Studies on the characterizations of functionalities in the rice miso products supplementary with beans and buckwheat at different fermentation periods

異なる熟成期間における豆類及び蕎麦を添加した米味噌製品の機能性に関する研究

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Content

Introduction

Diet and lifestyle are major factors thought to influence susceptibility to many diseases. Tobacco smoking, and alcohol drinking, as well as too much exercise, may also increase the risk of developing certain diseases [1]. Therefore, the term “lifestyle-related diseases” was proposed in 1996 by the Ministry of Health and Welfare’s Council on Public Health [2]. In recent years, dietary life of people began to consume more meat [3], dairy products [4], vegetable oils, tobacco, sugary foods, Coca-Cola, and alcoholic beverages during the latter half of the 20th century. People also developed sedentary lifestyles and greater rates of obesity. These unhealthy dietary habits are at high risk of developing a range of physical health conditions, such as diabetes [5], cardiovascular disease [6,7], obesity [8], cancer [9] and metabolic syndrome [10], and previously seen mainly in middle-aged and elderly people, occurs increasingly frequently in children and young people [11]. There is reported that people in developing or oriental countries, whose diets still depend largely on low-sugar starchy foods with little meat or fat have lower rates of these cancers [12]. In Japan, the National Health and Nutrition Survey of the Ministry of Health, Labour, and Welfare indicated that there is a tendency to increase the proportion of obese patients in recent years [13].

Soybeans and their fermented products are famous and popular in Asian diets [14], China is well known for its douchi [15], stinky tofu [16], and sufu [17]; Indonesia for the tempeh [18]; and Koreans for the cheonggukjang [19] and doenjang [20]. With the improvement of sensory characteristics and nutritious components, fermentation has become a beneficial and healthy method in food processing. Rice miso is a traditional

Japanese seasoning fermented by boiled soybean with salt and rice-malt, and high in protein and rich in vitamins and minerals played an important nutritional role in feudal Japan. A large number of studies have investigated the rice miso have all beneficial effects as lipid peroxidation-inhibiting action, anti-hypertension, anti-mutagen, and blood glucose level elevation-inhibiting action [21-23].

Black soybean is a variety of soybean (*Glycine max*), the origin is China, and used as a traditional Chinese medicine with beneficial effects as blood-activating, detoxifying and diuresis-promoting [24]. It is noted that contained a large amount of dietary fiber, flavonoid, and anthocyanin. Several studies have found that black soybean has various physiological effects such as strong antioxidant activity, glycolysis-inhibitory activity, anti-obesity, anti-cancer and hepatoprotective effect [25-29].

Kidney bean is a variety of the common bean (*Phaseolus vulgaris*), and the origin is Latin American, passed through Europe at the end of the 16th century, and transmitted to China and Japan in the 17th century. In 2017, kidney bean cultivated in Hokkaido was accounting for about 97% of common beans in Japan [30]. It contains a large amount of dietary fiber and anthocyanin with various physiological effects such as antioxidant activity, anti-inflammatory activity, anti-hypertensive and α-amylase inhibition [31-34].

Adzuki bean (*Vigna angularis*) is an annual vine widely cultivated throughout East Asia. The cultivars most familiar in northeast Asia have a uniform red color, however, white, black and variously mottled varieties also are known. In the present study, red adzuki bean was used. Adzuki bean is traditionally in Japan, usually used in food additives, such

as anko, amanatto, sekihan and other snacks due to a sweet taste. It is also known for health-promoting and nutritional properties, such as antioxidant, renal cortex protective, immunoregulatory activity, anti-obesity, anti-hypotensive, and hepatoprotective effects [35-38].

Buckwheat (*Fagopyrum esculentum*) is one of the most important functional foods throughout the world [39]. Buckwheat is a rich source of protein, dietary fiber, four B vitamins, and several dietary minerals, and also provide other positive health benefits. Buckwheat has a high level of antioxidant activity compared to other cereal crops, and this has been attributed to its high levels of flavonoid compounds [40]. It has been described that the consumption of buckwheat and buckwheat-enriched products is related to a wide range of biological and healthy activities: hypo-cholesterolemic, anti-oxidant, anti-fungal, anti-tumor, and anti-allergy activity [41-43].

Here we fermented rice miso products supplementary with black soybean (RM-BS), kidney bean (RM-KB), adzuki bean (RM-AB) and buckwheat (RM-BW) to development and utilization of Japanese traditional rice miso. In chapter one, peptide, reducing sugar, melanoidin, and polyphenol content were quantified and clarify these components to compared with rice miso (RM) at different fermentation periods. In chapter two, DPPH radical scavenging activity and ABTS radical scavenging activity were quantified and clarify the antioxidant activity to compared with rice miso (RM) at different fermentation periods. In chapter three, lipase inhibitory activity and α-glucosidase inhibitory activity were quantified and clarify the enzyme inhibitory activity to compared with rice miso (RM) at different fermentation periods.

Chapter One: Nutritional Components in Rice Miso Products

1. Materials and Methods

1.1. Materials

For the experiment, black soybean (*Glycine max(L)*), kidney bean (*Phaseolus vulgaris*), adzuki bean (*Vigna angularis*), soybean (*Glycine max*), buckwheat (*Fagopyrum esculentum*) were purchased from HosokawaSeian Co. Ltd. (Obihiro, Japan). The rice-malt purchased from MARUKOME Co., Itd. (Yasunori, Japan). The seed miso was purchased from the net-market of Koji-Za. The salt was purchased from the Salt Industry Center (Tokyo, Japan).

Folin-Ciocalteau reagent was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Catechin, DNS (3,5-dinitrosalicylic acid) was purchased from Sigma-Aldrich Co., LLC. (Tokyo, Japan). Glucose and glycine were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

1.2. Sample Preparation

The rice miso products were manufactured by industrial producing rice miso method [44]. Rice miso supplementary with black soybean (RM-BS) was prepared as the followings: First, soybean and black soybean of each 1.25 kg were washed and soaked in water for 16 hours at room temperature (beans: distilled water = 1: 3 (w/w)) (Table 1). Then, autoclaved soaked bean (KT-3045, ALP Co., Ltd., Japan) for 20 min at 110 ℃. After preparing the paste by crushing the steamed beans, mixed (KN1500, Taisho electric

MFG. Co., Ltd., Japan) with rice-malt (2.5 kg), salt (1 kg), seed miso (400 g) and some seed water (Table 2) to make the total weight of 10 kg. The mixture was packed in pickle barrels (Shinkigosei Co., Ltd. Japan) and fermented at 30℃. As mentioned above, rice miso with kidney bean (RM-KB), rice miso with adzuki bean (RM-AB) and rice miso with buckwheat (RM-BW) was manufactured in the same way. The products were sampled and stored in a -20℃ freezer (MF-U14A, MITSUBISHI electric. Co., Ltd., Japan) at 3, 6, 24, 36 months (M) after fermentation for experimental analysis.

Table 1. Amount of Raw Black Soybean, Kidney Bean, Adzuki Bean, Buckwheat, Soybean and Water for Rice Miso Production.

Materials	RM-BS	RM-KB	RM-AB	RM-BW	RM
Black Soybean (g)	1250	-	-	-	-
Kidney Bean (g)	-	1250	-	-	-
Adzuki Bean (g)	-	-	1250	-	-
Buckwheat (g)	-	-	-	1250	-
Soybean (g)	1250	-	-	1250	2500
Water (ml)	7500	7500	7500	7500	7500
Total (g)	10000	10000	10000	10000	10000

Abbreviations: RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso.

Table 2. Amount of Materials for Rice Miso Production.

Materials	RM-BS	RM-KB	RM-AB	RM-BW	RM
Boiled Beans (g)	5810	6000	6100	6100	5670
Rice-malt (g)	2500	2500	2500	2500	2500
Salt (g)	1000	1000	1000	1000	1000
Seed Miso (g)	400	400	400	400	400
Seed Water (ml)	290	100	-	-	430
Total (g)	10000	10000	10000	10000	10000

Abbreviations: RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso.

1.3. Extraction and Fractionation

The extraction of rice miso products was extracted by the method of Saito [26]. Each miso product (5 g) was mixed with 20 mL of 80% v/v ethanol, vortexed, and ultrasonicated for 30 min. The suspension was then centrifuged at 1,006 ×g for 10 min. As mentioned above, the same operation was repeated twice. Then, mixed with 20 mL of 70% v/v acetone and the aforementioned process was repeated thrice to take the extract as mixture extract. After that, according to the Ikeda's method [45], the mixture extract was concentrated by rotary evaporation in a vacuum and constant volume to 20 mL with distilled water. Then, added the same amount (20 mL) of n-hexane and ethyl acetate to delaminate the solution, and collected the hydrophilic fraction and the lipophilic fraction. Subsequently, a part of the hydrophilic fraction was purified by chromatography through a Diaion HP-20 column. The column was washed by distilled water (Fra. Water) and then eluted by methanol (Fra. Methanol). Moreover, the eluted methanol fraction was fractionated by Sephadex LH-20 column chromatography. The column was successively eluted with ethanol, methanol, and 60% acetone to collect fraction I, fraction II, and fraction III, respectively.

1.4. Melanoidin Content Determination

The content of melanoidin was determined by the method of Martins [46]. Briefly, 0.02 M of glucose and glycine were dissolved by 0.1 M phosphate buffer (pH 6.8), and heated at 120°C for 2 hours. Then, the solution was placed in a dialysis membrane (14000 MWCO, UC 36-32-100; EIDIA Corporation, Japan) and dialyzed against distilled water (7 days). The dialysate was lyophilized for 48 hours and took as a melanoidin standard.

Thereafter, the absorbance was measured using a UV-visible spectrophotometer (UVmini-1240, SHIMADZU Co., Kyoto, Japan) at 450 nm. The results were expressed as the mg melanoidin equivalents (ME) per gram DW miso ($y = 63.855x + 0.4273$, $R^2 = 0.9989$).

1.5.Peptide Content Determination

The peptide content of rice miso products was carried out by the BCA method [47]. First, Na_2CO_3 buffer (pH 11.25) containing 1% BCA and 0.4% $CuSO_4$ aqueous solution were mixed in a ratio of 50:1 as a reagent. Then, the samples (100μL) were mixed with 2 mL of the reagent, incubated at 37°C for 30 min, and centrifuged at 1,006 ×g for 10 min. Thereafter, the absorbance was measured using a UV-visible spectrophotometer (UVmini-1240, SHIMADZU Co., Kyoto, Japan) at 562nm. The albumin was taken as a standard, and results were expressed as the mg albumin equivalents (AE) per gram DW miso ($y = 59.298x – 0.4885$, $R^2 = 0.9968$).

1.6. Reducing Sugar Content Determination

The reducing sugar content of rice miso products was determined by the DNS method [26] with slight modifications. Specifically, the samples (50 μL) were diluted with 950 μL distilled water, and thoroughly mixed with 100 μL of 2N-NaOH, 100 μL of 1% DNS solvent, and reacted in boiling water bath for 10 min. Then, cooling it down to room temperature, and the absorbance was measured using a UV-visible spectrophotometer (UVmini-1240, SHIMADZU Co., Kyoto, Japan) at 540nm. The results were expressed as the mg glucose equivalents (GE) per gram DW miso ($y = 333.92x + 26.832$, $R^2 = 0.9922$).

1.7. Polyphenol Content Determination

The content of polyphenol was determined using the method of Folin-Ciocelteau [48] with slight modifications. Each extract of a sample (100 μL) was mixed with 300 μL of distilled water, 400 μL of 50% Folin-Ciocelteu reagent, and 400 μL of 10% Na_2CO_3 aqueous solution. The reaction solution was incubated at 30 ℃ for 30 min and centrifuged at 1,006 ×g for 10 min. Then, the absorbance was measured using a UV-visible spectrophotometer (UVmini-1240, SHIMADZU Co., Kyoto, Japan) at 760 nm, and results were expressed as the mg catechin equivalents (CE) per gram DW miso ($y = 11.834x - 0.9597$, $R^2 = 0.9938$).

1.8. Statistical Analysis

The experiments were repeated at least three times. Data were expressed as means ± standard deviation. Significant differences were determined by one-way ANOVA and Fisher's test (SAS v. 7.1, SAS Institute Inc., Cary, NC, USA). Differences were considered to be significant at $P < 0.05$.

2. Results and Discussions

2.1. Contents of Melanoidins and Related Components (Peptide and Reducing Sugar) Contained in Rice Miso Products at Different Fermentation Periods

The melanoidin is the products from a complicated reaction of Maillard reaction discovered by Louis-Camille Maillard in 1912 [49]. The melanoidin is aroma compounds and dark-brown polymeric compounds that produced from a mixture of amino acids and sugars [50]. The results of melanoidin content in a hydrophilic fraction of rice miso products were shown in Figure 1. Melanoidin content was increasing with prolonging the fermentation period of all rice miso products. The highest value of RM-BS was shown in fermented after 36 months (29.7 mg/g DW miso), and RM-KB was 26.5 mg/g DW miso, and highest value of RM-AB was shown in fermented after 24 months 20.2 mg/g DW miso, RM-BW was 23.7 mg/g DW miso, RM was 17.8 mg/g DW miso. Basically, the melanoidin content of rice miso supplementary with black soybean, kidney bean, adzuki bean, and buckwheat was significantly higher than rice miso (control). The ratios of melanoidin contained in RM-BS to compared with RM were increased from 1.1 to 1.8 folds at every fermentation period, and RM-KB was 1.1 to 1.6 folds, RM-AB was 0.9 to 1.2 folds, RM-BW was 1.0 to 1.4 folds, respectively.

Aspergillus oryzae is excellent in protein and starch degrading ability to decompose into peptide [51] and reducing sugar [52] through the fermentation process of rice miso product, and is used not only for seasonings and sweeteners but also for brewing of sake [53]. The peptide contents in rice miso products were shown in Figure 2. Since the

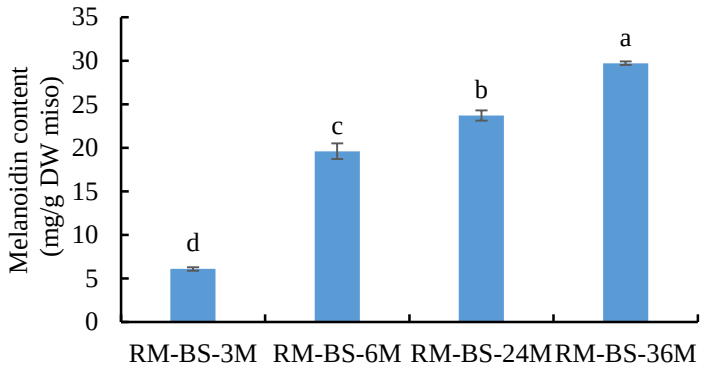
soybean protein decompose into peptide and amino acid, the peptide contents in rice miso products were increased from fermented after 3 months to 6 months, and used as a synthetic material for other substances and the contents were sharply declined at fermented after 24 months and 36 months, probably. To compared with RM, the ratios of peptide content contained in RM-BS were increased from 0.7 to 1.3 folds at every fermentation period, and RM-KB was 1.0 to 1.3 folds, RM-AB was 0.8 to 1.1 folds, RM-BW was 0.8 to 1.5 folds, respectively. Reducing sugar contents in rice miso products were shown in Figure 3. The increase trend of reducing sugar content is similar as peptide content in rice miso products, the reducing sugar contents in rice miso products were increased from fermented after 3 months to 6 months, and the contents were declined at fermented after 24 months and 36 months sharply. To compared with RM, the ratios of reducing sugar content contained in RM-BS were increased from 1.3 to 1.6 folds at every fermentation period, and RM-KB was 1.3 to 1.5 folds, RM-AB was 0.9 to 1.5 folds, RM-BW was 1.0 to 2.1 folds, respectively. Buckwheat and black soybean were mainly related to their protein content, kidney bean and red beans (*Vigna angularis*) contain more starch than soybeans [54,55]. Therefore, it is speculated that the RM-BS, RM-KB, RM-AB, and RM-BW have more peptide and reducing sugar than RM, and produce more melanoidin through the fermentation of rice miso product. As the above, the increase ratios of melanoidin content is similar as peptide and reducing sugar content, therefore, the melanoidin content were directly associated with peptide and reducing sugar in rice miso products.

