Desmutagenic and Bio-antimutagenic Effects of the Extracts from Japanese Miso in *Salmonella* Assay

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**Synopsis**

Using a *Salmonella* microsome assay, the antimutagenic effects of specific components of the extracts from Japanese Miso were investigated. Miso exhibited an antimutagenic activity against ethyl methanesulfonate (EMS)-induced mutagenicity. This activity was recognized in the fractions extracted with acetone and ether. The soybean-koji miso showed markedly antimutagenic effects with both ether layer (fraction 1) and water layer (fraction 2). No mutagenicity or toxicity was observed for *Salmonella typhimurium* TA100 using any of the extracts. To study the mechanism of the activity, the extracts of soybean-koji miso were employed further for desmutagenic test and bio-antimutagenic test. Fraction 1 extract was observed to exhibit both the desmutagenic effect and the bio-antimutagenic effect, but fraction 2 was observed to exhibit only the desmutagenic effect. It is suggested that miso is composed of multiple components with the antimutagenic activities.

**Introduction**

Miso, a kind of fermented food having a history over 1300 years in Japan, has very much to do with Japanese daily life. The consumption of miso is in abundance for miso soup and others. The raw materials used for the preparation of miso are soybeans, wheat, barley and rice. Miso is prepared by microbial fermentation of the mixture of raw materials for a long period till it changes to the peculiar components of ripe miso.

There are some reports about the antimutagenic effects of miso. Yamamoto *et al.* have reported the suppression of the 3-amino-1-methyl-5H-pyrrolo[4,3-b] indol (Trp-P-2) and 3,4-benzpyrene (B[e]P) induced mutagenesis in *Salmonella* assay by free unsaturated fatty acids such as linoleic acid, linolenic acid and oleic acid in miso[1]. Asahara *et al.* investigated the binding of mutagenic pyrolysates and subsequent antimutagenic activities of microbial strains of miso. Bacteria and yeast showed the largest antimutagenic effect towards Trp-P-2, but most strains tested had no effective antimutagenic activity against 3-amino-1-methyl imidazo [4,5-f] quinoline (IQ)[2]. The effect of miso diet on induction of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) mediated gastric tumors in rats have been reported. It was reported that compared to the group taking the solution of NaCl with MNNG the incidence of gastric tumors in the group taking miso with MNNG was reduced[3].

In this paper, we report the desmutagenic and bio-antimutagenic effects of the ether and water layers of the acetone extracts of miso on ethyl methanesulfonate (EMS) mediated induction of His+ colonies of *Salmonella typhimurium* TA100.

**Materials and Methods**

*MATERIALS AND REAGENTS.* The commercial products of rice-koji miso (white type, light yellow type and dark yellow type), barley-koji miso and soybean-koji miso, were purchased from a department store in Osaka city, Japan and stored at 4°C until use. Ethyl methanesulfonate (EMS) was obtained from Aldrich chemical Co. Bacto-agar was obtained from...
Dişco Laboratories. Oxoid nutrient broth No.2 was purchased from Unipath Ltd. All other chemicals and reagents used were of the commercially highest quality.

**Preparation of the extracts from Miso.** Two hundred grams of the miso was extracted with 800 ml of acetone (4°C, 24h) and then filtered. After the filtrate was evaporated at 40°C under reduced pressure to yield 1/20 of the original volume, the concentrated solution was extracted three times with diethyl ether of twice the volume of the concentrated solution. The solution was separated into the diethyl ether layer and the water layer and each layer was collected separately. The diethyl ether layer was evaporated at about 50°C under reduced pressure and the dry residue was dissolved in 10 ml of DMSO (fraction 1). The water layer was also evaporated at about 50°C under reduced pressure until 2 ml. The solution was then dissolved in 8 ml of DMSO (fraction 2). These solutions were used after filtering using a membrane filter (0.45 μm) for sterilization.

**Assay of antimutagenicity.** The suppression of mutagenicity test was investigated using *Salmonella typhimurium* TA100, with slight modification of the mutagenic test as described by Maron and Ames. The mixture of 0.1 ml of the overnight culture of *S. typhimurium* TA100, 50 μl of 8% EMS and the extracts of miso were thoroughly spread onto the minimal glucose plates. The plates were allowed for incubation at 37°C for 48h in an incubator and the number of His+ colonies were counted.

