Antimutagenicity of Water and Methanol Extracts of Soymilks Fermented by Bifidobacteria and Lactic Acid Bacteria

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An antimutagenic effect of the aqueous and 80% methanol extracts of soymilk was observed on N-methyl-N'-nitrosoguanidine (MNNG) mutagenesis toward *Escherichia coli* B/r WP2 trp⁻. Addition of both extracts to the plate decreased the mutation frequency through the dose amount of 60 μ l. However, there was a tendency for the mutation frequency to increase with the addition of more than 80 μ l. The effect of the extract prepared from soybeans soaked in water was increased by heating them at 100°C for more than 40 min. The 80% methanol extracts of soymilk fermented by *Bifidobacteria adolescentis*, *B. breve*, *B. longum* and *Streptococcus lactis* increased the antimutagenic effect compared with that of unfermented soymilk. The extract of fermented soymilk with *B. infantis* enhanced the MNNG mutagenesis toward *E. coli*. The antimutagenic effects of the fermented milk with *B. bifidum*, *S. thermophilus* and *Lactobacillus acidophilus* were lower than that of unfermented soymilk.

Extracts of vegetables and fruits exhibit an antimutagenic effect against chemical mutagens such as 3-amino-1, 4-dimethyl-5 H-pyrido [4,3-b] indole (Trp-p-1), 3-amino-1-methyl-5 H-pyrido [4,3-b] indole (Trp-p-2) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on the mutants of *Escherichia coli* and *Salmonella typhimurium*¹⁾⁻⁶⁾. Suppressive effects were also observed to occur in the process of food preparation. OBANA *et al.*⁷⁾ reported that freshly brewed coffee extracts suppressed the UV-light or chemical mutagenesis in *S. typhimurium* TA 1535/psk 1002, but green coffee-bean extracts did not. In addition, HOSONO *et al.*⁸⁾ reported that the antimutagenic activities of cow's skim milk against 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) in the streptomycin dependent strains of *Salmonella typhimurium* TA 98 increased by fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus*

and *Streptococcus salivarius* subsp. *thermophilus*. The acetone and ethyl acetate extracts from cow's milk fermented by *Streptococcus thermophilus* and/or *Lactobacillus bulgaricus* suppressed the mutagenesis of 4-nitroquinoline-N-oxide (4 NQO) and 2-aminofluorene (2-AF) in *S. typhimurium* TA 98 and TA 100⁹⁾.

The preparation of yogurt-like products from soymilk has been studied for the improvement of acid production and sensory quality by several workers¹⁰⁾⁻¹⁴⁾. However, antimutagenic activity of the fermented soymilk has not been reported so far and the clarification of this activity is of significance for the selection of bacterial strains as starter in the fermentation of soymilk.

In this study, the fermented soymilks were prepared by culturing with several strains of lactic acid bacteria and bifidobacteria, and the effects of each extracts on the chemically in-

duced mutagenesis toward *Escherichia coli* B/r WP 2 trp⁻ were examined.

Materials and Methods

Reagents and materials

The soybeans used were Miyagishirome produced at Wakutani-cho, Toda-gun, Miyagi prefecture. A chemical mutagen, MNNG, and dimethylsulfoxide (DMSO) were obtained from Aldrich Chem. Co. and Dojin Chemical Lab., respectively.

Bacterial strains and culture conditions

The following strains of bacteria for fermentation were obtained from the Institute of Physical and Chemical Research (Wako-shi, Saitama); *Bifidobacterium adolescentis* 1275, *B. bifidum* 1255, *B. breve* 1192, *B. infantis* 1222, *B. longum* 1217, *Streptococcus lactis* 208, *S. thermophilus* 419, *Lactobacillus acidophilus* 8102, *L. bulgaricus* 126.

The stock cultures and pre-cultures of these bacteria were prepared using GAM-semi liquid agar medium containing 0.2% agar (GAM Broth Nissui, Nissui Pharm. Co.) for bifidobacteria and 10% skim milk medium for lactic acid bacteria. For the cultivation of *L. acidophilus*, 10% skim milk + 1% glucose + 1% yeast extract (Difco Lab.) was used.

The strain of *Escherichia coli* B/r WP 2 trp⁻ (IFO 14195) was obtained from the Institute for Fermentation (Yodogawa-ku, Osaka). The bacterial suspension of *E. coli* was prepared by growing overnight in 0.8% nutrient broth (Difco Lab.) and then 0.7 ml of DMSO was added to 8.0 ml of the culture. The culture was dispensed in 0.2 ml aliquots into screwcap vials and kept at -80°C.

