Journal of Food Protection, Vol. 70, No. 4, 2007, Pages 1025–1028

Research Note

Possible Mechanisms of Antimutagenicity in Fermented Soymilk Prepared with a Coculture of *Streptococcus infantis* and *Bifidobacterium infantis*

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MS 06-320: Received 9 June 2006/Accepted 1 October 2006

ABSTRACT

The possible mechanisms of antimutagenicity against 4-nitroquinoline-*N*-oxide (4-NQO; a direct mutagen) and 3,2′-dimethyl-4-amino-biphenyl (DMAB; an indirect mutagen) were examined in fermented soymilk prepared with a coculture of *Streptococcus thermophilus* and *Bifidobacterium infantis.* The antimutagenicity in the fermented soymilk was not due to the bioantimutagenic effect of modulation of DNA repair processes. The mutagenicity of DMAB decreased with increased preincubation of fermented soymilk and the DMAB metabolite but not with intact DMAB or an S9 mixture. Mutagenicity of 4-NQO was not affected by preincubation of fermented soymilk with this mutagen. Mutagenicity of both 4-NQO and DMAB was reduced when *Salmonella* Typhimurium TA 100 was pretreated with fermented soymilk, indicating that fermented soymilk affected the function of the bacterial cell, which might also lead to reduced mutagenicity of the tested mutagens. Desmutagenic and blocking effects were the main mechanisms of antimutagenicity in the fermented soymilk against DMBA. In contrast, the antimutagenic effect of the fermented soymilk on 4-NQO was primarily due to a blocking effect.

Lactic acid bacteria and bifidobacteria are probiotic microorganisms that exert a beneficial effect on the health and well-being of the host *(12)* and thus are used commonly in the preparation of foods *(9).* Soybean is generally considered a food material with high nutritive quality despite its undesirable bean odor and the presence of stachyose and raffinose, which contribute to flatulence *(2).* In an attempt to develop a probiotic diet adjunct that overcomes the disagreeable bean flavor and to reduce the flatulence factor, a series of studies on the fermentation of soymilk (the water extract of soybean) with a probiotic culture of lactic acid bacteria and bifidobacteria have been conducted in our laboratory *(4, 6, 16–18).* The fermented soymilk possessed reduced stachyose and raffinose concentrations and contained probiotic bacteria *(18).* It also contained a significantly higher concentration of the bioactive isoflavone aglycone and had a greater effect than its unfermented counterpart on ascorbate antioxidation inhibition, reducing activity, and superoxide anion radical scavenging *(4, 17).* The increased antimutagenicity of fermented soymilk, which differed with the starter organism and the type of mutagen tested, was linked to the growth of the starter organism and the antimutagenic factors formed during fermentation *(6).*

The objective of the present study was to further explore possible mechanisms of antimutagenicity in fermented soymilk prepared with *Streptococcus thermophilus* and *Bifidobacterium infantis.* Soymilk fermented with these two bacteria had the highest antimutagenicity against 4-nitroquinoline-*N*-oxide (4-NQO) and 3,2′-dimethyl-4-amino-biphenyl (DMAB) among the various fermented soymilks examined *(6).*

MATERIALS AND METHODS

Chemicals and bacterial strains. The direct mutagen 4-NQO and the indirect mutagen DMAB, which requires liver microsomal activation, were purchased from Sigma-Aldrich Co. (St. Louis, Mo.). An S9 microsomal fraction (S9 mix) of rat liver was obtained from ICN Pharmaceuticals (Costa Mesa, Calif.). The 4-NQO and DMAB were dissolved in dimethylsulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at concentrations of 1.0 and 80 μg/ml, respectively.

Salmonella Typhimurium TA 100, *B. infantis* CCRC 14633, and *S. thermophilus* CCRC 14085 were all obtained from the Culture Collection and Research Center (Hsinchu, Taiwan). Tests of histidine requirement, *rfa* mutation, *uvrB* mutation, and R-factor were performed to confirm the genotypes of *Salmonella* Typhimurium TA 100. Prior to each mutagenicity and antimutagenicity assay, *Salmonella* Typhimurium TA 100 was grown in fresh nutrient broth no. 2 (Oxoid, Basingstoke, UK) at 37°C overnight *(11).*

Preparation of fermented soymilk. Fermented soymilk was prepared with a coculture of *S. thermophilus* and *B. infantis.* Fermentation was conducted at 37°C for 32 h. Detailed procedures for the preparation of fermented soymilk were described previously *(16).*

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TABLE 1. *Bioantimutagenic effect of fermented soymilk on 4-NQO and DMAB assayed in* Salmonella *Typhimurium TA 100*[a]

Sample	4-NQO		DMAB	
	No. of revertants[b]	% of control	No. of revertants	% of control
Control[c]	453 ± 40 A	100.0	229 ± 15 A	100.0
Fermented soymilk	429 ± 11 A	97.5 ± 5.0	242 ± 14 A	106.19 ± 10.42

[a] Results are presented as means ± SD for three experiments. Within a column, means with the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple range test.

