Japanese Soybean Paste Miso Scavenges Free Radicals and Inhibits Lipid Peroxidation

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Summary Japanese soybean paste miso, which has been reported to prevent gastric and mammary cancer and chronic nephritis, was demonstrated by electron spin resonance spectrometry using 5,5'-dimethyl-1-pyrroline-*N*-oxide as a scavenger of free radicals. Fifty mg/ml of miso scavenged 100% of 1,1-diphenyl-2-picrylhydrazyl radicals (3.9×10^{15} spins/ml); 45 mg/ml quenched 92% of hydroxyl radicals (7.9×10^{16} spins/ml); and 50 mg/ml quenched 50% of superoxide anion (6.7×10^{16} spins/ml). In the system of rat cerebral cortex homogenate supplemented with 2 mM each of Fe^{2+} and ascorbic acid, 90% and 82% of the hydrogen and carbon-centered radicals having 1.7×10^{13} spins/ml and 3.9×10^{13} spins/ml, respectively, were quenched by 180 mg/ml of miso. The thiobarbituric acid-reactive substances, an index of lipid peroxidation in the brain, was inhibited by 10 mg/ml of miso. These results showed that miso acts as an antioxidant by scavenging free radicals.

Key Words soybean paste miso, antioxidant, free radicals, free radical scavenger, lipid peroxidation, electron spin resonance spectrometry

Antioxidative properties have been found in Oriental foods, specifically, soybean (1), and fermented soybean products such as natto (a traditional Japanese food) (2) and tempeh (a traditional Indonesian food) (3). Soybean paste or miso is another traditional product that is consumed in Asian countries as seasoning or pickling material. It is prepared by fermenting a mixture of steamed soybeans, wheat or malt, yeasts such as *Saccharomyces cerevisiae* and *Pediococcus halophilus*, salt and "koji" (a 48-h incubated mixture of steamed soybeans and fungi *Aspergillus oryzae*) (4). However, no information is available on the antioxidant activity or free radical scavenging action of miso.

By electron spin resonance (ESR) spectrometry, which is considered to be the least ambiguous method for the detection of free radicals (5), and using a spin trap 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO), we examined the antioxidant/scave-

nging action of miso on free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide, hydrogen and carbon-centered radicals. We analyzed also the thiobarbituric acid-reactive substances (TBARS)—a marker of lipid peroxidation—in rat cerebral cortex homogenate.

METHODS

Miso. A yellow variety of commercially available miso from Kaneko-miso Ltd. (Tokushima, Japan) was used.

Animals. Male adult Sprague-Dawley rats from CLEA Japan Inc. (Tokyo, Japan) weighing 240–260 g were housed for at least 1 week at 25°C and humidity of $50 \pm 5\%$ and were allowed free access to water and a standard diet MF (Oriental Yeast, Tokyo, Japan). The rats were sacrificed by cervical decapitation. The cerebral cortex was immediately dissected on an ice plate and homogenized (0.05% w/v) in 0.1 M Tris/HCl buffer, pH 7.4.

Chemicals. Hypoxanthine, DPPH and diethylenetriaminepentaacetic acid (DETAPAC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); xanthine oxidase from Boehringer Mannheim GmbH (Germany); DMPO from Daiichi Pure Chemical Co. (Tokyo, Japan); vitamin C from Wako Pure Chemical Ind. Ltd. (Osaka, Japan); and vitamin E from Katayama Chemical (Osaka, Japan).

Radical analysis. The following free radicals were analyzed by ESR spectrometer (JEOL, JES-FE1XG, Tokyo, Japan).

DPPH: To 100 μ l of 30 μ mol of DPPH in ethanol was added 100 μ l of sample, which was then vortexed, placed in a special spectrometry flat cell (JEOL, Tokyo, Japan) and analyzed exactly after 60 s.

Hydroxyl: Seventy-five microliters of 1 mM FeSO₄ and 1 mM DETAPAC, 75 μ l of 1 M H₂O₂, 50 μ l of sample (in water), and 20 μ l of 0.092 M DMPO were vortexed and the DMPO-OH spin adducts were analyzed at 40 s after addition of DMPO.