Assay of antimutagenic activity

The semi-enriched minimal agar medium (SEM) was prepared according to the method of KADA and KANEMATSU¹⁵⁾. Top agar containing 0.6% NaCl and 0.6% Difco bacto agar was also prepared and kept at 43°C before use. The stock strain of *E. coli* was grown in 0.8% nutrient broth (Difco Lab.) and the pre-culture was incubated at 37°C for 16 h in B-2 broth medium. The cells obtained by centrifugation were washed twice with 1/15 M phosphate

buffer at pH 7.0 ($1-2 \times 10^9$ cells/ml). Twenty five ml of the cell suspension was added to the same volume of MNNG solution (120 µg/ml of the buffer). The cell-mutagen mixture was incubated at 37°C for 30 min with slow shaking. The treated cells obtained by centrifugation were washed three times with the buffer and resuspended in 25 ml of the buffer. A mixture of 0.1 ml of the suspension containing mutagenized bacteria and a certain volume of the extract was added to 3 ml of the top agar and poured onto the SEM plate. The suspension of mutagenized cells was also diluted with the buffer by a factor of 10^6 , and a 0.1 ml portion was poured in the same way as described above. Revertants and viable cells were counted after incubation at 37°C for 3 days.

Preparation of fermented soymilk

Soybeans were soaked overnight with 3 times their weight of deionized water at room temperature and dehulled. The soaked soybeans were blended for 10 min in a mixer (Hitachi Co., VA-W 35) by adding 6 parts of boiling water to one part of dry soybeans. The slurry was filtered through 4 thicknesses of gauze. The filtrate was heated at 85°C for 20 min and used as soymilk. One hundred ml of the soymilk (pH 6.8) was inoculated with 1 ml of the culture of lactic acid bacteria. For bifidobacteria, the surface of the inoculated soymilk was covered with sterile liquid paraffin to make an anaerobic condition. The inoculated soymilk was incubated at 37°C for 24 hrs. The titratable acidity and pH value of the fermented soymilks were measured.

Preparation of extracts from soybean and fermented soymilk

The slurry prepared from the soybeans soaked with water as described above was heated in a boiling water bath for 0 to 60 min and centrifuged at 18 000 rpm for 30 min. The supernatant was filtered through a polysulfone aseptec unit with a 0.45 µm membrane (Advantec) and the filtrate was used as the water extract of soybean for assay. One gram of the lyophilized soymilk and fermented milk was added to 10 ml of water and the mix-

ture was stirred for 5 h at room temperature. After pH adjustment to 4.7 and centrifugation, the water extracts were obtained as described above. The methanol extracts were also prepared from a mixture of 1 g of the lyophilizates and 15 ml of 80% methanol.

Results and Discussion

Antimutagenic activity of aqueous extract from soymilk

The antimutagenic effect of an aqueous extract of the soymilk on the mutagenesis of MNNG toward *E. coli* B/r WP 2 Trp⁻ is shown in Table 1. The numbers of mutant (Trp⁺) colonies decreased by addition of the extract to the plate, and the mutation frequency was also decreased by a dose amount of 60 μ l. However, a dose amount of more than 80 μ l tended

to increase the mutation frequency.

Influence of heat treatment of water-soaked soybean on antimutagenic activity

In order to observe the influence of heat treatment of the extract on the antimutagenic effect, aqueous extracts were prepared from soybeans soaked in water and heated in boiling water for different times. The antimutagenic effect of these extracts on the MNNG mutagenesis is shown in Table 2. The number of mutant (Trp⁺) colonies did not change in the extracts heated for 0 up to 30 min. However, the extracts heated for 40 and 60 min lowered the number of mutant (Trp⁺) colonies and suppressed the mutagenesis of MNNG toward *E. coli* by 13–18%. MORITA *et al.*¹⁾ reported that the desmutagenic factor in a water extract prepared from cabbage was sensitive to

Table 1 Antimutagenic effect of the aqueous extract of soymilk on the mutagenesis of MNNG toward *Escherichia coli* B/r WP 2 Trp⁻

