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Changes in Toxicity of *Porphyra tenera* Precontaminated with Fluoranthene During Processing

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Fluoranthene으로 오염시킨 김의 가공처리중 독성변화

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ABSTRACT

Due to increasing marine pollution there is a great possibility that seaweed is contaminated with polycyclic aromatic hydrocarbons (PAHs). To investigate the effect of processing on PAH removal from *Porphyra tenera* (laver) contaminated PAH, laver was contaminated with fluoranthene known to have a strong photoinduced toxicity, followed by washings and drying, which are usual processes for dried laver preparation. Sample at each step was collected and its toxicity was evaluated using cultured animal cells as well as analyzing PAH contents. Fluoranthene level in laver was significantly lowered by sequential washings with sea water and distilled water, but not by drying. Fluoranthene content in raw laver right after contamination was 221 ppm and decreased to 130 ppm by washings with seawater plus distilled water while its level was not further lowered by drying process. Cytotoxicity and photoinduced cytotoxicity in mammalian cells were significantly elevated in laver extracts containing fluoranthene. Cellular arylhydrocarbon hydroxylase (AHH), one of the biomarkers for cellular accumulation of PAH, was greatly induced by laver extract contaminated with fluoranthene. These results suggest that photoinduced toxicity and AHH activity can be used to monitor contamination of seafood by PAHs. Fluoranthene accumulated in laver was efficiently removed by sequential washings with seawater and tap water for 24 hrs and 12 hrs, respectively.

INTRODUCTION

Some polycylic aromatic hydrocarbons (PAHs) such as fluoranthene are easily accumulated by organisms and transformed into toxic forms when irradiated with ultraviolet (UV) light (1). For instance, it is well established that fluoranthene, one of PAHs, is highly toxic to animals in itself and its toxicity is dramatically enhanced by UV irradiation (2). Accurate evaluation of PAH toxicity for foodstuffs has been hampered by not only practical difficulty of analyzing all kinds of PAHs present in foodstuffs but the lack of knowledge about enhancement of its

toxicity by UV light. Furthermore photoinduced toxicity is variable depending on compounds. Certain PAHs in the biological tissue are much more toxic when irradiated with UV light whereas the cytotoxicities of some PAHs such as phenanthrene are not affected by UV exposure. We reported a way to evaluate photoinduced toxicities of PAHs present in foodstuffs all at once using cultured mammalian cells (3). This method has advantage of requiring relatively low labor and time in assay although it does not provide information about the kind and content of PAH.

Recent marine pollution by increased oilspill accidents poses major threat to the health of Korean population with relatively abundant consumption of marine food. The presence of PAHs in non-processed foods is associated with environmental pollution from both human and industrial activities, whereas contamination of processed foods can be caused by certain preservation and processing procedures (4). However PAH content in contaminated marine foods may be reduced during processing such as drying, washing and canning. Laver, one of the korean popular foodstuffs, has a good possibility to be exposed to oilspill as it is cultivated at littoral area where oilspill is common. It is usually processed into dry product prior to consumption. In this study laver contaminated artificially with fluoranthene were subjected to the processing for preparing dry laver and its possible hazard was evaluated by determining fluoranthene content in laver at each processing stage, photoinduced cytotoxicity and induction potential of cellular phase I enzyme of laver extracts.

MATERIALS AND METHODS

1. Samples and cells

Fluoranthene was obtained from Sigma (St Louis, MO, USA). Hepa1c1c7 cells (mouse hepatoma) were from Korean Cell Line Bank (Seoul, Korea). Laver was collected in Wando, Chonnam, Korea and stored at −70°C before use. Cell culture media and fetal bovine serum were from Gibco BRL (Gaitherburg, MD, USA).