Superoxide: Fifty microliters of 2 mM hypoxanthine, 35 μ l of 11 mM DETAPAC, 50 μ l of sample (in water), 15 μ l of DMPO, and 50 μ l of freshly prepared xanthine oxidase suspension (81.6 μ l in 5 ml of phosphate buffer, pH 7.8) were vortexed and the DMPO-O₂⁻ spin adducts were analyzed at 50 s after addition of DMPO.

Hydrogen and carbon-centered radicals: To 0.5 ml of rat cerebral cortex homogenate in Tris/HCl buffer (pH 7.4), 0.45 ml of 0.1 M Tris/HCl buffer, and 0.05 ml of 2 mM FeCl₂-ascorbic acid solution was added 200 mg of miso, which was then mixed for 10 s and incubated at 37°C for 15 min. To 200 μ l of the incubated medium was added 20 μ l of DMPO, which was then mixed for 10 s, then placed in an ESR spectrometry flat cell. The radicals were measured at 50 s after addition of DMPO under the following conditions: magnetic field, 335 ± 5 mT; response, 0.03 s; sweep time, 0.5 min; amplitude, 2×10^3 ; modulation amplitude, 0.08 mT; and

power, 8 mV. The spin number was calculated by comparing the signal heights of samples with those of known quantities of 2,2,6,6-tetramethyl-4-hydroxylpiperidine-1-oxyl as standard.

Lipid peroxidation. The peroxidation of lipid was induced by adding 2 mM of FeCl_2 and ascorbic acid in rat cerebral cortex homogenate as was done in the determination of hydrogen and carbon-centered radicals. After 15-min incubation at 37°C , TBARS was measured as described by Ohkawa *et al.* (6) using malondialdehyde as the standard. The fluorescence was analyzed at excitation 515 nm and emission 532 nm using tetramethoxy propane standard.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test.

RESULTS

Table 1 shows (qualitatively) that miso, vitamin C and vitamin E scavenged DPPH, hydroxyl and superoxide radicals. Figure 1a and 1c illustrate that at 50 mg/ml of miso, 100% and 50% of the DPPH radicals (3.9×10^{15} spins/ml) and superoxide (6.7×10^{16} spins/ml) were quenched, respectively. Whereas, 45 mg/ml of miso scavenged 92% of the hydroxyl radicals (7.9×10^{16} spins/ml), as shown in Fig. 1b. ESR hyperfine splitting constants of the hydroxyl radicals are $A_N/G = A_H/G = 15.1$, close to that obtained by Buettner (5). The spin adducts of the superoxide show ESR parameters of $A_N/G = 14.6$ and $A_H/G = 11.4$ and $A_\gamma/G = 1.2$. Figure 2 depicts the dose-response of the free radical quenching ability of miso at various concentrations. As shown in Fig. 3, 180 mg of miso scavenged about 90% and 82% of the hydrogen radicals (1.7×10^{13} spins/ml) and carbon-centered radicals (3.9×10^{13} spins/ml) which were generated in the homogenate of rat cerebral cortex after the addition of 2 mM each of FeCl_2 and ascorbic acid. ESR parameters of the spin adducts are $A_N/G = 16.6$ and $A_H/G = 22.5$ for DMPO-H and $A_N/G = 15.5$ and $A_H/G = 23.0$ for DMPO-C. Figure 4 shows the inhibitory effect of 10 mg/ml of miso on TBARS in the homogenate of rat cerebral cortex.

Table 1. Concentration of antioxidants required to quench 50% (IC_{50}) of the free radicals.

Antioxidant/ scavenger ($\mu\text{g/ml}$)	Radicals		
	1,1-Diphenyl-2- picrylhydrazyl ($\times 10^{14}$ spins/ml)	Hydroxyl ($\times 10^{15}$ spins/ml)	Superoxide ($\times 10^{15}$ spins/ml)
Vitamin C	0.80	35	24
Vitamin E	2.84	37	*
Soybean paste miso	5,000	4,000	50,000

Values are means of 5–6 determinations. *Delayed quenching action observed after 6–9 min, in lieu of the usual analytical time of 50 s.

Fig. 1. Dose-response of the scavenging action of soybean paste miso on a) 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), b) hydroxyl radicals, and c) superoxide radicals. The values are the means ($n=6-8$). * $p < 0.001$ vs. control, ** $p < 0.025$ vs. control.

