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Note

Suppressive Effects of the Extract from Sake Cake on Chemical Mutagen-Induced SOS Response in *Salmonella typhimurium*

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The suppressive effects of the extract from sake cake on the SOS response of *Salmonella typhimurium* TA 1535/pSK1002 induced by AF-2, 4NQO, Trp-P-1, Trp-P-2, IQ and MeIQx were investigated for the purpose of finding antimutagenic substances in sake cake. The water-extract and methanol-extract from sake cake strongly suppressed SOS response induced by IQ in the presence of S9 mix in a dose dependent manner. The molecular weight of a suppressive compound in the water-extract was less than 3000 in an analysis of gel filtration. Several suppressive compounds also seemed to exist in the methanol-extract from sake cake.

Keywords: sake cake, SOS response, suppression, antimutagenicity

There have been many reports in recent years on the important functions of food other than its nutritional function as a source of physiologically active substances that are effective in the metabolic control of the living body, prevention of aging, and anticarcinogenicity. A number of studies on the antimutagenicity of foods such as fruits, crops, vegetables and some fermented foods have been reported. It is known that isoflavone in miso and tempe, fermented products from soybean, has suppressive effects on the SOS response induced by Trp-P-1 (Kiyosawa *et al*., 1995). The water and methanol-extracts of soymilk fermented by bifidobacteria suppressed the mutagenicity induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Kiyosawa *et al*., 1992). The acetone extract from yogurt, a fermented product of milk, had an antimutagenicity against MNNG and 3-2′-dimethyl-4-aminobiphenyl (Bakalinsky *et al*., 1996). These reports indicated that there are unknown antimutagenic compounds in fermented products but not in raw materials. Sake cake (sakekasu), a by-product of sake-brewing from rice in Japan, is used to a limited extent in the food-processing industry as a raw material for the preparation of pickles, a substitute for Shubo-kakemai in sake-brewing and a resource for protein production. Therefore an effective way of utilization of sake cake is anticipated not only as a food material but also as a source of unknown physiologically active substances. As to the physiological function of sake cake, it has been reported that serum cholesterol was decreased by the intake of fiber in the cake (Ashida *et al*., 1997, Mochida *et al*., 2000), but the antimutagenicity of sake cake has not been investigated. Therefore, we attempted to examine if an extract of sake cake has antimutagenic activity by following the suppressive effect on the SOS response of *Salmonella typhimurium* induced by several chemical mutagens. β-Galactosidase activity of the bacterium corresponding to the *umu* C gene expression against DNA damage was measured to estimate the suppressive effect of the extract of sake cake in this work.

Materials and Methods

Chemicals and materials Mutagens were obtained from Wako Pure Chemical Industries, Osaka. S9 mix was obtained from Oriental Yeast Industries, Tokyo. The other chemicals were analytical grade reagents from Nacalai Tesque, Inc., Kyoto, and Wako Pure Chemical Industries, Osaka. A fresh sake cake (sakekasu) containing about 50% water and 8% ethanol prepared from Junmai-sake brewing was obtained from Tamanohikari Sake Brewing Co. Ltd., Kyoto. It was stored at 4°C until use.

Microorganism and cultivation *Salmonella typhimurium* TA1535/pSK1002 was made available by the gift of Dr. Yoshimitsu ODA, the Osaka Prefecture Institute of Public Health, Osaka. *S. typhimurium* was cultured at 37°C for 16 h with LB agar plate (10 g bactotrypton, 5 g yeast extract, 5 g NaCl and 50 mg ampicillin in 1000 ml of water, pH 7.0). An aliquot of the bacterial culture was then inoculated to TGA medium (10 g bactotrypton, 5 g NaCl, 2 g glucose and 20 mg ampicillin in 1000 ml of water) and further cultivated at 37°C to an appropriate bacterial density (absorbance at 600 nm: 0.2–0.25). The cell suspension was used for the *umu* test as described below.

Preparation of water-extract and methanol-extract from sake cake A three fold volume of distilled water was added to 100 g of sake cake and mixed at room temperature for 5 min and the mixture was centrifuged at 5000 rpm for 10 min. The supernatant solution was lyophilized and 10.1 g of lyophilized powder was obtained from 100 g of the cake. The lyophilized powder was dissolved in water (50% w/w), and it was used as a water-extract for the *umu* test unless otherwise stated. The residue of sake cake obtained by centrifugation after water extraction was lyophilized and about 11.0 g of lyophilized powder was obtained

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from 100 g of sake cake. The lyophilized sample was suspended in a 20 fold volume of methanol and mixed for 10 min at room temperature. The suspension was centrifuged at 5000 rpm for 10 min, and the supernatant methanol solution was concentrated to dryness by evaporation under reduced pressure. The concentrate was dissolved in the same volume of dimethylsulphoxide (DMSO) and applied to an *umu* test as a methanol extract.

