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Antimutagenic Effects of Doenjang(Korean Soy Paste)

Kun-Young Park†, Suk-Hee Moon*, Hong-Sik Cheigh and Hyung-Suk Baik**

Dept. of Food Science and Nutrition, Pusan National University, Pusan 609-735, Korea
**Dept. of Food and Nutrition, Kyungnam Junior College, Pusan 617-701, Korea*
***Dept. of Microbiology, Pusan National University, Pusan 609-735, Korea*

Abstract

Antimutagenic effect of Doenjang(Korean soy paste) on various carcinogens in *Salmonella typhimurium* strains of TA98 and TA100 were studied. By the addition of methanol extract of Doenjang to aflatoxin B_1 (AFB_1) in the experimental system, the mutagenicity of AFB_1 on the strains of TA98 and TA100 was completely inhibited. The methanol extract of the Doenjang also inhibited the mutagenicities induced by direct mutagens such as N-methyl-N'-nitro-N-nitroguanidine(MNNG) and 4-nitroquinoline-1-oxide(4-NQO), and another indirect mutagens of benzo(a)pyrene(BaP) and dimethylnitrosamine(DMN). From the solvents and thin layer chromatographic(TLC) fractionations, free fatty acid(s), especially linoleic acid in Doenjang seemed to be one of the active antimutagenic compounds.

Key words: antimutagenicity, Doenjang, free fatty acid

INTRODUCTION

Soybean is an important protein source for oriental Doenjang(fermented soy paste) is one of the most important fermented foods in Korea. Doenjang has been manufactured for centuries at home by traditional ways in which natural microflora, especially storage fungi in addition to *Bacillus subtilis*, participate in the fermentation. Much concern had been centered on Doenjang manufactured this way because of the possible contamination of mycotoxins, particularly aflatoxins.

Aflatoxin B_1(AFB_1) is one of the most potent carcinogens or mutagens, and is known to be produced by *Aspergillus flavus* and *Aspergillus parasiticus* as a secondary metabolite when they are contaminated in food or feed stuffs(1). Crane et al.(2) reported that the high incidence of stomach cancer in Koreans is probably due to the aflatoxin contaminated Doenjang.

Aflatoxins could be produced on Meju during fermentation of the Doenjang manufactured traditionally when *A. parasiticus* inoculated on the Meju cake on purpose(3,4). However, the toxins were degraded almost completely during the long period of ripening due to some factors such as NH_3 production, malanoidin color formation and charcoal addition etc.(5,6). Thus Doenjang does not seem to have any significant harmful effect because of the possible contamination by aflatoxins(3).

Many studies indicated that adequate nutritions protect significantly against the carcinogenesis(7). It was reported that some of the components of soybean, especially trypsin inhibitor, isoflavones, saponin and phytic acid etc. showed anticancer functions(8-12). So high consumption of Miso(Japanese soy paste) decreased the rate of death from the incidence of stomach cancer(13).

In this study, we hypothesized that Doenjang might have antimutagenic effect rather than exhibiting mutagenic activity. Thus the effect of Doenjang was investigated for its possible antimutagenic activity on the mutagenesis of AFB_1 on *Salmonella typhimurium* strains TA98 and TA100 in the Ames test. The antimutagenic activities of Doenjang were also evaluated on the mutagens such as N-methyl-N'-nitro-N-nitroguanidine(MNNG), 4-nitroquinoline-1-oxide(4-NQO), benzo(a)pyrene(BaP) and dimethylnitrosamine(DMN) mediated mutagenicities. And the antimutagenic compound(s) found in Doenjang were tentatively identified by solvent and TLC fractionations.

MATERIALS AND METHODS

Preparations of Doenjang by traditional method

Raw soybeans(var. *Namcheon*) were obtained from

†Corresponding author

the Research Center of Korea Agriculture Development, Suwon, Korea. the manufacturing methods of traditional Doenjang is diagrammed in Fig. 1(14). Soybeans were selected, washed and soaked in water overnight. The soaked raw soybean was autoclaved for 2~3 hrs at 100°C. The cooked soybeans were cooled to 40°C and crushed in a mortar. The paste was molded into cubical shapes, called Meju cake, and the cakes were dried in the sun during the daytime and storing at room temperature in the night. The dried Mejues were stored for 2 months hanging from a ceiling with straw rope during which resulting in microbial growth on the surface of the Meju. Salt and water were added to the fermented Meju. The mixed proportion was about 1 part of Meju, 1 part of NaCl and 2 parts of water. The Meju-brine mixture was ripened for 3 months. The paste was separated from the mixture and then ripened 3 months and used as Doenjang samples.

