Radical Scavenging Activity of the Japanese Traditional Food, Amazake

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The traditional Japanese beverage amazake was evaluated as a possible treatment for oxidative stress. For this purpose, the radical scavenging activities of four kinds of amazake were investigated by determining diphenylpicrylhydrazyl radical, galvinoxyl radical and superoxide anion radical quenching activities using a spectrophotometric method and an electron spin resonance (ESR) technique. ESR measurements were used to assess the superoxide anion radical scavenging activity by employing dimethylpyrroline N-oxide as a spin trapping reagent. The ferricyanide-reducing activity and chelating activity were evaluated spectroscopically. The results indicated that amazake made from brown rice had stronger radical scavenging, metal chelating and metal reducing activities than amazake produced from white rice and sake cake (by-product of sake).

Keywords: amazake, radical-scavenging activity, metal-chelating activity, metal-reducing activity, fermentation

Introduction

Amazake, a sweet beverage made from fermented rice, has been consumed in Japan for more than a thousand years (Yamamoto and Matsugo, 2008). Steamed rice, rice-koji and water are used as the raw materials for making traditional amazake (Shinmura, 1998; Murakami, 1986; Nakano, 1967). These three components are mixed together and kept at 55 – 60°C for 15 – 18 h. During this period, rice starch is saccharized by the enzymes present in rice-koji, with traditional amazake containing approximately 20% glucose (Yamashita, 2009). Overall, the saccharification process is the same as that of sake (an alcoholic beverage originating in Japan); however, the alcoholic fermentation process is not used in amazake production. The high temperature used to make amazake is the main factor that prevents yeast propagation; therefore, traditional amazake contains no alcohol.

Recently, drinking of traditional amazake has increased because of the tendency for consumers to prefer natural foods. Although the normal raw material of traditional amazake is polished rice, brown rice is sometimes used. Since the 1970s, a different kind of amazake has been manufactured using sake cake, which is obtained as a by-product of sake. This kind of amazake is simply made by mixing the sake cake and sugar in hot water. Generally, this type of amazake is called instant amazake. Sake cake is the condensate of rice protein and contains alcohol (Yamamoto and Matsugo, 2008; Kagawa, 2009). Recently, instant amazake has become popular because of its low price.

In spite of a long history of traditional amazake, the characteristics of amazake as a functional food are not well examined. It has become apparent that foods fermented by koji have various physiological functions including antioxidative activities. The physiological functions of soy sauce, miso, sake and mirin (alcoholic seasoning in Japan) are well studied (Soy Sauce Information Center, 2004; Japan Miso Promotion Board, 1999; Ohta et al., 1992; Ishizaki et al., 2006); however, no comprehensive study exists comparing the characteristics of traditional and instant amazake.

Many different functional nutrients are produced from raw materials during the fermentation process. We have chosen to examine the functionality of amazake by focusing on its antioxidative activity. Antioxidative activities are generally evaluated based on the radical scavenging activity of the compound of interest. Several methods to evaluate radical scavenging activity have been developed; however, the re-
Results are sometimes dependent on the radical species examined (Yamada and Matsuda, 2009; Kikuchi et al., 2004; Park et al., 2002). Four kinds of amazake found in the Japanese market were used in this study. Three of them are categorized as traditional amazake and the other is instant amazake. We evaluated the antioxidative activities of these amazakes against several radical species including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, galvinoxyl radical and superoxide anion radical (O$_2^\cdot$). Furthermore, we also evaluated the reducing and chelating activities of these amazakes by spectroscopic analyses using ferric-ferrous metal complexes.

Materials and Methods

Samples Two traditional amazakes made from brown rice (BRA and BRA2) were used in this study. The other traditional amazake (WRA) was made from white rice. Instant amazake (SCA) was made from sake cake. The characteristics of these amazakes, which are labeled according to the Japan Agricultural Standards Act and the Food Sanitation Act, are shown in Table 1. The nutritional composition of these amazakes was examined according to the conventional procedure. The data summarized in Table 2 were obtained with technical help from Ishikawa Health Service Association and Industrial Research Institute of Ishikawa. Free amino acids were analyzed using the L-8900 analyzer (Hitachi, Tokyo, Japan) after dilution with distilled water. The results shown in Table 3 were obtained with the help of Industrial Research Institute of Ishikawa.