To clarify the distributions of melanoidin polarity in rice miso products, the hydrophilic fraction was purified by chromatography through Diaion HP-20 column to eluted the water fraction (Fra. Water) and methanol fraction (Fra. Methanol). The results of

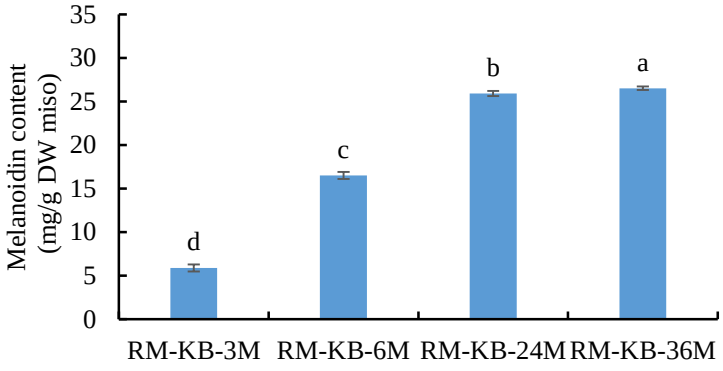
melanoidin content in Fra. Water and Fra. Methanol was shown in Figure 4. The increasing trends of melanoidin content in Fra. Water and Fra. Methanol was similar as in the hydrophilic fraction. Moreover, we also counted the ratios of melanoidin content between Fra. Water and Fra. Methanol, and the ratio of RM-BS was decreased from 0.4 to 0.3 folds, RM-KB was 0.5 to 0.3 folds, RM-AB was 0.5 to 0.3 folds, RM-BW was 0.5 to 0.2 folds, and RM was 0.3 to 0.2 folds, respectively. Therefore, the melanoidin was increasing with prolonging the fermentation of all rice miso products, and there is a high ratio of the melanoidin content contained in Fra. Water at fermented after 3 months, and with the extension of the fermentation period, the melanoidin contents of rice miso products were mainly contained in Fra. Methanol, and recognized as hydrophobic substance.

Furthermore, the RM-BS that contained the highest melanoidin content was used as a representative to clarify the distributions of melanoidin in Fra. Methanol. The Fra. Methanol was fractionated by Sephadex LH-20 column chromatography and eluted with ethanol, methanol, and 60% acetone to collect Fra. I, Fra. II, and Fra. III, respectively. The result of melanoidin content in fractions after the LH-20 column was shown in Figure 5. There was a trace melanoidin content contained in RM-BS-3M and mainly contained in Fraction I and II. On the contrary, a trace amount of melanoidin content were detected in Fraction I of RM-BS-6M, RM-BS-24M, and RM-BS-36M, but mainly contained in Fraction II and III. Moreover, the melanoidin content in Fraction. III was significantly increased with the extension of the fermentation period of miso. According to Saito [26], Fra. II and Fra. III contained oligo-polymeric functional compounds. Therefore, we estimate that the molecular weight of melanoidin was increased with the extension of the RM-BS fermentation.

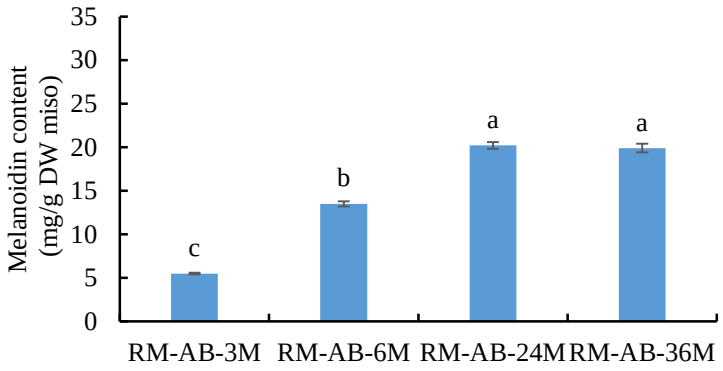
A


Melanoidin content (mg/g DW miso)
35
30
25
20
15
10
5
0
d
c
b
a
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


Melanoidin content (mg/g DW miso)
35
30
25
20
15
10
5
0
d
c
b
a
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


Melanoidin content (mg/g DW miso)
35
30
25
20
15
10
5
0
c
b
a
a
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

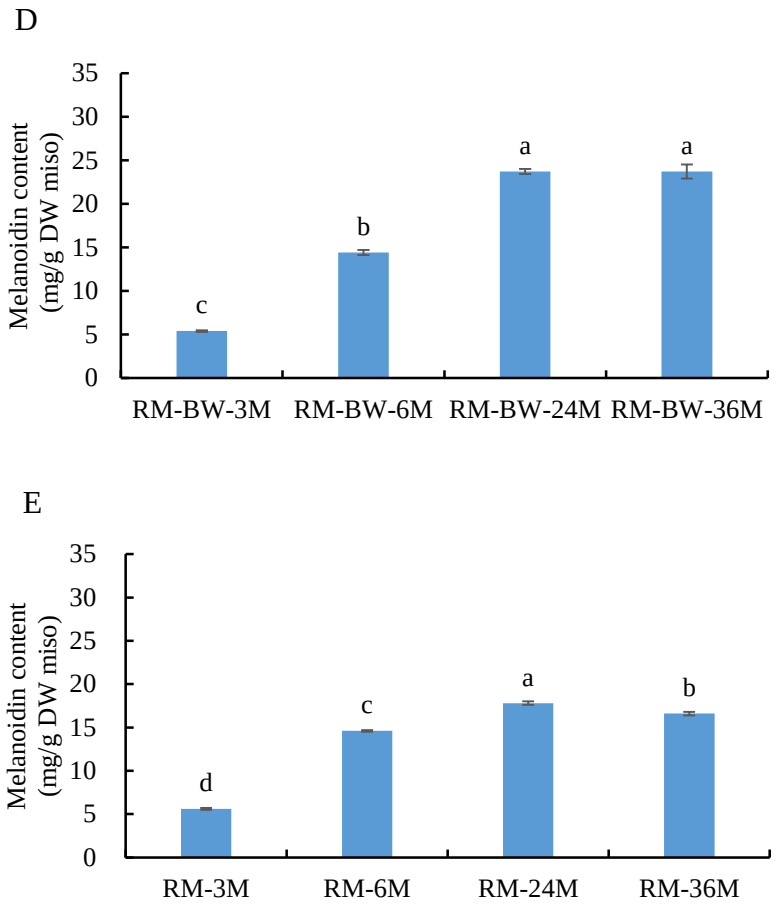
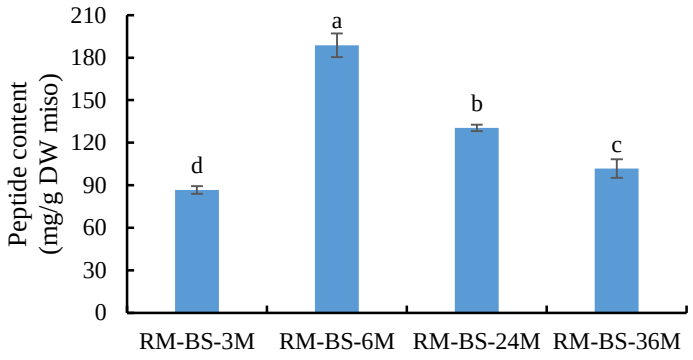
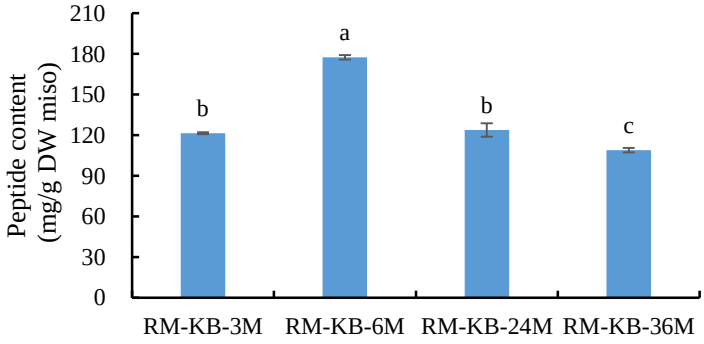


Figure 1. Melanoidin content in the hydrophilic fraction of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters are significant at $p < 0.05$.

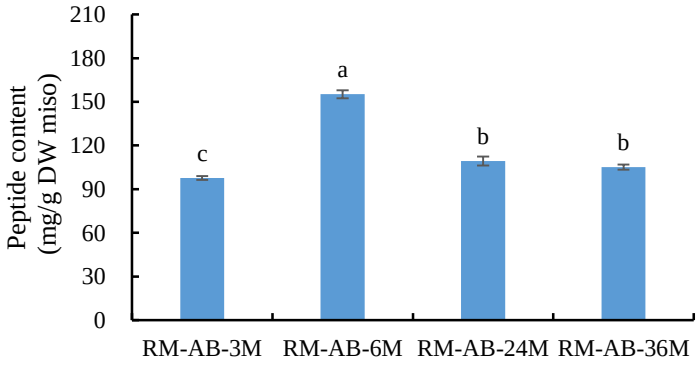
A


Peptide content (mg/g DW miso)
210
180
150
120
90
60
30
0
a
b
c
d
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


Peptide content (mg/g DW miso)
210
180
150
120
90
60
30
0
a
b
b
c
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


Peptide content (mg/g DW miso)
210
180
150
120
90
60
30
0
a
b
b
c
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

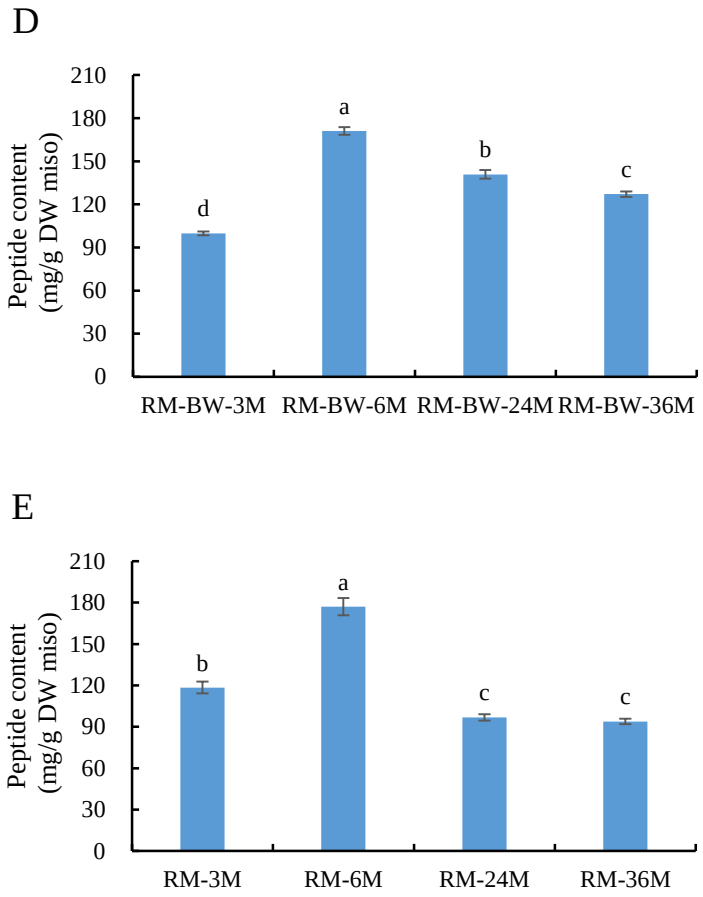
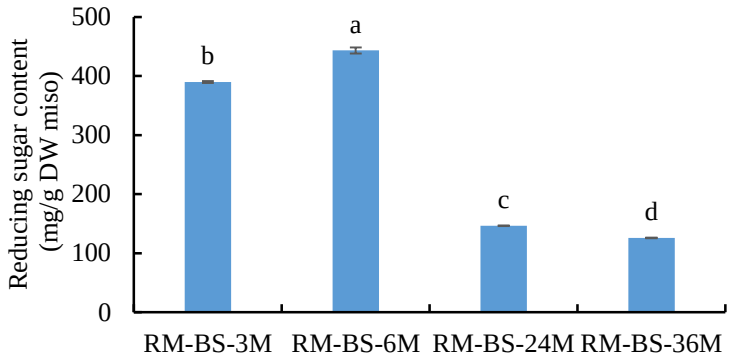
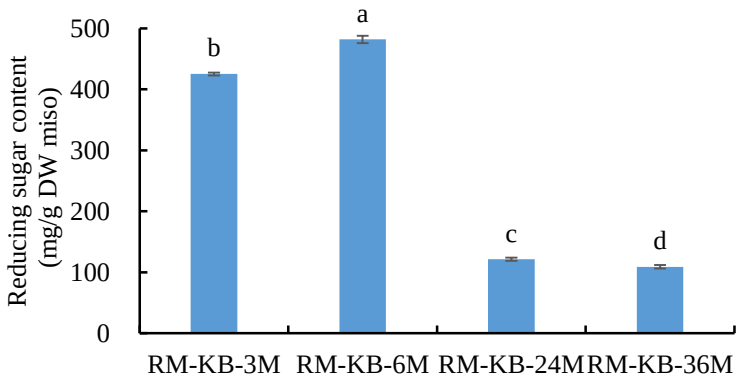


Figure 2. Peptide content in the hydrophilic fraction of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters are significant at $p < 0.05$.