Preparations of solvent extracts from Doenjang

Doenjang was ground and extracted in methanol(1 : 10g/v) for 7hrs three times. Each extract was separated by centrifugation at 10,000rpm for 10min. The residues were further extracted by chloroform-methanol mixture(1 : 1 v/v) and separated by the centrifugation (15). The extracts were dried by using vacuum evaporator(Büchi RE121, Switzerland) and then transferred to vials, and dissolved in DMSO to the same level of original weight of Doenjang(100%).

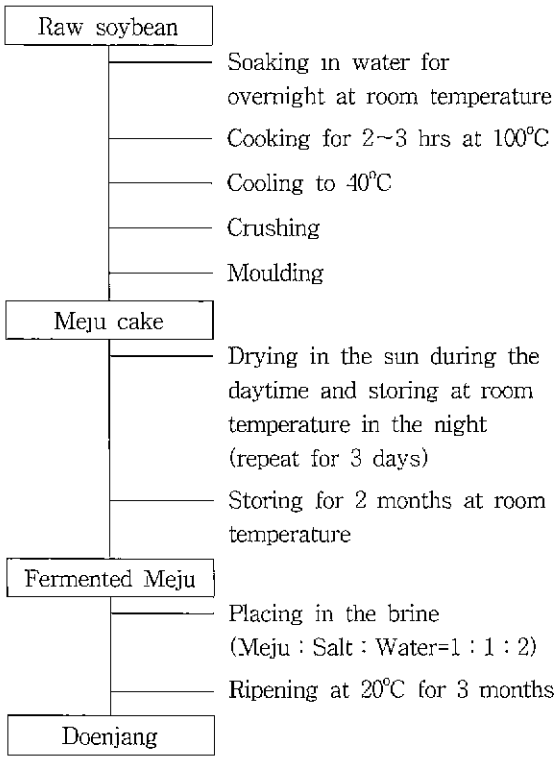

Fig. 1. Preparations of Doenjang(Korean soy paste) by traditional method.

Mutagens/Carcinogens

AFB_1, MNNG, 4-NQO, BaP and DMN were employed as mutagens/carcinogens for this study.

AFB_1, BaP and DMN were purchased from Sigma Chemical Co., St. Louis, Mo, USA. The standard AFB_1 solution was prepared according to AOAC method(16). AFB_1 was weighed and dissolved in methanol to make 250ppm solution. The appropriate amount of the standard solution was taken, dried and dissolved in spectrophotometric dimethylsulfoxide(DMSO, Aldrich Co., Milwaukee, WI, USA) for the mutagenicity test. The appropriate amounts of BaP and DMN were weighed and then dissolved in the DMSO and 95% methanol, respectively.

MNNG and 4-NQO were obtained from Aldrich Chemical Co., Milwaukee, WI, USA. The mutagens were dissolved in distilled water and 95% ethanol, respectively.

Ames mutagenicity test

Bacterial strains:

Salmonella typhimurium strains TA98 and TA100, histidine requiring mutants, were provided by Dr. B.N. Ames, Univ. of California, Berkeley, CA, USA and were maintained as described by Maron and Ames(17). The genetypes of tester strains were checked routinely for their histidine requirement, deep rough(*rfa*) character, UV sensitivity(*uvr* B mutation) and for the presence of R factor.

S9 fraction and S9 mix:

Sprague-Dawley male rats and Syrian hamsters were injected intraperitoneally with Aroclor 1254 dissolved in corn oil(500mg/kg of body wt.). Five days after the injections, the rats/hamsters were sacrified, livers were removed and minced in 0.15M KCl, and then homogenized with a Potter-Elvehjem apparatus. The homogenates were centrifuged at 9000g for 10min in a refrigerated centrifuge and the supernatant S9 fraction was distributed in 1.8~2.0ml portions in Nunc tubes, and stored at -80°C until used for mutagenic studies. In order to prepare the S9 mix, S9 fraction was thawed

immediately before being used for the preparation of S9 mix. Ten percent of S9 fraction in S9 mix was used as S9 mix for the experiment.