[b] Number of revertants reported is the total His^+ revertants minus spontaneous His^+ revertants per plate. Spontaneous revertants were obtained without mutagen or fermented soymilk.

[c] The control contained mutagen but not fermented soymilk.

Evaluation of bioantimutagenic effect. The bioantimutagenicity test was conducted essentially according to methods described by Sato et al. *(14)* and Chen and Yen *(3).* An overnight culture of *Salmonella* Typhimurium TA 100 (5.0 ml) was washed twice by centrifugation with a cold 1/15 M phosphate buffer (PB, pH 7.0) at 4°C and then resuspended in cold PB (4.0 ml). This cell suspension (3.0 ml) was added to 0.5 ml of 4-NQO or 0.5 ml of DMAB and incubated at 37°C for 1 h with gentle shaking. For DMAB, S9 mix (0.5 ml) also was added. The treated bacteria were washed twice by centrifugation and resuspended in cold PB. The treated *Salmonella* Typhimurium TA 100 (0.1 ml) was mixed in a tube with fermented soymilk (0.1 ml) or sterile distilled water (0.1 ml). After 20 min of incubation at 37°C in a rotary shaker, the molten top agar (2.0 ml) was added. The mixture was poured onto a minimal glucose agar plate. Colonies were counted after 48 h of incubation at 37°C.

Evaluation of desmutagenic effect. The procedures described by Yen and Hsieh *(19)* were followed to investigate the desmutagenicity of fermented soymilk by examining the effect of fermented milk on intact mutagen, S9 mix, and DMAB metabolites. To examine the effect on intact mutagen, fermented soymilk or sterile distilled water (0.1 ml) and 4-NQO (0.1 ml) or DMAB (0.1 ml) were incubated in a tube at 37°C for 0, 20, or 40 min. The tube contents were then added to the overnight culture of *Salmonella* Typhimurium TA 100 (0.1 ml) and PB (0.7 ml). For DMAB, the S9 mix (0.2 ml) also was added. These mixtures were then incubated at 37°C with gentle shaking for 20 min. After adding top agar (2.0 ml) and vortexing, the tube contents were pour plated onto a minimal glucose agar plate. Colonies were counted after 48 h of incubation at 37°C.

To investigate the effect on DMAB metabolites, the metabolites were first prepared by preincubation of DMAB (0.1 ml) and S9 mix (0.5 ml) at 37°C for 20 min with gentle shaking. Fermented soymilk or distilled water (0.1 ml) was then added to the reaction mixture (DMAB metabolites) and incubated for 0, 20, or 40 min at 37°C. *Salmonella* Typhimurium TA 100 (0.1 ml) was then added, and the culture was incubated at 37°C for another 20 min with gentle shaking. Further cultivation and colony counting were performed as described above.

To examine the effect on S9 mix, fermented soymilk or distilled water (0.1 ml) and S9 mix (0.5 ml) were preincubated together at 37°C for 0, 20, or 40 min. *Salmonella* Typhimurium TA 100 (0.1 ml) and DMAB (0.1 ml) were then added and incubated at 37°C for another 20 min. The culture was then assayed for antimutagenicity as described above.

Evaluation of the blocking effect. To evaluate the blocking effect of fermented soymilk, *Salmonella* Typhimurium TA 100 (5.0 ml) and fermented soymilk or distilled water (5.0 ml) were first incubated together at 37°C for 1 h. The bacteria were then washed three times with nutrient broth no. 2 with centrifugation. Mutagenicity of 4-NQO and DMAB was assayed with the treated or untreated *Salmonella* Typhimurium TA 100.

