Bioluminescent Bacterial Genotoxicity Test for Fatty Acid Derivatives and Heated Oils

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The Mutatox test (the commercial name for a bioluminescent bacterial genotoxicity test), which has been proved to be a good alternative to the Ames test, was used in this study to test the genotoxicity of fatty acid derivatives and heated oils. This test allows either pure or complex compounds to be rapidly screened for genotoxicity. Five different column fractions isolated from autoxidized methyl linoleate or autoxidized methyl linolenate were not genotoxic with or without the activation by the S-9 (microsomal) fraction. Chlorinated fatty acids including chloroethyl caprate, laurate, palmitate, and linoleate were genotoxic in the presence of S-9 activation. When heated at 180 °C, after 32 h, soybean oil exhibited genotoxic activity with or without the S-9 activation. The addition of 1% cholesterol into the soybean oil increased the onset of the genotoxicity during heating.

Keywords: *Mutatox test; genotoxicity test; fatty acid hydroperoxides; chlorinated fatty acids*

INTRODUCTION

The possibility that consumption of oxidized fats may be detrimental to health has stimulated extensive research about the toxicity of lipid oxidation products. Cortesi and Privett (1972) found that methyl linoleate hydroperoxide, when injected intravenously in the rat at a level of 30 mg/100 g body weight, killed the animal within 24 h. An investigation (Cutler and Schneider, 1973) was made of the embryotoxicity in mice of purified linoleic acid, oxidized linoleic acid, or purified linoleic acid hydroperoxide applied directly to the ovaries. After the treatment with linoleic acid hydroperoxide, an increase in fetal malformations occurred in litters of the first generation, and second-generation litters showed an increase in embryonic resorptions. The incidence of malformations after treatment with linoleic acid was similar to that occurring in the group of untreated controls. Oxidized linoleic and linolenic acids, their purified monohydroperoxides, and secondary oxidation products have been shown to be weakly mutagenic in the Ames test (Yamaguchi and Yamashita, 1979, 1980; MacGregor et al., 1985). However, studies by Scheutwinkel-Reich et al. (1980) and Gardner et al. (1983) did not support the mutagenicity of fatty acid hydroperoxides.

Toxicity studies in animals have been conducted on fats oxidized for long periods at very high temperatures. Fat subjected to such extreme processing conditions caused severe irritation of the gastrointestinal tract, growth retardation, and death in experimental animals (Kubow, 1990). Some researchers have also found elevated liver and kidney weights, cellular damage in various organs, and altered fatty acid composition of tissue lipids after administration of heated oils and fats subjected to normal usage (Alexander et al., 1987). There has been considerable concern over the mutagenic potential of continually reused fats. Studies by Taylor et al. (1983), Hageman et al. (1988), and Saleh et al. (1986) indicated that repeatedly used frying fats were mutagenic in the Ames test.

In recent years, much attention has been focused on the degradation products of cholesterol. Several cholesterol autoxidation products have undesirable biological effects, which were related to feedback inhibition of cholesterol biosynthesis, cytotoxicity, angiotoxicity, mutagenicity, and carcinogenicity (Smith, 1981; Finocchiaro and Richardson, 1983; Maerker, 1987). Substantial amounts of cholesterol oxidation products have been detected in deep-fried foods, dehydrated milk, and egg products (Addis, 1986; Nourooz-Zadeh and Appelqvist, 1987). Hubbard et al. (1989) conducted both in vivo and in vitro studies with products of oxidized cholesterol. They found that the oxidation products of cholesterol are highly toxic to cultured aortic smooth muscle cells of the rabbit and can induce arterial injury with 24 h of administration by gavage to rabbits. An investigation of the effect of feeding oxysterols at levels estimated to be the average U.S. dietary intakes was conducted by Jacobson et al. (1985). The effect of oxysterol feeding in White Carneau pigeons was a five times increase in coronary artery atherosclerosis after three months, compared with birds given pure cholesterol. Some of the products of autoxidized cholesterol and cholesterol hydroperoxides have been shown to be weakly mutagenic in the Ames test (Ansari et al., 1982).

Although the Ames test has been widely used as a screening test, alternative tests for genotoxicity have been developed. The bioluminescence test (BLT) for genotoxic agents uses dark mutants of luminous bacteria to determine the ability of the tested agent to restore luminescence by inducing mutation (Ulitzur, 1986). The restored luminescence can be measured by

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using photometric instrumentation. Sun and Stahr (1993) reported that results obtained for some mycotoxins and heterocyclic amines with the Mutatox test, a commercial version of the BLT, agreed well with results that had been obtained with the *Salmonella*/mammalian microsomal test and other genotoxicity tests. The Mutatox test can be performed rapidly, and by serially diluting the compound being tested, dose response data plus toxicity data can be generated for a number of samples simultaneously (Sun and Stahr, 1993).

