Desmutagenic Effect of Pheophytin b against Trp-P-2 in the *Salmonella* Assay

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Modifications of the standard Ames test and *umu* test were employed to study the “desmutagenic” response exerted by pheophytin b against the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2). The mixture from which pheophytin b separated was activated with S9 mix and was assayed to determine the remaining mutagenicity of Trp-P-2. The decrease in the His⁺ revertant colonies of *Salmonella typhimurium* TA98 was dependent on the increase in pheophytin b concentrations. Binding of Trp-P-2 to DNA and the subsequent induction of the SOS response were investigated using the *umu* test employing *Salmonella typhimurium* NM 2009. Trp-P-2 induced induction of β -galactosidase was suppressed when pheophytin b was included in the test. In order to evaluate the mode of action of pheophytin b in the mixture of Trp-P-2, Trp-P-2 was analyzed using HPLC. Compared with a solution of Trp-P-2 alone, the absorbance peak of Trp-P-2 in the HPLC profile of the mixture of Trp-P-2 and pheophytin b was observed to be decreased. Also a new peak was not shown in the HPLC profile. To investigate the possible effect of pheophytin b on the S9 mix, the HPLC profile of a mixture containing Trp-P-2, S9 mix and pheophytin b was compared with a mixture containing only Trp-P-2 and S9 mix. The absorbance peak of the hydroxylated Trp-P-2 in the reaction mixture containing pheophytin b was observed to be smaller than the peak of the mixture without pheophytin b. From these results, it is suggested that pheophytin b limits the formation of hydroxylated Trp-P-2.

Keywords: desmutagenicity, pheophytin, *Salmonella typhimurium* TA98, *Salmonella typhimurium* NM2009, Ames test, *umu* test

Many investigations have been carried out to identify naturally occurring and synthetic compounds capable of suppressing mutagenicity and carcinogenicity. Several literature reports point out the existence of compounds in daily food (Yoshikawa *et al.*, 1985; Yoshikawa & Ishii, 1987) which are capable of suppressing the formation of mutagens or carcinogens and/or are capable of inactivating mutagens or carcinogens. The factors which inactivate mutagens or carcinogens, particularly those from vegetables, fruits and dietary materials of plant origin, have been considered to exhibit important inhibitory effects on the development of human cancer. However, the exact mechanisms of inactivation of mutagens or carcinogens are not yet sufficiently understood.

High heat treatment or cooking of food materials rich in protein results in the formation of mutagenic heterocyclic amines with high potential for carcinogenicity (Sugimura *et al.*, 1977). Morita *et al.* (1978) have reported the suppression of heterocyclic amines induced mutagenesis by vegetables such as cabbages, broccoli, eggplants and others in the *Salmonella*/microsome assay. These extracts have been demonstrated to suppress the effects of heterocyclic amines such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) as shown by induced mutagenesis using the Ames test. Peroxidase in cabbage was identified by Inoue *et al.* (1981) as an agent which plays an important role. Yoshikawa *et al.* (1990) found that the mutagenicity of broiled fish was inhibited by crude extracts of vegetables such as burdock,

eggplant, broccoli and others. Japanese eggplant extract has been demonstrated to suppress 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) induced mutagenesis in *Salmonella typhimurium* TA98 (Yoshikawa *et al.*, 1996a), and one of the mutation-suppressing compounds was identified as pheophytin (Yoshikawa *et al.*, 1996b).

The present paper deals with the desmutagenic mechanism of mutation suppression by pheophytin b, a plant porphyrin.

Materials and Methods

Bacterial strains and chemicals The histidine-requiring strain of *S. typhimurium* TA98 was generously provided by Dr. T. Kada (National Institute of Genetics, Mishima). The *S. typhimurium* NM2009 used to study induction of the *umu* operon was a generous gift from Dr. Y. Oda (Osaka Prefectural Institute of Public Health, Osaka).

The chemicals used in this study were from the following sources: the S9 was prepared from Sprague-Dawley male rats treated with phenobarbital and benzoflavone, and the co-factors were purchased from the Oriental Yeast Co., Tokyo; 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), pheophytin b from chlorella were purchased from Wako Pure Chemical, Osaka. All other chemicals and reagents used were of the highest quality commercially available.