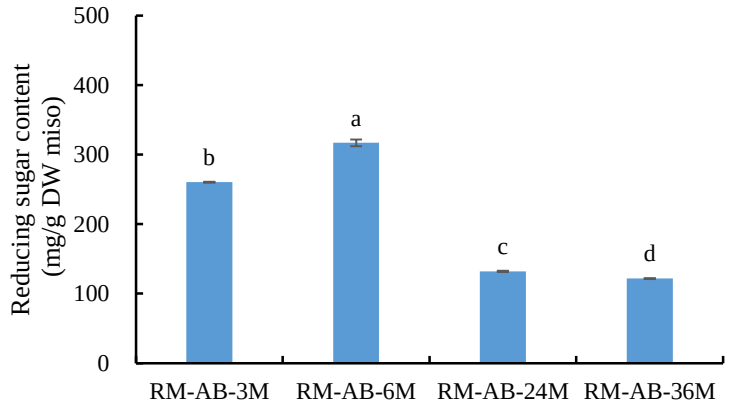
A


Reducing sugar content (mg/g DW miso)
500
400
300
200
100
0
b
a
c
d
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


Reducing sugar content (mg/g DW miso)
500
400
300
200
100
0
b
a
c
d
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


Reducing sugar content (mg/g DW miso)
500
400
300
200
100
0
b
a
c
d
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

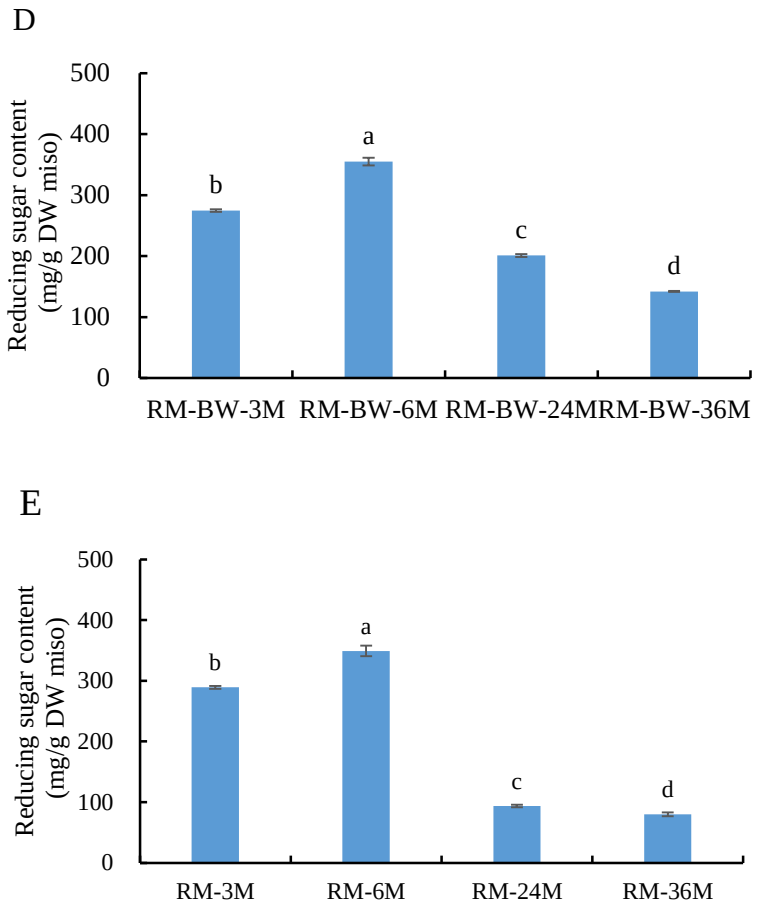
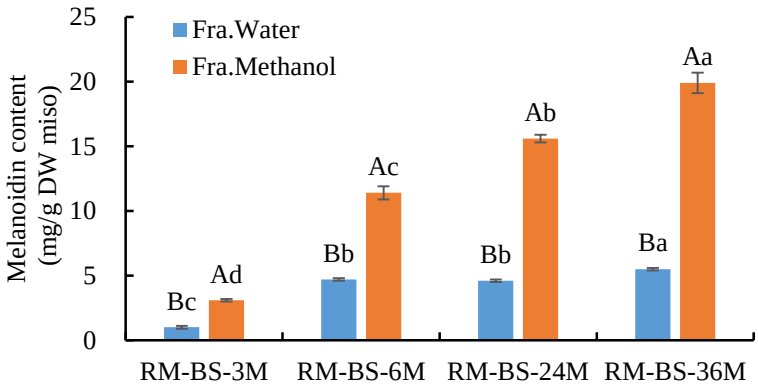
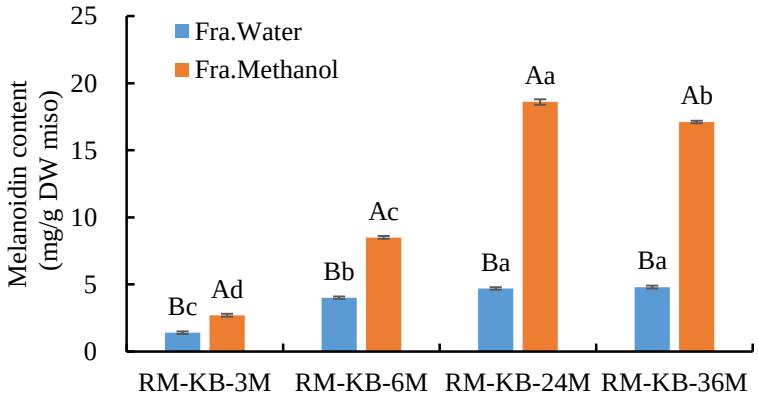


Figure 3. Reducing sugar content in the hydrophilic fraction of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters are significant at $p < 0.05$.

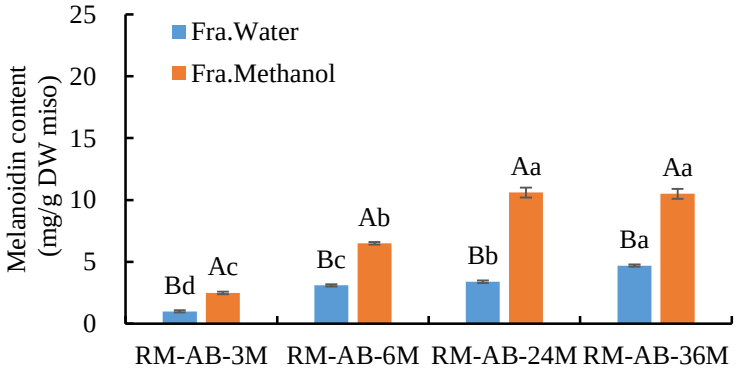
A


Fra.Water
Fra.Methanol
Melanoidin content (mg/g DW miso)
25
20
15
10
5
0
Bc
Ad
Bb
Ac
Bb
Ab
Ba
Aa
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


Fra.Water
Fra.Methanol
Melanoidin content (mg/g DW miso)
25
20
15
10
5
0
Bc
Ad
Bb
Ac
Ba
Aa
Ba
Ab
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


Fra.Water
Fra.Methanol
Melanoidin content (mg/g DW miso)
25
20
15
10
5
0
Bd
Ac
Bc
Ab
Bb
Aa
Ba
Aa
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

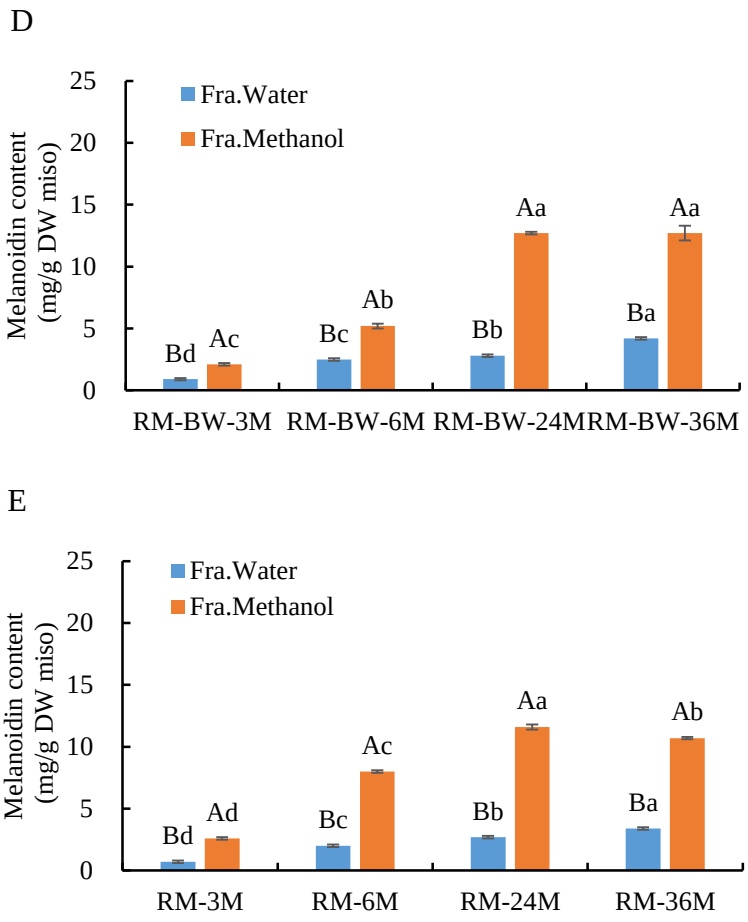


Figure 4. Melanoidin content in the Fra. Water and Fra. Methanol of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters and values within a column followed by different capital letters are significant at $p < 0.05$.

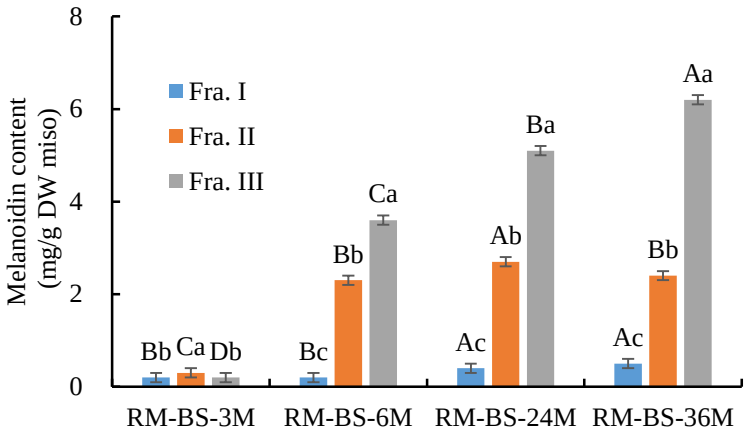


Figure 5. Melanoidin content in the fractions after LH-20 Column of RM-BS with different fermentation period. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean. Data represent the mean ±SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

2.2. Polyphenol Content in Rice Miso Products at Different Fermentation Periods

Humans pay more attention to various of functional properties of polyphenols contained in beans in the recent years [56], especially large number studies reported that beans with deep colored coat have significantly higher polyphenol than those beans with slight colored coat [57, 58]. Therefore, as an improved product of fermented rice miso, it is necessary to clarify the polyphenol content, and the relationship with other antioxidant substances contained in rice miso products supplementary with black soybean, kidney bean, adzuki bean, and buckwheat. The results of polyphenol content in the hydrophilic fraction of rice miso products were shown in Figure 6. The increasing trend of polyphenol content is similar to melanoidin content in rice miso products, polyphenol content was increasing with prolonging the fermentation period. The highest value of RM-BS was shown in fermented after 36 months (12.9 mg/g DW miso), and RM-KB was 11.5 mg/g DW miso, RM-AB was 10.5 mg/g DW miso, RM-BW was 11.8 mg/g DW miso, and the highest value of RM was shown in fermented after 24 months (9.2 mg/g DW miso). The ratios of polyphenols contained in RM-BS to compared with RM were increased from 1.2 to 1.6 folds at every fermentation period, and RM-KB was 1.1 to 1.7 folds, RM-AB was 1.0 to 1.3 folds, RM-BW was 0.8 to 1.4 folds, respectively.

In the present study, we used Folin-Ciocelteau phenol reagent to determine the polyphenol content, actually, it is a reagent not only for the determination of total polyphenol but also including protein and other antioxidants. Therefore, to clarify the pure polyphenol content in rice miso products, the hydrophilic fraction was purified by chromatography through Diaion HP-20 column to eluted the fraction water (Fra. Water) and fraction methanol (Fra. Methanol). The results of polyphenol content in Fra. Water

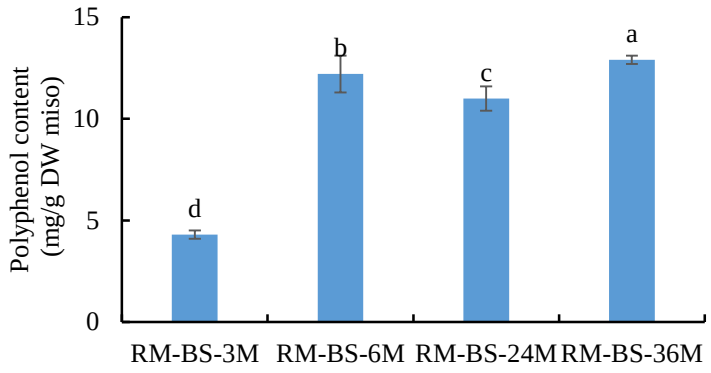
was shown in Figure 7. The increasing trends of polyphenol content in Fra. Water was similar as peptide and polyphenol content in the hydrophilic fraction, the ratios of polyphenols contained in RM-BS to compared with RM were increased from 1.3 to 2.1 folds at every fermentation period, and RM-KB was 1.3 to 2.0 folds, RM-AB was 0.9 to 1.6 folds, RM-BW was 0.8 to 1.6 folds, respectively, which proved the examined polyphenol content was not only the pure polyphenol, but also including the antioxidant effect of peptide in the hydrophilic fraction, and mainly contained in Fra. Water purified by chromatography through Diaion HP-20 column. The polyphenol content contained in Fra. Methanol was significantly less than that in Fra. Water, and the ratios of RM-BS between polyphenol content contained in Fra. Methanol and Fra. Water (Figure 7) was increased from 0.2 to 0.4 folds, RM-KB was 0.1 to 0.5 folds, RM-AB was 0.2 to 0.4 folds, RM-BW was 0.2 to 0.5 folds and RM was 0.3 to 0.6 folds. Therefore, although the polyphenol content contained in Fra. Methanol is less, the proportion of Fra. Methanol was increased with the consumption of antioxidant effect substance (peptides) contained in Fra. Water with the extension of the fermentation period.

In addition, we counted the ratios between polyphenol content and melanoidin content in Fra. Methanol to clarify the relationship between polyphenol content and other antioxidant substances contained in the rice miso products. The ratio between melanoidin content and polyphenol content contained in Fra. Methanol of RM-BS was increased from 3.4 to 5.9 folds, RM-KB was 4.5 to 6.1 folds, RM-AB was 4.2 to 4.6 folds, RM-BW was 4.0 to 4.5 folds, and RM was 3.3 to 5.6 folds, respectively. There are similar results of total phenolic contents in soybean fermented products were increased with the extension of fermentation [59, 60]. Therefore, the polyphenol and melanoidin content were both increased in the rice miso products with the prolonging fermentation period, however, the

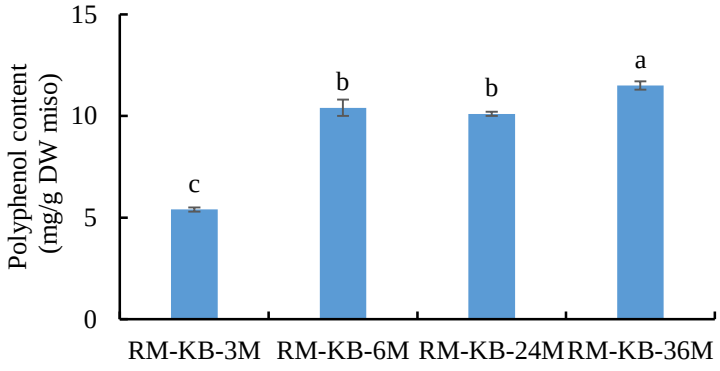
ratio between polyphenol content and melanoidin content contained in each rice miso product was decreased from approximately 1/3 to 1/6, and we estimated that the mainly antioxidant substance in rice miso product was melanoidin.

The result of the polyphenol content of RM-BS in the fractions after the LH-20 column was shown in Figure 8. The polyphenol content contained in fractions was similar to melanoidin content, and there was a significantly higher polyphenol content contained in Fra. I of RM-BS-3M, and trace contained in Fra. II. On the contrary, the polyphenol content was not detected in Fraction I of RM-BS-6M, RM-BS-24M, and RM-BS-36M, but mainly contained in Fraction II and III. Moreover, the ratio of polyphenol content in Fraction. II was decreased, and that contained in Fraction. III was significantly increased with the extension of the rice miso fermentation.