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**Materials** DPPH radical, xanthine oxidase, phenol reagent (Folin reagent), and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Nacalai Tesque (Kyoto, Japan). Disodium 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(4-disulfophenyl)-2H-tetrazolium (WST-1) and sodium 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfate

**Table 1.** Raw materials and manufacturer of amazake samples used in this study.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Raw Materials</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRA</td>
<td>brown rice, koji made from brown rice</td>
<td>Ayumasamune-shuzo</td>
</tr>
<tr>
<td>BRA2</td>
<td>brown rice, koji made from white rice</td>
<td>Yamato-soysauce-miso</td>
</tr>
<tr>
<td>WRA</td>
<td>white rice, koji made from white rice</td>
<td>Shinozaki</td>
</tr>
<tr>
<td>SCA</td>
<td>sugar, sake cake, koji made from white rice, sodium chloride, acidifier</td>
<td>Morinaga(seller)</td>
</tr>
</tbody>
</table>

**Table 2.** Nutrition components in amazakes.

<table>
<thead>
<tr>
<th>Component</th>
<th>BRA</th>
<th>BRA2</th>
<th>WRA</th>
<th>SCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (g)</td>
<td>75.80</td>
<td>71.10</td>
<td>74.70</td>
<td>84.40</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>95.00</td>
<td>113.00</td>
<td>112.00</td>
<td>66.00</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>1.80</td>
<td>2.00</td>
<td>1.70</td>
<td>0.80</td>
</tr>
<tr>
<td>Lipid (g)</td>
<td>0.10</td>
<td>0.20</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>21.80</td>
<td>25.80</td>
<td>25.20</td>
<td>14.50</td>
</tr>
<tr>
<td>Reducing sugar (g)</td>
<td>13.00</td>
<td>20.00</td>
<td>17.20</td>
<td>4.60</td>
</tr>
<tr>
<td>Mineral</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na (mg)</td>
<td>35.00</td>
<td>8.00</td>
<td>2.00</td>
<td>63.00</td>
</tr>
<tr>
<td>K (mg)</td>
<td>54.00</td>
<td>20.00</td>
<td>3.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Mg (mg)</td>
<td>29.00</td>
<td>18.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>P (mg)</td>
<td>73.00</td>
<td>23.00</td>
<td>6.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Vitamin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 (mg)</td>
<td>0.06</td>
<td>0.07</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>B2 (mg)</td>
<td>0.03</td>
<td>0.06</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>pH</td>
<td>5.98</td>
<td>5.96</td>
<td>5.66</td>
<td>4.40</td>
</tr>
</tbody>
</table>
to the DPPH radical was measured using a JASCO V-550 spectrometer (JASCO Corporation, Tokyo, Japan). The experiment was repeated three times. DPPH radical scavenging activity was calculated using the following equation:

\[
\text{DPPH radical scavenging activity (\%) = } \left\{1 - \frac{(A_1 - A_3)}{(A_2 - A_3)}\right\} \times 100 \quad (1)
\]

\(A_1\) = absorbance at 527 nm in the presence of amazake  
\(A_2\) = absorbance at 527 nm in the absence of amazake  
\(A_3\) = absorbance at 527 nm in the presence of 2 mM ascorbic acid (blank test)

Electron spin resonance (ESR) spectra of DPPH radical were measured using a JEOL JES-FR30EX Free Radical Monitor (JEOL Ltd., Tokyo, Japan). The measurement conditions are as follows: magnetic field, 336.0 ± 7.5 mT; power, 4 mW; sweep time, 1 min; modulation, 100 kHz, 0.32 mT; amplitude, 790; time constant, 0.3 s. A typical example of an ESR figure is depicted in Fig. 1 (a). The measurement of ESR spectra was repeated five times. DPPH radical scavenging activity estimated by ESR spectra was calculated using the following equation. Signal intensity was compared based on the ratio against the magnetic marker (standard of ESR, ferrozine) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Gallic acid was purchased from Kanto Chemical (Tokyo, Japan) and galvinoxyl radical was purchased from Tokyo Chemical Industry (Tokyo, Japan).