Aqueous extract added (μ l)	Mean number of mutant (Trp ⁺) colonies per plate	Mean number of survival (Trp ⁻) colonies per plate ($\times 10^5$)	Mutation frequency ($\times 10^8$)	Percentage inhibition (%)
0	1 136	102	1 113	0
20	934	112	833	25.2
40	926	112	827	25.7
60	886	112	791	28.9
80	873	110	794	28.7
100	916	104	881	20.8

Table 2 Changes in the antimutagenicity of the aqueous extracts of soybeans heated at 100°C for different times on the mutagenesis of MNNG toward *Escherichia coli* B/r WP 2 Trp⁻

Heated time (min)	Mean number of mutant (Trp ⁺) colonies per plate	Mean number of survival (Trp ⁻) colonies per plate ($\times 10^5$)	Mutation frequency ($\times 10^8$)	Percentage inhibition (%)
Control	1 072	94	1 140	0
0	1 102	97	1 136	0.4
10	1 112	98	1 135	0.4
20	1 128	100	1 128	1.1
30	1 124	101	1 113	2.4
40	930	100	930	18.4
50	978	102	959	15.9
60	997	101	987	13.4

Assay was carried out using 100 μ l of water (control) and the extract.

heating in boiling water. SHINOHARA *et al.*³⁾ suggested that heat stable antigens exist in the aqueous dialyzates of vegetables. In the aqueous extract of soybeans, the antimutagenic effect was increased by heating them at 100°C for more than 40 min. This suggests that the antimutagenic activity in the extract of soybeans could not be lost by the heat treatment for the preparation of soymilk.

Antimutagenic activity of 80% methanol extract of soymilk

The antimutagenic effect of 80% methanol extracts on the mutagenesis of MNNG toward *E. coli* is shown in Table 3. The MNNG mutagenesis was suppressed by 22–24% by the extract of soymilk at a dose amount of 20–60 μ l. However, there was a tendency for the antimutagenic effect to be reduced at dose amount of more than 80 μ l as observed with the aqueous extract. A marked difference in the antimutagenic effect was not observed between the aqueous and 80% methanol extracts.

Antimutagenic activities of 80% methanol extracts from fermented soymilks

The pH values and titratable acidities of soymilks fermented by strains of bifidobacteria and lactic acid bacteria are shown in Table 4. The extent of fermentation in the soymilks was clearly different among the bacterial strains. The strains other than *S. thermophilus*, *L. acidophilus* and *L. bulgaricus* lowered the pH value to less than 5.0. The acidity increased significantly in the soymilk fermented by *B.*

breve and *B. infantis*. A bacterial strain having the ability to increase the acidity at more than 0.7 is thought to be suitable for the preparation of fermented soymilk.

The antimutagenic effect of the 80% methanol extracts from fermented milks is shown in Table 5. The mutation frequencies in the fermented soymilks with bifidobacteria and lactic acid bacteria other than *B. infantis* were lower than that of the control containing only MNNG. The soymilks fermented by *B. adolescentis*, *B. breve*, *B. longum* and *S. lactis* showed higher antimutagenic effect than unfermented soymilk. The extracts of fermented soymilks by *B. longum* and *B. breve* strongly suppressed the mutagenesis of MNNG toward *E. coli* compared with those by other strains.

Table 4 Titratable acidities and pH values of soymilks fermented by bifidobacteria and lactic acid bacteria at 37°C for 24 h

Species	pH	Acidity(%)
Control	6.80	0.20
<i>B. adolescentis</i>	4.51	0.78
<i>B. bifidum</i>	4.96	0.59
<i>B. breve</i>	4.55	0.87
<i>B. infantis</i>	4.51	0.87
<i>B. longum</i>	4.91	0.66
<i>S. lactis</i>	4.63	0.81
<i>S. thermophilus</i>	5.44	0.30
<i>L. acidophilus</i>	5.44	0.40
<i>L. bulgaricus</i>	6.09	0.21

Table 3 Antimutagenic effect of the 80% methanol extract of soymilk mutagenesis of MNNG toward *Escherichia coli* B/r WP 2 Trp⁻

Methanol extract added (μ l)	Mean number of mutant (Trp ⁺) colonies per plate	Mean number of survival (Trp ⁻) colonies per plate ($\times 10^7$)	Mutation frequency ($\times 10^8$)	Percentage inhibition (%)
0	1168	106	1102	0
20	928	109	851	22.8
40	856	102	839	23.9
60	770	106	726	24.1
80	854	100	854	22.5
100	916	100	916	16.9