In the current study, we used the Mutatox system, directly and with the S-9 activation system, to screen several food-lipid related systems or compounds. Our goal was to further elucidate the genotoxicity of some materials that can be derived from food lipids during processing and storage. Specifically, we sought to determine whether the products of oxidized fatty acids, the 2-chloroethyl esters of fatty acids, and soybean oil heated with and without added cholesterol are genotoxic with the Mutatox system.

EXPERIMENTAL PROCEDURES

Mutatox Test. The Mutatox test, which was developed by Microbics Corp. (Carlsbad, CA), is the commercial version of the bioluminescent bacterial genotoxicity test described by Ulitzur (1986). The Microbics Corp. provided all the reagents that were needed for the Mutatox test which included the bacterial culture and assay medium. The Mutatox test for each sample was conducted according to the protocol described by Sun and Stahr (1993).

Preparation of Autoxidized Fatty Acids. Methyl linoleate (18:2) and methyl linolenate (18:3) (95%) were purchased from Sigma Chemical Co. (St. Louis, MO). Methyl linoleate and linolenate (5 g of each) were autoxidized in a water bath at 37−40 °C in the dark by bubbling air into the fatty acids for 144 and 72 h, respectively. The autoxidized fatty acid sample was fractionated on a silica gel column (2 × 15 cm) with diethyl ether/hexane eluants into five different fractions. Fraction one was eluted with 200 mL of 1:9 diethyl ether/hexane. The following oxidation products were then eluted with 100 mL portions of diethyl ether/hexane mixtures of the volume proportions indicated: fraction 2 (2:8), fraction 3 (3:7), fraction 4 (5:5), and fraction 5 (7:3). The remaining oxidation products were eluted with 100% diethyl ether. The eluted solvent mixture was evaporated by vacuum evaporator. The fractions were redissolved in methanol, at concentrations ranging from 0.001 to 50 μg/mL. Samples were stored at −20 °C until tested for genotoxicity. For every genotoxicity test, pure fatty acid was always prepared as a control and methanol was used as a solvent control.

Chlorinated Fatty Acids. The chlorinated fatty acids, which include 2-chloroethyl esters of capric, lauric, palmitic, and linoleic acids, were isolated and provided by Dr. D. L. Heikes. The chlorinated fatty acids were dissolved in methanol to give concentrations ranging from 10 to 100 μg/mL.

Heated Oil Samples. Soybean oil was purchased from a local retail grocery store in Ames, IA. The cooking oil (100 mL) was heated at 180−190 °C in a beaker on a hot plate for 8 h each day on 5 consecutive days. Aliquots of the heated cooking oil were taken out at the end of heating on each day. The oil aliquots were dissolved in acetone to give concentrations ranging from 0.01 to 150 μg/mL. The samples were stored at −20 °C until tested. For each genotoxicity test, a fresh oil sample without heating was always prepared as a control. In a separate experiment, 1% (w/v) pure crystalline cholesterol (Sigma, Grade I) was dissolved in the cooking oil, which was then treated as described for the soybean oil.

RESULTS

There are two systems that have been designed for the Mutatox test: (1) direct assay, tested chemicals without the activation by S-9 fraction, and (2) S-9 assay, tested chemicals with activation by S-9 fraction. The positive controls selected for the direct assay were 3,6-diaminoacridine (proflavine) or *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG or NTG), while for the S-9 assay they were benzo[*a*]pyrene (B(a)P) or aflatoxin B_1 (AFB_1). From the experiments with proflavine, MNNG, B(a)P, and AFB_1, the positive controls were established for further study with other chemicals to be tested. Table 1 summarizes the results of all the positive controls.

Table 1. Genotoxicity of Proflavine, MNNG, Aflatoxin B_1, and B(a)P

chemical	direct assay	S-9 assay	concentration range for positive response (μg/mL media)	toxic concentration (μg/mL media)
proflavine	+	NT[a]	0.3−7.0	9.0
MNNG	+	NT	0.03−0.10	0.3
aflatoxin B1	−	+	0.10−5.00	8.0
B(a)P	−	+	0.001−0.700	1.0

[a] NT = not tested.

