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Note

Effects of *koji* Production and Saccharification Time on the Antioxidant Activity of *amazake*

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***Amazake* was prepared using yellow-*koji* (*Aspergillus oryzae*) or white-*koji* (*Aspergillus kawachii*). In both cases, when rice-*koji* and water were mixed in a ratio of 1: 2, saccharification was completed in 6 h and reducing sugar was formed. Changes in antioxidant activity were investigated for each *amazake* saccharification process. In both *amazake* preparations, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity increased for the first 2 h of the saccharification period, and then gradually decreased. The lipid peroxidation inhibitory activity with β-carotene, however, did not change with saccharification time in either *amazake* preparation, and the antioxidant activity was roughly constant during saccharification. Next, both types of *amazake* were prepared by saccharifying for 6 h at 55°C, with the respective rice-*koji* obtained with different *koji* production times (42, 48 and 54 h). Antioxidant activity was then compared. It was found for both types of rice-*koji* that longer *koji* production time yields *amazake* with higher DPPH radical-scavenging activity and lipid peroxidation inhibitory activity.**

Keywords: *amazake,* DPPH radical-scavenging activity, lipid peroxidation inhibitory activity, rice-*koji*.

Introduction

Amazake is a sweet beverage made from rice that is unique to Japan. It is one of several traditional Japanese fermented products prepared from polished rice, rice-*koji,* and water. Rice-*koji* is produced by adding mycelia, a type of mold, to steamed rice. *Amazake* is prepared by adding water to rice-*koji* and heating the mixture for several hours. The main sweet component in *amazake* is glucose, which is formed from the decomposition of rice starch by the amylase secreted from the mycelia. Oligosaccharides are also formed during this process. On the rice surface, there are many proteins, and so large amounts of amino acids and peptides are formed by the proteolytic enzymes of the mycelia. Proliferation of mycelia produces vitamins and amino acids and large amounts of these dissolve in the *amazake*. Therefore, *amazake* is sometimes referred to as a Japanese-style yogurt, and is currently undergoing consideration as a nutritious health beverage. In recent years, the beneficial effects of *amazake* have been widely studied, and blood pressure-lowering effects, antiobesity effects, liver-protecting effects, and antiamnesic effects have been reported (Ohura, 2003). One of our aims is to establish production technology to make *amazake* that has beneficial effects. Kitagaki *et al.* (1999) proposed 7 substances- tannic acid, gallic acid, ferulic acid, protocatechuic acid, (−)-epicatechin, 3,5-dimethoxy-4-hydroxycinnamic acid, and *p*-hydroxycinnamic acid- that have the free radical-scavenging ability naturally contained in *sake*. Ohta *et al.* (1992) reported on antioxidative substances with lipid peroxidation inhibitory activity in *sake,* and proposed that they were peptides and ferulic acid produced from rice by *koji* enzymes. *Amazake* may contain these antioxidative substances. In this paper, the effects of *koji* production time and *amazake* saccharification time using yellow-*koji* (normally used for *amazake*) and white-*koji* (usually used for *shochu*) on the antioxidant activity of *amazake* are discussed.

Materials and Methods

Materials DPPH was purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). β-Carotene was purchased from Sigma Chemical Co. (St. Louis, MO). 6-Hydroxy- 2,5,7,8 -tetramethylchroman- 2 -carboxylic acid (Trolox) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Butylated hydroxytoluene (BHT) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade. *Tane-koji* for making rice-*koji* were purchased from Kawauchi genichiro shouten Co., Ltd. (Kagoshima, Japan).

Rice-koji production Polished rice (200 g) was soaked in water for 20 min, and the water was then drained for 2 h. Following this, the rice was steamed for 40 min with a steamer, and was allowed to stand until the temperature decreased to 40°C. Next, 0.2 g of *tane-koji,* either yellow- or white-*koji,* was added to the steamed rice, and the mixture was stirred until it became uniform. Except for the steaming process, the entire procedure was performed under sterile conditions. The steamed rice was then transferred to a petri dish and packed with a spatula. A

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filter paper was inserted inside the cover, and the mixture was incubated at 30℃. The rice-*koji* was stirred every 12 h, and three types of rice-*koji* (culture time: 42, 48, or 54 h) were prepared.

Preparation of amazake *Amazake* was prepared by adding 100 ml of deionized water to 50 g of either yellow- or white-*koji,* followed by saccharification for 6 h in a constant-temperature bath at 55℃. Total reducing sugar content was determined using the method of Somogyi-Nelson (Somogyi, 1952; Nelson, 1944).

