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Article

Antimutagenic Effect of *Bacillus natto* Isolated from *Natto*

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***In vitro* antimutagenicity of *Bacillus natto* islolated from *Natto*, Japanese traditional fermented food, was investigated using *umu*-test. Mutagenicity of S9-activated metabolites of Trp-P-2 and IQ for *Salmonella typhimurium* TA 1535/pSK1002 was remarkably inhibited by addition of bacterial cells and their cytoplasmic fraction. Desmutagenicity by cytoplasmic fraction increased with increasing concentration of the fraction. Bioantimutagenic effect of cytoplasm on *Salmonella typhimurium* SD-100 did not show bioantimutagenic activity against mutated bacterial cells induced by Trp-P-2. Cytoplasmic fraction exhibited 17% bioantimutagenicity due to desmutation caused by IQ.**

Key words: *Antimutagenicity, Bacillus natto, desmutagenicity, pyrolysate*

To reduce the risks of cancer in human, much research has studied the conditions that increase or decrease the activation of environmental mutagens. Pyrolysates are potent mutagens in the presence of an appropriate activation system[1] and carcinogenic heterocyclic amines that form when protein-rich animal foods are cooked. For example, very strong mutagenic pyrolysates of 3-amino-1methyl[5H]oyrido(4,3-b)inodole (Trp-P-2) and 2-amino-3-methyl-inidazo(4,5-f)quinoline (IQ) etc.[2] It is therefore important to control mutagenesis and carcinogenesis by identifying the hazardous agents and removing them from our daily food and environment, as well as preventing the interactions of chemical mutagens and carcinogens with DNA.[3]

The *ame*-test is widely used to assess antimutagenicity. It uses *Salmolnella typhimurium* frameshift mutant strain TA98 and base-pair substitution mutant strain TA 100.[4,5] However, the *ame*-test is influenced by proteinase and amino acid-rich samples. Oda *et al.*[6] developed a new system to evaluate environmental carcinogens and mutagens called the *umu*-test. *S. typhyimurium* TA1535/pSK1002 is used as the tester strain in the *umu*-test, which enables one to monitor the level of *umu*-operon expression by measuring in bacterial cells. The method is a simple, rapid, and very sensitive test.

Many studies have been performed on mutagens, carcinogens, and antimutagenicity of human foodstuffs such as fermented milk products, juices, *Miso*, and vegetable fibers, and some bacteria isolated fermented foods such as *Miso* and cheese.[7-11] However, little information is available on the antimutagenicity of *Bacillus natto* or *Natto*, which is recognized as a health food in Japan.

The object of this study was to learn more about the use of *Natto*, a fermented Japanese health food. The *in vitro* antimutagenicity of *Natto* and *Bacillus natto* isolated from *Natto* was examined using *umu*-test.

Materials and Methods

Preparation of mutagen solution. Trp-P-2 (3-amino-1-methyl-5H-pyrido-(4,3-*b*)indole) and IQ (IQ type, 2-amino-3 methylimidazo-(4,5-*f*)quinoline) were obtained from Wako Pure Chemical Industry Co. Ltd. (Osaka, Japan) for use as mutagens. Each mutagen (0.5 mg) was dissolved in 1 m*l* dimethyl sulfoxide (DMSO) and stored in a refrigerator at –4°C prior to use. All chemicals used in this experiment were purchased from Wako Pure Chemical Industry Co. Ltd.

Media and strains used. LB medium consisted of 10 g bacto tryptone, 5 g Yeast extract, and 5 g sodium chloride, with pH adjusted to 7.0. TGA medium consisted of 10 g bacto tryptone, 5 g sodium chloride, 2 g glucose, 50 mg ampicillin, with pH adjusted to 7.0. B-2 broth (g/*l*) consisted of 10 g polypeptone, 10 g meat extract, 5 g sodium chloride, and 50 mg ampicillin, with pH adjusted to 7.0. Z-buffer[12] consisted of 0.04 M $NaH_2PO_4.2H_2O$, 0.06 M NaH_2PO_4, 0.01 M KCl, and 0.001 M $MgSO_4.7H_2O$ and was added with 0.05 M 2-β-mercaptoethanol before use. Z-buffer and S9 were obtained from Osaka City University, and *Salmonella typhimurium* TA 1535/pSK 1002 was a gift from Osaka Prefecture Institute of Public Health, Japan. Eight separate colonies were isolated from the mixture of two commercial *Natto* samples and measured their desmutagenicity to Trp-P-2 and IQ. The colonies were tentatively identified using the methods described by Sneath *et al.*[13]

