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Inhibition of Benzo[*a*]pyrene-induced Mouse Forestomach Neoplasia by a Principal Flavor Component of Japanese-style Fermented Soy Sauce[1]

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ABSTRACT

A refined diet supplemented with Japanese-style fermented soy sauce (shoyu) inhibits benzo[*a*]pyrene-induced forestomach neoplasia in mice (Cancer Res., *51:* 2940–2942, 1991). In the present study, soy sauce was extracted with ethyl acetate. The soluble fraction contained flavor/aroma compounds and antioxidants, whereas amino-carbonyl compounds that impart color were concentrated in the ethyl acetate insoluble fraction. Both fractions inhibited benzo[*a*]pyrene-induced forestomach neoplasia in a protocol in which the test material was fed following exposure to the carcinogen. A principal flavor/aroma component of soy sauce, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone, was fed to mice following benzo[*a*]pyrene administration and found to inhibit the subsequent development of forestomach neoplasia. 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone was effective when fed at 4 mg/kg body weight/day, indicating that it is a potent anticarcinogen.

INTRODUCTION

Japanese-style soy sauce (shoyu) is produced through a complex microbial fermentation of soy beans and wheat (1). During its manufacture, characteristic flavors and aromas develop due to microbial metabolism and reactions between various chemical substances (1, 2). The characteristic brown color results from the formation of amino-carbonyl compounds, which are products of condensation reactions between reducing sugars and amino acids (3).

Soy sauce was reported to contain substances that under acidic conditions may react with nitrite to generate direct-acting bacterial mutagens (4). It was proposed that the formation of such mutagens within the digestive tract might be a contributing factor to the high incidence of stomach cancer in Japan. Other reports, however, provide data that do not support this hypothesis (5–7).

We found that feeding Japanese-style soy sauce to mice inhibits BP[3]-induced forestomach neoplasia (8, 9). In the first study (8) using aged soy sauce, nitrite administered in the drinking water enhanced the anticarcinogenic effect. In the second study (9) using freshly prepared soy sauce, nitrite neither enhanced nor reduced the effect. It was hypothesized that the anticarcinogenic effect was due to antioxidants in soy sauce that oxidized during prolonged storage but that could be regenerated by the action of nitrite, a reducing agent without antioxidant activity.

The present investigation was undertaken to further explore the chemical components of soy sauce that are involved in mediating the observed anticarcinogenic effect. Soy sauce was extracted with ethyl acetate. The soluble fraction contained flavor/aroma compounds and antioxidants, whereas amino-carbonyl compounds that impart color were concentrated in the ethyl acetate insoluble fraction. Both fractions were fed to mice following exposure to BP and shown to inhibit the subsequent development of forestomach neoplasia.

HEMF, a major flavor/aroma component of soy sauce (2, 10), was then studied. HEMF was found to be an antioxidant. It was fed to mice following BP exposure and shown to inhibit the subsequent development of forestomach neoplasia. The data indicate that HEMF contributes to the anticarcinogenic effect of soy sauce. HEMF was effective when fed at 4 mg/kg body weight/day, indicating that it is a potent anticarcinogen in the test system.

MATERIALS AND METHODS

Materials. BP (98% pure), potassium oxalate, bathophenanthroline, EDTA, and sodium acetate were purchased from Sigma Chemical Company (St. Louis, MO). *N*-Amyl acetate and nitric acid were purchased from Aldrich Chemical Company (Milwaukee, WI). Acetic acid was obtained from Fisher Scientific (Fair Lawn, NJ). Absolute ethanol was from Aaper Alcohol and Chemical Company (Shelbyville, KY). Ferric nitrate was from Mallinckrodt, Inc. (Paris, KY). Ethyl acetate was from J. C. Baker Chemical, Inc. (Phillipsburg, NJ). HEMF, purity greater than 97%, was purchased from Tokyo Kasei Kogyo Company (Tokyo, Japan). Soy sauce (shoyu) was supplied by Kikkoman Foods, Inc. (Walworth, WI); it contained no added antioxidants or other added ingredients.