Ames test The desmutagenic effect of pheophytin b was assayed using *S. typhimurium* TA98. The reaction mixture, adjusted to 1.0 ml by the addition of phosphate buffer (pH 7.0, 37°C), containing 10 nmol of Trp-P-2 and

different concentrations of pheophytin b dissolved in dimethyl sulfoxide (DMSO), was allowed to react for 30 min in air at 37°C with gradual shaking. The reaction mixture was then heated at 100°C for 3 min. The mixture was filtered using membrane filters (0.45 μ m), and the filtrate was assayed. The assay procedure was as described in Mitscher *et al.* (1986), Shankel and Clarke (1990) and Yoshikawa *et al.* (1991). The mutagen used requires S9 for metabolic activation. The mutagenicity of the mutagen in the absence of pheophytin b is defined as 100% remaining mutagenicity. Each set of experiments was run in triplicate and repeated at least 3 times.

Umu test The *umu* test was carried out as described by Oda *et al.* with slight modification using *S. typhimurium* NM 2009 (Oda *et al.*, 1985, 1995). The bacterial strain was grown in Luria broth medium containing ampicillin (25 μ g/ml) at 37°C for 16 h with shaking. The overnight culture was transferred to TGA medium (1% Bactotryptone, 0.5% NaCl, 0.2% glucose, 20 mg/ml ampicillin) at a 50-fold dilution and was then cultured at 37°C till the absorbance at 600 nm reached 0.3. In another test tube, 1.7 ml of this culture, 0.3 ml of S9 mix, 10 μ l of Trp-P-2 (1 μ g/ml) and 10 μ l of pheophytin b (of different concentrations) were added, and the mixture was incubated for 2 h at 37°C with shaking for the induction of the *umu* operon. The bacterial density at 600 nm was measured, and β -galactosidase activity was assayed using 2-nitrophenyl- β -D-galactopyranoside as the substrate. The activity was determined by the method of Miller (1972) as modified by Oda *et al.* (1985).

HPLC analysis in the mixture of Trp-P-2 and pheophytin b In the reaction mixture of 1.0 ml, 0.1 ml each of 0.2 mM Trp-P-2 (in water) and 0.4 mM pheophytin b (in DMSO) were added in a phosphate buffer, pH 7.0. The reaction was allowed to proceed for 30 min at 37°C and was stopped by the addition of 1.0 ml cold acetone. The reaction mixture was then centrifuged (8000 $\times g$, 4°C for 10 min). The supernatant was evaporated under reduced pressure at 36°C using a rotary evaporator until completely dry. The dried residue was then dissolved in 1.0 ml of 50 mM disodium phosphate-25 mM citrate buffer, pH 4.0. The solution was diluted 10 times, and 20 μ l was employed for HPLC analysis. The result of the HPLC analysis was shown by the absorbance at UV 254 nm (Arimoto & Hayatsu, 1989) using a Shimadzu 10AS liquid chromatography apparatus with a Wakosil 5C18 (4.6 ϕ $\times$ 200 mm) column. The mobile phase used was acetonitrile and ammonium sulfate (1:3, v/v) with a flow rate of 1.0 ml/min.

Effect of pheophytin b on N-hydroxylation of Trp-P-2 The N-hydroxylation of Trp-P-2 was investigated by analyzing the HPLC profile by a method similar to that as described for the HPLC analysis of the mixture of Trp-P-2 and pheophytin b. To this mixture, 0.5 ml of S9 mix was added prior to the incubation, and the peak corresponding to the N-hydroxy Trp-P-2 was analyzed.

Results

Ames test Using *S. typhimurium* TA98, the mutation-suppressing ability of pheophytin b was assayed by the Ames test. The remaining mutagenicity of Trp-P-2 on co-incubating pheophytin b is shown in Fig. 1. The addition of 3.0 nmol of

pheophytin b to the test plate resulted in 36% suppression of the mutagenicity induced by 10 nmol of Trp-P-2, and 39% suppression was recognized when 4.0 nmol of pheophytin b was added. Further increases in the concentration of pheophytin b did not result in any more suppression: addition of 8.0 nmol resulted in 40% inhibition.

Umu test Using *S. typhimurium* NM2009, an investigation was undertaken to demonstrate that pheophytin b prevents the binding of the ultimate mutagenic metabolite of Trp-P-2 to the DNA molecule which then induces the *umu* operon. The Trp-P-2 induced levels of β -galactosidase in the *S. typhimurium* NM2009 were confirmed to decrease as a result of the addition of pheophytin b. The β -galactosidase activity was observed to decrease by 23% on co-incubating Trp-P-2 with 1.5 nmol/ml of pheophytin b (Fig. 2). Further increases in the concentration of pheophytin b seem to slightly increase the β -galactosidase activity; however, the β -galactosidase activity remained lower than the value attained without pheophytin b. Higher concentrations of pheophytin b could not be employed in the test because of the disturbances of green color on the absorbance at 420 nm and 550 nm. These results indicate that the decrease in β -galactosidase activity is due to a decrease in the induction of the

Fig. 1. Effect of pheophytin b on Trp-P-2 (10 nmol/plate) induced mutagenesis in *Salmonella typhimurium* TA98. Mean colony counts $\pm$ standard deviation of Trp-P-2 (10 nmol/plate)-induced His⁺ revertants were 1462 $\pm$ 91 ($n=9$), and mean colony counts $\pm$ standard deviation of spontaneous His⁺ revertants were 25 $\pm$ 10 ($n=9$).