Metabolic activation of Trp-P-2 and IQ. Fifty micrograms of Trp-P-2 or IQ in 50 μ*l* DMSO solution was diluted with 0.45 m*l* deionized water. The mutagen solution was mixed with 0.5 m*l* S-9 mix, incubated at 37°C for 30 min with

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shaking, and used immediately.

Activation mixture. S-9 fraction was prepared from livers of male rats pretreated with phenobarbital and 5,6-benzoflavone. S-9 mixture, necessary as an activator for mutagens, was prepared by adding 1 m*l* S9 (Oriental Yeast Co., Tokyo) to 9 m*l* cofactor and filter-sterilized (ϕ, 0.45 μm, Roshi, Tokyo, Japan). Co-factor (mg/vial) consisted of 16.3 $MgCl_2 \cdot 6H_2O$, 24.6 KCl, 17.0 Glucose-6-phosphate, 36.2 NADPH, 30.5 NADH, 119.6 Na_2HPO_4, and 24.7 $NaH_2PO_4 \cdot 2H_2O$.[3]

Desmutagenicity assay. Desmutagenic effect was investigated through *umu*-test system using *S. typhimirium* TA 1535/pSK1002. The strain was grown in either Luria broth (LB) or TGA medium supplemented with 50 μg/m*l* ampicillin. The bacterial cells grown at 37°C for 12 h were diluted 5-fold with TGA broth, and incubated at 37°C with shaking at 180 rpm until the cell density reached 0.25-0.3 at OD 600. The culture was divided into 2.8 m*l* portions in test tubes.

The mutagen mixture (25 μg mutagens in 50 μ*l* DMSO solution) containing 5 mg test samples and 0.5 m*l* S9 microsobmal fraction (for metabolic activation) was incubated at 37°C for 20 min and centrifuged at 15,000 × g for 20 min. The supernatant of 0.2 m*l* test samples was added to each culture tube. After 2 h incubation at 37°C for 20 min with gentle shaking, the level of β-galactosidase activity in the cells was assayed. The inhibition of β-galactosidase activity was evaluated as follows. Levels of the controls were determined as above but excluding cells and mutagens. The mixture was incubated at 37°C for 25 min with shaking. The mixture was centrifuged at 1,500 × g for 15 min at 4°C, and the supernatant (200 μ*l*) was used for determination of β-galactosidase activity, which was measured by Oda's methods.[6] The degree of β-galactosidase activity (desmutagenicity) inhibition was calculated as follows:

Desmutagenicity (%) =
(β-gal. activity of control − β-gal. activity of sample)/
(β-gal. activity of control)

Bioantimugenic effect assay. *S. typhimurium* SD-100 provided by Prof. Ebata of the Faculty of Science of Living at Osaka City University was used as a tester strain to detect the mutation. This strain is streptomycin-dependent but not histidine-dependent, and is not influenced by amino acids existing in the examined samples. The B-2 broth supplemented with 20 μg/m*l* streptomycin was used to culture the bacterial cells. For the counting of surviving cells, 0.1 m*l* each sample was plated on B-2 medium supplemented with 20 μg/m*l* streptomycin.