Extraction of Soy Sauce. Three hundred ml of soy sauce were extracted twice with ethyl acetate (400 ml × 2). The soluble fraction was mixed with anhydrous sodium sulfate, filtered, and subjected to vacuum at room temperature to remove the ethyl acetate. The residue was then dissolved in 300 ml distilled water thus bringing it to a volume equivalent to the original starting volume. The insoluble fraction was also subjected to vacuum at room temperature to remove ethyl acetate, then brought to 300 ml with distilled water. Both fractions were filtered through sterile Acrodisc filters (0.45 μm; Gelman Science, Ann Arbor, MI) and then stored at 5°C. The procedure was repeated until 30 liters of each fraction were produced. Limited analysis of the ethyl acetate soluble fraction was accomplished by high performance liquid chromatography using a Beckman system (Arlington Heights, IL). The conditions were as follows: Altex Ultrasphere-ODS reversed phase column (10 × 25 cm); water/acetonitrile (0 to 100%) gradient; flow rate 3.0 ml/min at room temperature; dual detection at 230 and 285 nm.

Determination of Antioxidant Activity. Antioxidant activity was determined by the method described by Tsen (11). Bathophenanthroline/iron reagent was mixed with each sample. The absorbance of the top pink layer was measured in a Beckman DU-7 spectrophotometer at 534 nm.

Animals. One hundred fifty (experiment 1) and 120 (experiment 2) 5-week-old female ICR mice were purchased from Sprague-Dawley (Madison, WI). The mice were housed (5/cage) in plastic cages in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Bedding material (pine shavings) was changed twice weekly. Semipurified diet (TD86348; Teklad Test Diets, Madison, WI) and

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[3] The abbreviations used are: BP, benzo[*a*]pyrene; HEMF, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (tautomer ratio about 3:2).

water were available *ad libitum*; food cups were changed 3 times/week. Since food spillage was minimal, food intake was determined by measuring the amount of food that remained in a cup and then dividing the amount by the number of mice in the cage. Body weights were determined by weighing tared cages and dividing by the number of mice per cage.

Induction of Forestomach Neoplasia. Forestomach neoplasia was induced in a similar manner to that described previously (8). At 9 weeks of age, the animals were given by stomach tube 1.5 mg BP in 0.2 ml corn oil. This dose was repeated once a week for 4 weeks. After the last BP dose, the animals were switched to an experimental diet. In experiment 1, the mice were fed the semipurified diet supplemented with the ethyl acetate soluble fraction (10–30%) or the ethyl acetate insoluble fraction (20%); control diet contained 20% water. In experiment 2, the diet contained HEMF (0–75 ppm) dissolved in water (a constant level of 4 parts diet to 1 part water was maintained). Surviving animals were killed at 211 days of age by CO_2 suffocation. The stomachs were removed and fixed in an expanded state produced by i.g. injection of 4% formalin; 24 h later, the stomachs were split longitudinally. Tumors of the forestomach that were 1 mm or larger were counted under a dissecting microscope. Subsequently the tumors were confirmed histologically.

Statistical Methods. Data are expressed as means ± SEM and analyzed for statistical significance using Student's *t* test.

RESULTS AND DISCUSSION

Soy sauce was extracted with ethyl acetate. The soluble fraction contained considerable antioxidant activity (Table 1), in agreement with our previous findings (9). It was also apparent that this fraction contained flavor/aroma compounds characteristic of soy sauce (2). The soluble fraction was chemically characterized to a limited extent by high performance liquid chromatography (for example, a substance with the retention time and UV spectral characteristics of HEMF was observed) (data not shown).

The ethyl acetate insoluble fraction contained a much greater mass than the soluble fraction but considerably less antioxidant activity (Table 1). Amino-carbonyl compounds, which impart a dark color to soy sauce (3), were concentrated in this fraction. The insoluble fraction was not subjected to further chemical analysis.