Statistical analysis. The mean values and standard deviations (SDs) were calculated from the data obtained from three separate experiments. These data were then compared using Duncan's multiple range method *(13).*

TABLE 2. *Effects of preincubation of fermented soymilk with DMAB, S9 mix, DMAB metabolites, or 4-NQO for various periods on the mutagenicity of DMAB and 4-NQO*[a]

Incubation time (min)[b]	No. of revertants[c]		% inhibition[d]
	Control	Fermented soymilk	
DMAB			
0	594.8 ± 96.5	197.3 ± 38.3	66.3 ± 3.7 A
20	517.5 ± 72.5	210.7 ± 23.7	60.0 ± 9.6 A
40	552.8 ± 103.9	169.3 ± 23.5	69.7 ± 4.0 A
S9 mix			
0	336.5 ± 43.1	134.3 ± 36.1	61.5 ± 7.1 A
20	307.0 ± 14.1	159.7 ± 29.7	45.6 ± 3.3 A
40	298.5 ± 28.0	142.7 ± 18.1	47.6 ± 8.7 A
DMAB metabolites			
0	405.0 ± 60.1	186.3 ± 25.0	54.9 ± 2.2 A
20	371.3 ± 22.2	152.3 ± 31.0	54.7 ± 2.2 A
40	376.7 ± 10.0	120.7 ± 4.2	65.7 ± 3.7 B
4-NQO			
0	366.3 ± 8.1	101.5 ± 5.0	74.4 ± 9.5 A
20	348.0 ± 10.1	119.5 ± 6.4	69.0 ± 15.5 A
40	338.0 ± 29.5	130.3 ± 15.6	63.9 ± 4.7 A

[a] Results are presented as mean ± SD for three experiments.

[b] Fermented soymilk was preincubated with DMAB, S9 mix, DMAB metabolite, or 4-NQO for 0 to 40 min before the antimutagenicity assay was performed.

[c] Number of revertants reported in the total His^+ revertants minus spontaneous His^+ revertants per plate. Spontaneous revertants were obtained without mutagen or fermented soymilk.

[d] Inhibition (%) = $[1 - (A - E)/(B - D)] \times 100$, where A and B are numbers of mutagen-induced revertants in the presence and absence of sample, respectively, and D and E are numbers of spontaneous revertants observed in the sample and control, respectively. Within the column, means with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

TABLE 3. *Mutagenicity of 4-NQO and DMAB in* Salmonella *Typhimurium TA 100 treated with fermented soymilk*[a]

Sample	4-NQO		DMAB	
	No. of revertants[b]	% of control	No. of revertants	% of control
Control[c]	359 ± 23 A	100.0	301 ± 47 A	100.0
Fermented soymilk	52 ± 11 B	14.2 ± 2.4	97 ± 12 B	29.5 ± 3.5

[a] Results are presented as means ± SD for three experiments. Within a column, means with the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple range test. *Salmonella* Typhimurium TA 100 and fermented soymilk were preincubated at 37°C for 1 h. The bacteria were washed three times with nutrient broth and were then mixed with 4-NQO or with DMAB and S9 mix at 37°C for 20 min before the mutagenesis assay was performed.

[b] Number of revertants reported is the total His^+ revertants minus spontaneous His^+ revertants per plate. Spontaneous revertants were obtained without mutagen or fermented soymilk.

[c] The control contained mutagen but not fermented soymilk.

RESULTS AND DISCUSSION

Bioantimutagenic effect of fermented soymilk. According to Kada et al. *(7)* and Chen and Yen *(3),* bioantimutagens suppress the effects of mutagens by modulating cellular mutagenic processes, i.e., by mainly acting on DNA replication and repair processes after DNA is damaged by the mutagen. In the present study, the bioantimutagenic effect of fermented soymilk on DMAB and 4-NQO was evaluated according to the method described by Sato et al. *(14).* The number of revertants of the test organism was determined after the mutated *Salmonella* Typhimurium TA 100 was treated with the fermented soymilk. Table 1 shows the bioantimutagenic effect of soymilk fermented with *S. thermophilus* and *B. infantis* on the mutagenicity of 4-NQO and DMAB. The fermented soymilk did not reduce the number of revertants; the result on the plate containing fermented soymilk was similar to that for the control. Thus, mutagenicity of both mutagens tested was not diminished by the fermented soymilk in the bioantimutagenic test, which indicates that DNA damaged by 4-NQO or DMAB was not repaired by treatment with fermented soymilk and that the antimutagenic activity of the fermented soymilk previously observed *(6)* was not due to a bioantimutagenic effect.