S. typhimurium SD-100 was grown at 37°C for 20-25 h in B-2 broth supplemented with 20 μg/m*l* streptomycin. The bacterial cells were washed three times with 50 m*l* sodium phosphate buffer (pH 7.0), and the cells were resuspended in the same buffer. To mutate the tester strain of *S. typhimurium* SD-100, 3 m*l* bacterial suspension was incubated with 2 m*l* solution containing activated metabolites mutagens at 37°C for 40 min, and the supernatant was discarded. The cells were washed three times with the buffer to remove the mutagens, and the cells were suspended in 9 m*l* of 50 mM sodium phosphate buffer. The bacterial solution (0.1 m*l*) was mixed with 0.1 m*l* each of various concentrations of the strain No. 3, which tentatively identified as *Bcillus natto,* cytoplasm (the cytoplasm was filter-sterilized (ϕ, 0.45 μm, Toyo Roshi Co. Ltd.). Control consisted of buffer (0.1 m*l*) added with 0.1 m*l* bacterial solution. The mixture (100 μ*l*) was spread on the B-2 plates and incubated at 38°C for 3 days. The numbers of SM^{ind} revertant colonies, which had been induced by N-OH-Trp-P-2 or N-OH-IQ, and surviving colonies (SM^d) were counted.

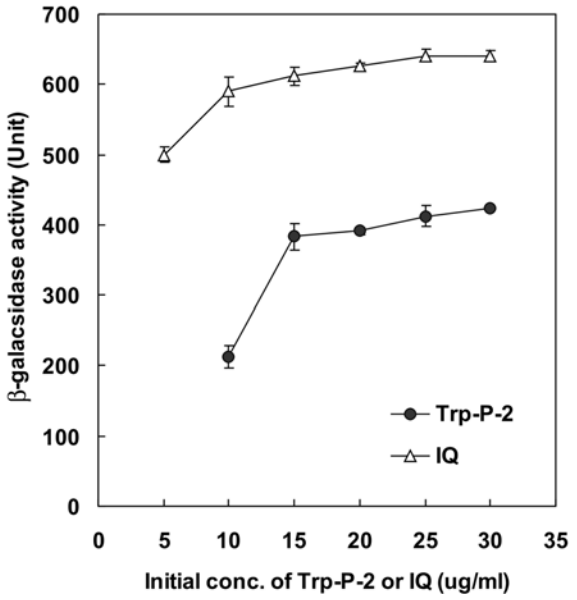

Fig. 1. The relationship of concentration of Trp-P-2 and IQ with the activity of β-galactosidase induced in the cells of *Salmonella typhimurium*. The error bars are standard deviation.

Mutagen measure. The mutagens were quantified through HPLC (Toyo Soda UV Model 2, Tokyo, Japan) equipped with a UV monitor and a reverse-phase column (TSK gel ODS-120T, 7.8 mm × 30 cm, Toyo Soda, Tokyo, Japan). A mobile phase of acetonitril/H_2O triethylamine (50 : 50 : 0.05) was used at a flow rate of 1 m*l*/min. Absorbance at 254 nm was recorded using an integrator.

Results and Discussion

Desmutagenic activities of *Bacillus natto* bacterial cells against Trp-P-2 and IQ. The eight isolates obtained from *Natto* and tentatively identified as *Bacillus natto* markedly inhibited the mutation of bacterial strains caused by Trp-P-2 and IQ (Table 1). The percentages of desmutagenicity towards Trp-P-2 and IQ were around 55-75 and 69-85%, respectively, depending on the isolates used. The bacterial cells manifest very high desmutagenic activity. Therefore, the desmutagenic activities of the bacterial cells fractions were tested (Table 2). The cytoplasm exhibited a significantly high desmutagenic activity, and was higher than that of the whole cell. The reactions of desmutagenicity were around 70-80 and 80-90%,

Table 1. Desmutagenicity of isolates obtained from *Natto* to Trp-P-2 and IQ

No. of isolates	Desmutagenicity (%) to	
	Trp-P-2	IQ
3	55.5 ± 0.78	69.2 ± 1.06
4	66.1 ± 0.90	71.3 ± 1.17
5	66.6 ± 0.72	78.3 ± 0.64
6	66.7 ± 1.35	81.1 ± 0.85
8	71.6 ± 0.95	79.8 ± 1.17
10	72.6 ± 0.78	84.1 ± 0.85
11	74.5 ± 1.06	81.7 ± 0.92
15	72.6 ± 1.22	83.3 ± 0.81

respectively, for Trp-P-2 and IQ.