Fig. 2. Effect of pheophytin b on Trp-P-2 (50 ng) induced induction of *umu* operon in *Salmonella typhimurium* NM2009.

Fig. 3. HPLC profiles of the Trp-P-2 reaction mixture. a: without pheophytin b, b: with pheophytin b. Trp-P-2 (20 nmol) was incubated with 0.4 mM pheophytin b at 37°C for 30 min and then analyzed on HPLC as mentioned in the text. The peak at 6.89 (6.90) retention time corresponds to Trp-P-2.

Fig. 4. HPLC profiles of the S9-mediated hydroxylation reaction mixture. a: without pheophytin b, b: with pheophytin b. Trp-P-2 (20 nmol) was incubated with 0.4 mM pheophytin b and 0.5 ml S9 mix at 37°C for 30 min and then analyzed on HPLC as mentioned in the text. The peaks at 6.18 (6.21) and 6.89 retention times correspond to *N*-hydroxy Trp-P-2 and Trp-P-2, respectively.

umu operon and confirm that pheophytin b can suppress mutability.

HPLC analysis of the mixture of Trp-P-2 and pheophytin b To ascertain whether or not pheophytin b reacted directly with Trp-P-2, the remaining Trp-P-2 in solution was estimated by HPLC. It was recognized that compared to the HPLC peak of 2.0 nmol Trp-P-2 alone at retention time (Rt) 6.90 (Fig. 3a), the HPLC peak of Trp-P-2 reacted with pheophytin b at Rt 6.89 drastically decreased (Fig. 3b). This result indicates that the amount of free Trp-P-2 molecules in the reaction mixture containing pheophytin b decreases by a considerable amount.

Effect of pheophytin b on *N*-hydroxylation of Trp-P-2 To confirm the *N*-hydroxylation of Trp-P-2 resulting from reactions with the S9 mix, HPLC analysis of the mixture of Trp-P-2, S9 and pheophytin b was performed. The HPLC profile at 254 nm of the reaction mixture containing Trp-P-2 and the S9 mix exhibited a peak at Rt 6.21 min for the *N*-hydroxy metabolite of Trp-P-2, and a peak at Rt 6.89 min for Trp-P-2 alone (Fig. 4a), as reported by Arimoto *et al.* (1989). When 42.2 nmol of pheophytin b was added to the mixture containing 20 nmol of Trp-P-2, shown in Fig. 4b, the peak for Trp-P-2 (Rt, 6.89) produced a greater peak area and the peak for the *N*-hydroxy metabolite of Trp-P-2 (Rt, 6.18) produced a smaller peak area, compared to the reaction mixture without pheophytin b. The comparatively small peak area for the *N*-hydroxy metabolite of Trp-P-2 in the reaction mixture with pheophytin b indicates a decrease in the enzymatic formation of the *N*-hydroxy metabolite.

Discussion

From the cancer-chemoprevention point of view, it is

considered that the mutation-suppressing compounds in our daily diet may deter the onset of cancer. This idea is based on the isolation and identification of mutation-suppressing compounds from vegetables and fruits and the epidemiological data showing a correlation between dietary habits and susceptibility to carcinogenesis (Block *et al.*, 1992).

In earlier reports, it was demonstrated that Japanese eggplant extracts suppressed the mutagenicity induced by some mutagenic heterocyclic amines, and one of the compounds exhibiting desmutagenicity towards Trp-P-2 induced mutagenicity was identified as pheophytin (Yoshikawa *et al.*, 1996b). In the present study, the desmutagenicity of pheophytin b was investigated employing the *umu* test, and the speculative desmutagenic mechanism was investigated.