2. Cell cultures

Hepa1c1c7 cells were plated at density of 5×10^5 cells per plate in 10 ml of α-MEM supplemented with 10% charcoal-treated fetal bovine serum (α-MEM/FBS). Cells were routinely cultured in humidified incubator in 5% CO_2 at 37°C and passaged every 3~4 days. Hepa1c1c7 cells was used in this study because of relatively ease to handle and similar characteristics in photoinduced cytotoxicity to other epithelial cells (not published).

3. Preparation of laver extract

Two kilograms of thawed laver was contaminated in fifty liter sea water containing 2 ppm fluoranthene for 2 weeks at 5°C. After contamination the aliquot of sample was alloted for the assay and rest of sample was subjected to washings with sea water and distilled water, sun-drying (5 hrs) or oven drying (45°C, 2 hrs) with forced air-flow. Sample collected at each processing stage was dried in an oven and powdered. Ten gram of dry sample powder was extracted with 200 ml of dichloromethane overnight. Dry laver extract remained after solvent removal by by rotary evaporation was redissolved in 2 ml of dichloromethane and used for the future assays.

4. HPLC analysis of fluoranthene

Laver extract (50 ul) redissolved in dichloromethane was injected into HPLC equipped with Waters 474 scanning fluoranthene detector and Supelcosil LC-PAH column (150 mm × 4.6 mm, Supelco). Fluoranthene was separated and detected in the conditions described previously (5).

5. Photoinduced toxicity of laver extracts on mammalian cells

5×10^3 cells per 96-plate well with 0.2 ml of α-MEM/FBS were plated and incubated for 4 hrs, followed by removing medium and addition of fresh medium containing 0~2 ul of laver extract dissolved in dichloromethane. Cells cultured for 24 hrs were

irradiated with UVA for 3 min using UV radiator (National Biological Corporation, Ohio, USA), and incubated until control cells not exposed to laver extract reached confluence. Cell survival rate was determined by MTT assay (6) and expressed as percentage of control cells

6. Enzyme assay

2×10^5 cells per plate were plated with 10 ml of α-MEM/FBS and incubated for 48 hrs, followed by adding the extract equivalent to 25 mg dry laver per ml medium and incubation for another 24 hrs prior to enzyme assay.

AHH activity was assayed as described previously (7). The microsomal pellet was resuspended in 30% glycerol-0.25 M potassium phosphate buffer (pH 7.25) by gentle homogenization to give a final protein concentration of 8~12 mg/ml. The one-milliliter reaction mixture included 50 umoles of postassium phosphate buffer (pH 7.25), 0.36 umoles of NADPH, 0.39 umoles of NADH, 0.1 ml of microsomal homogenate, and 80 nmol of benzo(a)pyrene (in 40 ul of methanol). The mixture was shaken at 37°C, for 10 min and the reaction was stopped with 4.25 ml of cold hexane-acetone (3.25 : 1.00). One milliliter aliquots of organic phase were added to 3.0-ml volumes of 1 N NaOH. The 3-hydroxy-benzo(a)pyrene was immediately determined spectrofluorometrically (Ex 396 nm, Em 522 nm) and relative AHH activity (%) was calculated. Protein concentrations were determined by the method of Lowry (8).

7. Effect of washing on fuoranthene removal from laver

Fifty grams of raw laver were incuabated in a solution containing 2 ppm fuoranthene at 4°C for 3 days. Contaminated laver was subjected to various washing steps where one liter of sea water or distilled water was used, and dried in a 45°C dry oven. Fluoranthene contents in dry samples were analyzed by HPLC as described above.