Measurement of DPPH radical-scavenging activity *Amazake* was added to the same volume of deionized water, and the mixture was centrifuged (3,000 rpm, 10 min). The resulting supernatant was then mixed with the same volume of ethanol, and the mixture was again centrifuged. The obtained supernatant was thus 4-fold diluted *amazake* containing 50% ethanol. This *amazake* was further diluted 4-fold with 50% ethanol, and this final 16-fold diluted *amazake* containing 50% ethanol was used for analysis. The radical-scavenging activity was measured based on the method of Suda *et al.* (2000) and Wang *et al.* (2004). DPPH radical-scavenging activity was evaluated in terms of Trolox equivalent (μM).

Measurement of lipid peroxidation inhibitory activity Lipid peroxidation inhibitory activity was measured based on the method of Kudo *et al.* (2000) using β-carotene. For analysis, 16-fold diluted *amazake* containing 50% ethanol was prepared in the same way as analysis of DPPH radical-scavenging activity. Lipid peroxidation inhibitory activity was evaluated in terms of BHT equivalent (μM).

Assay of glucoamylase activity Glucoamylase activity was assayed using the official analytical method of the National Tax Administration Agency of Japan (1993). One unit of glucoamylase activity was defined as the amount of enzyme that produced 1 mg of glucose in the rice-*koji* in 60 min.

Results and Discussion

When *amazake* was prepared with yellow- or white-*koji* obtained with a *koji* production time of 48 h, the amount of reducing sugar gradually increased for 6 h of saccharification at 55℃ in both cases. Sugar formation was not seen beyond 6 h (Fig. 1).

Although both yellow- and white-*koji* were prepared under the same conditions, more reducing sugar was formed in *amazake* prepared from white-*koji*. Under these conditions, changes in antioxidant activity during the 6-h saccharification process were investigated for each *amazake* (Fig. 2).

DPPH radical-scavenging activity significantly increased with saccharification in yellow-*koji amazake* ($p<0.01$) and white-*koji amazake* ($p<0.01$) for the first 2 h of saccharification, after which it gradually decreased. Furthermore,

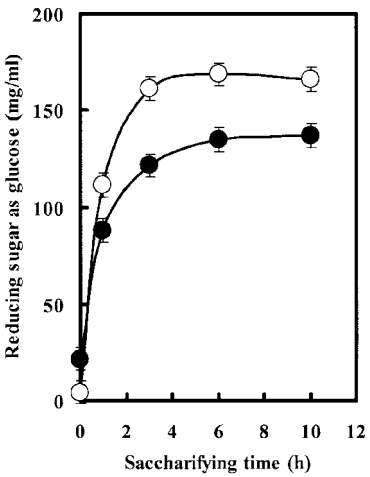

Fig. 1. Change in reducing sugar during *amazake* making. Symbols: ○, white-*koji*; ●, yellow-*koji*.

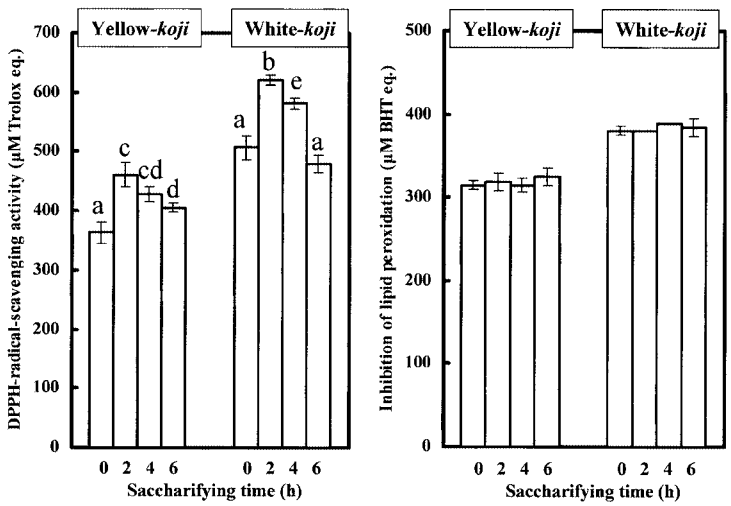

Fig. 2. Effects of saccharification time on antioxidant activity of *amazake*.
Antioxidant activity was evaluated by DPPH radical-scavenging activity (left) and inhibition of lipid peroxidation (right). Each value is the mean±standard deviation of three replicates. Statistical difference was analyzed by a multiple comparison post test using Tukey's method. Values in columns followed by different superscript letters in each *amazake* (a-b, a-c, a-cd, and a-e) are significantly different ($p<0.01$). Also, each *amazake* (a-d, c-d, and b-e) is significantly different ($p<0.05$) as well.

