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Suppression on the mutagenicity of 4-nitroquinoline-*N*-oxide by the methanol extracts of soybean koji prepared with various filamentous fungi

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Abstract

In this study, solid fermentation of soybean with various GRAS filamentous fungi including *Aspergillus sojae* BCRC 30103, *Aspergillus oryzae* BCRC 30222, *Aspergillus awamori*, *Actinomucor taiwanesis* and *Rhizopus* sp. was performed to prepare various soybean kojis. Toxicity, mutagenicity and suppression on the mutagenesis induced by a direct mutagen, 4-nitroquinoline-*N*-oxide (4-NQO) on *Salmonella typhimurium* TA 100, by the various methanol extracts of the prepared soybean koji and unfermented soybean were determined and compared.

Results revealed that methanol extracts of unfermented soybean and kojis show no toxicity and mutagenic activity within the dose levels examined on test organism. On the other hand, antimutagenic activity against 4-NQO was observed with the extract of unfermented soybean. Furthermore, fermentation, regardless of the starter organism employed, resulted in an enhanced antimutagenic effect on the mutagenesis of 4-NQO by the extracts of the soybean koji. Across the dose range (0.625–5.0 mg/plate) tested, a dose-dependent antimutagenic activity was observed. Antimutagenic activities of the koji extracts varied with starter organism, with *A. awamori*-prepared koji extract exhibiting the highest rate of suppression on the mutagenicity of 4-NQO. Further study with *A. awamori* also revealed that fermentation temperature affected the antimutagenic activity of the prepared koji extract. In general, the extract of the *A. awamori*-soybean koji prepared at 30 °C showed a higher antimutagenic activity than those prepared at 25 or 35 °C.

Keywords: Mutagenicity; Antimutagenicity; 4-nitroquinoline-*N*-oxide; Soybean koji; Toxicity

1. Introduction

Maron and Ames (1983) indicated that there is a close correlation between mutagenesis and carcinogenesis. Other researchers have suggested that to avoid unhealthful exposure to mutagens and carcinogens, the intake of antimutagens may decrease the rate of mutation and thus reduce the incidence of cancer in humans (Kim et al., 2000). In Asian countries, the lower rate of breast, prostate and colon cancer has been attributed to the consumption of soy based foods which contain antimutagenic factors (Setchell et al., 1984; Adlercreutz, 1990; Barnes et al., 1990). Given these findings, much effort has been devoted to develop food formula possessing antimutagenic factors so that the rate of mutation and incidence of cancer can be reduced. Previously, many constituents of natural foods such as ascorbic acid, chlorophyll, ellagic acid, oleic acid, tocopherol, and vitamin A, have been found to exhibit antimutagenic activity (Abdelali et al., 1995). In addition, various components of soybean, such as isoflavones, trypsin inhibitor, saponin, and phytic acid, possess antimutagenic and antitumorial activity (Yavelow et al., 1983; Weed et al., 1985; Jing and Waxman, 1995; Rao and Sung, 1995; Miyazawa et al., 1999). Kim et al. (2000) reported that soybean fermented with either *Phellinus igniarius* or *Agrocybe cylindracea* inhibited the mutagenicity of 4-nitro-*o*-phenylemdiamine, NaN_3, 2-aminoflurorene and benzol[*a*]pyrene. Park et al. (2003) also noted a marked antimutagenic activity in doenjang, a Korean fermented soypaste.

In oriental countries, traditional fermented foods are usually prepared with solid fermentation of steamed soybean, rice, barley, wheat and mixtures of wheat flour, with microorganisms such as *Aspergillus* sp., *Rhizopus* sp., and *Bacillus natto*. These

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microorganisms possess high proteolytic, saccharolytic enzyme activity, and may also impart special flavors and color to the fermented products. Besides, some of these microorganisms have also been reported to produce physiological substances associated with antioxidant and antibacterial activity (Santiago et al., 1992; Esaki et al., 1997; Berghofer et al., 1998; Yen and Chang, 2003). For example, miso, tempeh and natto, fermented soybean products prepared with *A. oryzae*, *R. oligosporum* and *B. natto*, respectively, possess higher antioxidative activity than unfermented steamed soybean (Santiago et al., 1992; Berghofer et al., 1998).

In the present study, mutagenesis and antimutagenic effects of soybean koji, prepared with various filamentous fungi commonly used as starter organism for the production of oriental fermented foods, were examined and compared with those of unfermented soybean. In addition, the effect of cultivation temperature on these activities of *A. awamori*-soybean koji was investigated.