Antimutagenicity test:

Plate incorporation test was performed to determine the mutagenic activities of MNNG and 4-NQO(17). A modified plate incorporation test(18) in which 30min liquid preincubation of the organisms with the test compounds was employed to determine the antimutagenic effects of Doenjang extract on mutagenesis of AFB_1, BaP and DMN. In the preincubation test, 0.5ml of S9 mixture. was distributed in sterile capped tubes in ice bath and then 0.1ml of testers from overnight culture($1\sim2\times10^9$ cells/ml) and 0.1ml of test compounds were added. The tubes were vortexed gently and preincubated at 37°C for 30min. Two ml of the top agar in each tube kept at 45°C were added and vortexed for 3 seconds. The resulting entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 hrs and then the revertant bacterial colonies on each plate were counted. Dose response tests of the mutagens on the tester strains were carried out to determine the regions of revealing mutagenicity induced by the mutagens. Toxicity and mutagenicity tests for the different levels of Doenjang extracts and fractions were also carried out(17) and the Doenjang samples for the mutagenic test in this study did not show any toxicity and mutagenicity to the tester strains.

Tentative identification of antimutagenic compound(s) from methanol extracts of Doenjang

Solvent fractionation of methanol extracts from Doenjang:

Methanol extracts of Doenjang were fractionated by sequential extractions using hexane, chloroform, ethylacetate and butanol(Fig. 2). Each extraction was repeated for 3 times and dissolved to dimethylsulfoxide (DMSO) for the mutagenicity test.

Further fractionation of hexane fraction from methanol extract of Doenjang:

To determine the antimutagenic active compound(s) of Doenjang, the active hexane fraction of methanol extract from Doenjang was further fractionated by thin layer chromatography on silica gel GF(TLC-plastic sheet, Merk Co., Germany) with solvent system containing petroleum ether-diethyl ether-acetic acid, 80:20:1(v/v/v). After finishing development, spots were identified by spraying with 50% H_2SO_4 and subsequent charring for 20min at 150°C(19). Each fraction which had same R_f value on thin layer chromatogram was scrapped and extracted with chloroform. The silica gel in the samples was removed by filtration. The chloroform was evaporated and dissolved to appropriate solvents for mutagenicity test. The 4 fractions obtained from the TLC plates were tested for their antimutagenic effects on AFB_1 induced mutagenicity. The spot which showed the antimutagenic activity was tentatively confirmed.

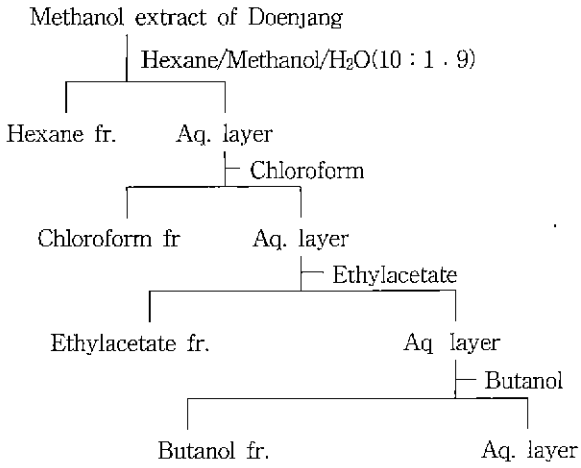

Fig. 2. Fractionation of methanol extract of Doenjang.

Statistical analysis

Statistical analysis was performed by analysis of variance. Significant differences between treatment means were determined by using Duncan's multiple range test(20).

RESULTS AND DISCUSSION

From the dose response test of AFB_1 1μg of AFB_1 per plate was employed to evaluate the antimutagenic effect of methanol extract of Doenjang on the AFB_1 induced mutagenesis. As shown in Table 1, strong antimutagenic activity was observed in methanol extract of Doenjang which was prepared by Korean traditional method.

The mutageneses in both strains of TA98 and TA 100 were completely inhibited at the level of 50% of the Doenjang extract. Since the antimutagenic effect of raw soybeans was not so significant compared to Doenjang(21), the higher antimutagenic activity probably

Table 1. Effect of methanol extract of Doenjang on the mutagenesis of aflatoxin B_1 in *Salmonella typhimurium* strains of TA98 and TA100

	TA98	TA100
	Revertants/plate	
Aflatoxin B_1[1)]	2251±69[a3)]	956±45[a3)]
Aflatoxin B_1+Doenjang[2)]	56± 2[b]	133±13[b]
Spontaneous	66± 5[b]	132± 9[b]

[1)]The level of aflatoxin B_1 employed was 1μg/plate in the Ames test system
[2)]The concentration of the Doenjang extract used was 50% when compared with the original weight of the Doenjang
[3)]The values are mean±standard deviation of three samples
[a,b]The different letters beside the data are significantly different at the 0.01 levels of significance as determined by Duncan's multiple range test

resulted from some end products produced by the action of microorganisms on soybeans during the fermentation of the Doenjang.