Table 5 Antimutagenic effect of 80% methanol extract from fermented soymilk by bifidobacteria or lactic acid bacteria on the mutagenesis of MNNG toward *Escherichia coli* B/r WP 2 Trp⁻

Species	Mean number of mutant (Trp ⁺) colonies per plate	Mean number of survival (Trp ⁻) colonies per plate ($\times 10^7$)	Mutation frequency ($\times 10^8$)	Percentage inhibition (%)
Control 1	1 036	99	1 047	0
Control 2	939	114	824	21.3
<i>B. adolescentis</i>	879	109	806	24.0
<i>B. bifidum</i>	933	94	993	5.2
<i>B. breve</i>	404	72	561	46.4
<i>B. infantis</i>	1 284	110	1 167	-11.5
<i>B. longum</i>	550	81	679	35.1
<i>S. lactis</i>	1 144	149	768	26.6
<i>S. thermophilus</i>	1 234	145	851	18.7
<i>L. acidophilus</i>	1 126	119	943	9.9
<i>L. bulgaricus</i>	1 136	139	837	20.1

Control 1: added 50 μ l of 80% methanol to the medium.

Control 2: added the extract of unfermented soymilk.

In the water extracts of soymilk fermented by *B. longum*, *B. breve* and *B. adolescentis*, the antimutagenic effect was also observed at almost the same level as those of the 80% methanol extracts. The extract of soymilk fermented by *B. infantis* increased the MNNG mutagenesis toward *E. coli*. This bacteria may produce an effector to enhance the MNNG mutagenesis. It is apparent that the antimutagenic activity is not always related to the extent of fermentation indicated by pH value and titratable acidity of the fermented soymilk.

According to HOSONO *et al.*⁸⁾, cow's milks cultured with lactic acid bacteria such as *L. bulgaricus* and *S. thermophilus* have strong antimutagenic activity against AF-2, 4 NQO and MNNG. They also observed that the antimutagenic activities were lost by heating the cultured milk to temperatures higher than 55°C. In the extracts of fermented soymilks, the antimutagenic activities were not as strong as those of the fermented cow's milks obtained by them. The difference can be ascribed to the preparation of the sample used for the assay and the composition of the cow's milk and soymilk. To compare the antimutagenic act-

ivity of fermented soymilk with that of fermented cow's milk, it is further necessary that the antimutagenic activity is assayed for the sample of both milks prepared by the same procedure. Our observations were limited to the use of 80% methanol extracts from fermented soymilks. However, the antimutagenic activity related to the fermented cow's milk has also been observed in the cell walls of certain lactic acid bacteria¹⁶⁾ and acetone or ethyl acetate extracts from the milk⁹⁾.

According to EBATA *et al.*¹⁷⁾ the β -glucosidase produced by *Rhizopus oligosporus* could be involved in the formation of antioxidants in the medium containing soybean paste. The increase in the antimutagenic activity of 80% methanol extract from fermented soymilk is expected to be associated with the action of β -glucosidase produced by the bacterial strain during the fermentation on the components of soymilk such as isoflavones. Further work is needed to elucidate the antimutagenic factor formed during the fermentation of soymilk.

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ビフィズス菌および乳酸菌による発酵豆乳の水
およびメタノール抽出物の抗突然変異性

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大豆ならびにビフィズス菌および乳酸菌発酵豆乳の水および 80% メタノール抽出液の抗突然変異性について調べた。抽出液の抗突然変異性は、*Escherichia coli* B/r WP2 trp⁻ の N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG) による誘発突然変異に対する阻害性によって観察した。加熱した水浸漬大豆の水抽出液では、100℃、40 分以上加熱すると抗突然変異性が高まった。豆乳の 80% メタノール抽出液を 1 プレート当たり 20~60 μ l 添加した場合、22~24% の抗突然変異性を示した。発酵豆乳の 80% メタノール抽出液について、*Bifidobacterium breve* および *B. longum* ではそれぞれ 46.4 および 35.1% となり、未発酵豆乳の 21.3% より高い抗突然変異性を示した。*B. adolescentis* および *B. bifidum* ではそれぞれ 24.0 および 5.2% となった。しかし、*B. infantis* では突然変異を高める傾向が認められた。乳酸菌では、*Streptococcus lactis* が 26.6% となり、未発酵豆乳より高かった。*S. thermophilus*, *Lactobacillus acidophilus* および *L. bulgaricus* では、いずれも低くなった。