2. Materials and methods

2.1. Test organism

First, filamentous fungi which are commonly used as starter organisms for the preparation of traditional, oriental fermented food products are used to prepare soybean koji. They include: *A. oryzae* BCRC 30222, *A. sojae* BCRC 30103, *A. taiwanensis* BCRC 31160, *A. awamori* and *Rhizopus* No. 2. The test organism in tests for toxicity, mutagenicity and antimutagenicity was *Salmonella typhimurium* TA 100. All the test organisms were obtained from the Bioresources Collection and Research Center (BCRC), Hsinchu, Taiwan except *A. awamori* and *Rhizopus* No. 2, which were provided by Professor Yu, Graduate Institute of Food Science and Technology, National Taiwan University.

In preparation, the starter organisms were activated by two successive transfers to Potato dextrose agar (PDA, Difco, Detroit, MI, USA) slants and incubated at 30 °C for 3 days. To prepare the inocula of starter organism, the test organism was inoculated into PDA and incubated at 30 °C for 3 days. Spores of the test organism were harvested by flooding the surface of the agar with sterile distilled water containing 0.1% Tween 80 (Icatayama Chemical,Osaka, Japan). The spore suspension was adjusted with sterile distilled water to a concentration of ca 10^6/ml and served as inocula for the fermentation of soybean.

Tests of histidine requirement, *rfa* mutation, *uvrB* mutation and R-factor were performed to confirm the genotypes of *S. typhimurium* TA100. Prior to each test, *S. typhimurium* TA100 was grown in fresh Oxoid nutrient broth No. 2 (Oxoid, Basingstoke, Hampshire, UK) at 37 °C overnight (Maron and Ames, 1983).

2.2. Preparation of soybean koji

Whole soybeans were washed and then soaked overnight in distilled water that was six times the soybeans weight at room temperature. After decanting the water, the soybeans were cooked in an autoclave (121 °C, 15 min) and then cooled. Solid state fermentation was performed by evenly spraying 1.0 ml spore suspension into the steamed soybean substrate (50 g). After mixing thoroughly, the inoculated soybean substrate was placed on a round screen with 60-mesh. They were then incubated for 3 days at 30 °C and 95% RH. During the cultivation period, the soybean was stirred and mixed after 17 and 25 h of cultivation to accelerate the release of fermentation heat.

2.3. Preparation of methanol extracts of soybean koji and unfermented steamed soybean

After drying at 60 °C for 24 h, the prepared soybean koji and the unfermented steamed soybean were pulverized to pass 30-mesh screen. Samples of the ground soybean koji powder were extracted with methanol (1 : 10, w/v) by refluxing at ca 65 °C for 3 h. After filtering through Whatman No. 1 filter paper, the extract was vacuum concentrated and dried by a freeze-dryer (77500-00 M, Labconco Co., MO, USA).

Yield of methanol extract was 5.30% for the non-fermented soybean. While koji fermented with *A. sojae*, *A. oryzae*, *A. awamori*, *A. taiwanensis*, and *Rhizopus* sp. was found to contain 8.45%, 7.00%, 9.00%, 6.70% and 16.80%, respectively, of methanol extract.

2.4. Toxicity test

The method described by Chou et al. (2002) was followed to examine the toxicity of koji extract. An aliquot of 0.1 ml dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) containing 0.625–5.0 mg sample was combined with 0.8 ml phosphate buffer (0.2 M, pH 7.4) and 0.1 ml activated culture of *S. typhimurium* TA 100 (ca 10^8 CFU/ml). The mixture was incubated at 37 °C, at 120 rpm, for 20 min. Serial dilutions were made with phosphate buffer and then 1.0 ml of the aliquot was plated onto nutrient agar (Difco). After incubation at 37 °C for 48 h, the number of colonies was counted. A toxicity effect was confirmed if the viable count of the test sample was determined to be significantly lower than that of the control.

2.5. Antimutagenicity and mutagenicity tests

In this study, the antimutagenic activity of soybean koji extracts and unfermented soybean extract against 4-nitroquinoline-*N*-oxide (4-NQO), a mutagen not requiring liver microsomal activation, was examined. The Ames test pre-incubation method was followed (Maron and Ames, 1983). Briefly, 0.1 ml DMSO containing 0.625–5.0 mg of sample was added with 0.7 ml of sodium phosphate buffer (0.2 M, pH 7.4) and 0.1 ml of the overnight activated culture of *S. typhimurium* TA 100 (histidine-requiring). Then, 0.1 ml DMSO was combined both with and without 0.1 μg 4-NQO. The entire mixture was then pre-incubated at 37 °C for 20 min before 2.0 ml molten top agar containing NaCl (LAB-SCAN, Labscan Asia Co., Patumwan, Thailand), 5.0 g l^{-1}; L-histidine (Sigma), 0.025 mM; biotin (Sigma), 0.025 mM and agar (Difco), 6.0 g l^{-1} was added. The mixture was poured onto a Minimal glucose agar plate. The His^+ revertant colonies were counted after incubation at 37 °C