0.05) and ** (p<0.01) refer to significant and highly significant differences between samples and positive control.

Although these results were attributed to the presence of free histidine in the mushroom extracts which apparently promotes reversion from histidine auxotrophy to prototrophy, we report here strong inhibitory effects of both aqueous and organic extracts on mutagenicity elicited by B[a]P. This inhibition was dose-dependent especially in reaction mixtures containing S-9 from untreated rats. B[a]P is a mutagen which is activated by cytochrome P 450 (CYP) and the reaction mixtures without S-9 had a similar number of revertants as the control. To clarify whether this antimutagenicity was due to inhibition of the CYP1A-dependent metabolic activation of B[a]P or not, we tested the effect of these extracts on CYP1A1-dependent EROD activity and CYP1A2-dependent MROD activity. Both aqueous and organic extracts at 5 to 50 mg inhibited EROD activity, while the MROD activity was inhibited only at high doses. These results implied that the antimutagenic effect of PC mushroom extracts in this experiment may be due to direct suppression of the activity of the CYP1A subfamily, especially CYP1A 1.

The constituent(s) responsible for the antimutagenic activity of edible PC mushroom has not been identified. An analysis of the mushroom revealed that it contained (1-3) β -D-glucan, ergosterol, mannitol (angiotensin converting enzyme (ACE)) inhibitors, phenolic compounds, linoleic acid, peptides, and carbohydrates²⁾, most of which are antioxidants. Feng et al.⁷⁾ found that, the polyphenolic compounds (flavonoids, phenolic acids and their derivatives, and coumarin and its derivative) have antimutagenic and EROD-inhibiting effects. They also added that both antimutagenicity and suppression of CYP1A by phenols appeared to be dependent on alkyl chain lengths, which suggested a dependence on lipophilicity. Kanazawa et al.¹⁴⁾ showed

that the steric structure of flavonoids (those with a hydrophobic and coplanar stereostructure are EROD inhibitors), not the hydroxyl number, is important for the inhibition of CYP 1A. Lee et al.¹⁵⁾ reported that flavonoids with C5, C7 and C4-hydroxyl groups are potent CYP1A inhibitors, while Siess et al.²⁴⁾ suggested that flavonoids with hydroxyl groups only on the A ring are powerful inhibitors. Taken together, we are not able to conclude which components are responsible for the inhibition of B[a]P mutagenesis in this study. However, we can say that inhibition of CYP1A activity plays an important role in antimutagenesis and phenols are the only compound found in this mushroom reported to inhibit CYP1A.

We also investigated the effect of PC mushroom extract on oxidative DNA damage induced by reactive oxygen species (ROS) which can be generated within mammalian cells. Oxidative damage to DNA is believed to play an important role in degenerative processes such as carcinogenesis and aging¹⁹⁾. Antioxidants have attracted substantial attention during the last decade. Antioxidants are chemical species capable of terminating radical chain reactions. Natural antioxidants are believed to be of importance to human diet particularly in relation to the prevention of cancer²²⁾. A potent reactive species, the \bullet OH radical was generated by short-term treatment with hydrogen peroxide in V79 cells. It is assumed that H_2O_2 crosses biological membranes and penetrates the nucleus, where it reacts with ions of iron or copper forming \bullet OH radicals via the Fenton reaction. An attack by \bullet OH radicals on mammalian DNA at the sugar residue leads to fragmentation, base loss, and strand breaks^{23,11)}.

The aqueous extract of PC mushroom was more antigenotoxic than the organic extract in our Ames results. Furthermore, we

used the Comet assay to test the aqueous extract. The aqueous extract of PC mushroom had a marked, dose-dependent, protective effect on the oxidative DNA damage caused by H₂O₂. As has been stated earlier, β-D-glucan and mannitol are two constituents of PC mushroom, and are contained in the aqueous extract. Further study is needed to determine which of the constituents are responsible for these effects.

We concluded that extracts from this mushroom can inhibit CYP1A activities and prevent the metabolic activation of mutagens such as B[a]P. These effects are more potent with the aqueous than organic extracts. Pretreatment of V79 cells with the aqueous extract of PC mushroom significantly prevented DNA damage from H₂O₂ indicating that this mushroom also has an antioxidative effect. Therefore, the antigenotoxic effects of PC mushroom are due to inhibition of metabolic activation by CYP1A and the inhibition of ROS-induced DNA damage.

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