**Table 3. Free amino acid composition of amazakes (mg/100 g).**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>BRA</th>
<th>BRA2</th>
<th>WRA</th>
<th>SCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>316.6</td>
<td>464.0</td>
<td>310.0</td>
<td>120.6</td>
</tr>
<tr>
<td>Asp</td>
<td>20.8</td>
<td>36.8*</td>
<td>17.9</td>
<td>6.3</td>
</tr>
<tr>
<td>Thr</td>
<td>13.1*</td>
<td>17.9</td>
<td>11.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Ser</td>
<td>18.0</td>
<td>29.3</td>
<td>17.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Asn</td>
<td>16.2**</td>
<td>6.9</td>
<td>12.0**</td>
<td>10.2**</td>
</tr>
<tr>
<td>Glu</td>
<td>22.1</td>
<td>42.7*</td>
<td>20.5</td>
<td>7.7</td>
</tr>
<tr>
<td>Gln</td>
<td>3.6</td>
<td>22.3*</td>
<td>37.5**</td>
<td>1.5</td>
</tr>
<tr>
<td>Gly</td>
<td>9.7</td>
<td>17.2</td>
<td>11.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Ala</td>
<td>25.5</td>
<td>35.7</td>
<td>21.1</td>
<td>14.8**</td>
</tr>
<tr>
<td>Val</td>
<td>20.8</td>
<td>29.1</td>
<td>17.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Cys</td>
<td>1.7</td>
<td>2.3</td>
<td>3.1</td>
<td>1.8*</td>
</tr>
<tr>
<td>Met</td>
<td>4.8</td>
<td>7.6</td>
<td>4.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Ile</td>
<td>16.2</td>
<td>21.4</td>
<td>13.1</td>
<td>4.9</td>
</tr>
<tr>
<td>Leu</td>
<td>31.4</td>
<td>43.6</td>
<td>25.8</td>
<td>9.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>20.3</td>
<td>31.5</td>
<td>20.0</td>
<td>6.7</td>
</tr>
<tr>
<td>Phe</td>
<td>23.5*</td>
<td>25.8</td>
<td>15.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Trp</td>
<td>9.6**</td>
<td>8.3</td>
<td>5.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Lys</td>
<td>14.6*</td>
<td>16.5</td>
<td>16.7**</td>
<td>7.9*</td>
</tr>
<tr>
<td>His</td>
<td>5.4</td>
<td>7.4</td>
<td>6.1*</td>
<td>2.5*</td>
</tr>
<tr>
<td>Arg</td>
<td>20.5</td>
<td>45.0*</td>
<td>19.2</td>
<td>9.7</td>
</tr>
<tr>
<td>Pro</td>
<td>7.0</td>
<td>11.5</td>
<td>7.7</td>
<td>2.3</td>
</tr>
<tr>
<td>4-amino butanoic acid</td>
<td>11.9**</td>
<td>5.0</td>
<td>7.3*</td>
<td>3.3*</td>
</tr>
</tbody>
</table>

** remarkably high value by comparison with the proportion of the four amazakes  
* slightly high value by comparison with the proportion of the four amazakes

(ferrozine) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Gallic acid was purchased from Kanto Chemical (Tokyo, Japan) and galvinoxyl radical was purchased from Tokyo Chemical Industry (Tokyo, Japan).
taining ultrapure water and ethanol (80:20, v/v). The amount of unreacted galvinoxyl radical was determined by the absorbance at 430 nm. The experiment was repeated three times. Galvinoxyl radical scavenging activity based on the absorbance at 430 nm was calculated by the following equation:

\[
\text{Galvinoxyl radical scavenging activity (\%) = } \frac{1 - (A_1 - A_3)}{(A_2 - A_3)} \times 100
\]

\(A_1 = \text{Absorbance at 430 nm in the presence of amazake}\)
\(A_2 = \text{Absorbance at 430 nm in the absence of amazake}\)
\(A_3 = \text{Absorbance at 430 nm in the presence of 2 mM ascorbic acid}\)

The amount of unreacted galvinoxyl radical was also evaluated by the intensity of the ESR spectrum. The measurement conditions were as follows: magnetic field, 336.0 ± 7.5 mT; power, 4 mW; sweep time, 1 min; modulation, 100 kHz, 0.32 mT; amplitude, 630; time constant, 0.3 s. A typical example of an ESR spectrum is depicted in Fig. 1 (b). The experiment of the ESR spectra was repeated five times. Galvinoxyl radical scavenging activity measured by ESR spectra was calculated using the following equation:

\[
\text{Galvinoxyl radical scavenging activity (\%) = } (1 - h_1/h_2) \times 100
\]