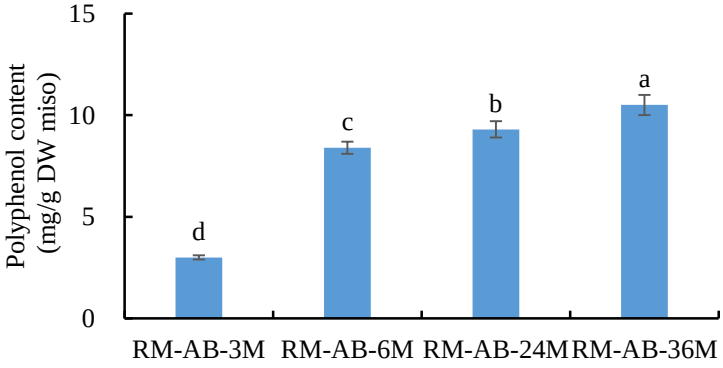
A


15
10
5
0
Polyphenol content (mg/g DW miso)
d
b
c
a
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


15
10
5
0
Polyphenol content (mg/g DW miso)
c
b
b
a
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


15
10
5
0
Polyphenol content (mg/g DW miso)
d
c
b
a
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

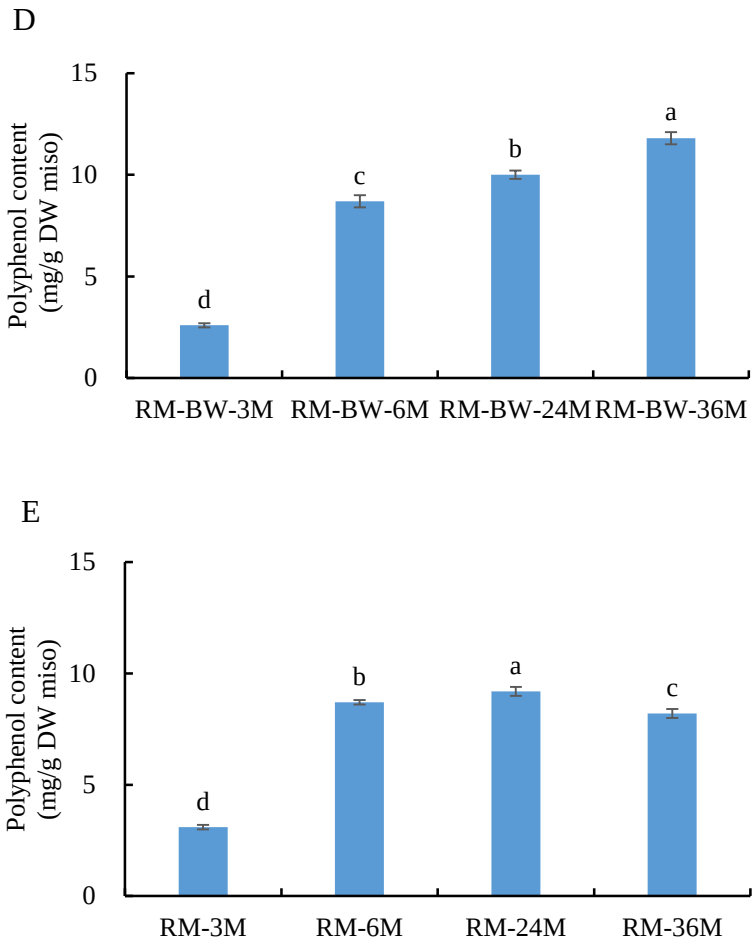
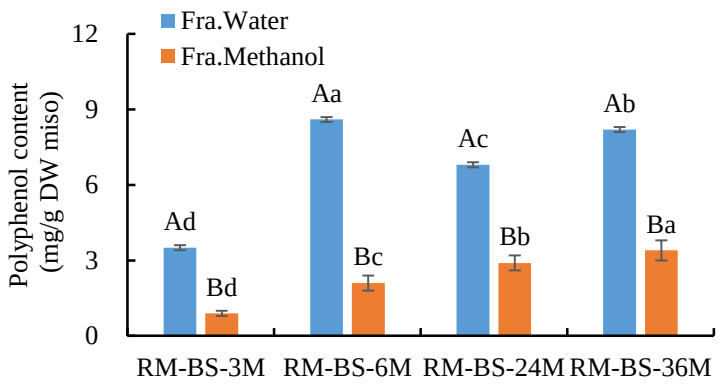
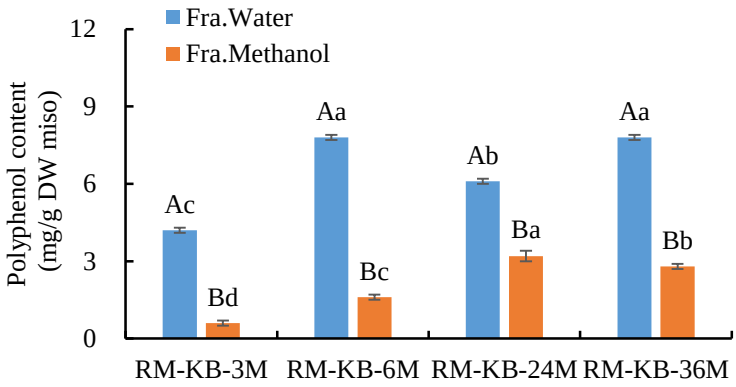


Figure 6. Polyphenol content in the hydrophilic fraction of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters are significant at $p < 0.05$.

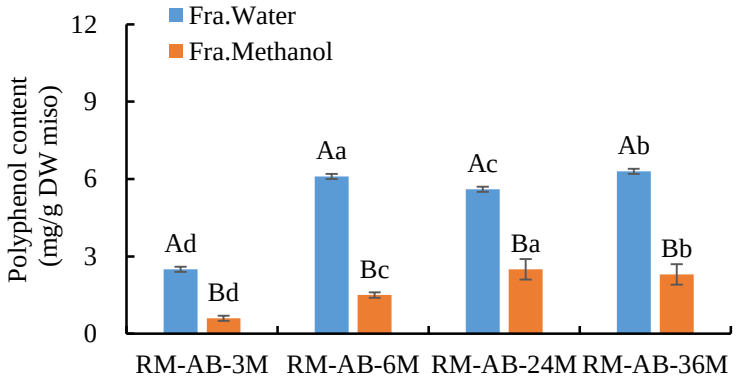
A


Fra.Water
Fra.Methanol
Polyphenol content (mg/g DW miso)
12
9
6
3
0
Ad
Bd
Aa
Bc
Ac
Bb
Ab
Ba
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


Fra.Water
Fra.Methanol
Polyphenol content (mg/g DW miso)
12
9
6
3
0
Ac
Bd
Aa
Bc
Ab
Ba
Aa
Bb
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


Fra.Water
Fra.Methanol
Polyphenol content (mg/g DW miso)
12
9
6
3
0
Ad
Bd
Aa
Bc
Ac
Ba
Ab
Bb
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

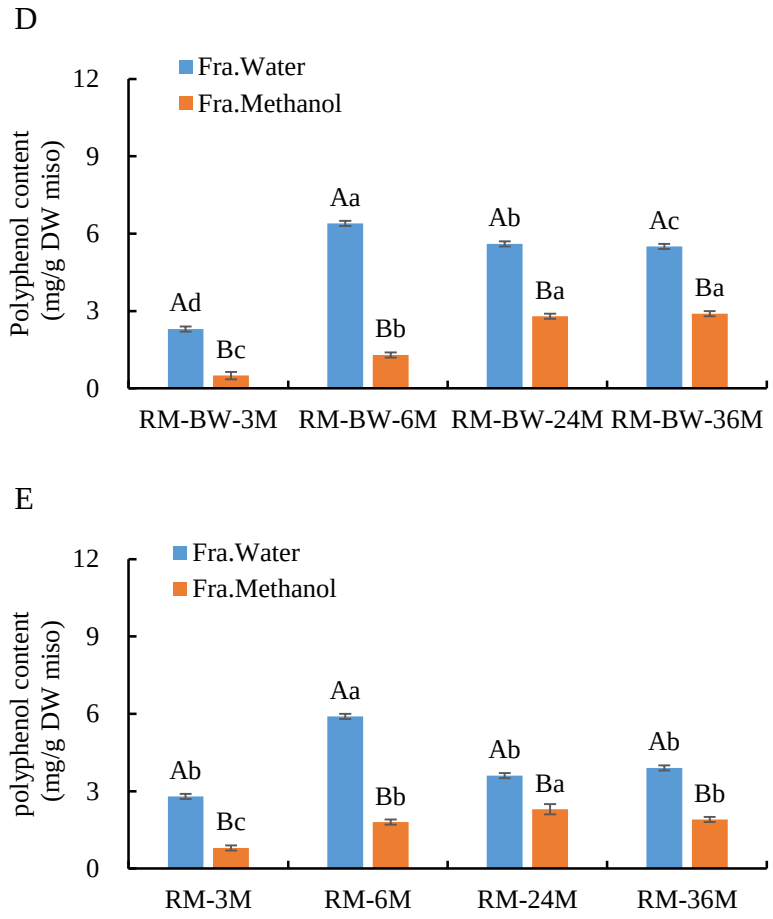


Figure 7. Polyphenol content in the Fra. Water and Fra. Methanol of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters and values within a column followed by different capital letters are significant at $p < 0.05$.

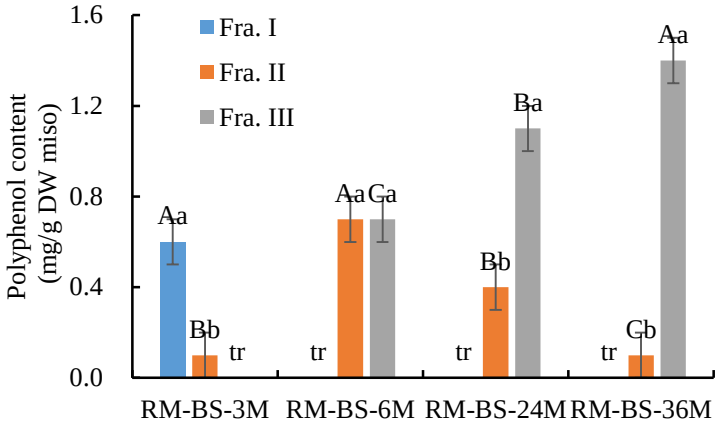


Figure 8. Polyphenol content in the fractions after LH-20 Column of RM-BS with different fermentation period. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean. Data represent the mean ±SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Chapter Two: Antioxidant Activity in Rice Miso Products

Introduction

The antioxidant is one of the food functionalities, not only used as food additives to help guard against food deterioration [61], but also considered important nutraceuticals on account of many health benefits, such as anti-hangover, hepatoprotective action, chronic diseases [62-64]. In recent years, in view of increasing the risk of carcinogenicity of synthetic antioxidant BHA [65], people are more inclined to natural antioxidants [66]. In the present study, we determined the DPPH radical scavenging activity to evaluated the antioxidant activity contained in rice miso products supplementary with black soybean, kidney bean, adzuki bean, and buckwheat at different fermentation periods. The DPPH radical scavenging activity assay was the simplest method developed by Blois in 1958 [67], and determine the antioxidant activity by using a stable free radical with an unpaired valence electron at one atom of nitrogen bridge [68].

ABTS radical scavenging activity is one another determination of antioxidant activity with a similar principle of interaction between free radicals to show direct evidence for antioxidants to scavenge free radical. Moreover, $ABTS^{+}$ radicals are more reactive than DPPH radicals, and has been extensively used to evaluate the antioxidant activity of complex mixtures and individual compounds [69], and several studies reported that the high-pigmented and hydrophilic antioxidants were significantly better reflected by ABTS than DPPH radical scavenging activity [70], some amino- and amido-thiols were also capable detected by ABTS radical scavenging activity [71]. Therefore, in the present study, we also determined ABTS radical scavenging activity to clarify the similarities and

differences with DPPH radical scavenging activity in the representative sample (RM-BS, RM-KB, and RM).

1. Materials and Methods

1.1. Materials

Detailed information is available in Chapter One.

DPPH (2, 2-diphenyl-1- picrylhydrazyl) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Potassium persulfate was purchased from Kanto Chemical Co., INC. (Tokyo, Japan). Trolox was purchased from Sigma-Aldrich Co., LLC. (Tokyo, Japan). The other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

1.2. Sample Preparation

Detailed information is available in Chapter One.

1.3. Extraction and Fractionation

Detailed information is available in Chapter One.

1.4. DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity was determined by the method of Brand-Williams [37]. The extract (50 μL) was added to a microplate and mixed with 100 μL of 99.5% v/v ethanol and 150 μL DPPH solution. The solution was kept in the dark for 15 min after which its absorbance was determined at 520 nm by a microplate reader. Ten-fold diluted 2 mM Trolox was used as the standard and the results were expressed as μmol Trolox equivalents (TE) per gram DW miso ($y = -13.555x + 30.842$, $R^2 = 0.9955$).

1.5. ABTS Radical Scavenging Activity Assay

Determination of antioxidant activity has used the method of ABTS radical scavenging [72]. First, the ABTS reagent was prepared by adding 88 μL of 140 mM $K_2S_2O_8$ to 5 mL of 7 mM ABTS aqueous solution and placed in the dark for 16 hours at room temperature. The samples were diluted with ethanol to 1180 μL and mixed with 20 μL of ABTS reagent. Three minutes later, the absorbance was measured at 734nm, and results were expressed as μmol Trolox equivalents (TE) per gram dry weight miso. ($y = -46.013x + 39.518$, $R^2 = 0.9981$).

1.6. Statistical Analysis

The experiments were repeated at least three times. Data were expressed as means ± standard deviation. Significant differences were determined by one-way ANOVA and Fisher's test (SAS v. 7.1, SAS Institute Inc., Cary, NC, USA). Differences were considered to be significant at $P < 0.05$.

2. Results and Discussions

2.1. DPPH Radical Scavenging Activity in Rice Miso Products with Different Fermentation Periods

The results of DPPH radical scavenging activity in the hydrophilic fraction of rice miso products were shown in Figure 9. As the increasing trend of functional components, DPPH radical scavenging activity was increasing with prolonging the fermentation period of all rice miso products. The highest value of RM-BS was shown in fermented after 36 months (9.1 μmol/g DW miso), RM-AB was 8.5 μmol/g DW miso, RM-BW was 8.5 μmol/g DW miso, and the highest value of RM-KB and RM was shown in fermented after 24 months (8.6 μmol/g DW miso; 8.4 μmol/g DW miso). Basically, the DPPH radical scavenging activity of RM-BS, RM-KB, RM-AB, and RM-BW was significantly higher than RM (control). The ratios of DPPH radical scavenging activity contained in RM-BS to compared with RM were increased from 1.0 to 1.3 folds, and RM-KB was 1.0 to 1.3 folds, RM-AB was 0.9 to 1.2 folds, RM-BW was 0.8 to 1.2 folds, respectively.