Fig. 1 shows the incidence of neoplasms, and neoplasms per animal, for control mice and mice fed diets containing the ethyl acetate soluble and insoluble fractions from soy sauce. Both fractions were inhibitory. The data of Fig. 2 confirm that for the ethyl acetate soluble fraction, food intake and body weight were not affected by the feeding protocol. Hence, the anticarcinogenic effect was due to the specific action of one or more chemical constituents in the fraction rather than to hypothetical effects on calorie intake or utilization. For the ethyl acetate insoluble fraction, this issue is less clear since there appeared to be a small but significant decrease in body weight (but not in food intake) among treated animals.

A major flavor/aroma component of Japanese-style fermented soy sauce is HEMF (Fig. 3) (2, 10). It is present in soy sauce at a level of 230 ppm (12). Synthetic HEMF was assayed for antioxidant activity and compared with soy sauce, the ethyl acetate soluble and insoluble soy sauce fractions, and ascorbic acid. Table 1 shows the results from a representative experiment. HEMF displayed greater antioxidant activity than ascorbic acid and appears to account for a substantial fraction of the antioxidant activity of soy sauce.

We tested HEMF for anticarcinogenic activity. As shown in Fig. 4, HEMF is an effective inhibitor of BP-induced mouse

Table 1 *Antioxidant activity of soy sauce, soy sauce fractions, and HEMF*[a]

Sample	Nano-equivalents[b] /ml sample	Wt (mg solids/ml)	Specific activity[c] (nano-equivalents /mg)
Soy sauce	64.6	358	0.18
Ethyl acetate-soluble soy sauce fraction	46.7	1.7	27.8
Ethyl acetate-insoluble soy sauce fraction	32.3	301.2	0.1
HEMF, 250 ppm in water	3.8	0.25	15.2
Ascorbic acid, 10,000 ppm in water	101.0	10.0	10.1

[a] Reported values are the means of duplicate assays.
[b] Nanoequivalents of antioxidant activity.
[c] Specific activity, nanoequivalents of antioxidant activity/mg solids.

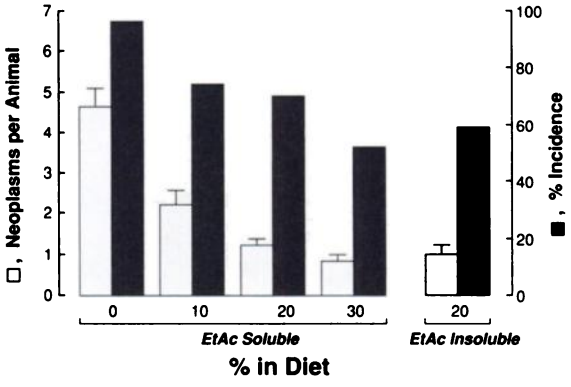

Fig. 1. Inhibition of BP-induced forestomach neoplasia by the ethyl acetate-soluble and -insoluble soy sauce fractions. For neoplasms per animal, all treatment groups were significantly different from the untreated control group ($P \leq 0.05$). Data are expressed as means with SEM shown where they exceed the size of the symbols. n = 26–27 mice/group.

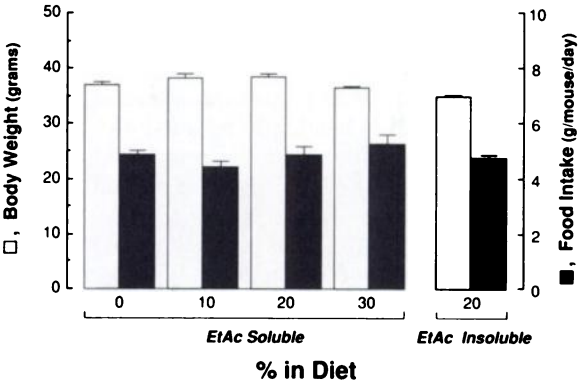

Fig. 2. Body weights and food intake for animal subjects of Fig. 1. Except for the body weights of animals fed the ethyl acetate (EtAc)-insoluble fraction, none of the body weight or food intake data for treated animals are significantly different from those of their respective controls ($P \leq 0.05$).