\(h_1 = \text{relative height in the presence of amazake}\)
\(h_2 = \text{relative height in the absence of amazake}\)

**Determination of \(O_2^•−\) scavenging activity**

WST-1 (250 \(\mu\)M, 1.0 ml) and hypoxanthine (94 \(\mu\)M, 1.0 ml) were added to the phosphate buffer (100 mM, pH 7.4, 2.4 ml) and allowed to stand for 10 min at 30°C. An aliquot of the sample solution (200 \(\mu\)l) and 37.5 mU/ml xanthine oxidase (400 \(\mu\)l) was added to the WST-1 and hypoxanthine solution at room temperature. The mixture was shaken vigorously and allowed to stand for 30 min at 30°C. The absorbance at 438 nm assignable to formazan derivative formation was measured. The scavenging activity for \(O_2^•−\) was calculated using the following equation:

\[
\text{\(O_2^•−\) scavenging activity (\%) = } \frac{1 - (A_1 - A_3)}{A_2} \times 100
\]

\(A_1 = \text{absorbance at 438 nm in the presence of amazake}\)
\(A_2 = \text{absorbance at 438 nm in the absence of amazake}\)
\(A_3 = \text{absorbance at 438 nm in the presence of ultrapure water}\)

The \(amazake\) sample was centrifuged twice and used in the experiment without any dilution. DMPO (1 M, 50 \(\mu\)l) and hypoxanthine (2 mM, 50 \(\mu\)l) were added to phosphate buffer (200 mM, pH 7.4, 50 \(\mu\)l) and allowed to stand for 1 min at 30°C. After adding xanthine oxidase (1 U/ml, 50 \(\mu\)l)

![Fig. 1. Representative ESR spectra of (a) DPPH radical, (b) galvinoxyl radical, and (c) DMPO-OOH spin adduct.](image)
and shaking, the ESR spectrum of DMPO-OOH spin adduct was measured. The measurement conditions were as follows: magnetic field, 337.0 ± 5.0 mT; power, 4 mW; sweep time, 1 min; modulation, 100 kHz, 0.32 mT; amplitude, 2000; time constant, 0.3 s. A typical example of an ESR spectrum is depicted in Fig. 1 (c). The experiment was repeated three times. The scavenging activity for $O_2^\cdot^-$ was calculated using the following equation:

$$O_2^\cdot^-\text{ scavenging activity} (%) = \left(1 - \frac{h_1}{h_2}\right) \times 100 \quad (6)$$

$h_1 =$ relative height in the presence of amazake
$h_2 =$ relative height in the absence of amazake

Measurement of total phenol content Total phenol content in amazake was determined according to the Folin-Denis method (Singleton and Rossi, 1965). The amazake sample was centrifuged twice and used in the experiment without any dilution. To the solution containing the sample solution (400 μl), sodium carbonate (10 wt%, 400 μl) and ultrapure water (2.0 ml) were added with the phenol reagent (0.2 M, 200 μl). After keeping the solution in the dark for 1 h, the absorbance at 700 nm was measured. Total phenol content was determined using gallic acid as a standard.

Measurement of ferricyanide-reducing activity Ferricyanide (Fe(III))-reducing activity of the amazake samples was examined according to the following procedure (Yen and Chen, 1995). The diluted sample solution was prepared according to the procedure described in the determination of DPPH radical scavenging activity. The diluted sample solution of amazake (300 μl) was mixed with phosphate buffer (200 mM, pH 6.6, 300 μl) and 1.0 wt% potassium ferricyanide (300 μl). This mixture was kept for 20 min at 50°C. After the addition of 10 wt% trichloroacetic acid (300 μl) and stirring, the reaction mixture was centrifuged at 6700 × g for 10 min. An aliquot of the supernatant (800 μl) was diluted with ultrapure water (800 μl) and 0.1 wt% ferric chloride solution (800 μl) was added to the reaction mixture. Ferricyanide is reduced by the antioxidative substance to ferrocyanide (reduced form). Ferrocyanide forms a complex with the Fe(III) cation and the resulting complex has an absorption band at 700 nm. Ferricyanide-reducing activity was evaluated using the following equation:

$$\text{Ferricyanide-reducing activity} = \frac{A_1 - A_2}{A_1} \quad (7)$$

$A_1 =$ absorbance at 700 nm in the presence of the sample
$A_2 =$ absorbance at 700 nm in the absence of the sample