Moreover, based on the results shown in Figure 1, 6 and 9, Figure 10 was depicted to the correlation between melanoidin, polyphenol content and DPPH radical scavenging activity. We found there was a high positive relationship between melanoidin content and DPPH radical scavenging activity (correlation coefficient; R = 0.9345) in Figure 10A. The antioxidant activity is increasing with the prolonging the wheat miso fermentation period and greatly involved with produced coloring component [73]. Moreover, increased melanoidin content was responsible for the rise in anti-oxidative activity of Zhenjiang Aromatic Vinegar after the decoction [74]. Melanoidin produced from an amino acid-

sugar model system have been associated with the formation of compounds with strong antioxidant activity [75]. Moreover, the composition of melanoidin also affects the strength of antioxidant activity. The high molecular weight reaction products resulting from the interaction of lysine with hexanal showed a higher radical scavenging activity than the glycine/glucose model [76]. There was also a positive relationship between polyphenol content and DPPH radical scavenging activity (correlation coefficient; R = 0.8533) in Figure 10B. The polyphenol content of perilla miso product is increasing with DPPH radical scavenging activity [77]. Isoflavones in fermented soybean have a strong antioxidant activity [78]. Therefore, the antioxidant activity contained in rice miso products provided by melanoidin and polyphenol that produced through the fermentation of rice miso products.

To clarify the distributions of DPPH radical scavenging activity in rice miso products, the hydrophilic fraction was purified by chromatography through Diaion HP-20 column to eluted the fraction water (Fra. Water) and fraction methanol (Fra. Methanol). The results of DPPH radical scavenging activity in Fra. Water and Fra. Methanol was shown in Figure 11. The increasing trends of DPPH radical scavenging activity in Fra. Water and Fra. Methanol was similar as in the hydrophilic fraction. Moreover, we also counted the ratios of DPPH radical scavenging activity between Fra. Water and Fra. Methanol and the ratio of RM-BS at every fermentation period was decreased from 0.7 to 0.3 folds, RM-KB was 0.7 to 0.3 folds, RM-AB was 0.6 to 0.3 folds, RM-BW was 1.0 to 0.3 folds, and RM was 1.0 to 0.2 folds, respectively. Therefore, the DPPH radical scavenging activity was increasing with prolonging the fermentation period of all rice miso products, and there is a high ratio of the DPPH radical scavenging activity contained in Fra.

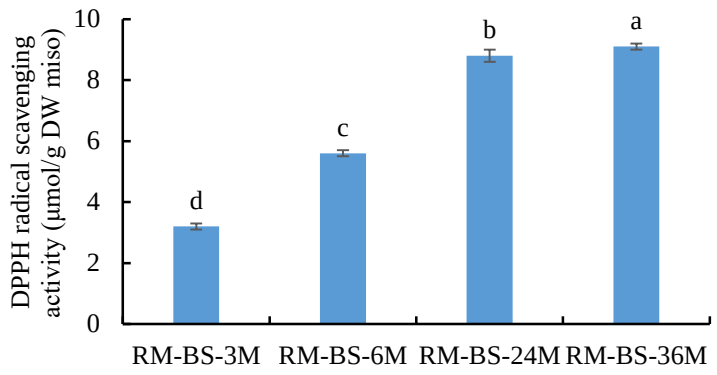
Methanol as melanoidin content, and we estimated that the mainly antioxidant substance contained in rice miso products was melanoidin produced through fermentation period.

In addition, based on the results shown in Figure 4, 7 and 11, Figure 12 was depicted to further clarify the relationship between melanoidin, polyphenol content and DPPH radical scavenging activity. We also found there was a high positive relationship between melanoidin content and DPPH radical scavenging activity (correlation coefficient; R = 0.9250) in Figure 12A, and a positive relationship between polyphenol content and DPPH radical scavenging activity (correlation coefficient; R = 0.9554) in Figure 12B. Therefore, although the polyphenol content contained in rice miso products was less than the melanoidin content, and provided less antioxidant activity, the much higher correlation coefficient between polyphenol content and DPPH radical scavenging activity showed that the unit content of polyphenol provides much more antioxidant activity than melanoidin.

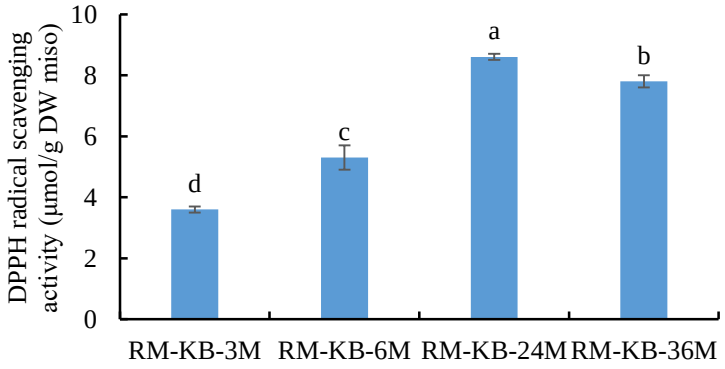
The result of DPPH radical scavenging activity of RM-BS in the fractions after the LH-20 column was shown in Figure 13. The DPPH radical scavenging activity contained in fractions was similar to melanoidin content, and there was a significantly higher DPPH radical scavenging activity contained in Fra. I of RM-BS-3M, and trace contained in Fra. II. On the contrary, the DPPH radical scavenging activity was not detected in Fra. I of RM-BS-24M and 36M, but mainly contained in Fra. II and III. Moreover, the ratio of DPPH radical scavenging activity in Fra. II and that contained in Fra. III was significantly increased with the extension of the RM-BS fermentation. Therefore, we can conclude that the antioxidant activity contained in rice miso products was provided by the high molecular substance and that mainly substance was melanoidin. There are reports that melanoidin above 3kDa shows particularly a complex increase in the antioxidant activity

of blood plasma [79], and melanoidin from barley malt showed 3-fold higher capacity to scavenge radicals than the lower molecular weight colorants by the metmyoglobin assay [80].

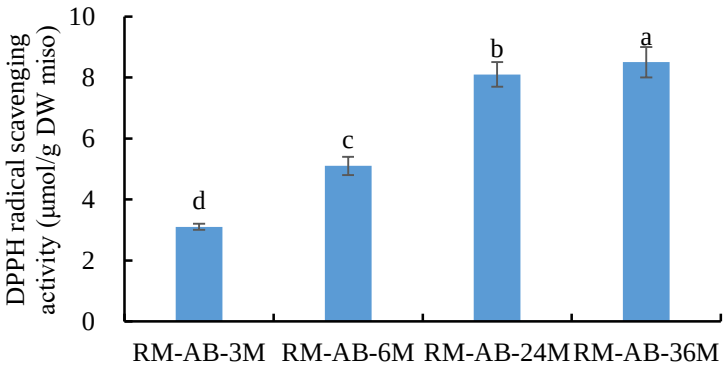
A


DPPH radical scavenging activity (μmol/g DW miso)
10
8
6
4
2
0
d
c
b
a
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


DPPH radical scavenging activity (μmol/g DW miso)
10
8
6
4
2
0
d
c
a
b
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


DPPH radical scavenging activity (μmol/g DW miso)
10
8
6
4
2
0
d
c
b
a
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

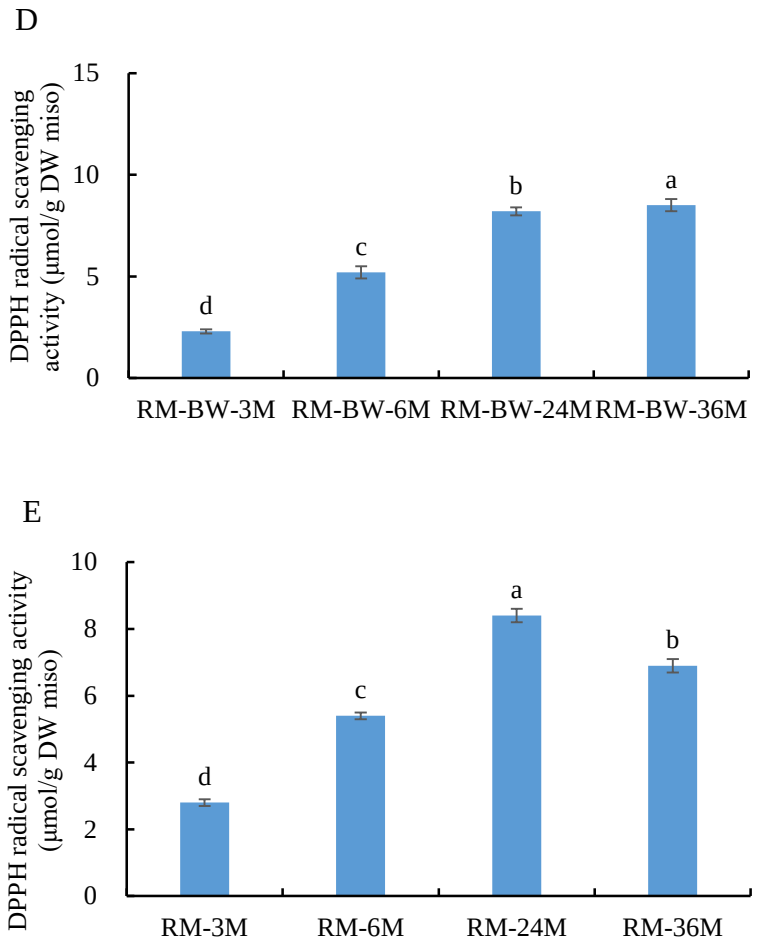


Figure 9. DPPH radical scavenging activity in the hydrophilic fraction of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters are significant at $p < 0.05$.

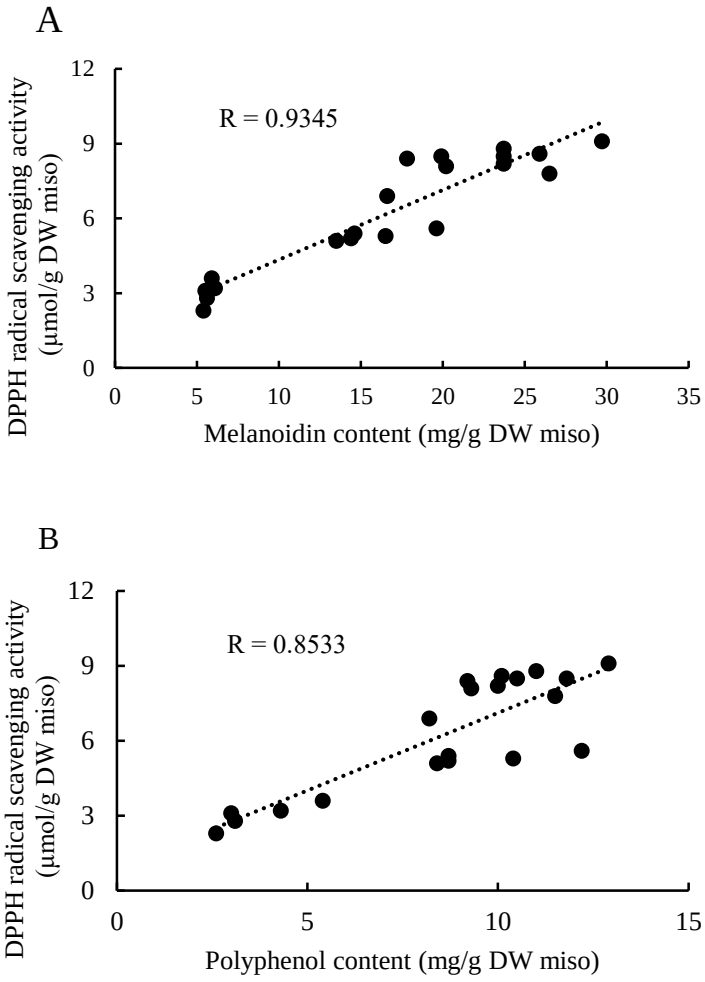
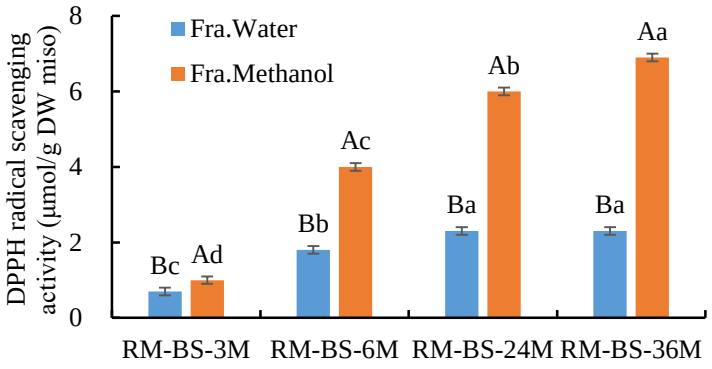
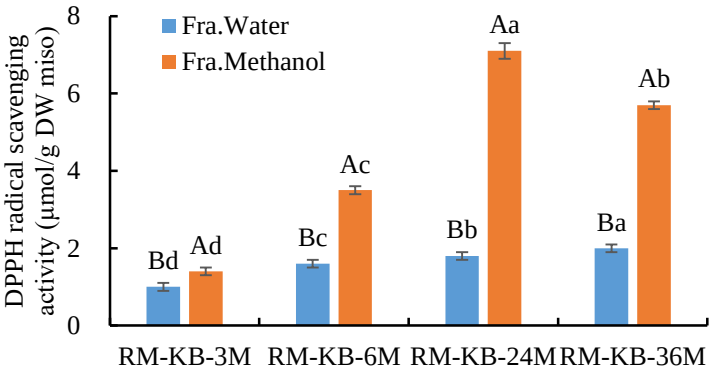


Figure 10. Relationship between melanoidin, polyphenol content and DPPH radical scavenging activity contained in hydrophilic fraction of rice miso products with different fermentation period. A, melanoidin content and DPPH radical scavenging activity. B, polyphenol content and DPPH radical scavenging activity.

