Original paper

Comprehensive Evaluation of the Antioxidant Activity of Miso by the Myoglobin Method

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Antioxidant activities of ten different commercially available misos were measured against four reactive oxygen species using our developed Myoglobin method and the conventional DPPH method. Antioxidant activities were characterized using 5-axe cobweb charts. The examined misos showed high antioxidant activities against peroxyl radical, hypochlorite ion, and peroxynitrite ion. Samples were classified according to antioxidant activity against peroxynitrite ion into high, medium, and low activity groups. Miso soup prepared with miso and bonito-dashi, and miso soups prepared with miso, bonito-dashi, and vegetables showed higher antioxidant activities than miso. These results suggested that miso, bonito-dashi, and vegetables contribute additively to the antioxidant activities of miso soup.

Keywords: miso, fermentation, maturation, antioxidant activity, DPPH

Introduction

Recent studies have revealed that oxidative stresses generated within living organisms enhance the aging process and/or trigger life-style related diseases such as cancer, diabetes, arteriosclerosis, and so on (Madamanchi et al., 2005, Vina et al., 2011, Chen and Keaney 2012). Since human anti-oxidative potency decreases with age, the intake of foods rich in antioxidant activity is strongly recommended (Saba et al., 2012). Thus, evaluation of the antioxidant activities of foods is a very important issue for the food sciences. The USDA-adopted ORAC (Oxygen Radical Absorption Capacity) has become popular, and the ORAC database was released on the USDA webpage in 2007 (Wu et al., 2004a, 2004b). Many food companies use the ORAC value as an indicator of the antioxidant activity of their products. The USDA, however, shut down the ORAC database in 2012, stating that the ORAC does not properly express the antioxidant activities of foods (Niki 2012). Various reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in living organisms (Villamena 2013, Sharma 2013). Foods contain various kinds of antioxidants and their activities against ROS and RNS differ. For example, vitamin C shows different antioxidant activities against different ROS and RNS. Many new methods have been proposed by researchers to overcome this challenging issue. Our group has proposed a new method based on the structural change of myoglobin by reaction with ROS or RNS (Terashima et al., 2007a, 2007b, 2009, 2010, 2013). This method, termed the Myoglobin method, successfully evaluates the antioxidant activities of a substance against peroxyl radical, hydroxyl radical, hypochlorite ion, and peroxynitrite. Our group also proposed a comprehensive analysis using 5-axe cobweb charts. Differences in the antioxidant activities of a sample against five different ROS and RNS can be discussed using this chart, because antioxidant activity analyzed by the conventional DPPH method and those analyzed by the Myoglobin method are plotted on the same chart. Antioxidant activities of various antioxidant substances and vegetables were successfully characterized by this protocol (Terashima et al., 2010, 2013).

In this work, we attempted to evaluate the antioxidant activities of miso. Miso is a traditional Japanese seasoning prepared
from soybeans and fermented with salt and the fungus Aspergillus oryzae. Various types of miso are produced by local miso breweries in Japan. Rice miso, barley miso, and soybean miso are prepared with rice malt, barley malt, and soybean malt, respectively. During the fermentation process, various peptides and amino acids are generated by the hydrolysis of proteins. A number of health benefits are reported with miso (Du et al., 2014, Lee et al., 2014), since miso contains various kinds of nutrients such as amino acids, peptides, vitamins, minerals, and polyunsaturated fatty acids. We attempted to characterize the antioxidant activities of 10 commercially available brands of miso using our method.

Materials and Methods

Materials Ten commercially available brands of miso were used (Miso A - G, rice miso; Miso H, soybean miso; Miso I and J, barley miso). All samples were kindly donated by the Central Miso Research Institute (Tokyo, Japan). Two brands of dried bonito used to prepare broth and vegetables (onion and spinach) were purchased at a local supermarket. Myoglobin (equine skeletal muscle, 95 – 100%) and peroxynitrite were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Dojin Chemicals (Kumamoto, Japan), respectively. All other chemicals used were of reagent grade.

Preparation of samples Hot water extracts of miso, bonito broth, miso soup prepared with bonito broth, and miso soup with bonito broth containing vegetables were prepared by the following protocols.

a) Hot water miso extracts Twelve grams of miso were added to boiled Milli-Q water (180 mL) and mixed well. After the mixture was centrifuged at 10,000 rpm for 10 minutes (Tomy Seiko Co., Ltd, Tokyo, Japan; TX201), the supernatant was recovered and filtered (Advantec, Tokyo, Japan; pore size, 0.65µm).

b) Bonito broth Bonito broth was prepared according to the manufacturer’s instructions. Dried bonito (30 g) was added to the boiling Milli-Q water (1,000 mL), and left to stand for 6 minutes until the dried bonito sunk to the bottom. After the solid materials were filtered out with gauze, the solution was filtered (Sartorius Stedim, Tokyo, Japan; pore size, 0.2 µm).

c) Miso soup with bonito broth Twelve grams of miso were added to the bonito broth (180 mL) and mixed well. After the mixture was centrifuged at 10,000 rpm for 10 minutes (Tomy Seiko Co., Ltd; TX201), the supernatant was recovered and filtered (pore size, 0.65 µm).

d) Miso soup with bonito broth containing vegetables Onion (45 g) was added to the boiled bonito broth (147 mL) and simmered for a few minutes to cook. After turning off the electric stove, 11.6 g of miso was added. Then, the miso soup containing onion was homogenized with a mixer (Matsushita Electric Industrial, Osaka, Japan; MX-945). The homogenate was centrifuged at 10,000 rpm for 10 minutes (Tomy Seiko Co., Ltd; TX201), and the supernatant was recovered and filtered (pore size, 0.65µm). For the miso soup containing spinach, 14 g of spinach were added instead of onion.