There was a report that Meju which was fermented soybean cake before the Doenjang making showed an antimutagenic effect on AFB_1(22). The rats which were fed AFB_1 containing Meju did not cause tumor, however, rats without Meju but AFB_1 fed developed tumor(personal *communication*).

Thus Meju seemed to prevent cancer development. Our experiment also confirmed the fact that the mutagenicity of AFB_1 was inhibited in the presence of Doenjang extract.

The traditional Doenjang contained about 10% of NaCl and the NaCl was extracted from the Doenjang during the methanol extraction. It is known that NaCl in foods plays a cocarcinogenic role in the presence of MNNG(23). However, the antimutagenic effect of Doenjang showing in this study was so strong that the mutagenic effect of NaCl was blocked. This result indicated even if the AFB_1 is contaminated in Doenjang(this possibility is very low) the mutagenicity induced by AFB_1 can be highly inhibited.

It was curious whether this antimutagenic effects is also effective to other carcinogens/mutagens, such as direct mutagens of MNNG and 4-NQO, and indirect mutagens of BaP and DMN.

Direct mutagens such as MNNG and 4-NQO are showing strong mutagenic activity toward TA100 strain without metabolic activation system. The dose response tests were performed with the plate incorporation test recommended by Ames et al.(24) and Maron and Ames (17). One μg of MNNG/plate resulted in revertant numbers of 1650 and 0.5μg of 4-NQO/plate revealed 1500 of the revertants. These concentrations were employed to study antimutagenic effect of Doenjang toward these mutagens.

Dose response effects of indirect mutagens of BaP and DMN were performed with the preincubation test recommended by Yahagi et al.(25) and Maron and Ames (17). DMN was dissolved in 95% methanol instead of DMSO because DMSO had been known to inhibit the mutagenic effect of DMN(25). It was known that DMN induced reverse mutation in *Salmonella typhimurium* in the presence of mouse, rat and hamster liver microsomal fractions *in vitro*, however, the hamster liver system was the most effective to detect the mutagenicity induced by DMN(26,27). Maximum level of 1300 revertants were obtained when 10mg of DMN/plate was used in the system. However, maximum revertant number was about 500 at the dose level of 10μg of BaP/plate(Fig. 3). 3000μg of DMN and 10μg of BaP/plate were employed to study the antimutagenic effect of Doenjang toward these indirect mutagens.

In order to confirm the antimutagenic effects of Doenjang toward other mutagens, the effects of Doenjang

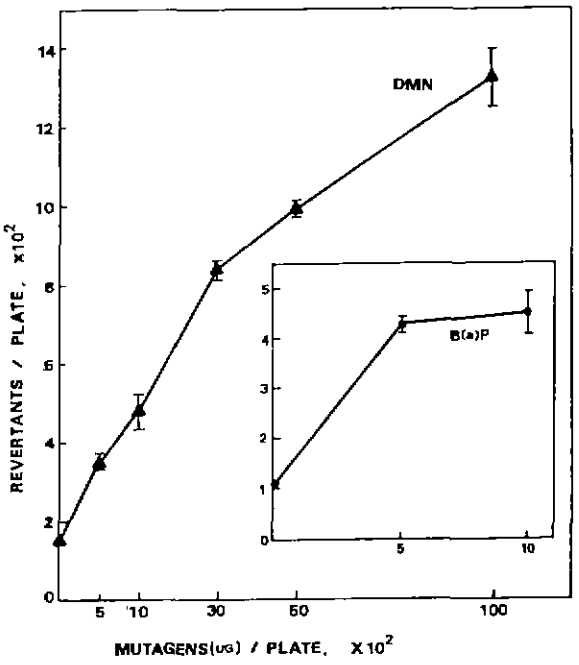

Fig. 3. Dose response effect of dimethylnitrosamine(DMN) and benzo(a)pyrene(BaP) in *Salmonella typhimurium* TA100 strain.
The vertical bars represent one standard deviation of three samples.