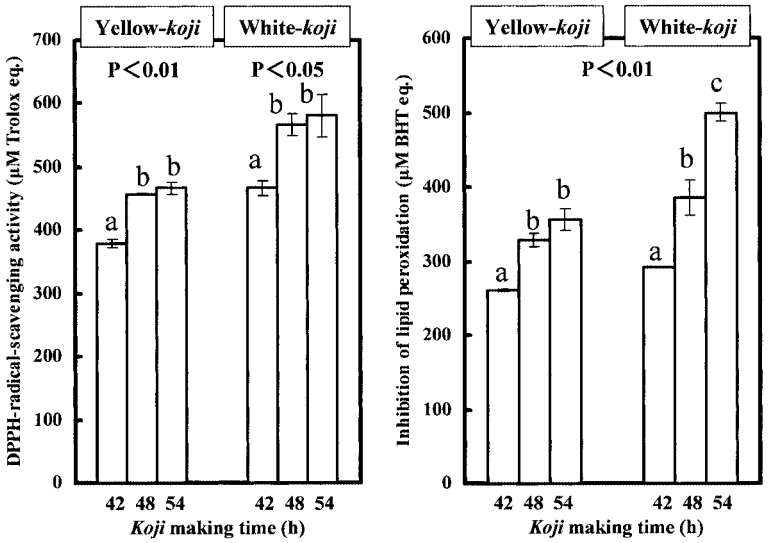

Fig. 3 Effects of *koji* production time on antioxidant activity of *amazake*.
Antioxidant activity was evaluated by DPPH radical-scavenging activity (left) and inhibition of lipid peroxidation (right). Each value is the mean±standard deviation of three replicates. Statistical difference was analyzed by a multiple comparison post test using Tukey's method. Bars topped by different letters are significantly different.

the DPPH-radical-scavenging activity of *amazake* was 400–600 μM-Trolox eq., which is the same as the fresh weight of Nigaure, Papaya and Hechima (Maeda *et al.*, 2006). On the other hand, Kudo *et al.* (2001) reported that the peptides produced from casein during lactic acid fermentation have lipid peroxidation inhibitory activity. In addition, they suggested that it is important to produce peptides with specific structures to induce lipid peroxidation inhibitory activity, and that lipid peroxidation inhibitory activity may decrease following decomposition of the peptides by proteolytic enzymes.

The change in lipid peroxidation inhibitory activity during lactic acid fermentation was very similar to the change in DPPH radical-scavenging activity during the saccharification of *amazake*. From these findings, it was conjectured that antioxidant substances with some specific structure were released in *amazake* during the first 2 h of saccharification; after that, the structures changed. Therefore, the radical-scavenging activity of *amazake* increased during the first 2 h, after which it gradually decreased. On the other hand, lipid peroxidation inhibitory activity remained largely unchanged during the saccharification process for *amazake* prepared from either yellow- or white-*koji*. It was inferred from these results that the lipid peroxidation inhibitory activity of *amazake* is due to materials formed during the *koji* production process. These materials were probably not affected by the enzymes in rice-*koji* during the saccharification period. Furthermore, the lipid peroxidation inhibitory activity of *amazake* was 250–500 μM, which was 5–50 times higher than that of the sweet potato yogurt reported by Kudo *et al.* (2001). When the radical-scavenging and lipid peroxidation inhibitory activity of *amazake* prepared from yellow- and white-*koji* was compared, *amazake* prepared from white-*koji* exhibited more activity at all time points. Next, *amazake* was prepared from yellow- or white-*koji* with *koji* production times of 42, 48, or 54 h and saccharification of 6 h. The antioxidant activities of the obtained *amazake* were compared (Fig. 3). The DPPH radical-scavenging activity of *amazake* prepared from either yellow-*koji* ($p<0.01$) or white-*koji* ($p<0.05$) significantly increased with *koji* production time (42, 48 h and 42, 54 h). However, there was almost no difference between 48 h and 54 h. Similarly, the lipid peroxidation inhibitory activity of *amazake* prepared from both yellow- and white-*koji* significantly increased ($p<0.01$) with *koji* production time (42, 48 h and 42, 54 h). However, unlike DPPH radical-scavenging activity, a significant difference ($p<0.01$) was observed between *koji* production times of 48 h and 54 h in *amazake* prepared using only white-*koji*. The relationship between the antioxidant activity of six types of *amazake* and the glucoamylase activity of their respective rice-*koji* were investigated (Fig. 4).