Measurement of the chelating activity The chelating activity of the amazake samples for Fe(II) cation was evaluated using ferrozine (Decker and Welch, 1990). Ferrous chloride solution (2 mM, 100 μl) was diluted with ultrapure water (3.0 ml) and an aliquot of diluted solution of amazake (800 μl) and ferrozine solution (5 mM, 100 μl) was added. This reaction mixture was allowed to stand for 10 min. Fe(II) cation forms the complex by reacting with ferrozine. The resulting complex has an absorption maximum at 562 nm. Chelating activity was calculated using the following equation:

$$\text{Chelating activity} (%) = \left(1 - \frac{A_1}{A_2}\right) \times 100 \quad (8)$$

$A_1 =$ absorbance at 562 nm in the presence of the sample
$A_2 =$ absorbance at 562 nm in the absence of the sample

Results

The nutritional components of amazake used in this study are summarized in Table 2. Amazake is a calorie-rich food because of its high carbohydrate content. For BRA, BRA2 and WRA (traditional amazake), most of the carbohydrates originate from the rice-derived glucose. The carbohydrate in SCA (instant amazake) is sucrose added during the production procedure. Two kinds of traditional amazake made from brown rice (BRA and BRA2) have an appreciably large quantity of minerals compared with those of WRA and SCA. Of note is that the large quantity of Na in SCA comes from the sodium chloride included in the raw material.

BRA2 had the highest protein content, followed by BRA and WRA, both of which contained much more than SCA. The protein content in BRA, BRA2 and WRA is derived from rice protein and that of SCA is derived from sake cake. The protein components that contribute to the radical scavenging activity are soluble amino acids and peptides. The free amino acid compositions of amazake are summarized in Table 3. The total free amino acid amount decreased in order from BRA2 >> BRA = WRA >> SCA. The free amino acid amount in BRA and WRA is approximately two thirds that of BRA2. If the amino acid distribution is the same among the three amazakes, Asn, Trp, and 4-aminobutanoic acid in BRA and Asn, Gln, and Lys in WRA are found at higher concentrations than in BRA2. Thr, Phe and Lys in BRA, and His and 4-aminobutanoic acid in WRA were found at higher concentrations than calculated. BRA2 contains the largest amount of free amino acids, although Asp, Glu, Gln and Arg were not found at high concentrations.

Instant amazake is made by mixing the sake cake and carbohydrate in hot water. Generally, sake cake contains about 8 wt% alcohol. To be compliant with the liquor tax law in Japan, as amazake is sold as a soft drink, the alcohol content has to be lower than 1 vol%. Thus, sake cake can only be used in small quantities during the production process and as a result, SCA has the lowest amount of free amino acids. Although the total free amino acids in SCA were low, the proportions of Asn and Ala were high and Cys, Lys, His and 4-aminobutanoic acid were moderately high.

The DPPH radical scavenging activity of amazake calcu-
lated by absorbance at 527 nm and by ESR spectra is shown in Fig. 2. The radical scavenging activity of each type of amazake showed first-order dependence on the ratio of concentrations. BRA and BRA2 showed the strongest radical scavenging activity toward DPPH radical. The radical scavenging activity of WRA was weaker than that of BRA and BRA2, but it was stronger than that of SCA. When the ratio of the concentration was 1.0, which represents no dilution, WRA showed more than 80% of maximum scavenging activity, while SCA showed only ca. 30%.

Galvinoxyl radical scavenging activity of amazake was evaluated by absorbance at 430 nm and ESR spectra (Fig. 3). A similar trend was observed. The order of galvinoxyl radical scavenging activity was BRA = BRA2 > WRA >> SCA. BRA and BRA2 scavenged almost 100% of the galvinoxyl radical at C/C₀ = 0.3. On the other hand, even at C/C₀ = 1.0, WRA scavenged ca. 80% and SCA scavenged only ca. 25%.