After the reaction mixture of Trp-P-2 and pheophytin b was heated and filtered, the filtrate was activated with the S9 mix, and the mixture was assayed to determine the remaining mutagenicity of Trp-P-2 using *S. typhimurium* TA98. The decrease in the His⁺ revertant colonies depends on the pheophytin b concentration from 1.0 nmol to 3.0 nmol per plate. The inhibition of His⁺ revertant colonies was calculated to be about 40% on addition of more than 4.0 nmol of pheophytin b per test plate (Fig. 1). Chlorophyllin, a man-made salt of chlorophyll containing either sodium or copper as the central chelating metal, has been observed to inhibit the mutagenic activity of amino- α -carboline, 2-amino-6-methyl-dipyrido[1,2a:3',2'-d]imidazol, 2-amino-6-dipyrido[1,2a:3',2'-d]imidazol, aminomethyl- α -carboline, 3-amino-3,4-dimethyl-5H-pyrido[4,3-b]indole, and Trp-P-2 in *Salmonella*/microsome assay with TA98 in the presence of S9 (Arimoto *et al.*, 1980, 1993; Dashwood *et al.*, 1991; Dashwood & Guo, 1993). In an earlier report, we observed that pheophytin b inhibits the mutagenicity of Trp-P-2 to a similar extent as that observed with chlorophyll in *Salmonella*/microsome assay with TA98 in the presence of S9 (Yoshikawa *et al.*, 1996b).

Furthermore, the binding of the electrophilic metabolite of Trp-P-2 to DNA and the subsequent induction of the SOS response of *S. typhimurium* NM 2009 were investigated using the *umu* test. Trp-P-2 induced induction of β -galactosidase was recognized to be suppressed on addition of pheophytin b in the test (Fig. 2). Slight increases in β -galactosidase activity were observed with increasing concentrations of pheophytin b. The exact cause of this effect is not clear, but it is assumed that the enhancing expression of β -galactosidase may be due to the green color of pheophytin b which shows the absorbance at 550 nm. The decrease in β -galactosidase activity suggests that pheophytin b inhibits the binding of the electrophilic metabolite of Trp-P-2 (acetylated form) to the DNA of *S. typhimurium* NM 2009, thus inhibiting the expression of the *umu* operon. This strain contains plasmid pSK 1002 having an *umu C' lac Z* fusion gene whose expression is regulated in the same manner as that of the *umu* operon (Okai *et al.*, 1996).

From the results of the Ames and *umu* tests, it is suggested that pheophytin b exhibits desmutagenicity towards Trp-P-2 induced mutagenesis by preventing the binding of the ultimate mutagenic metabolite of Trp-P-2 to the DNA. In order to further confirm the mechanism of desmutagenicity of pheophytin b, pheophytin b was allowed to react with

Trp-P-2, and HPLC analysis of the reaction mixture was carried out. Compared to the control without pheophytin b, the UV 254 nm absorbance peak corresponding to Trp-P-2 decreased in the reaction mixture containing pheophytin b (Fig. 3). The decrease in the absorbance peak indicated the decrease in free Trp-P-2 molecules. The absence of any additional new peak indicated that these two reacting molecules did not form a complex, as in the case of chlorophyll (Arimoto *et al.*, 1993). We believe that these two molecules are stacked on each other and exhibit some sort of spatial arrangement which is not detected under the present analytical conditions.

It is known that promutagenic Trp-P-2 is enzymatically biotransformed to its hydroxylated form by microsomal protein (Hashimoto *et al.*, 1980; Guengerich, 1988; Guengerich & Shimada, 1991), and the hydroxylated metabolite is further metabolized to the ultimate mutagenic electrophilic metabolite (acetylated form) by *O*-acetyltransferase (Kato & Yamazoe, 1988; Watanabe *et al.*, 1990). From the results of HPLC analysis of the pheophytin b and Trp-P-2 reaction mixture, it is thought that the suppression of His⁺ revertant colonies of *S. typhimurium* TA 98 and the reduced induction of the *umu* operon of *S. typhimurium* NM 2009 are due to the interception of Trp-P-2 by pheophytin b, hence inhibiting activation by S9. When we investigated the effects on the S9 mix mediated hydroxylation of Trp-P-2, the HPLC profile of the reaction mixture containing Trp-P-2, S9 mix and pheophytin b, the UV 254 nm absorbance peak associated with hydroxylated Trp-P-2 decreased, whereas the absorbance peak associated with Trp-P-2 alone increased, compared to that of the reaction mixture with Trp-P-2 and the S9 mix (Fig. 4). This clearly suggests that pheophytin b inhibits the formation of hydroxylated the Trp-P-2 by the inhibition of S9 mix mediated hydroxylation.

Based on these studies, it is suggested that pheophytin b exhibits desmutagenicity towards Trp-P-2 induced mutagenesis by limiting Trp-P-2 for the enzymatic metabolism to its hydroxy-form, most probably by intercepting Trp-P-2 molecules.

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