Preparation of hexane-extract from methanol-extract The methanol-extract from sake cake described above was dissolved in a mixture of water and *n*-hexane (1:6 by volume) and was shaken for 5 min at room temperature. The water phase and *n*-hexane phase were separated. The water phase was lyophilized, and was dissolved in the same volume of water as methanol-extract before separation to make a methanol-water extract. The *n*-hexane phase was evaporated under reduced pressure and the residue was dissolved in the same volume of DMSO as methanol-extract to make a hexane-extract.

Assay of the suppressive SOS response (umu test) The suppression of SOS response was measured by a slightly modified method of the *umu* test (Oda *et al*.,1985). The standard assay mixture for the suppressive effect on the SOS response contained 20 μl of direct or indirect mutagens (see below), 0.1 ml of each extract prepared from sake cake and 1.9 ml of bacterial suspension as previously described. For the assay using indirect mutagens, 1.6 ml of the cell suspension was mixed with 0.3 ml of S9 mix solution. After incubation at 37°C for 2 h, the mixture was diluted with 0.85% NaCl solution, and the cells were collected by centrifugation at 3000 ×*g* for 20 min. The cells were resuspended in 0.85% NaCl solution, and then β-galactosidase activity of the cell suspension was assayed (*A* unit). The control solution containing 0.1 ml of water in place of the extract from sake cake was also assayed for the enzyme activity (*B* unit). The percentage suppression of the SOS response was calculated from the expression of $(1-A/B) \times 100$. β-Galactosidase activity of the bacterial suspension was assayed as follows: Two hundred microliters of bacterial suspension in 0.85% NaCl was vigorously mixed with 1.8 ml of Z-buffer (0.06 M $Na_2HPO_4 \cdot 12H_2O$, 0.04 M $NaH_2PO_4 \cdot 2H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$ and 0.05 M β-mercaptoethanol) in the presence of 50 μl of 0.1% sodium dodesyl sulfate and 10 μl of chloroform. The enzyme reaction was initiated by the addition of 0.2 ml of 2-nitrophenyl-α-D-galactopyranoside solution (4 mg/ml in 0.1 M phosphate buffer, pH 7.2), incubated at 28°C for 20 min and stopped by adding 1.0 ml of 1 M Na_2CO_3.

β-Galactosidase activity was calculated as follows (Miller, 1972);

$$\beta\text{-Galactosidase unit} = 1000(C - 1.75 \times D)/\ 0.2 \times E,$$

where *C* and *D* represent the absorbance at 420 nm and 550 nm, respectively, of the enzyme reaction mixture of the β-galactosidase assay, and *E* shows the absorbance at 600 nm of the bacterial suspension grown in the *umu* test. The experimental results were expressed as the average of two experiments with triplicate assays.

In the *umu* test, furylfuramide (AF-2), and 4-nitroquinoline-1-oxide (4NQO) were used as direct mutagens without activation to induce mutagenicity, while 2-aminoanthracene (2AA), 2-amino-3-methylimidazo (4,5-f) quinoline (IQ), 2-amino-3, 8-dimethylimidazo (4,5-f) quinoline (MeIQ), 3-amino-1,4-dimethyl-5*H*-pyrido (4,3-b) indole(Trp-P-1), and 3-amino-1,4-dimethyl-5*N*-pyrido (4,3-b) indole (Trp-P-2) were used as indirect mutagens which were activated by S9 mix during incubation. Each mutagen was used at the concentration which induced 500–1000 units of β-galactosidase activity.

Table 1. Suppressing effects of water-extract from sake cake on SOS response of *S. typhimurium* TA1535/pSK 1002.

Mutagen (μg/ml)		S9mix[a)]	Suppression (%)
AF-2	(0.03)	−	2.3±2.4
4NQO	(0.3)	−	3.8±2.0
2AA	(1.0)	+	21.6±1.8
IQ	(0.3)	+	51.9±2.0
MeIQ	(0.03)	+	22.9±3.4
Trp-P-1	(3.0)	+	32.9±2.4
Trp-P-2	(3.0)	+	34.9±1.1

[a)] + added, − not added.
Each data is shown by means±S.E. (n=6). Water extract equivalent to 1 g of sake cake was used in the *umu* test. In *umu* test, concentration of water-extract was 2.5%.

Results and Discussion

Induction of β-galactosidase activity by extracts from sake cake First we examined the mutagenicity of sake cake in an induction system of β-galactosidase activity of the bacterium by the extracts in the absence of mutagens in reaction system for the *umu* test. β-Galactosidase activity of the test bacterium which was induced in the presence of water-extract (98.6±2.5 unit) and methanol-extract (103.7±0.1 unit) was almost the same value as that in the presence of water (101.6±0.6 unit) and methanol (103.5±1.3 unit). Therefore, it was confirmed that neither extract from sake cake had mutagenicity by itself. As the absorbance of 600 nm measured as an indicator of cell number in the presence or absence of both extracts was almost unchanged, neither extract had toxicity for the cell.