RESULTS AND DISCUSSION

Raw laver contaminated with fluoranthene was processed into dry laver. Fluoranthene content in laver sample after contamination was 221.3 mg/kg dry matter and decreased to 129.8 mg/kg dry matter by consecutive washings with seawater and distilled water. This result indicates that washing process can eliminate a significant amount of PAH from contaminated laver. However, drying process had little

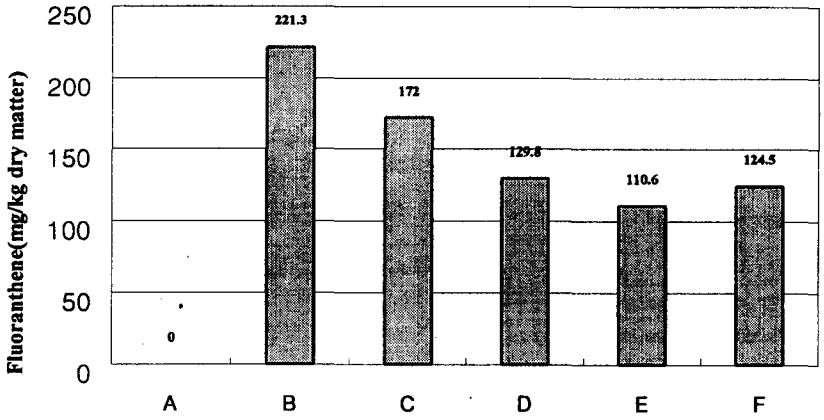

Fig. 1. Change of fluoranthene content during preparation of dry laver. Raw laver was artificially contaminated in a solution containing 2 ppm fluoranthene and processed into dry laver. Laver sample was collected and its fluoranthene content was determined by HPLC at the following processing stages; before contamination (A), after treatment (B), washed with ten volumes of seawater (C), washed with seawater plus distilled water (D), oven-dried (E) or sun-dried (F).

effect on fluoranthene content in laver. PAH, easily bioaccumulated in the organism, is known to be extremely resistant to chemical and biological removal regimens (9). But this study demonstrated that certain PAHs can be effectively eliminated by washing treatment only. Two factors, lipid and organic carbon, control to a large extent the partitioning behavior of PAHs in sediment, water, and tissue; the more hydrophobic a compound, the greater the partitioning to these phases. These two factors, along with the octanol-water partition coefficient, are the best predictors of this partitioning and can be used to determine PAH behavior and its bioavailability in the environment. It is well known that the lipid of organisms contains the highest levels of hydrophobic compounds such as PAHs, and that organic carbon associated with sediment or dissolved in water can have the greatest influence on PAH bioavailability (10).

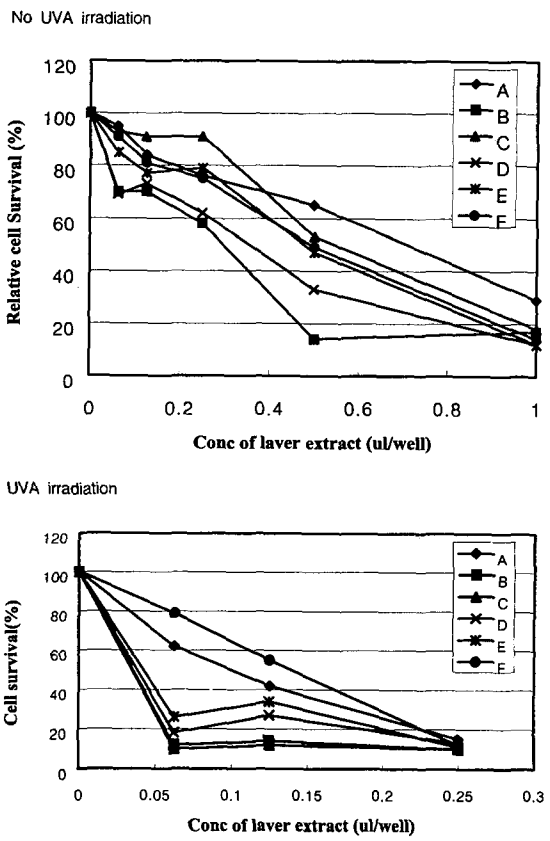

Fig. 2. Cytotoxicity of laver extract from different processing stages. Cells were incubated in the presence of 0~1 ul laver extract per well containing 200 ul media (upper graph) or preincubated in the presence of laver extract for 24 hrs and irradiated with UVA for 3 minutes (lower graph). After control cells reached confluence, cell density in 96-well plate was measured by MTT assay. Extract was prepared from laver at the following processing stages; A: before contamination (control), B: after treatment, C: washed with ten volumes of seawater, D: washed with seawater plus distilled water, E: oven-dried, F: sun-dried.