Desmutagenicities of *Natto* sample, soybean, and VPM (viscous polymeric materials) towards IQ were investigated (Table 3). VPM showed the highest desmutagenic activity (30.5%), followed by soybean sample (18.3%) and *Natto* (3.8%). The desmutagenicities of VPM obtained from *Natto* and soybean were lower than those of bacterial cells and cell fraction (Table 2). Results of several investigations on the binding activity and antimutagenicity of various bacteria isolated from fermented foods to mutagens and carcinogens suggest daily consumption of fermented food can reduce the risk of carcinogenesis caused by mutagens and carcinogens. [7,9,11,14]

Desmutagenicity of cytoplasmic fraction towards Trp-P-2 and IQ. When the concentration of cytoplasm increased, desmutagenicity also increased (Fig. 2). The results indicated that the bacterial cells and cytoplasm possess high desmutagenicity towards the mutation of tester strain caused by Trp-P-2 and IQ. Cell wall also showed desmutagenic activities, compared to which *Natto* sample and VPM exhibited weak activities. However, whether this suppressive effect of cytoplasm is due to its bioantimutagenicity or desmutagenicity is yet unclear.[15]

Bioantimutagenic effect assay. The bio-antimutagenicity of isolate No-3, which was tentatively identified as *Bacillus natto,* cytoplasm on tester bacterial cells mutated by the activated metabolites of Trp-P-2 and IQ is shown in Table 4. The induced mutation frequency was expressed as the ratio of the number of revertant colonies (SM^{ind}) versus the number of surviving colonies (SM^{d}). A decrease in the value of mutation frequency reflects the bioantimutagenic effect of the cytoplasm examined.

Table 3. Desmutagenicity of VPM, *Natto* and soybean samples to IQ

	VPM*	Natto	Soybean
Desmutagenicity (%)	30.5 ± 0.60	18.2 ± 0.85	3.8 ± 1.06

*viscous polymeric materials

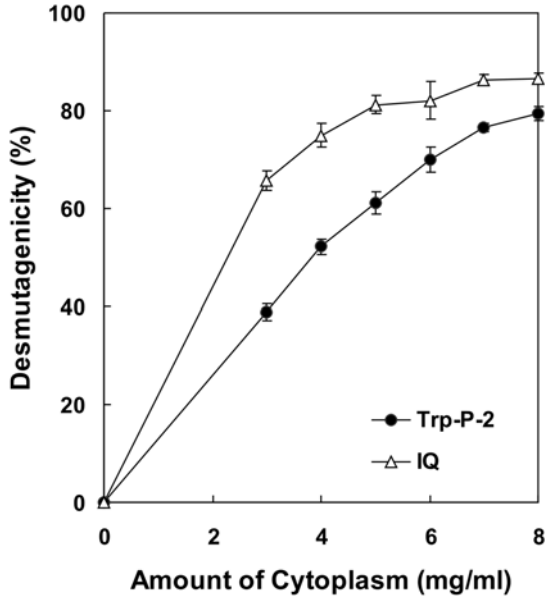

Fig. 2. Desmutagenicity of bacterial cells of *Bacillus natto* to Trp-P-2 and IQ. The error bars are standard deviation.

In the case of Trp-P-2, SM^{d} remained almost constant, and SM^{ind} also did not decrease with the increasing amount of cytoplasm. The frequency on plates without cytoplasm was 976×10^{-8} and remained unchanged with the addition of 2 and 4 mg of cytoplasm. The frequencies of 2 and 4 mg cytoplasm were 976×10^{-8} and 971×10^{-8}, respectively, which is an indication that the cytoplasm of strain B6-3 did not exhibit bioantimutagenicity on bacteria mutation caused by the activated metabolite of Trp-P-2.