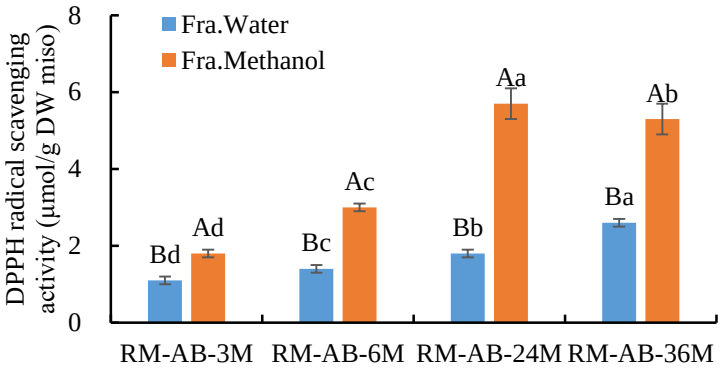
A


DPPH radical scavenging activity (μmol/g DW miso)
Fra.Water
Fra.Methanol
0
2
4
6
8
Bc
Ad
Bb
Ac
Ba
Ab
Ba
Aa
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M

B


DPPH radical scavenging activity (μmol/g DW miso)
Fra.Water
Fra.Methanol
0
2
4
6
8
Bd
Ad
Bc
Ac
Bb
Aa
Ba
Ab
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M

C


DPPH radical scavenging activity (μmol/g DW miso)
Fra.Water
Fra.Methanol
0
2
4
6
8
Bd
Ad
Bc
Ac
Bb
Aa
Ba
Ab
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

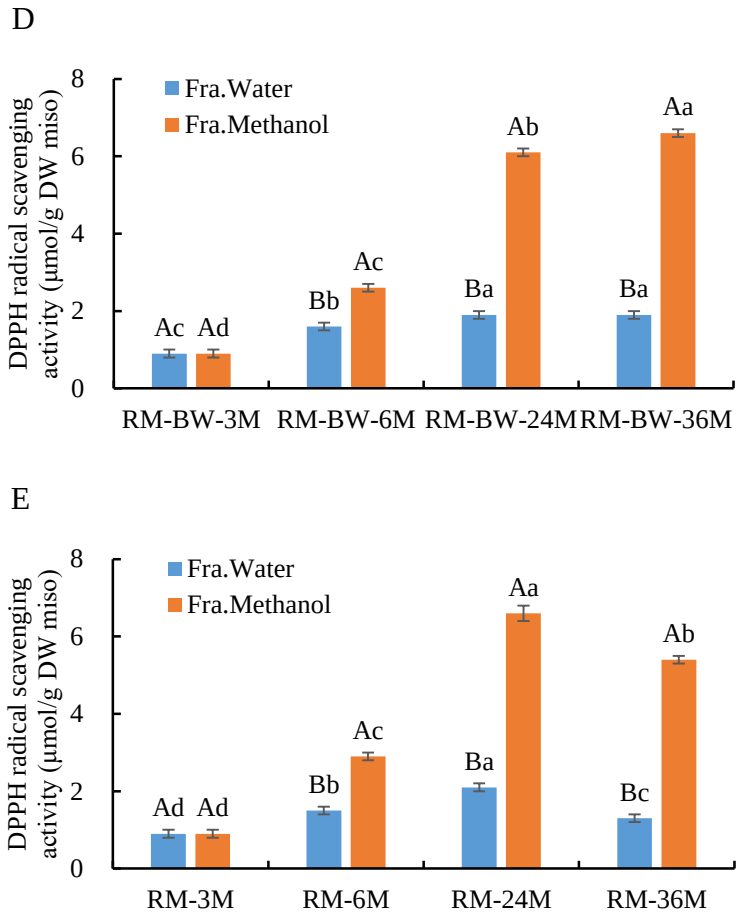


Figure 11. DPPH radical scavenging activity in the Fra. Water and Fra. Methanol of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters and values within a column followed by different capital letters are significant at $p < 0.05$.

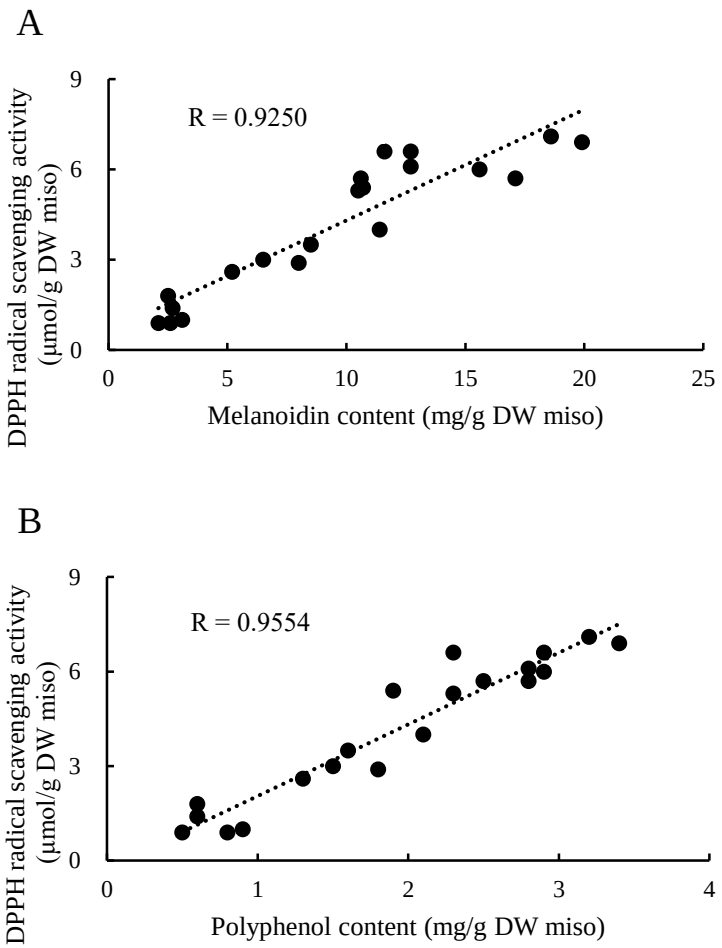


Figure 12. Relationship between melanoidin, polyphenol content and DPPH radical scavenging activity contained in Fra. Methanol of rice miso products with different fermentation period. A, melanoidin content and DPPH radical scavenging activity. B, polyphenol content and DPPH radical scavenging activity.

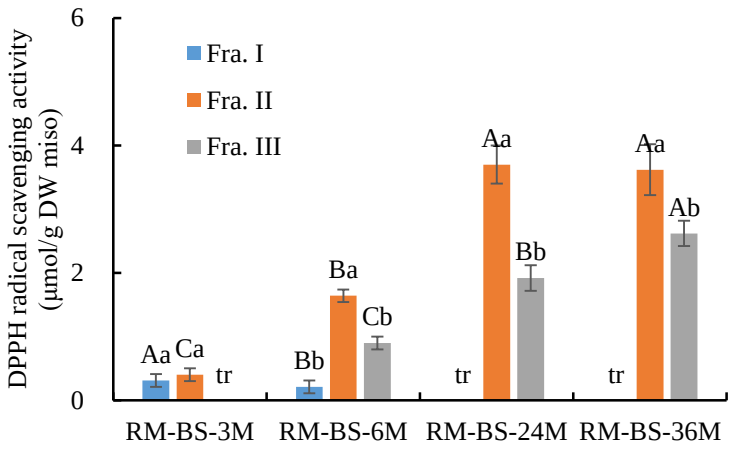


Figure 13. DPPH radical scavenging activity in the fractions after LH-20 Column of RM-BS with different fermentation period. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

2.2. ABTS Radical Scavenging Activity in Rice Miso Products with Different Fermentation Periods

The results of ABTS radical scavenging activity in the hydrophilic fraction of RM-BS, RM-KB, and RM were shown in Figure 14. However, ABTS radical scavenging activity was increasing with prolonging the fermentation period of all rice miso products, the highest value of RM-BS, RM-KB, and RM was showed at fermented after 24 months. The highest value of RM-BS was 17.2 μmol/g DW miso, RM-KB was 14.7 μmol/g DW miso, and RM was 11.2 μmol/g DW miso. On the other hand, the antioxidant activity of RM-BS, RM-KB and RM with different fermentation period determined by ABTS radical scavenging activity (4.6 to 17.2 μmol/g DW miso; 4.5 to 14.7 μmol/g DW miso; 4.5 to 11.2 μmol/g DW miso) was significantly higher than DPPH radical scavenging activity (shown in Table 13, 3.2 to 9.1 μmol/g DW miso; 3.6 to 8.6 μmol/g DW miso; 2.8 to 8.4 μmol/g DW miso), respectively. Therefore, compared with DPPH radical scavenging activity, ABTS radical scavenging activity can detect more antioxidant active substances in rice miso products. ABTS radical scavenging activity of rice miso supplementary with black soybean and kidney bean were also significantly higher than rice miso (control). The ratios of DPPH radical scavenging activity contained in RM-BS to compared with RM were increased from 1.0 to 1.8 folds, and RM-KB was 1.0 to 1.5 folds, respectively.

Moreover, based on the results shown in Figure 1, 6 and 14, Figure 15 was depicted to the correlation between melanoidin, polyphenol content and ABTS radical scavenging activity. There was also a high positive relationship between melanoidin content and ABTS radical scavenging activity (correlation coefficient; R = 0.9363) in Figure 15A. Melanoidin could have the antioxidant capacity, and the most antioxidant melanoidin was

from coffee in Spanish diets [81]. The Maillard reaction products from the glucose-lysine system were not only improved antioxidant activity but also led to a significantly higher intake of antioxidant activity [82]. Besides, not only the high molecular weight fraction of melanoidin apparent strong antioxidant activity but aromatic amino acids in low molecular weight during Maillard reaction might also have a contribution to the greater $ABTS^+$ scavenging activity [83]. Moreover, MRPs prepared from chito-oligomer solution have a time-dependent increase in $ABTS^+$ scavenging activity [84]. There was also a positive relationship between polyphenol content and ABTS radical scavenging activity (correlation coefficient; R = 0.8587) in Figure 15B. The fermentation process significantly increased total phenolic content and antioxidant activities of oats [85]. Another similar study reported that there is also a good correlation between antioxidant activities and total phenolic content contained in fermented tubers of *Bletilla formosana* [86]. Therefore, there are high correlations between melanoidin, polyphenol content and DPPH radical scavenging activity, as well as good correlations with ABTS radical scavenging activity contained in rice miso products at different fermentation period, either.

The results of ABTS radical scavenging activity in Fra. Water and Fra. Methanol was shown in Figure 16. The increasing trends of ABTS radical scavenging activity in Fra. Water and Fra. Methanol was similar as in the hydrophilic fraction. Moreover, we also counted the ratios of ABTS radical scavenging activity between Fra. Water and Fra. Methanol and the ratio of RM-BS at every fermentation period was decreased from 1.2 to 0.6 folds, RM-KB was 1.3 to 0.6 folds, and RM was 1.5 to 0.5 folds, respectively. Therefore, the proportion of ABTS radical scavenging activity contained in Fra. Water was gradually decreasing, and the proportion of that in Fra. Methanol was increased with

prolonging the fermentation period, and we clarified that the antioxidant activity was mainly contained Fra. Methanol since the fermentation continues.

In addition, based on the results shown in Figure 4, 7 and 16, Figure 17 was depicted to further clarify the relationship between melanoidin, polyphenol content and ABTS radical scavenging activity. We also found there was a high positive relationship between melanoidin content and ABTS radical scavenging activity (correlation coefficient; R = 0.9212) in Figure 17A, and a positive relationship between polyphenol content and ABTS radical scavenging activity (correlation coefficient; R = 0.9338) in Figure 17B. Therefore, although the polyphenol content contained in rice miso products was less than the melanoidin content, there has the same high correlation between polyphenol content and ABTS radical scavenging activity. Moreover, to compare with hydrophilic fraction, the correlation coefficients contained in the Fra. Methanol has been significantly improved, indicating that the antioxidant substances contained in rice miso products with different fermentation period have been refined and purified.

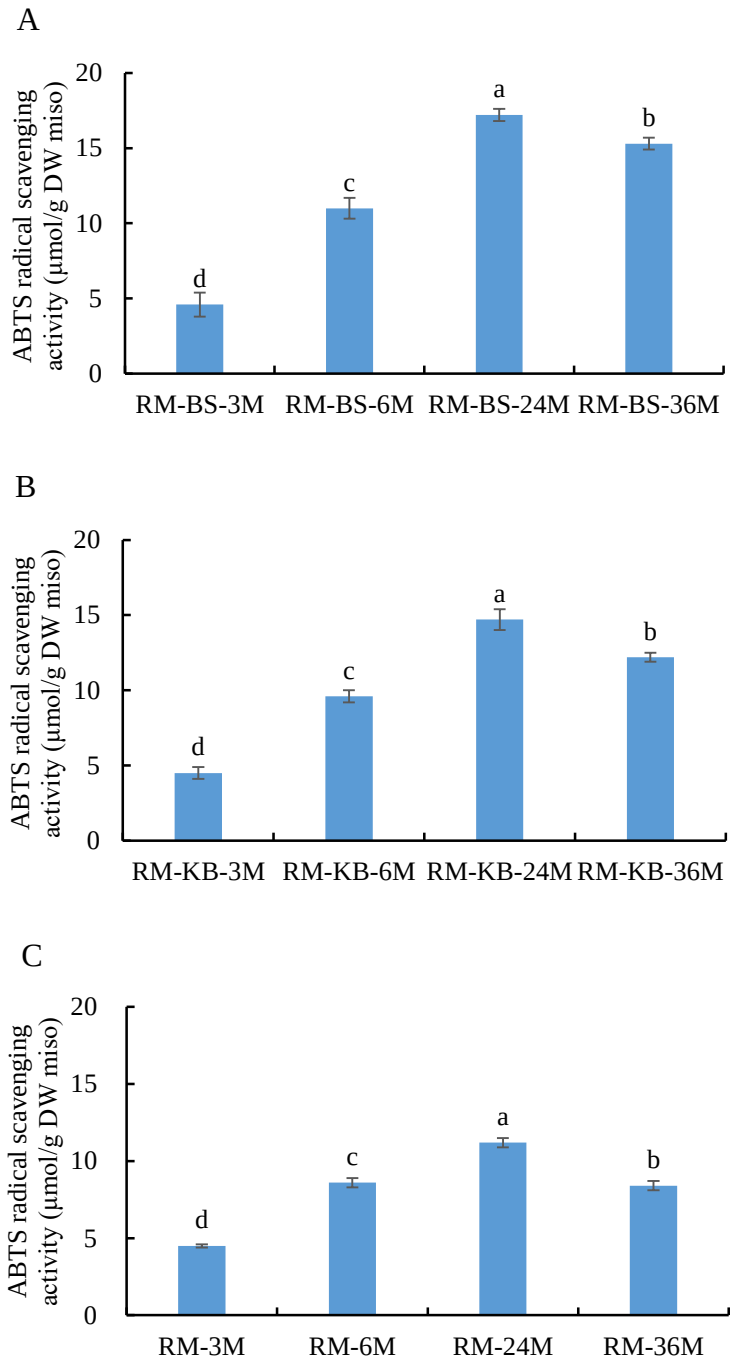


Figure 14. ABTS radical scavenging activity in the hydrophilic fraction of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters are significant at $p < 0.05$.

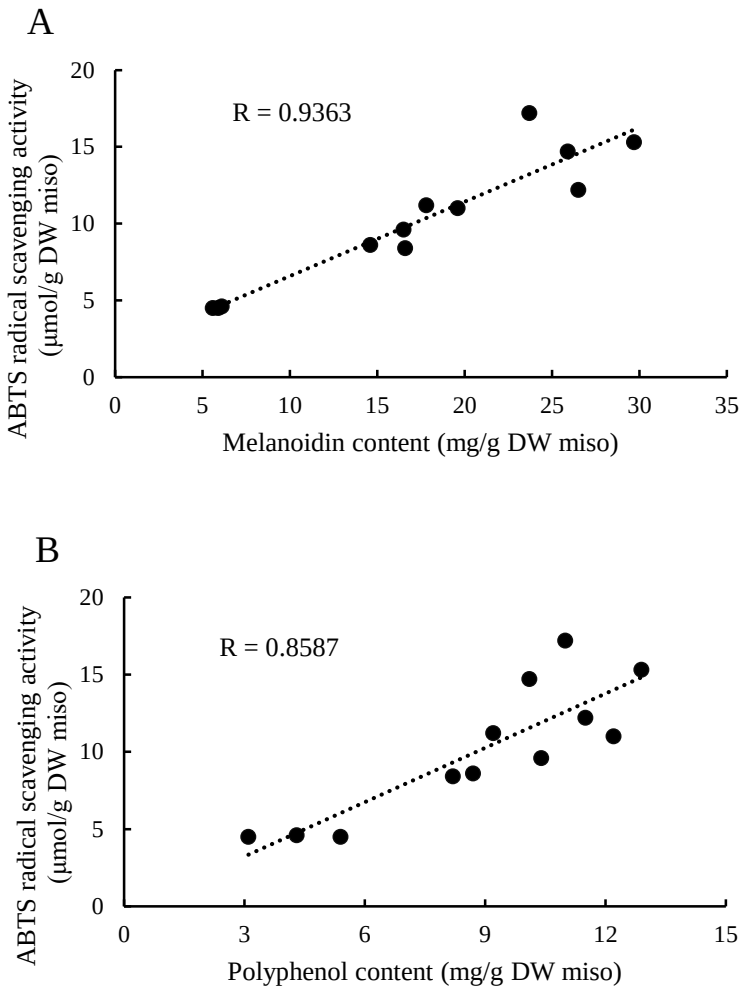


Figure 15. Relationship between melanoidin, polyphenol content and ABTS radical scavenging activity contained in hyd rophilic fraction of rice miso products with different fermentation period. A, melanoidin content and ABTS radical scavenging activity. B, polyphenol content and ABTS radical scavenging activity.