Measurement of antioxidant activity Antioxidant activities of the samples against peroxyl radical, hydroxyl radical, hypochlorite ion, and peroxynitrite were measured by our Myoglobin method (Terashima et al., 2010, 2013). In the Myoglobin method, antioxidant activities are evaluated based on the decrease in absorbance at 409 nm of myoglobin due to reaction with ROS (peroxyl radical, hydroxyl radical, hypochlorite ion) or RNS (peroxynitrite). The myoglobin protection ratio(%) for the sample was defined by the following equation:

\[ \text{Myoglobin protection ratio (\%)} = \left(1 - \frac{ABS^0 - ABS^R(\text{with antioxidant})}{ABS^0 - ABS^R(\text{without antioxidant})}\right) \times 100 \quad \cdots \text{Eq. 1} \]

where \(ABS^0\) is the absorbance of myoglobin solution (control), \(ABS^R(\text{without antioxidant})\) is the absorbance of the measurement solution containing only ROS or RNS, and \(ABS^R(\text{with antioxidant})\) is the absorbance of the measurement solution containing both the test sample and ROS or RNS. Average values were obtained from triplicate measurements.

A relative concentration of 1.0 was defined as the undiluted concentration of ingredients in the prepared sample. The prepared sample was serially diluted with Milli-Q water to examine the effect of relative concentration on the antioxidant activity.

Antioxidant activity against the stable radical DPPH was measured as previously described (Terashima et al., 2007a). The antioxidant activity of a specimen was expressed as ascorbic acid equivalent concentration (mM VC).

Evaluation of antioxidant activity using cobweb charts In order to comprehensively evaluate the antioxidant activities against five different ROS and RNS, myoglobin protective ratio (%) for peroxyl radical, hydroxyl radical, hypochlorite ion, peroxynitrite, and the ascorbic acid equivalent concentration were plotted on 5-axe cobweb charts.

Results and Discussion

Myoglobin protective ratio of miso hot water extracts The miso hot water extracts examined in this work showed relatively high antioxidant activities against peroxyl radical, hypochlorite ion, and peroxynitrite. Effects of the relative concentration of the examined misos on myoglobin protective ratio against peroxyl radical, hydroxyl radical, and hypochlorite ion are shown in Figs. 1-4; average values are shown with error bars. Myoglobin protective ratios of Misos A - E against peroxyl radical were increased with the relative concentration, and reached 50 – 60% at a relative concentration of 1.0 (Fig. 1a). Myoglobin protective ratios of Misos F - J against peroxyl radical were increased with the relative concentration (Fig. 1b). The myoglobin protective ratio...
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The antioxidant activity of miso G at a relative concentration of 1.0 was 59.1%, while those for Misos F, H, I, and J were in the range of 30 – 50%.

Myoglobin protective ratios of Misos B, D, and E against hydroxyl radical at a relative concentration of 1.0 were 4 – 5%, and those of Misos A and C were almost zero (Fig. 2a). Myoglobin protective ratios of Misos F and G against hydroxyl radical at a relative concentration of 1.0 were 1 – 4%, and those of Misos H, I, and J were almost zero (Fig. 2b). These results indicate that the examined misos do not have antioxidant effects against the hydroxyl radical.

Fig. 1a. Myoglobin protection ratio of miso hot water extracts against peroxyl radical. Miso A (○), Miso B (△), Miso C (▼), Miso D (□), Miso E (◆).

Fig. 1b. Myoglobin protection ratio of miso hot water extracts against peroxyl radical. Miso F (●), Miso G (▲), Miso H (▼), Miso I (■), Miso J (◇).

Fig. 2a. Myoglobin protection ratio of miso hot water extracts against hydroxyl radical. Miso A (○), Miso B (△), Miso C (▼), Miso D (□), Miso E (◆).

Fig. 2b. Myoglobin protection ratio of miso hot water extracts against hydroxyl radical. Miso F (●), Miso G (▲), Miso H (▼), Miso I (■), Miso J (◇).

Myoglobin protective ratios of Misos A - E against hypochlorite ion increased with the relative concentration (Fig. 3a). At a relative concentration of 1.0, myoglobin protective ratios of B, D, and E were 70 – 90%, while that of Miso A was 52%. Myoglobin protective ratio of Miso I was 83%, and those of Misos F, G, H, and J were 67 – 75% at a relative concentration of 1.0 (Fig. 3b).