The scavenging activity of amazake toward O₂⁻ (using the WST-1 method) is depicted in Fig. 4. The scavenging activity was related in a non-linear fashion to the concentration ratio, in contrast to the scavenging activities toward DPPH and galvinoxyl radicals. Using the WST-1 method, there was no significant difference of O₂⁻ scavenging activity between BRA, BRA2, and WRA. However, SCA showed the weakest scavenging activity among the four types of amazake. We determined the EC₅₀ value, which is the concentration ratio to scavenge 50% of the O₂⁻, as 0.07 ± 0.02 (BRA), 0.11 ± 0.02 (BRA2), 0.14 ± 0.02 (WRA) and 0.68 ± 0.02 (SCA) (Fig. 4). The O₂⁻ scavenging activity was also examined using an ESR spin trapping technique (Fig. 5). The O₂⁻ scavenging activity of BRA was not statistically higher than that of BRA2 (p > 0.05). A significant difference between BRA and WRA was not observed in this study (p > 0.05). The radical scavenging activity of SCA was statistically lower than that of BRA and BRA2 (p < 0.01). The O₂⁻ scavenging activity of SCA was almost half of the other amazakes.

Next, we examined the total phenol content of amazake to examine the relationship between the radical scavenging activity of amazake and the total phenol content (Fig. 6). BRA, BRA2, WRA and SCA showed significantly different phenol content profiles (p < 0.001). The phenol content of SCA was one third of BRA and half of WRA. The order of

![Fig. 2](image1)  ![Fig. 3](image2)

**Fig. 2.** DPPH radical scavenging activity of amazakes measured (a) by UV-vis absorption spectra; n = 3, and (b) by ESR spectra; n = 5.

**Fig. 3.** Galvinoxyl radical scavenging activity of amazakes measured (a) by UV-vis absorption spectra; n = 3, and (b) by ESR spectra; n = 5.
total phenol content was BRA2 > BRA > WRA > SCA \( (p < 0.001) \).

Discussion

Measurement of the DPPH radical scavenging activity of two kinds of traditional amazake produced from brown rice (BRA and BRA2) indicated they had strong radical scavenging activity, while the other traditional amazake made from white rice (WRA) showed moderate radical scavenging activity. The DPPH radical scavenging activity of each amazake showed a first-order dependency on the ratio of concentration. When the ratio of the concentration was 0.2 (Fig. 2 (a)), the activities of BRA and BRA2 were four and three times larger than that of WRA, respectively \( (p < 0.001) \). The activity of SCA was only one-third of the activity of WRA \( (p < 0.001) \) without dilution.

Based on the ESR experimental results, the concentration of amazake necessary to scavenge 50% of DPPH radical corresponded to about a 10-fold dilution of the undiluted supernatant \( (C/C_0 = 0.1) \) in the case of BRA and BRA2. The concentration of WRA corresponded to about 2-fold dilution \( (C/C_0 = 0.5) \). Even undiluted SCA could not scavenge 50% of the DPPH radical (Fig. 2 (b)). The 50% DPPH radical scavenging activity of \( \text{L-ascorbic acid} \) was calculated to be 7.6 \( \mu \text{M} \). Thus, the \( \text{L-ascorbic acid} \) index of BRA, BRA2, and WRA was calculated as 33.5 mg eq./100 ml, 23.9 mg eq./100 ml, and 6.4 mg eq./100 ml, respectively. According to a previous report (Ishiwata et al., 2000), the radical scavenging activity (mg ascorbic acid equivalent/100 ml) of coffee beverage, cocoa beverage, tea beverage, fruit juice and beverage, vegetable juice, milk beverage, soybean milk beverage, carbonated beverage, and near-water beverage were in the range of 248.0±7.7 to 132.0±5.2, 193.0±6.7 (only one), 178.0±1.9 to 1.8±1.0, 368.2±17.5 to 28.7±2.6, 347.0±24.0 to 10.0±0.8, 35.3±0.3 to 6.9±0.3, 90.8±6.1 to 8.1±0.9, 103.1±11.8 to 1.2±0.2, and 180.5±10.5 to 1.0±0.6, respectively. Although a direct comparison is quite difficult as a number of beverages contain ascorbic acid added as an antioxidant, the values of BRA and BRA2 were almost the same as vegetable juice and milk beverage, but were smaller than the values of coffee beverage, cocoa beverage, and tea beverage. Ishiwata et al. (2001) also reported that sake showed practically no DPPH radical scavenging activity. Thus, it is likely that the fermentation procedure used for amazake production generates radical scavenging substrates.