A positive correlation was observed between the DPPH radical-scavenging activity of *amazake* and the glucoamylase activity of rice-*koji*. However, no correlation was observed between the lipid peroxidation inhibitory activity of *amazake* and the glucoamylase activity of rice-*koji*. Recently, it was reported that ferulic acid with antioxidant activity binds arabinose, which is the side chain of xylan, the structural material of hemicellulose in plant cell walls, and that ferulic acid was released from rice on addition of takadiastase, originating from *Aspergillus oryzae* (Koseki *et al.*, 1994). Furthermore, Koseki *et al.* (1996) reported that the ferulic acid released from rice was changed to 4-vinyl guaiacol by acid and heating during the *shochu* distillation process. From these findings, we conjectured that the release of ferulic acid by the increase of glucoamylase activity might cause a increase in DPPH radical-scavenging activity of *amazake* with increasing *koji* production time. Furthermore, we hypothesize that ferulic acid is oxidized during saccharification and its

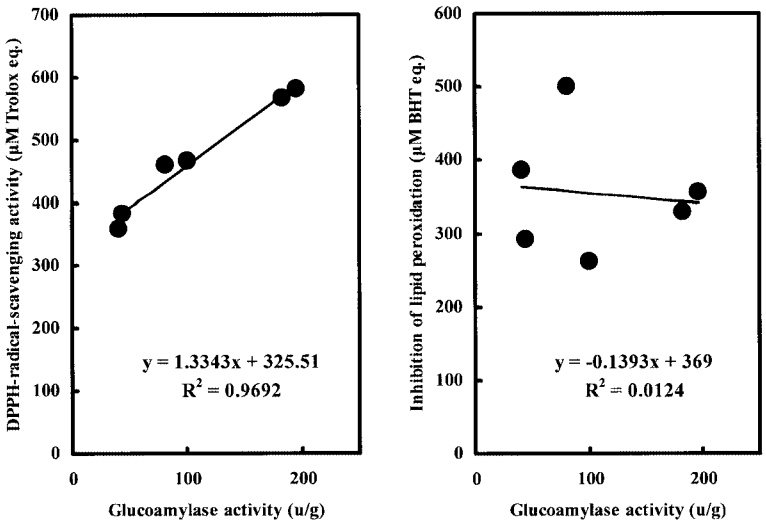

Fig. 4 Relationship between glucoamylase activity of rice-*koji* and antioxidant activity of *amazake*. *Amazake* was prepared from yellow-*koji* or white-*koji* obtained using *koji* production times of 42, 48 or 54 h (saccharification time: 6 h).

structure might be changed; accordingly, radical-scavenging activity might be decreased. In addition, we believe that the substances with lipid peroxidation inhibitory activity in *amazake* might be formed in mycelia during the *koji* production process, and might increase with the growth and proliferation of mycelia. These substances may not be affected by the enzymes in rice-*koji,* or by oxidization during saccharification. Chen *et al.* (1998) reported that peptides with lipid peroxidation inhibitory activity did not have DPPH radical-scavenging activity. It is also possible that the antioxidant substances in *amazake* need to be identified, as they may be different substances.

In this study, *amazake* was prepared from two types of rice-*koji*: yellow- and white-*koji*. Antioxidant activity was higher for *amazake* made from white-*koji* than from yellow-*koji*. While the same *koji* production conditions were used in this study; optimal conditions were not used. The antioxidant activity of *amazake* may be affected by enzyme activity. Thus, the conditions for mycelial proliferation and enzyme production for white-*koji* may have been closer to optimal than those for yellow-*koji*. In conclusion, it was confirmed that antioxidant activity is affected by *koji* production conditions, saccharification conditions, and *tane-koji* species. Therefore, the antioxidant activity of *amazake* can be controlled by altering production conditions. In order to prepare highly functional *amazake,* future investigations will aim to determine the components involved in the antioxidant activity of *amazake,* and to establish technology that can produce sufficient amounts of these components.

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