In the case of IQ, the frequency on plates without cytoplasm

Table 2. Desmutagenicity of cell fractions of the isolates obtained from *Natto* to Trp-P-2 and IQ

No. of isolates	Trp-P-2		IQ	
	cytoplasm	cell wall	cytoplasm	cell wall
3	85.0 ± 0.90	36.1 ± 0.65	90.3 ± 1.03	23.3 ± 0.71
4	79.8 ± 1.22	40.0 ± 0.80	85.5 ± 0.81	19.3 ± 0.95
5	68.7 ± 0.86	42.0 ± 0.59	80.5 ± 0.91	24.5 ± 1.01
6	80.4 ± 0.95	43.3 ± 0.59	86.5 ± 1.01	12.3 ± 0.82
8	79.8 ± 0.90	49.3 ± 1.19	83.6 ± 0.87	16.7 ± 0.66
10	78.6 ± 1.22	47.4 ± 0.95	85.4 ± 0.95	40.5 ± 0.86
11	76.5 ± 0.67	47.3 ± 0.95	86.6 ± 0.75	41.6 ± 0.79
15	71.7 ± 0.90	30.5 ± 0.97	90.2 ± 0.92	12.4 ± 0.90

Table 4. Bioantimugenicity of cytoplasm of the isolate No-3 tentatively identified as *Bacillus natto* on bacteria mutated by the activation metabolites of Trp-P-2 and IQ

Added amount of Cytoplasm (mg/plate)	Mean number of surviving colonies* ($\times 10^6$/plate)	Mean number of induced colonies** (mean No./plate)	Frequency of induced SM^{ind} (revertant/10^8 survivors)
(for Trp-P-2)			
0	89.3 ± 0.95	30.1 ± 0.90	976.3 ± 1.53
2 mg	91.3 ± 0.55	45.9 ± 1.41	976.9 ± 0.90
4 mg	87.1 ± 0.85	43.9 ± 1.53	971.0 ± 0.90
(fro IQ)			
0	101.1 ± 0.86	34.0 ± 1.13	1402.1 ± 0.85
2 mg	87.3 ± 0.85	44.0 ± 1.53	1300.9 ± 0.65
4 mg	93.1 ± 0.81	46.9 ± 0.95	1180.5 ± 0.50

All values are mean+SDs (n = 3)
*The treated bacterial suspension (0.1 m*l*) was plated for observation of mutation induction. From the mean number of induced colonies appearing per plate, the mean number of spontaneous mutant colonies per plate was subtracted.
**Induced mutation frequency *induced revertants/10^8 viable surviving cells). Inhibitory rate (%) = [induced mutation frequency of control minus induced mutation frequency of test]/[induced mutation frequency of control]
*surviving colonies means SM^d
**induced colonies means SM^{ind}

was 1402×10^{-8}. Upon the addition of 2 and 4 mg cytoplasm, SM^{ind} decreased slightly. The frequency on the plates with 2 mg cytoplasm was 1300×10^{-8}, showing 7.2% bioantimutagenicity of the mutagenesis induced by IQ. On the plates with 4 mg cytoplasm, the frequency decreased to 1180×10^{-8}, exhibiting 15% bioantimutagenicity of the mutagenesis induced by IQ.

In general, IQ is a stronger mutagenic compound compared to other known mutagens such as Trp-P-2 and Trp-P-1.[2,16] However, no report had been published on IQ bioantimutagenicity even though it showed high binding activity with some microorganisms and fibers such as lactic acid bacteria, yeast, and fungi.[3,8] The present study revealed that the cytoplasm fraction extracted from *Bacillus natto* has 17% bioantimutagenicity.

From the results obtained, the suppressive effect against the mutagenesis of tester bacterial strains caused by pyrolysates, Trp-P-2, and IQ can be ascribed to their desmutagenicity rather than bioantimutagenicity. The results further suggest that the antimutagenic activity of *Natto* products and the bacterial cells existing in the products towards other types of mutagens and carcinogens, which are usually formed both in foods and intestinal tracts, would be possible.

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