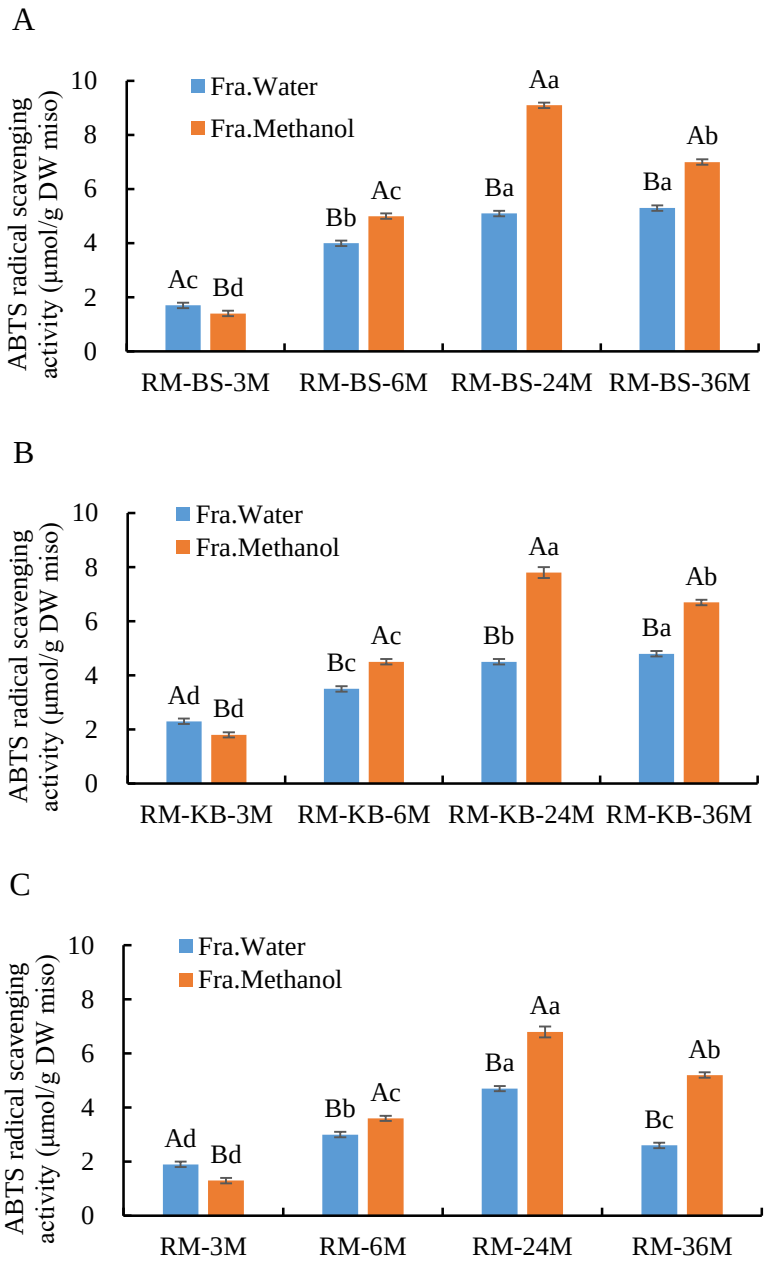


Figure 16. ABTS radical scavenging activity in the Fra. Water and Fra. Methanol of rice miso products with different fermentation period. A, RM-BS. B, RM-KB. C, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row

followed by different small letters and values within a column followed by different capital letters are significant at $p < 0.05$.

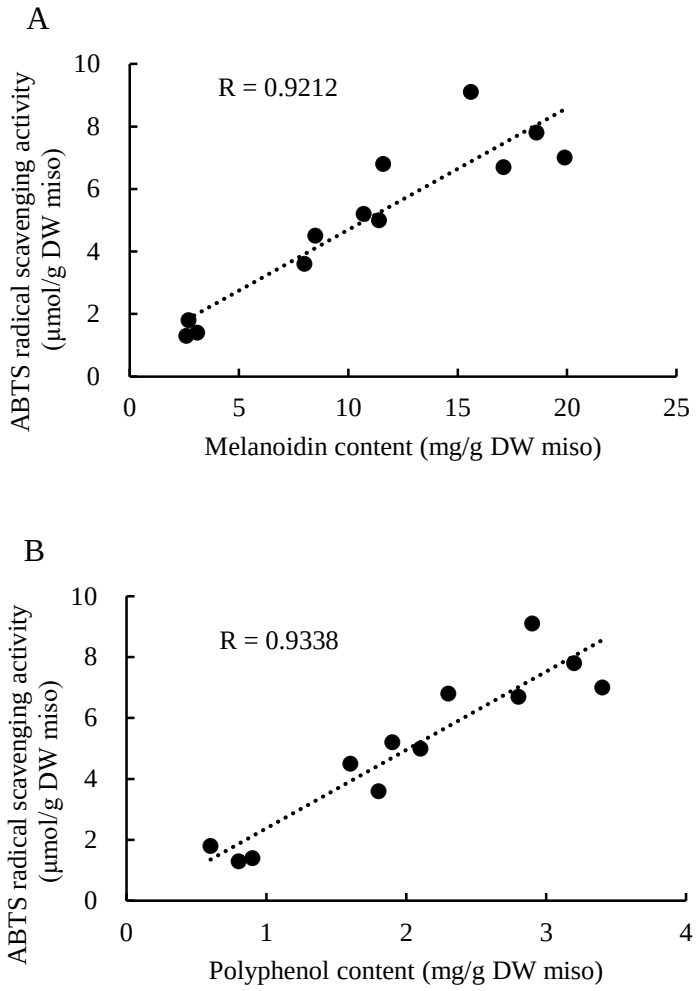


Figure 17. Relationship between melanoidin, polyphenol content and ABTS radical scavenging activity contained in Fra. Methanol of rice miso products with different fermentation period. A, melanoidin content and ABTS radical scavenging activity. B, polyphenol content and ABTS radical scavenging activity.

Chapter Three: Enzyme Inhibitory Activity in Rice Miso Products

Introduction

In recent years, food energy consumption has been recognized as a primary factor which may be related to obesity, and It has also changed significantly over time [87]. More than half of the older men in the US (51.6%) and 43.1% of the women are overweight. In Japan, only 16.0% of older men and 20.2% of the women are overweight [88]. Lipase is a key enzyme in lipid metabolism that catalyzes the hydrolysis of fats [89]. The inhibition of lipase can be beneficial used to inhibit absorption of lipids, and treat obesity [90, 91]. In the present study, lipase inhibitory activity was determined by an improved method of Han to measuring the rate of release of oleic acid from triolein [92].

Diabetes mellitus is a well-known metabolic disorder, which is characterized by an abnormal postprandial increase of blood glucose level. The control of postprandial hyperglycemia is believed to be important in the treatment of diabetes mellitus [93]. The α-glucosidase is the enzyme involved in the digestion and absorption of carbohydrates, as break down starch and disaccharides into glucose and increase glucose concentration in the body [94]. The inhibition of α-glucosidase activity can be effective in retarding carbohydrate digestion and glucose absorption to suppress postprandial hyperglycemia [95, 96].

1. Materials and Methods

1.1. Materials

Detailed information is available in Chapter One.

DNS (3, 5-dinitrosalicylic acid) and lipase were purchased from Sigma-Aldrich Co., LLC. (Tokyo, Japan). Glucose was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Lecithin was purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). The other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

1.2. Sample Preparation

Detailed information is available in Chapter One.

1.3. Extraction and Fractionation

Detailed information is available in Chapter One.

1.4. Lipase Inhibitory Activity Assay

Lipase inhibitory activity was determined by the improved method of Han [97]. The substrate solution was prepared by adding 10 mg lecithin, 80 mg triolein, and 5 mg cholic acid to 9 mL TES buffer (pH 7.0), and sonication. Add 240 μL of sample extracts with different concentration (0.05, 0.1, 0.2 g), 80 μL lipase solution and 80 μL substrate solution into a glass tube, incubate at 37℃ for 30 min. Then, add 2 mL copper reagent and 4 mL chloroform, stir and centrifuge at 1,006 ×g for 5 min. Transfer 2.4 mL of chloroform layer to a new glass tube and add 400 μL of 0.1% DDTC-butanol solution

and measure the absorbance at 440 nm. Standard curves were using linoleic acid and expressed as lipase inhibition (%).

$$\text{Lipase Inhibitory (\%)} = \left[1 - \left(\frac{A_{sample}}{A_{control}}\right)\right] \times 100\%$$

where A_{sample} is the absorbance of the mixture of sample, substrate solution, enzyme and DDTC-butanol solvent; $A_{control}$ is the absorbance of the mixture of buffer (instead of sample), substrate solution, enzyme and DDTC-butanol solvent.

1.5. α-Glucosidase Inhibitory Activity Assay

α-Glucosidase inhibitory activity was determined by the improved DNS method [26]. A mixture of 0.5 mL of sample extract with different concentration (0.05, 0.1, 0.2 g) and 0.5 mL of the α-glucosidase solution was pre-incubated at 37°C for 10 min to prepare solution I. A mixture of 50 μL of 0.4% sucrose solution, 625 μL of 0.1M Na_2PO_4 buffer (pH 6.8) and 125 μL of 1% NaCl was pre-incubated at 37°C for 10 min to prepare solution II. Then, 200 μL of solution I was mixed with solution II and incubated at 37°C for 30 min. The enzyme reaction was stopped by adding 125 μL of 2 N NaOH (added 2 N NaOH before incubation for blank). DNS solvent (1%, 125 μL) was added and reacted in boiling water bath for 10 min. Absorbance was measured at 540 nm. Standard curves were using glucose calibration and expressed as α-glucosidase inhibition (%).

$$\alpha - \text{Glucosidase Inhibitory (\%)} = \left[1 - \left(\frac{A_{sample} - A_{blank}}{A_{control} - A_{test}}\right)\right] \times 100\%$$

where A_{sample} is the absorbance of the mixture of sample, sucrose solution, enzyme and DNS solvent; A_{blank} is the absorbance of the mixture of sample, sucrose solution and DNS solvent without enzyme; $A_{control}$ is the absorbance of the mixture of buffer (instead of

sample), sucrose solution, enzyme and DNS solvent; A_{test} is the absorbance of the mixture of buffer (instead of sample), sucrose solution and DNS solvent without enzyme.

1.6. Statistical Analysis

The experiments were repeated at least three times. Data were expressed as means ± standard deviation. Significant differences were determined by one-way ANOVA and Fisher's test (SAS v. 7.1, SAS Institute Inc., Cary, NC, USA). Differences were considered to be significant at $P < 0.05$.

2. Results and Discussions

2.1. Lipase Inhibitory Activity in Rice Miso Products with Different Fermentation Periods

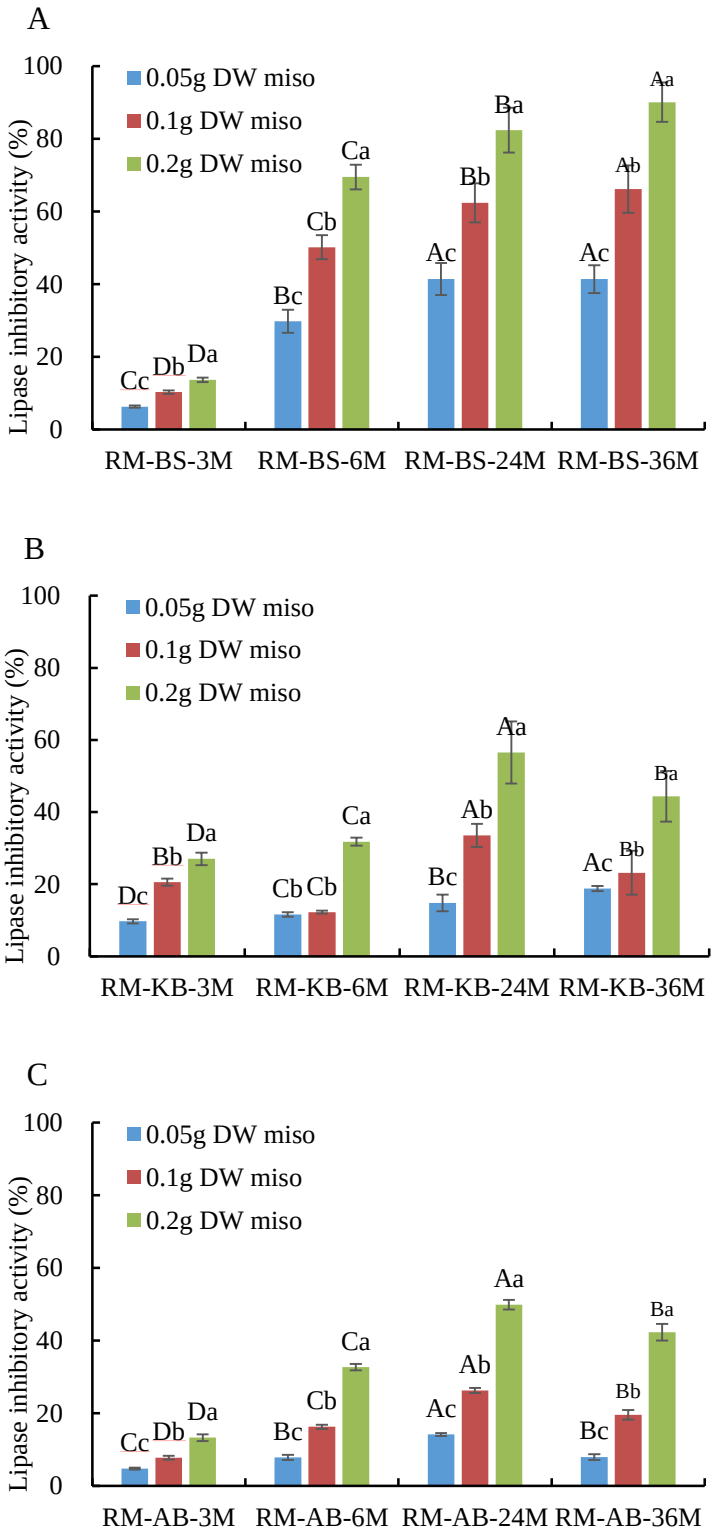
The lipase inhibitory activity in different weights of rice miso products was shown in Figure 18. The lipase inhibitory activity in rice miso products were significantly increased with prolonging fermentation period. However, the highest lipase inhibitory activity of RM-BS was at fermented after 36 months (90.1%/ 0.2g DW miso), RM-KB showed the highest value at fermented after 24 months 56.1%/ 0.2g DW miso, RM-AB was 49.9%/ 0.2g DW miso, RM-BW was 84.1%/ 0.2g DW miso and RM was 28.4%/ 0.2g DW miso, respectively. Moreover, Lipase inhibitory activity of RM-BS, RM-KB, RM-AB, and RM-BW were significantly higher than RM (rice miso; as control) at different fermentation period, respectively. Therefore, we speculated that the traditional rice miso supplementary with black soybean, kidney bean, adzuki bean, and buckwheat were rich and increased the antioxidant and lipase inhibitory substances.