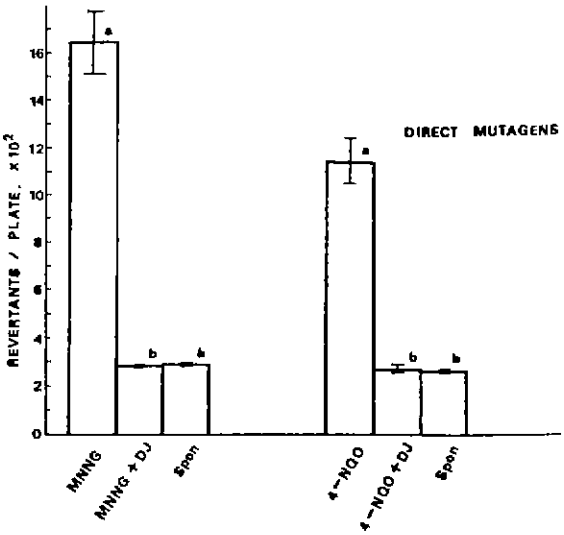

Fig. 4. Effect of methanol extract of Doenjang(DJ) on the mutagenesis of direct mutagens of N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) and 4-nitroquinoline-1-oxide(4-NQO) in *Salmonella typhimurium* TA100 strain.
The vertical bars represent one standard deviation of three samples. The different letters surmounted on the bars in panel are significantly different at the 0.01 levels of significance as determined by Ducan's multiple range test.

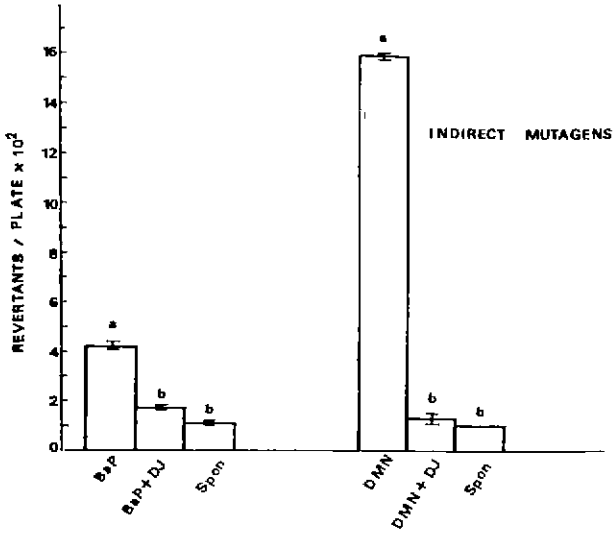

Fig. 5. Effect of methanol extract of Doenjang(DJ) on the mutagenesis of indirect mutagens of benzo(a)pyrene(BaP) and dimethylnitrosamine(DMN) in *Salmonella typhimurium* TA100 strain.
The vertical bars represent one standard deviation of three samples. The different letters surmounted on the bars in panel are significantly different at the 0.01 levels of significance as determined by Ducan's multiple range test.

on the mutageneses of other mutagens such as MNNG, 4-NQO, BaP and DMN were tested. As showed in Fig. 4, MNNG and 4-NQO induced mutageneses were completely inhibited in TA100 strain at the level of 100% of the Doenjang extract($p<0.01$).

80% and 98% of BaP and DMN induced mutageneses were also blocked respectively at the concentration of 100%(Fig. 5). The revertant numbers were significantly reduced when the Doenjang extract was added to the systems of the mutagens($p<0.01$). There were no significant differences between spontaneous revertants and the mutagens plus Doenjang extract($p<0.01$, Fig. 4 and 5).

Thus it can be concluded that the methanol extract of Doenjang showed strong antimutagenic activity not only to AFB_1 but also toward other known mutagens/carcinogens.

In order to identify the antimutagenic compound(s) in methanol extract of Doenjang, methanol extract of Doenjang was fractionated by sequential extractions using hexane, chloroform, ethylacetate and butanol. As shown in Table 2, sequential fractionation of methanol extract from Doenjang revealed that hexane fraction contained the antimutagenic active compound(s). Hexane fraction inhibited the AFB_1 induced mutagenicity in both tester strains almost same levels as the methanol extract, however, other fractions did not show any inhibition. Thus the antimutagenic active compound(s) of Doenjang was assumed non-polar substances.

To identify the antimutagenic compound(s) in the hexane fraction of Doenjang, hexane extract of Doenjang was further fractionated by thin layer chromatographic technique(19). As shown in Fig. 6, hexane fraction of methanol extract from Doenjang was refractionated 4 fractions(spots) according to their R_f values.