Cytotoxicity examination for extracts from laver collected at each processing stage showed that laver contaminated with fluoranthene is highly cytotoxic and its toxicity is further augmented by UVA exposure as shown Fig. 2, if cells were allowed to accumulate the substance for 24 h prior to irradiation. UV irradiation on cells in the presence of laver extract containing fluoranthene drastically reduced cell survival rate, causing about 10-fold increase in cytotoxicity. Extract from sun-dried laver showed relatively low photoinduced cytotoxicity. This may be resulted from the conversion of fluoranthene into less photosensitive metabolite (s) by sunlight. Meanwhile laver extract not contaminated with fluoranthene was

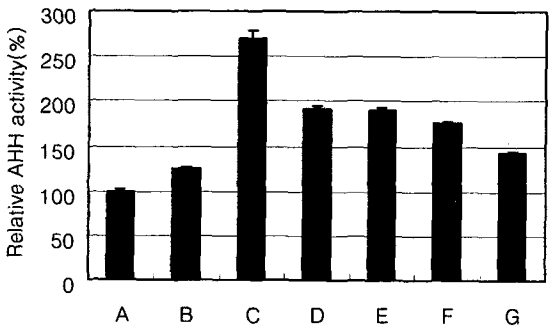

Fig. 3. Induction of cellular AHH activity by laver extract from different processing stages. Cells preincubated 48 hrs were exposed to 100 ul laver extract per 10 ml plate for 24 hrs, followed by microsomal AHH assay. Control plate (A) was grown in the absence of laver extract. A: control, B: not contaminated with fluoranthene, C: after treatment, D: washed with ten volumes of seawater, E: washed with seawater plus distilled water, F: oven-dried, G: sun-dried.

also cytotoxic although its cytotoxicity was not induced by UVA irradiation, suggesting the existence of cytotoxic natural components in laver.

Some PAHs are known to induce microsomal phase I enzyme such as arylhydrocarbon hydroxylase. Therefore induction of phase I enzyme in cells exposed to laver extract may indicate the existence of PAHs in the sample. To examine if phase I enzyme is sensitive enough to detect the amount of fluoranthene present in laver, hepa1c1c cells were incubated in the medium containing laver extract equivalent to 25 mg dry laver per ml medium for 24 hrs and subjected to micosomal AHH assay. The extract, prepared from laver contaminated with fluoranthene but not washed, induced AHH activity by 2.7 fold while extract from dry laver caused 1.4~1.7 fold induction of the enzyme. However cellular AHH activity seemed to be much less sensitive method to detect PAHs in foodstuffs than photoinduced cytotoxicity.

To examine the effect of washing on fluoranthene removal from laver, fifty gram of raw laver contaminated with fluoranthene for 24 hrs at 4°C were subjected to different washing processes, followed by the determination of fluoranthene content in oven-dried laver. The compound level was significantly decreased by elongated washing with sea water. Sequential washings with seawater and tap water resulted in further reduction in fluoranthene content of laver. That is, washing with seawater for 24 hrs in combination with tap water washing for 12 hrs removed approximately 62 percent of initial amount of the compound (Table 1).

Table 1. Removal of fluoranthene from laver precontaminated with fluoranthene by washings

Washing condition	Conc of fluoranthene (mg/kg dry laver)	Fluoranthene retained (%)
No washing	43	100
seawater, 24 hr	33	78
seawater, 48 hr	25	57
seawater, 24 hrs + tap water (6 hrs)	24	55
seawater, 24 hrs + tap water (12 hrs)	20	38

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