Similar results were obtained for the galvinoxyl radical scavenging activities of amazake (Fig. 3). The difference between BRA and BRA2 was significant \( (p < 0.01) \) at the concentration ratio of 0.2, however, it was not significantly different \( (p > 0.05) \) at the other concentration ratios. There
was a slight difference between DPPH and galvinoxyl radical scavenging activities, and the galvinoxyl radical scavenging activity of BRA and BRA2 was nearly equal. These results showed that traditional amazakes (BRA, BRA2 and WRA) have considerably high radical scavenging activities against DPPH and galvinoxyl radical compared with instant amazake (SCA). Amazakes produced from brown rice (BRA and BRA2) showed stronger DPPH and galvinoxyl radical scavenging activities than amazake produced from white rice (WRA).

The behavior of the O$_2^•−$ scavenging activity of amazake differed from those for the DPPH radical and galvinoxyl radical. Using the WST-1 method, the radical scavenging activity showed a non-linear relationship to the concentration ratio. This fact is explained by considering that the antioxidative components of amazake and WST-1 might undergo a competitive reaction against O$_2^•−$. The order of the radical scavenging activity was BRA ≥ BRA2 ≥ WRA >> SCA. A similar tendency was observed from the ESR spin trapping method. In the same spectroscopic experiment using gallic acid instead of amazake, the IC$_{50}$ value of gallic acid was 60.9 μM for the DPPH radical while it was 34.1 μM for O$_2^•−$. Devi and Arumughan (2007) reported that ferulic acid showed almost 22 times stronger scavenging activity than tricine toward DPPH radical but it was only two times stronger than tricine for scavenging O$_2^•−$. This result indicates that there are specific radical scavengers in amazake that vary in their abilities to scavenge DPPH, galvinoxyl radicals and O$_2^•−$. Though these components have not been identified, it is clear that traditional amazakes (BRA, BRA2 and WRA) have stronger O$_2^•−$ scavenging activity than instant amazake (SCA).

Ishiwata et al. (2001) reported that the DPPH radical scavenging activity and polyphenol content of commercially available alcoholic beverages were highly correlated. Several phenolic compounds in rice-koji have been identified, including pyrocatechol, hydroquinone, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, p-hydroxyphenylactic acid, p-hydroxyphenyllactic acid, p-coumaric acid, protocatechuic acid, vanillic acid and ferulic acid (Nakamura et al., 1969). Harukaze et al. (1999) have identified the phenolic components in rice as ferulic acid, p-coumaric acid, 5,5′-diferulic acid, 5,8′-diferulic acid benzofuran form and 5,8′-diferulic acid by HPLC. Recently, Tian et al. (2005) developed methods to separate 6′-O-feruloylsucrose, 6′-O-sinapoylsucrose, sinapinic acid, chlorogenic (3-caffeoylquinic) acid, caffeic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid and syringic acid. As traditional amazake is made from steamed rice and rice-koji, it is reasonable to consider that it might contain these types of phenolics. The order of total phenol content in amazake was BRA2 > BRA > WRA > SCA (Fig. 6). The order of DPPH and galvinoxyl radical scavenging activities was BRA = BRA2 > WRA >> SCA and that of O$_2^•−$ scavenging activity was BRA ≥ BRA2 ≥ WRA >> SCA. There was not a good correlation between the radical scavenging activity and phenol content, especially for BRA and BRA2. Thus, the amount of phenolic compounds is likely not the predominant factor governing the reduction of radicals.

To find other factors contributing to the radical scavenging activity, we also carried out studies on the metal reducing and chelating activities of these amazakes. A linear relationship between the ferricyanide-reducing activity and amazake concentration ratio was observed (Fig. 7). The order of reducing ability was BRA > BRA2 > WRA > SCA ($p < 0.01$) under the half-diluted conditions (C/C$_0$ = 0.5). The order of reducing activity agreed well with DPPH (Fig. 2) and galvinoxyl (Fig. 3) radical scavenging activities. Carbonyl compounds, SH compounds, amines, phenolic compounds and amino-carbonyl reaction products might participate in...
ferricyanide-reducing activity (Sato et al., 1968).