Against peroxynitrite, myoglobin protective ratios of Misos A - E increased with the relative concentration (Fig. 4a). Myoglobin protective ratios of Misos B and D were 58 – 67%, while those of Misos A, C, and E were in the range of 20 – 40% at a relative concentration of 1.0.
concentration of 1.0. The myoglobin protective ratio against peroxynitrite of Miso H was 60%, while those of Misos F, G, I, and J were 10–40% at a relative concentration of 1.0 (Fig. 4a).

These results suggest that the myoglobin protective ratio depends on the miso ingredients.

Comprehensive evaluation of miso hot water extracts using 5-axe cobweb charts
Myoglobin protective ratios against four ROS and RNS at a relative concentration of 1.0, and the ascorbic acid equivalent concentration (mM VC) for the DPPH method were plotted on 5-axe cobweb charts (Fig. 5). The cobweb charts clearly showed the antioxidant characteristics of the examined miso hot water extracts. Generally, the examined misos showed relatively high antioxidant activities against peroxyl radical, hypochlorite ion, and peroxynitrite, while they showed very low antioxidant activities against DPPH and hydroxyl radical.

Ten brands of miso were categorized into three groups based on the antioxidant activity against peroxynitrite: high activity group (Misos B, D, H), moderate activity group (Misos E, F, G), and low activity group (Misos A, C, I, J). In the redox reactions among biomolecules, various types of electron transfer reactions between ROS/RNS and antioxidants occur depending on the molecular structures of antioxidants and physiological conditions.
Although reaction kinetics for model compounds have been studied, reaction properties of foods containing a large variety of antioxidants have yet to be clarified, and must be estimated from fragmented information. The protective effects of miso against the different ROS/RNS shown in this study are due to synergy effects of the different reactivities of the antioxidants contained in the miso hot water extracts. Our current study revealed that the hot water extracts prepared from different misos could be classified into three groups based on the antioxidant activity against peroxynitrite. Other researchers have reported that flavonoids have protective effects against peroxynitrite (Sadowska-Bartoz 2014), and that thiol-containing compounds (Zeida et al., 2013) and tyrosine, tyrosylmethionine, and tyrosylphenylalanine (Zhang et al., 2009) are oxidized by peroxynitrite. Differences in the protective effects against peroxynitrite among the 10 miso brands might arise from differences in the content of flavonoids and thiol-containing compounds. Miso contains both soybean as well as various compounds produced by the fermentation process. Analysis of the principal components of miso hot water extracts is under study. Our preliminary analysis using HPLC suggested that flavonoids such as daidzin and daisein are not contained in the miso hot water extracts. Tyrosine and phenylalanine were found in miso B by amino acid analysis (data not shown). In order to understand the antioxidant properties of miso, it is necessary to analyze antioxidant properties of 70% ethanol extracts, in which most flavonoids of miso are extracted.

Since our protocol can differentiate the antioxidant properties of misos produced using different materials and processes, it can be applied to the analysis of the relationship between antioxidant activities and the miso manufacturing process.

**Antioxidant activities of bonito broth and miso soup with bonito broth** The cobweb charts of bonito broth, and miso soup prepared with Miso B and bonito broth are shown in Fig. 6. Bonito broth showed a high myoglobin protective ratio against peroxyl radical, hypochlorite ion, and peroxynitrite. The cobweb chart for miso prepared with Miso B and bonito broth was similar to that for Miso B (Fig. 5). These results suggest that the antioxidant activity of the miso soup can be mainly attributed to the antioxidant activity of miso.

**Antioxidant activities of bonito broth and miso soup with bonito broth containing vegetables** The cobweb charts of miso soup prepared with Miso B and bonito broth containing onion or spinach are shown in Fig. 7. Our previous study showed that fresh onion and spinach showed relatively high antioxidant activities against peroxyl radical, hydroxyl radical, hypochlorite ion, and peroxynitrite (Terashima et al., 2013). The myoglobin protective ratio of miso soup increased from 7.0% (Fig. 6) to 22% (onion) and 17% (spinach), as shown in Fig. 7. These results suggest that the vegetables in the miso soup also contribute to the antioxidant activity of the miso soup, and that the antioxidant activity of the miso soup can be improved by adding vegetables.

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Fig. 5. Antioxidant activity of misos
(AAPH: peroxyl radical, OH: hydroxyl radical, CLO: hypochlorite ion, ONOO: peroxynitrite)
Fig. 5. Antioxidant activity of misos (cont.)
(AAPH: peroxyl radical, OH: hydroxyl radical, CLO: hypochlorite ion, ONOO: peroxynitrite)
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Conclusion

Antioxidant activities of 10 commercially available miso brands against five ROS and RNS were evaluated by the Myoglobin method and the DPPH method. The characteristics of miso and miso soup were then comprehensively evaluated by plotting on 5-axe cobweb charts. Samples showed antioxidant activities against peroxyl radical, hypochlorite ion, and peroxynitrite. The examined misos were classified into three groups based on the activity against peroxynitrite. Antioxidant activities derived from the bonito broth and vegetables used to prepare miso soup also contributed to the antioxidant activities of miso soup.

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References