Suppressive effects of water-extract from sake cake on SOS response The suppressive effects of water-extract from sake cake on SOS response of *S. typhimurium* induced by direct or indirect mutagens were investigated. Table 1 shows that the

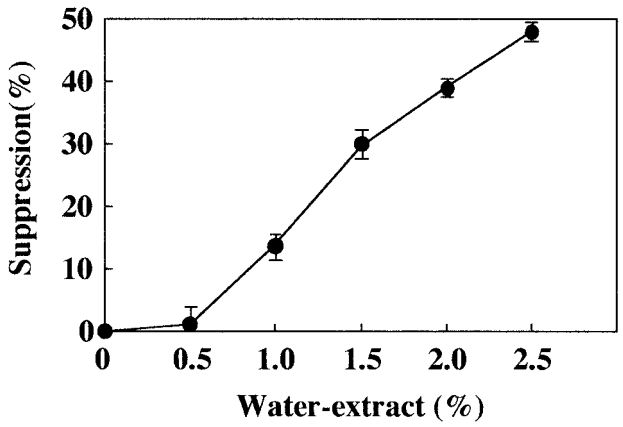

Fig. 1. The effect of concentration of water-extract from sake cake on suppression of SOS response induced by IQ in the presence of S9 mix. The concentration of water-extract prepared from sake cake as shown in Materials and Methods was changed in the *umu* gene expression reaction system. The concentration of IQ was 0.3 μg/ml. Each value is shown as the mean ±S.E.(n=6).

Table 2. Suppressing effects of water-extract from sake cake on SOS response of *S. typhimurium* TA1535/pSK 1002

Mutagen (μg/ml)		S9mix[a)]	Suppression (%)		
			Methanol-extract	Hexane-extract	Methanol-water-extract
AF-2	(0.03)	−	5.3±4.5	−	−
4NQO	(0.3)	−	11.3±6.0	−	−
2AA	(1.0)	+	55.4±5.1	45.1±6.8	n.s.[b)]
IQ	(1.0)	+	41.1±2.3	42.1±1.7	n.s.
MeIQ	(0.03)	+	45.3±1.3	49.8±2.0	n.s.
Trp-P-1	(3.0)	+	38.4±2.7	27.0±7.1	25.4±3.9
Trp-P-2	(3.0)	+	52.0±3.0	36.0±9.1	30.0±3.9

[a)] + added, − not added.
[b)] n.s.=not suppressed.
Each data is shown by means±S.E. (n=6). Methanol-, hexane- and methanol-water-extract prepared from 50 mg of sake cake was used in the *umu* test.

water-extract seemed to suppress SOS response induced by indirect mutagens which were activated by addition of S9 mix, especially for IQ. The effect of the concentration of water extract on the suppression of SOS response induced by IQ was found to increase almost linearly with the concentration of water-extract added in a range of 0.5 to 2.5 % (Fig. 1). The water-extract was then treated by gel filtration. The suppressive effect was found in a fraction with a molecular weight of more than 3000 (data not shown).

Suppressive effect of methanol-extract from sake cake on SOS response Investigation of the suppressive effects of methanol-extract from sake cake on the SOS response of *S. typhimurium* induced by direct and indirect mutagens showed that the methanol-extract had a suppressive effect on the SOS response of mutagens activated by S9 mix (Table 2). Relatively higher suppression rate was found for 2AA (55%) and Trp-P-2 (52%) among the mutagens tested. The suppressive effect of methanol-extract on SOS response induced by IQ increased almost linearly with the concentration of extract added (data not shown). Methanol-extract was further fractionated by a mixture of water and *n*-hexane to determine the suppressive effect of each fraction. Table 2 shows that the hexane-extract strongly suppressed the SOS response induced by 2AA, IQ, and MeIQx, the rate of suppression being 45.1, 42.1, 49.8%, respectively. On the other hand, the SOS response induced by Trp-P-1 and Trp-P-2 was suppressed by both hexane-extract and methanol-water-extract at the same level. Therefore, it seems that several suppressive compounds may exist in methanol-extract from sake cake.

This study has suggested that some antimutagenic compounds may be present in the water-extract of sake cake which is a by-product of sake brewing. As the fraction with a molecular weight of more than 3000 in the water-extract was positive in Molisch reaction, Fehling reaction and Ninhydrin reaction, respectively, sugar, peptide or protein may be involved in the suppression of the SOS response induced by mutagens. Both hydrophilic and hydrophobic compounds may exist in the methanol extract. Isolation and identification of the unknown compound in sake cake that shows a suppressive effect on SOS response are now under investigation. The mechanism of suppression on the SOS response by the water and methanol-extracts is speculated to be as follows: substances in the extract of sake cake may inactivate the metabolizing enzyme in S9 mix which activated certain mutagens, because both extracts suppressed indirect mutagens activated by S9 mix more than direct mutagens.

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