Fig. 2. Effect of soybean paste miso on electron spin resonance spectrum of a) 1,1-diphenyl-2-picrylhydrazyl (DPPH), b) hydroxyl radicals as spin adducts of DMPO-OH with hyperfine splitting constants of $A_N=A_H=15.1$, and c) superoxide radicals as spin adducts of DMPO-O₂⁻ with hyperfine splitting constants of $A_N=14.6$, $A_H=11.4$, and $A_\gamma=1.2$. Spectra are typical of 6 determinations.

DISCUSSION

Numerous reports on the nutritional benefits of soybean and soybean paste miso provide an interesting area in which the free radical theory is thought to apply. Soybean, the basic material of miso, has been demonstrated to inhibit mammary tumors in two models of breast cancer in rats using dimethylbenz[*a*]anthracene (DMBA) and *n*-methyl-*n*-nitrosourea (7) and ionizing radiation (8). Soybean also minimized the carcinogenicity of nitrosamine in mice (9). This anticarcinogenic property has been attributed to protease inhibitors of soybean (10). On one hand, approximately 80% of soybean paste miso produced in Japan is consumed as miso soup, the consumption of which has been shown by epidemiological studies to be strongly associated with a reduction in gastric cancer mortality in this country (11). Miso itself has been found to lower the incidence of chronic nephritis (12). It has

Fig. 3. Scavenging action of soybean paste miso on hydrogen radicals as spin adducts of DMPO and carbon-centered radicals as spin adducts of DMPO generated from rat cerebral cortex homogenate induced with equimolar mixture (2 mM) of FeCl_2 and ascorbic acid at 37°C . Hyperfine splitting constants of DMPO-H are $A_N/G=16.6$ and $A_H/G=22.5$. Hyperfine splitting constants of DMPO-C are $A_N/G=15.5$ and $A_N/G=23.0$.

Fig. 4. Thiobarbituric acid-reactive substances (TBARS) in a) rat cerebral cortex homogenate at 37°C , b) as in (a) but induced with 2 mM each of FeCl_2 and ascorbic acid at 37°C , c) as in (b) but treated with 10 mg/ml of soybean paste miso. The values are the means $\pm$ SEM ($n=6$). *C is significantly different from B; $p<0.001$.

also been reported to reduce the incidence and to delay the appearance of DMB-A-induced mammary adenocarcinomas in rats (13).

Free radicals have been found to be involved in both the initiation and promotion of carcinogenesis. However, antioxidants function as inhibitor at both initiation and promotion/transformation stages of carcinogenesis and protect cells against oxidative damage caused by free radicals (14). Our findings show that miso

has antioxidant property by scavenging free radicals such as DPPH, hydroxyl, superoxide, hydrogen and carbon-centered radicals. Miso inhibited TBARS formation in rat cerebral cortex, thereby preventing lipid peroxidative damage. Hydroxyl and peroxy radicals and lipid peroxides are known to participate in the initiation and propagation of lipid peroxidation.

The scavenging property of miso may be attributed to soybean, its basic component, which contains a number of known antioxidants such as vitamin E, isoflavones and saponins. Among these, vitamin E has been shown to correlate with the prevention of cancer (15) and iron-induced epileptiform discharges in rats (16). Vitamin E, a major lipid-soluble chain-breaking antioxidant, has been demonstrated to impart oxidative stability and antioxidant activity in soybean oil (17). Isoflavones from soybeans (18) have been found to have antioxidant action (1). Oral administration of saponins in rats prevented liver injury and hyperlipemia induced by high fat diet containing highly peroxidized corn oil—indicative of saponins' protective action *in vivo* and *in vitro* production of lipid peroxides (19). Recently, we found that baker's yeast *S. cerevisiae* scavenged DPPH, hydroxyl and superoxide, quenched hydrogen and carbon-centered radicals and inhibited TBARS in the rat brain homogenate which was induced with 2 mM each of FeCl₂ and ascorbic acid (20). Other antioxidants in miso may include fermentation products and other compounds undestroyed by heating in miso soup preparation. All these antioxidants in miso may be responsible for scavenging DPPH, hydroxyl, superoxide, hydrogen and carbon-centered radicals.

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