The whole autoxidized and unoxidized (pure) methyl linoleate and linolenate as well as five different fractions from each autoxidized sample acid were used for the Mutatox test. None of the compounds or fractions tested exhibited genotoxic activity for either the direct or S-9 assay. The toxic concentrations (concentration at which the chemical inhibits bacterial growth) were as follows: 15 μg/mL of media for the whole autoxidized fatty acid methyl esters and also for fraction 2 and 10 μg/mL of media for fractions 3, 4 and 5. Within the concentration ranges tested (0.001−50 μg/mL media), toxic concentations were not determined for the pure fatty acid methyl esters or fraction 1. On the basis of the report of Neff et al. (1981), the fractions might be expected to contain the following components: fraction 1, unoxidized methyl esters of the fatty acids; fraction 2, epoxy compounds; fraction 3, a mixture of hydroperoxides; fraction 4, a mixture of hydroperoxides and hydroperoxy-cyclic compounds; and fraction 5, unidentified polar compounds. We used thin-layer chromatography and gas chromatography/mass spectrometry to further verify the identity of components in the tested fractions. The characteristic masses obtained suggest that the fractions may contain the components noted above. Because none of the fractions tested positive for genotoxic materials, no further efforts were made to identify the specific components of the fractions.

As shown in Table 2, none of the four chlorinated fatty acids were genotoxic in the direct assay. However, in the presence of S-9 activation, all samples showed genotoxic activity between 10 and 100 μg/mL. Also, the toxic concentrations for both direct and S-9 assays for all chlorinated fatty acids were 150 μg/mL.

Table 3 summarizes the results of Mutatox test of heated oil. The fresh soybean oil without any heating and soybean oil heated for 8, 16, and 24 h gave negative responses for both direct and S-9 assays. After 32 and 40 h of heating, the soybean oil showed a positive response for genotoxic activity for both the direct and S-9 assays. For these samples, the concentration range for positive response was between 1 and 75 μg/mL media, while the toxic concentration was 100 μg/mL. The oil with 1% cholesterol responded positively to both direct and S-9 assays in the Mutatox test after 16 h of heating (Table 4). A positive response was obtained from 0.5 to 40 μg/mL, and the toxic concentration was

Table 2. Genotoxicity of Chlorinated Fatty Acids

chemical	direct assay	S-9 assay	concentration range for positive response (μg/mL media)	toxic concentration (μg/mL media)
2-chloroethyl caprate	−	+	10−100	150
2- chloroethyl laurate	−	+	10−100	150
2-chloroethyl palmitate	−	+	10−100	150
2-chloroethyl linoleate	−	+	10−100	150

Table 3. Genotoxicity of Heated Cooking Oil

hours of heating	direct assay	S-9 assay	concentration range for positive response (μg/mL media)	toxic concentration (μg/mL media)
0	−	−	ND[a]	ND
8	−	−	ND	ND
16	−	−	ND	ND
24	−	−	ND	120
32	+	+	1−75	100
40	+	+	1−75	100

[a] ND = not determined within the concentration range used.

Table 4. Genotoxicity of Heated Cooking Oil with Addition of 1% Cholesterol

hours of heating	direct assay	S-9 assay	concentration range for positive response (μg/mL media)	toxic concentration (μg/mL media)
0	−	−	ND[a]	ND
8	−	−	ND	ND
16	+	+	0.5−40	100
24	+	+	0.5−40	120
32	+	+	0.5−40	100
40	+	+	0.5−40	100

[a] ND = not determined within the concentration range used.

100 μg/ mL for oil heated for 16, 32, and 40 h and 120 μg/mL for oil heated for 24 h.

DISCUSSION

Sun and Stahr (1993) reported that results obtained with the Mutatox test agreed well with the results of the *Salmonella*/mammalian microsome test and other genotoxicity tests for several myctotoxins and heterocyclic amines. Our results also demonstrate the utility of this test for complex systems, as well as pure compounds. We demonstrated that soybean oil heated at 180−190 °C became genotoxic after 32 h of heating, while oil with 1% cholesterol became genotoxic after 16 h of heating. Genotoxicity was demonstrated both with the direct assay and with the S-9 system. The heat treatment given to oils in our experiment was similar to the standard frying conditions described by Hageman et al. (1990). Hageman et al. (1990) found that polar fractions of the repeatedly used frying fat significantly increased the number of revertants in *Salmonella typhimurium* strain TA 97 without the S-9 mix. In a previous study (Hageman et al., 1988), frying fat sampled at restaurants and tested by the same protocols also was found to have significant mutagenic activity. Our results with a different test system provide confirmation that genotoxic materials are developed in oil that has been repeatedly heated at temperatures suitable for deep-fat frying.