Among the 4 fractions(Fig. 6) from the hexane extract,

Table 2. Effect of solvents fractionated compounds from methanol extract of Doenjang on the inhibition of aflatoxin B_1 mutagenicity in *Salmonella typhimurium* TA98 and TA100

Fractionations of methanol extract	Revertants per plate	
	TA98 strain	TA100 strain
Aflatoxin B_1(AFB_1)	1936± 65	1896± 33
Spontaneous reversion	31± 4	107± 2
AFB_1+methanol extract	76± 7	227± 2
AFB_1+Hexane	104± 10	241± 13
AFB_1+Chloroform	1539±196	1648±278
AFB_1+Ethylacetate	1566± 77	1980± 78
AFB_1+Butanol	1598± 69	1942± 95
AFB_1+Aqueous layer	1703± 12	2342± 87

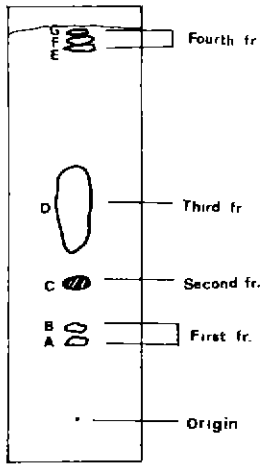

Fig. 6. Thin layer chromatographic fractionation of hexane fraction of methanol extract of Doenjang.
Solvent system; Petroleum ether: diethyl ether: acetic acid=80:20:1(v/v/v)
A: Monoglyceride B: Diglyceride
C: Fatty acid hydroperoxide C: Fatty acid
E: Triglyceride F: Unknown
G: Esterified sterol

Table 3. Effect of fractionated compounds by TLC from hexane extract of methanol extract of Doenjang on the inhibition of aflatoxin B_1 mutagenicity in *Salmonella typhimurium* TA98 and TA100

Fractionations of methanol extract	Revertants per plate	
	TA98 strain	TA100 strain
Aflatoxin B_1(AFB_1)	1440±101	1654±43
Spontaneous reversion	25± 3	104±13
AFB_1+hexane extract	169± 16	212±11
AFB_1+first fr.	1426±47	1574±54
AFB_1+second fr.	1298±79	1622±21
AFB_1+third fr.	185±26	220±19
AFB_1+fourth fr.	1334±45	1336±36

we can conclude that the third fraction contained the antimutagenic active compound(s) from the data shown in Table 3. Almost 88% and 93% of AFB_1 induced mutagenesis were inhibited in TA98 and TA100, respectively, by the third fraction of hexane extracts of Doenjang. Thus, antimutagenic active compound(s) of Doenjang is a kind of free fatty acids on considering R_f value and the shape of the spot. It was known from other reports that there were the changes of free fatty acid compositions during the fermentation process of soybean koji preparation for Doenjang. That is, total lipid of soybean koji consists of about 90.6% neutral lipid, 7.6% phospholipid and 1.8% glycolipid. The major component on non-polar lipid in soybean koji were 39.6% free fatty acids and 29.2% triglycerides. Free fatty acids increased as the triglycerides decreased during soybean koji preparation by hydrolysis of lipase reaction. Especially a considerable increase of linoleic acid in free fatty acid fraction was observed in soybean koji(28-30). The composition of fatty acids in crude lipid of soaked soybean is 57.2% of linoleic acid, 20.9% of oleic acid and 7.3% of linolenic acid(28). It is clear that the antimutagenic effect found in Doenjang was due to the presence of free fatty acids originated from soybeans. Thus it is concluded that one of major antimutagenic compound(s) in Doenjang is one or more than one of free fatty acids. We finally identified that linoleic acid was one of the major active antimutagenic compounds in Doenjang(31).

Hayatsu et al.(32,33) reported that linoleic acid and oleic acid in an ether extract of normal human feces inhibited the mutagenic activity of a number of chemicals for *Salmonella typhimurium*. The oleic acid in the acidic fraction of cooked beef was found to completely inhibit the mutagenicity of the basic fraction of cooked ground beef. Ha et al.(34,35) also reported that isomeric derivatives of linoleic acid which was isolated from grilled ground beef was effective in partially inhibiting the initiation of mouse epidermal carcinogenesis by 7,12-dimethylbenzo(a)anthracene and forestomach tumorogenesis induced by benzo(a)pyrene (36-38). Linoleic acid decreased growth of various human cancer cells(39) and trasplanted tumors in mice(40). Linoleic acid also enhanced the phagocytic activity and NBT reduction of peritoneal phagocyte of mice(41).

It is very interesting finding that Doenjang has antimutagenic effects to wide range of the carcinogens. Further study is needed on the identification of the active compound(s), the mechanisms of the antimutagenicity and *in vivo* anticancer experiment in detail.

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