The chelating activity was also examined using ferrozine (Fig. 8). The order was BRA > BRA2 > WRA = SCA \( (p < 0.001) \). When the ratio of the concentration was 0.5, BRA and BRA2 had about 7 and 4 times stronger chelating activity than that of WRA, respectively \( (p < 0.01) \). BRA and BRA2, both produced from brown rice, had strong chelating activity. No chelating activity was observed for SCA. Brown rice contains 1.03 – 1.17% phytin \( (7.5 – 14.5\% \text{ in rice bran} \) (Yoshii, 1971). Phytic acid is known to be a strong chelator (Graf and Eaton, 1990). As shown in Table 2, the phosphorus \( (P) \) content of amazake produced from brown rice (BRA and BRA2) is considerably high. It is likely that the high phytin content is decomposed by the phytase from Aspergillus oryzae, which increases the amount of phytic acid-P.

Melanoidin has chelating activity for transition metals (Gomyo and Horikoshi, 1976; Honma, 1991). Phenolic acids such as ferulic acid and sinapinic acid also have high chelating activity (Hynes and Coincianainn, 2002). Metallic ion chelating agents have the potential to be an antioxidant because transition metals often generate reactive oxygen species (ROS). Though it is not clear which component would act as a chelator, amazake produced from brown rice can be regarded as a strong scavenger of radicals.

BRA2 has a larger amount of phenolic compounds than BRA, while BRA has stronger metal reducing and chelating activities than BRA2. Although BRA2 had the highest total phenol content, amino acids and reducing carbohydrates, it did not have the strongest radical scavenging activity. When the free amino acid components were examined, Trp was found in the order of BRA2 > BRA > WRA > SCA. Contrary to this, BRA has larger amount of Asn and 4-aminobutanoic acid than BRA2, while BRA has the same amount of Lys and Phe as BRA2.

As amazakes have large quantities of free amino acids and reducing carbohydrates, one can study the possibility of the Maillard reaction, which includes formation of a Schiff base followed by an Amadori rearrangement. If the Maillard reaction occurs during the amazake producing process, an absorption band around 420 nm derived from the Maillard reaction products should be observed (Ukeda and Ishii, 1997; Murakami et al., 2002; Miyoshi et al., 2006). The absorbance of BRA and BRA2 at 420 nm was 0.78 and 0.54, respectively, and the absorbance of WRA and SCA was 0.14 and 0.09, respectively. There is a high possibility that amazakes produced from brown rice (BRA and BRA2) contain a significant amount of melanoidin as a result of the Maillard reaction. In the outside layer of brown rice, soluble albumin and globulin are found in large amounts, while insoluble prolamin and glutelin are rich in the inside layer. Free amino acids are also rich in the outside layer (Komuyama et al., 1969). Thus, it is possible that Maillard reaction products might participate, in some part, to the antioxidant activity observed in BRA and BRA2.

According to these results, DPPH and galvinoxyl radical scavenging activities are dependent on phenol contents and Maillard reaction products. These two kinds of compounds can also contribute to ferricyanide-reducing activity, making it a quick way to determine DPPH and galvinoxyl radical scavenging activities of amazake. It is apparent that amazake produced from brown rice has a greater capacity to be phenolic and melanoidin rich, which is advantageous for its overall organic radical scavenging activity.

Conclusion

Based on the experimental results reported herein, we ascertained that the type of raw material used in amazake production made a remarkable difference to the functionalities of amazake. Two types of amazake made from brown rice had extremely strong antioxidative activities compared with the others, which were made from white rice and sake. For the two amazakes derived from brown rice, the amount of phenolic compounds was not related to their antioxidative activities. Instead, the BRA amazake, which had a larger amount of Maillard reaction products, showed stronger ferricyanide-reducing activity and chelating activity. Thus, it is possible that Maillard reaction products are also related to the total antioxidative activities of amazake.

The results obtained in this study should help to better understand the characteristics of amazake. Further investigations are ongoing, including studies on the variations in antioxidative activities obtained after different methods of manufacturing.

References


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