**Assay of desmutagenicity.** The desmutagenic effect of the extracts of miso was assayed using the Ames test. The mixture of 150 μl of 8% EMS and 300 μl of miso extracts in DMSO was incubated for 30 min at 37°C with slight shaking, then heated at 100°C for 3 min. The mixture was then filtered using a membrane filter (0.45 μm) and the filtrate was assayed. The mixture of 0.1 ml of the overnight culture of *S. typhimurium* TA100 and 150 μl of the filtrate was thoroughly spread onto the minimal glucose plates. The plates were allowed for incubation at 37°C for 48h in an incubator and the number of His+ colonies were counted. The procedure was as described by Mitsher et al., Shankel and Clarke and Yoshikawa et al.

**Assay of bio-antimutagenicity.** After the mixture of 500 μl of the culture of *S. typhimurium* TA100 and 250 μl of 8% EMS was kept in water bath at 37°C for 30 min with shaking, the mixture was washed three times with phosphate buffer (PB, 0.067M KH₂PO₄ / 0.067M Na₂HPO₄, pH 7.0) by centrifugation (1,100 X g) to remove mutagen retained in suspension. The bacterial cells were harvested and suspended in 0.5 ml of PB. The Ames assay was performed by pouring the mixture of 0.1 ml of the suspension and 0.1 ml of the extracts of miso in DMSO onto minimal glucose plates. There plates were incubated at 37°C for 48h and the number of His+ colonies was counted.

**Calculations of % of remaining mutagenicity.** Antimutagenic, desmutagenic and bio-antimutagenic activities were expressed as % of remaining mutagenicity:

\[
\text{Remaining mutagenicity} (\%) = \frac{(A-C)}{(B-C)} \times 100
\]

where A and B are number of EMS-induced His+ revertants observed in the presence and absence of the extracts, respectively; and C is numbers of spontaneous His+ revertants observed in DMSO control.

**Results and Discussion**

The deleterious effect of ubiquitously occurring environmental mutagens and carcinogens have been investigated intensively. The suppression of formation of mutagens and carcinogens by fruits and vegetables lead to the understanding that the components of daily diet are capable of preventing the mutation leading to carcinogenesis. In this paper, an investigation was carried out to understand the antimutagenicity of miso, a common diet in Japan, towards EMS induced mutagenesis. The acetone extract of 5 different miso was fractionated to ether layer (fraction 1) and water layer (fraction 2) by the addition of ether after the complete evaporation of acetone. Antimutagenicity of these fractions towards the EMS induced mutagenesis in *Salmonella typhimurium* TA100 is shown in Table 1. The decrease in the revertant colonies of *S. typhimurium* TA100 was observed to be dependent on the concentration of test samples. In addition, toxicity was not observed towards *S. typhimurium* TA100 in the test concentration. Out
Table 1. Antimutagenicity of extracts of miso against the mutagenicity of EMS in Salmonella typhimurium TA100 test system

<table>
<thead>
<tr>
<th>Extract of miso</th>
<th>Rice-koji miso</th>
<th>EMS (33 nmol/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>white type</td>
<td>light yellow type</td>
</tr>
<tr>
<td></td>
<td>(µl/plate)</td>
<td></td>
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<tr>
<td><strong>Fraction 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>524±41.3 (55)</td>
<td>476±7.8 (48)</td>
</tr>
<tr>
<td>20</td>
<td>462±22.5 (46)</td>
<td>469±17.0 (47)</td>
</tr>
<tr>
<td>50</td>
<td>441±35.4 (44)</td>
<td>431±18.0 (41)</td>
</tr>
<tr>
<td><strong>Fraction 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>778±47.4 (77)</td>
<td>736±25.0 (72)</td>
</tr>
<tr>
<td>20</td>
<td>727±57.3 (71)</td>
<td>787±29.0 (78)</td>
</tr>
<tr>
<td>50</td>
<td>658±69.1 (62)</td>
<td>647±27.9 (61)</td>
</tr>
</tbody>
</table>

Values are mean colony counts±SD of three times. Remaining mutagenicity (%) of EMS-induced mutagenesis are in parentheses. Mean colony counts of EMS-induced His+ revertants in the absence of extracts were 829±46.0 (n=15) in experiments with fraction 1 and 927±50.4 (n=15) in experiments with fraction 2. Mean colony counts of spontaneous His+ revertants in the controls were 150±20 (n=15) in experiments with fraction 1 and 136±15.0 in experiments with fraction 2.