Desmutagenic effects of fermented soymilk. Desmutagens are antimutagenic because they directly inactivate mutagens or their precursors by suppressing the activity of metabolic enzymes *(3, 7, 8).* To investigate the mechanism underlying the desmutagenic activity against DMAB of soymilk fermented with *S. thermophilus* and *B. infantis,* fermented soymilk was preincubated with (i) DMAB, (ii) S9 mix, or (iii) metabolites of DMAB and S9 mix for 0 to 40 min, and the antimutagenic activities were then determined. In the preliminary tests, these reaction products had no toxic effect on *Salmonella* Typhimurium TA 100. Increasing the preincubation time of fermented soymilk with DMAB did not cause a significant change in antimutagenic activity (Table 2). Therefore, the antimutagenic activity of fermented soymilk tested should not be attributed to the interaction between fermented soymilk and DMAB directly. No increase in inhibition of mutagenesis was noted as the preincubation period of fermentation of soymilk and S9 mix was extended. Therefore, inactivation of the activity of the hepatic microsome is not likely to be the main cause of antimutagenic activity of the fermented soymilk observed. However, the antimutagenicity of fermented soymilk against DMAB increased when the preincubation time with DMAB metabolites increased. For example, 65.66% inhibition was noted when fermented soymilk was preincubated with DMAB metabolites for 40 min compared to 54.88% noted without preincubation. This finding indicates that the interaction of antimutagenic factors in fermented soymilk with metabolites of DMAB may contribute to the desmutagenic effect of fermented soymilk on DMAB. DMBA-3,4-diol-1,2-epoxide is the principal ultimate carcinogenic metabolite of DMBA *(5).* Reaction of the diol epoxide with the amino group of the protein and amino acid *(10)* present in the sample may result in a lower concentration of DMBA-3,4-diol-1,2-epoxide and thus lead to the reduced mutagenicity observed. However, the exact mechanism required further investigation.

4-NQO is a direct-action mutagen, i.e., it requires no activation to induce mutation. To evaluate the desmutagenicity of fermented soymilk against 4-NQO, the soymilk was preincubated with the mutagen for 0 to 40 min, and then the soymilk antimutagenic activity was determined. Preincubation of fermented soymilk with 4-NQO, regardless of the length of the preincubation period, did not result in a significant change of antimutagenicity (Table 2). This finding rules out the possibility that the antimutagenic activity of fermented soymilk against 4-NQO is due to a desmutagenic effect, i.e., a direct interaction of fermented soymilk with the mutagen.

Blocking effect of fermented soymilk. In addition to bioantimutagenesis and desmutagenesis, antimutagens may also exert a blocking effect, adjusting the function of bacterial cells to reduce the DNA mutation induced by the mutagen *(3).* For example, Ayrton et al. *(1)* reported that ellagic acid exerted a blocking effect on the mutagenicity of 2-amino-3-methylimidazo[4,5-*f*] quinoline by modifying the binding site of mutagens on DNA to avoid the DNA damage. Tajmir-Riahi et al. *(15)* reported that the DNA adducts with chlorophyll and chlorophyllin, which exhibited antimutagenic activity, reduced the DNA damage induced by mutagens.

To examine the possible blocking effect of fermented soymilk, *Salmonella* Typhimurium TA 100 was preincu-

bated with fermented soymilk for 1 h before mutation was induced by either 4-NQO or DMAB. *Salmonella* Typhimurium TA 100 treated with the fermented soymilk had of 52 revertant CFU per plate, which is significantly less ($P < 0.05$) than the 359 revertant CFU per plate observed with the control mutated with 4-NQO (Table 3). A similar phenomenon was also noted when mutagenicity of DMAB was examined. These results indicated that fermented soymilk might exert a blocking effect on the mutagens of both 4-NQO and DMAB by adjusting the function of the bacterial cell.

Based on the results obtained from the present study, we concluded that the antimutagenicity of the soymilk fermented with *S. thermophilus* and *B. infantis* may be due to a single or a combination of different distinctive mechanisms and can vary with the type of mutagen tested. Interactions of desmutagenic factors in fermented soymilk with DMAB metabolites and a blocking effect were the main mechanisms of the antimutagenicity of fermented soymilk against DMBA. In contrast, the antimutagenic effect of the fermented soymilk on 4-NQO was primarily due to a blocking effect.

ACKNOWLEDGMENT

This research was financially supported by the National Science Council, Republic of China (Taiwan) (NSC 94-2313-B-002-017).

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