The identity and significance for human health of the mutagens formed in frying fats remains to be established. Hageman et al. (1990) suggested that while the nature of the mutagens formed during deep-fat frying is not known, polymerized oxidation and degradation products of fatty acids are not likely to be responsible for the observed mutagenic activity. Other lipid oxidation products, including lipid hydroperoxides, have been tested for direct mutagenic effects in several systems, with varying results. Yamaguchi and Yamashita (1979) used the Ames test to demonstrate the weak mutagenicity of peroxidized fatty acids under certain test conditions. They also reported that the hydroperoxide fractions of peroxidized methyl linoleate and methyl linolenate showed mutagenicity at lower concentrations than did the crude preparations (Yamaguchi and Yamashita, 1980). Because cumene hydroperoxide and *tert*-butyl hydroperoxide were also found to be mutagenic, while peroxides, peracids, and hydrogen peroxide were not, Yamaguchi and Yamashita attributed the mutagenicity to the hydroperoxide group. However, Scheutwinkel-Reich et al. (1980) failed to find mutagenicity for linoleic acid hydroperoxide in the Ames test. At least a partial explanation may be due to differences in procedures. Yamaguchi and Yamashita (1979) reported that autoxidized linolenic acid did not elicit increased reversion rates unless the fatty acid was sonicated with either detergent or protein. On the basis of these results, a negative response would be predicted for tests, including those conducted by Scheutwinkel-Reich et al. (1980) and in the current studies, not using these procedures.

Contradictory results have also been observed when examining the mutagenicity of fatty acid epoxides by using the Ames test. Gardner et al. (1983) suggested that the epoxide fatty esters were not mutagenic at concentrations up to 2000 mg/test plate even though these esters had structural characteristics similar to certain potent mutagens. On the other hand, MacGregor et al. (1985) found that relatively high levels of epoxide fatty esters, as well as other pure oxidation products, were weakly mutagenic in strains TA97 and/ or TA100.

Our results with a bioluminescent genotoxicity test failed to reveal mutagenicity of oxidized fatty acid methyl esters or any of the column fractions from oxidized fatty acid methyl esters. While different assay procedures may account for the apparently contradictory results obtained in various studies, taken overall, the evidence to date indicates at most rather weak direct mutagenicity for fatty acid hydroperoxides and epoxides. Further clarification of the roles of these and other oxidative and thermal degradation products in the genotoxicity of heated oils is needed.

Our results indicate that genotoxic materials were formed when cholesterol was heated in soybean oil for 16 h at 180−190 °C, while soybean oil heated alone was not genotoxic at 16 h. The mutagenic activity of products of oxidized cholesterol has been demonstrated with the *Salmonella* test system (Smith et al., 1979; Ansari et al., 1982; Watanabe et al., 1988). Smith et al. (1979) heated cholesterol at 70 °C for several weeks. Watanabe et al. (1988) used temperatures ranging from 25 to 225 °C and reported that, with 7 h of heating, mutagens were detected only above 150 °C. These

results, taken together with ours, suggest that the temperatures used in deep-fat frying operations may be adequate to produce mutagens from cholesterol after heating for 8 to 16 h. Watanabe et al. (1988) did not incorporate the S-9 mixture into their test system and suggested that there might have been some cholesterol degradation products that were undetected with this assay system. We did not demonstrate differences in genotoxicity with and without the S-9 system. Further evaluation of the role of cholesterol as a precursor for genotoxic materials in foods is needed, as is assessment of the impact of these materials on human health.

The 2-chloroethyl esters of fatty acids have been identified in spice and food samples by using gas−liquid chromatography−mass spectrometry (Heikes and Griffitt, 1979). Chloroethanol (ethylene chlorohydrin) has been found in whole and ground spices after they were fumigated with ethylene oxide. The fumigant combines with moisture and natural inorganic chloride to form the corresponding chlorohydrin (Wesley et al., 1965). It has been postulated that the 2-chloroethyl esters were the product of the reaction of 2-chloroethanol and the natural fatty acids. The chlorinated fatty acids tested positive in the S-9 assay system. We believe that this is the first study conducted to evaluate the genotoxicity of these esters. Further evaluation of their impact on human health is needed.

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