See the text for detail of fractionation.

of 5 different miso investigated, Soybean-koji miso exhibited higher degree of suppression of the remaining mutagenicity of EMS. The remaining mutagenicity in 50 µl/plate of the fraction 1 and 50 µl/plate of the fraction 2 was observed to be 26% and 35%, respectively. Rice-koji miso and barley-koji miso are prepared by fermenting the mixture of boiled soybean, NaCl and koji prepared by inoculating koji mold to rice or barley, respectively. Soybean-koji miso is also prepared in similar way where soybean is used in preparation of koji. The comparative high antimutagenicity exhibited by soybean-koji miso is proposed to be due to the constituents of soybean.

Mutation suppressibility of any compound has been interpreted as desmutagenic or bio-antimutagenic depending upon the mode of its antimutagenicity. In order to elucidate the possible antimutagenicity of Soybean-koji miso, the time of incorporation of test sample in the Salmonella / microsome assay was altered and investigated. On employing the sample prepared by allowing fraction 1 to react with EMS for 30 min at 37°C in Salmonella / microsome assay, the revertant colonies of S. typhimurium TA100 were fewer than those observed in control plates, indicating possible desmutagenic effect. In addition to this, fraction 1 was also observed to decrease the revertants of S. typhimurium TA100 preincubated with EMS for 30 min at 37°C (Fig. 1.). From these results, it is thought that fraction 1 exhibits both the desmutagenic effect of inactivating EMS and bio-antimutagenic effect of destabilizing DNA damage induced by EMS. Fatty acids and lipids are thought to be the major components in the diethyl ether layer of an acetone extract of miso. In one of the report by Yamamoto et al., it has been reported that free fatty acids exhibits the mutation suppressibility. From the results of fraction 1 of soybean-koji miso, it is thought that different components might be acting to exhibit desmutagenic and bio-antimutagenic effect towards EMS induced mutagenesis.

The fraction 2 of soybean-koji miso was also investigated in the similar pattern as that of fraction 1. In fraction 2 the desmutagenic effect was observed prominently while bio-antimutagenic effect was not so prominent (Fig. 2). In one of the reports, it is reported that the external and internal dialysate of water soluble fraction of miso exhibits the antimutagenicity towards AF-2 induced mutagenesis. Isoflavones such as daidzein and genistein, which are found in considerable amount in the extracts of miso, have been reported to inhibit the SOS response induced by MNN and Trp-P-1 in
Extract of miso (µl) : Desmutagenicity of fraction 1

Bio-antimutagenicity of fraction 1

Fig. 1 Desmutagenic and bio-antimutagenic effects of miso (fraction 1) against the mutagenicity of EMS in Salmonella typhimurium TA 100

Extract of miso (µl) : Desmutagenicity of fraction 2

Bio-antimutagenicity of fraction 2

Fig. 2 Desmutagenic and bio-antimutagenic effects of extracts of miso (fraction 2) against the mutagenicity of EMS in Salmonella typhimurium TA 100

Salmonella typhimurium TA1535/pSK1002

At present further investigation is ongoing to understand the possible mechanism of suppression of mutagenicity by the fractions of miso with special attention on extraction and identification of antimutagenic compound of miso.

References


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味噌抽出液の変異異原効果と生物的抗変異原効果

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要約

未味噌、変味噌、豆味噌を用い、それぞれアセトン抽出後、抽出液をエーテル可溶分 (Fr. 1) とエーテル不溶分 (Fr. 2) に分画し、S. typhimurium TA100 を用いて Ames test 法でそれぞれの変異異原効果を検定した。それぞれの味噌の Fr. 1 と Fr. 2 とともに Ethyl methanesulfonate (EMS) に対して変異異原効果を示し、この効果は豆味噌で最も大きかった。豆味噌の Fr. 1 は比較的強い変異異原効果と比較的弱い生物的抗変異原効果を示したが、Fr. 2 には生物的抗変異原効果は認められず、変異異原効果のみが確認された。