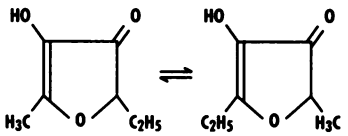

Fig. 3. HEMF, a major flavor/aroma component of Japanese-style fermented soy sauce. Both tautomeric forms are shown.

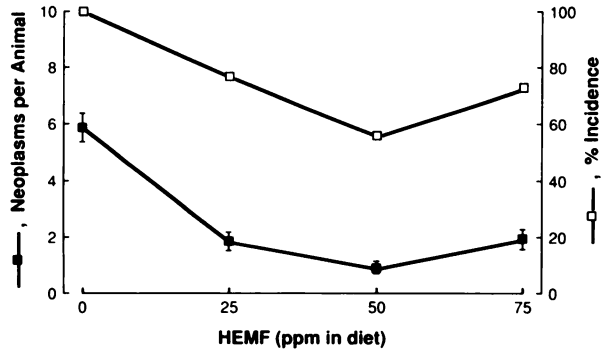

Fig. 4. Inhibition of BP-induced forestomach neoplasia by HEMF. For neoplasms per animal, all treatment groups were significantly different from the untreated control group ($P \leq 0.05$). Data are expressed as means with SEM shown where they exceed the size of the symbols. $n = 25$–27 mice/group.

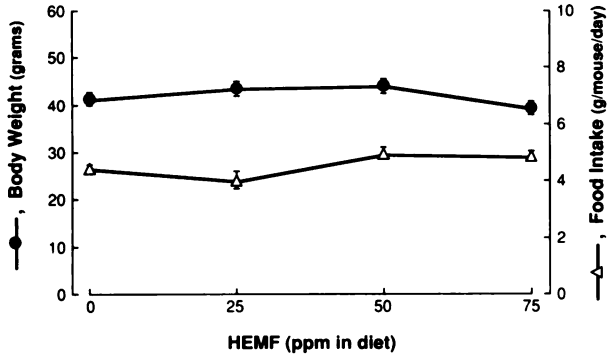

Fig. 5. Body weights and food intake for animal subjects of Fig. 4. None of the data for treated animals are significantly different from those of their respective controls ($P \leq 0.05$).

forestomach neoplasia. A dietary level of 25 ppm HEMF was sufficient to substantially reduce the incidence of neoplasia and the number of neoplasms per mouse. This amounts to a daily HEMF intake of about 4 mg/kg body weight, indicating that in the test system HEMF is a potent anticarcinogen. By comparison, the synthetic antioxidant 2-tert-butyl-4-hydroxyanisole was reported (13) to reduce the number of BP-induced mouse forestomach neoplasms by 75% when fed at 5400 ppm (about 1 g/kg body weight).

The data of Fig. 5 confirm that the addition of HEMF to the diet had no effect on food intake or body weight. Hence, the anticarcinogenic effect of HEMF was not due to calorie restriction.

Soy products are reported to contain several factors that are known to inhibit cancer in experimental animals, *e.g.*, protease inhibitors (14), or postulated to be effective in this regard, *e.g.*, phytoestrogens (15, 16). Based on the data of Figs. 4 and 5, the list of known inhibitors can now be expanded to include HEMF. Moreover, it is very likely that other antioxidant anticarcinogens are present in Japanese-style fermented soy sauce as well.

A central question concerns the possible mechanism of inhibition of carcinogenesis by HEMF and the other anticarcinogens that may be present in soy sauce. The animal challenge protocol that was employed involved feeding the test material after exposure to BP. Hence, inhibition was at the stage of tumor promotion. This observation has important implications since inhibitors of tumor promotion are likely to be effective against neoplasia induced by a range of initiating carcinogens (17). We are currently investigating the validity of this conclusion in experiments aimed at determining the biochemical basis for the mechanism of inhibition of tumor promotion by HEMF.

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