In addition, based on the results shown in Figure 4 and 18, Figure 19 was depicted to further clarify the relationship between melanoidin content and lipase inhibitory activity at the different weight of 0.05g, 0.1g, and 0.2g DW miso. We also found there was a positive relationship between melanoidin content and lipase inhibitory activity at 0.05g DW miso (correlation coefficient; $R = 0.6730$) in Figure 19A, a positive relationship at 0.1g DW miso (correlation coefficient; $R = 0.6888$) in Figure 19B and a positive relationship at 0.2g DW miso (correlation coefficient; $R = 0.7405$) in Figure 19C, respectively. Although in the physiological literature such MRPs are assigned as

advanced glycation end products [98] and may cause inflammatory reactions and other nutritional risks [99], they have also been associated with positive impacts on gut microbiota and human health [100, 101]. Dietary MRPs have been discussed Polymeric melanoidin exhibit serum cholesterol-lowering action, intestinal lactic acid bacteria improving action [102]. Brown pigment in soybean paste (miso) have strongly anti-trypsin activity except for sugar-digesting enzyme activity in vivo experiments [103]. Inhibition of lipid peroxidation was increased evidently with an increased heating time of all MRPs derived from chitosan-sugar model systems [104].

Moreover, based on the results shown in Figure 7 and 18, Figure 20 was depicted to further clarify the relationship between polyphenol content and lipase inhibitory activity at the different weight of 0.05g, 0.1g, and 0.2g DW miso. We also found there was a positive relationship between polyphenol content and lipase inhibitory activity at 0.05g DW miso (correlation coefficient; R = 0.6184) in Figure 20A, a positive relationship at 0.1g DW miso (correlation coefficient; R = 0.6732) in Figure 20B and a positive relationship at 0.2g DW miso (correlation coefficient; R = 0.7754) in Figure 20C, respectively. It is reported that there is a positive correlation between the total phenolics content and pancreatic lipase inhibitory activity contained in the fungi-fermented oats [105]. There is also reported that the intake of long-term fermented soybean pastes elevated the content of flavonoids and offered protection against fatty liver disease [106], and isoflavone from Korean soybean paste has remarkable anti-obesity activity *in vitro* and *in vivo* [107]. Therefore, it was recognized that the rice miso supplementary with black soybean, adzuki bean, and buckwheat could indeed improve the lipase inhibitory activity of traditional rice miso product, and the lipase inhibitory activity was involved with melanoidin and polyphenol produced through rice miso fermented. However, melanoidin and

polyphenol are in a mixed state, we will clarify the strength of the inhibitory activity of melanoidin and polyphenol in the future.


A
100
80
60
40
20
0
Lipase inhibitory activity (%)
0.05g DW miso
0.1g DW miso
0.2g DW miso
Cc
Db
Da
Bc
Cb
Ca
Ac
Bb
Ba
Ac
Ab
Aa
RM-BS-3M
RM-BS-6M
RM-BS-24M
RM-BS-36M
B
100
80
60
40
20
0
Lipase inhibitory activity (%)
0.05g DW miso
0.1g DW miso
0.2g DW miso
Dc
Bb
Da
Cb
Cb
Ca
Bc
Ab
Aa
Ac
Bb
Ba
RM-KB-3M
RM-KB-6M
RM-KB-24M
RM-KB-36M
C
100
80
60
40
20
0
Lipase inhibitory activity (%)
0.05g DW miso
0.1g DW miso
0.2g DW miso
Cc
Db
Da
Bc
Cb
Ca
Ac
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Aa
Bc
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Ba
RM-AB-3M
RM-AB-6M
RM-AB-24M
RM-AB-36M

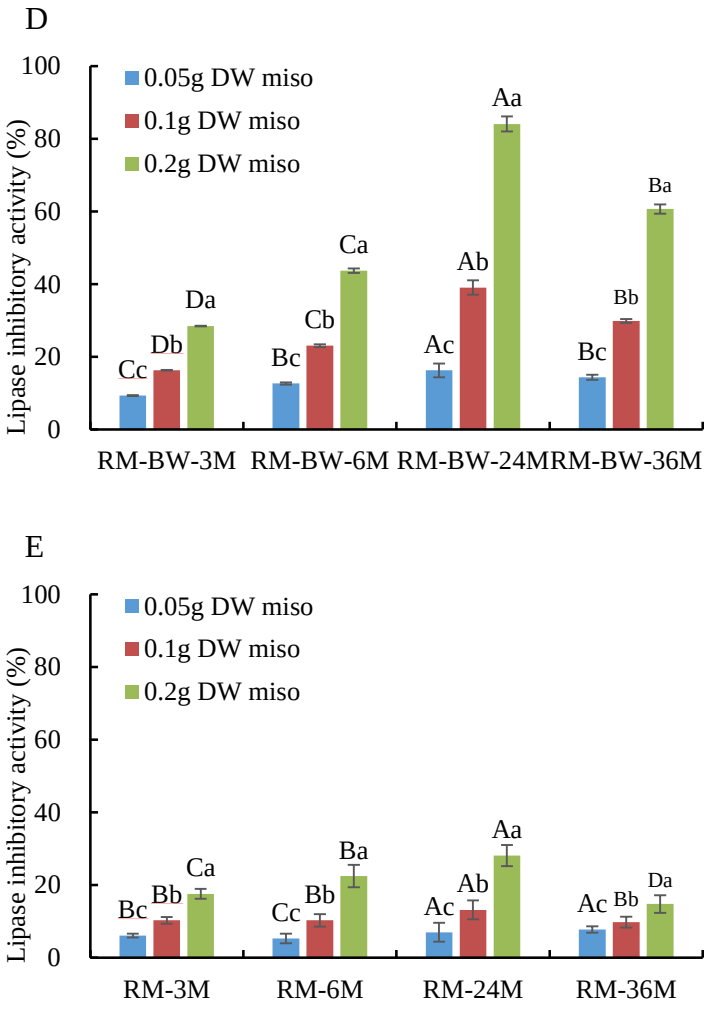

Figure 18. Lipase inhibitory activity in the Fra. Methanol of rice miso products with different weights and fermentation periods. A, RM-BS. B, RM-KB. C, RM-AB. D, RM-BW. E, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters and values within a column followed by different capital letters are significant at $p < 0.05$.

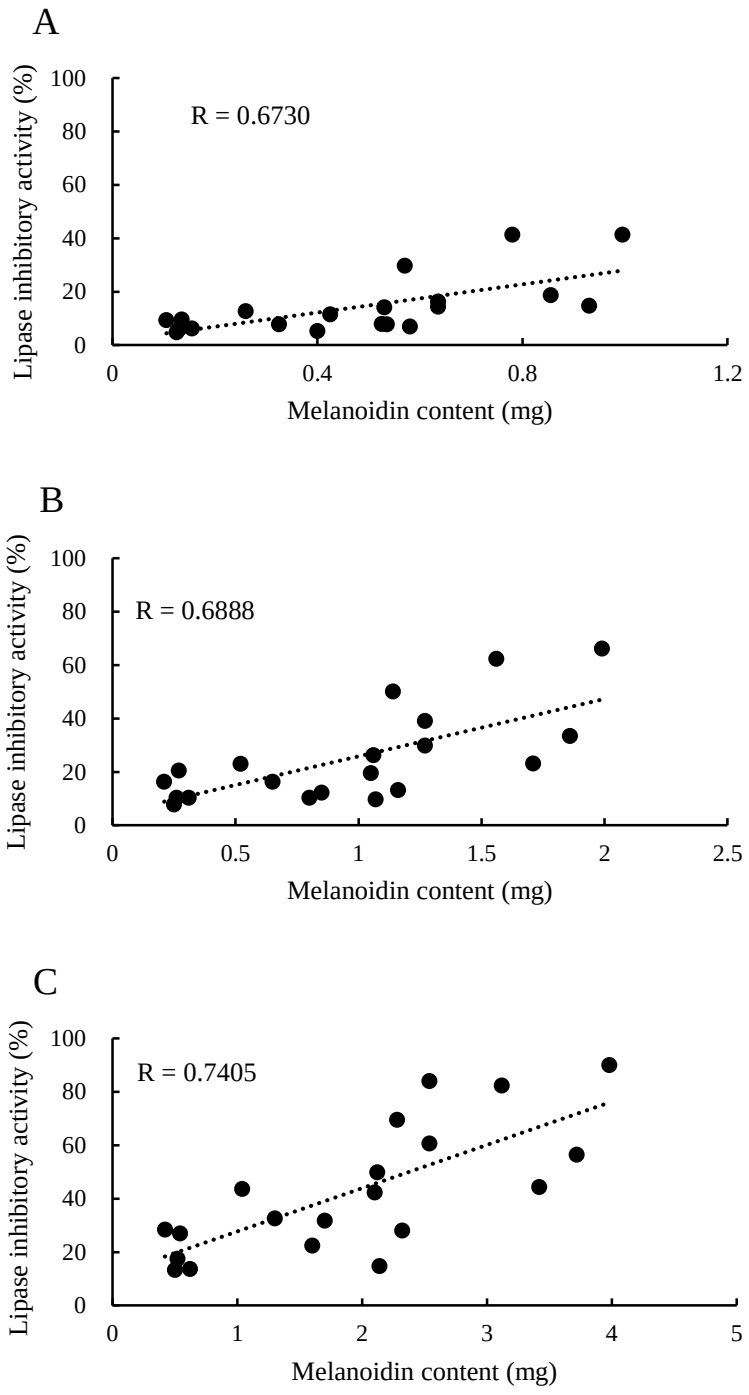

Figure 19. Relationship between melanoidin content and lipase inhibitory activity contained in Fra. Methanol of rice miso products with different fermentation period. A, 0.05g DW miso. B, 0.1g DW miso. C, 0.2g DW miso.

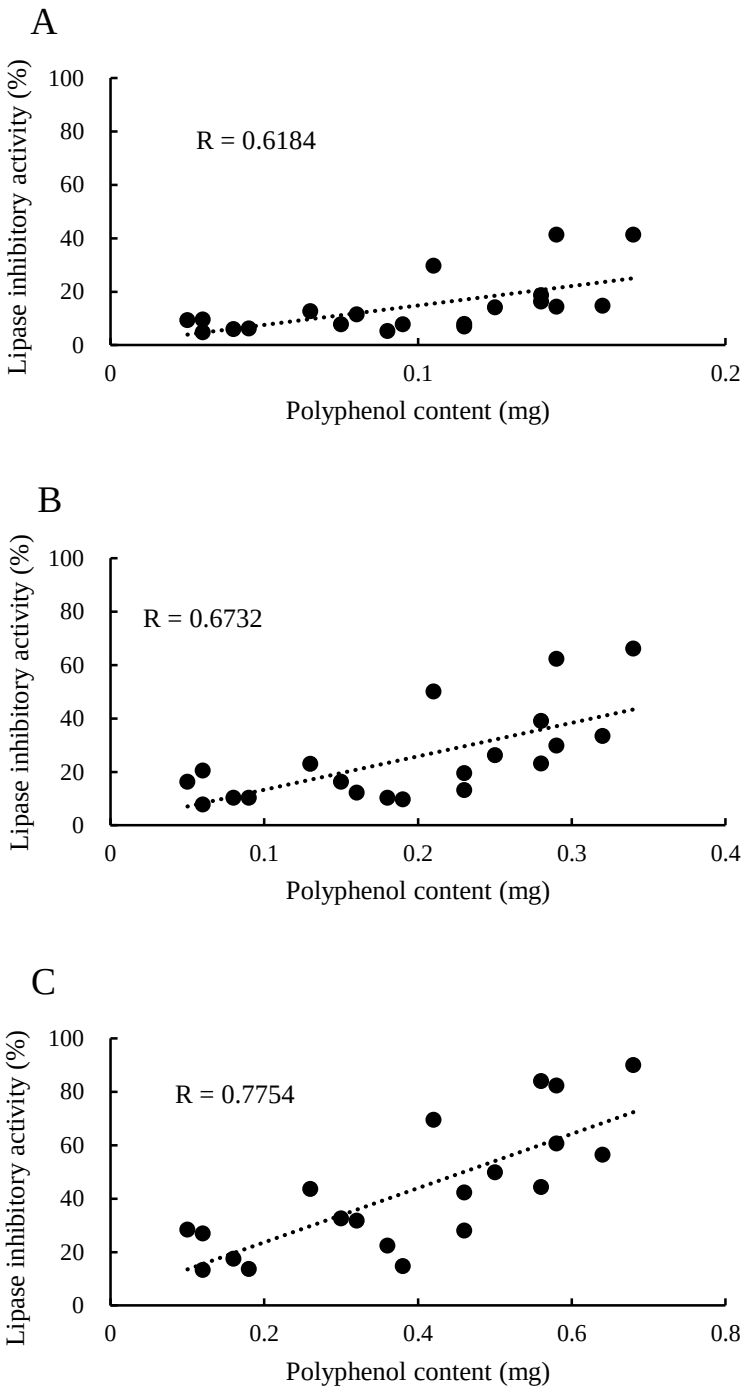

Figure 20. Relationship between polyphenol content and lipase inhibitory activity contained in Fra. Methanol of rice miso products with different fermentation period. A, 0.05g DW miso. B, 0.1g DW miso. C, 0.2g DW miso.

2.2. α-Glucosidase Inhibitory Activity in Rice Miso Products with Different Fermentation Periods

α-Glucosidase inhibitory activity in different weights of rice miso products was shown in Figure 21. The increased trend of α-glucosidase inhibitory activity was similar to lipase inhibitory activity, and the α-glucosidase inhibitory activity in rice miso products was increased with prolonging fermentation period. The highest value of RM-BS (78.2%/ 0.2g DW miso) was shown at fermented after 24 months, and RM-KB was 55.0%/ 0.2g DW miso and RM was 26.4%/ 0.2g DW miso. α-Glucosidase inhibitory activity of RM-BS and RM-KB were also significantly higher than RM (rice miso; as control) at different fermentation period, respectively. Therefore, we speculated that the functional substances produced during the fermentation of RM-BS, RM-KB, and RM also affect the inhibition of α-glucosidase activity.

In addition, based on the results shown in Figure 4, 7 and 21, Figure 22 was depicted to further clarify the relationship between melanoidin content, polyphenol content and α-glucosidase inhibitory activity at 0.2g DW miso. We also found there was a positive relationship between melanoidin content and α-glucosidase inhibitory activity (correlation coefficient; R = 0.6206) in Figure 22A, a positive relationship between polyphenol content and α-glucosidase inhibitory activity (correlation coefficient; R = 0.6314) in Figure 22B, respectively. Therefore, it was recognized that the α-glucosidase inhibitory activity was involved with melanoidin and polyphenol content produced through rice miso fermented. There is reported that in the glucose-amino acid model MRPs, fructose- and glucose- tyrosine showed stronger α-glucosidase inhibitory activity than that of others MRPs [108]. There are also reported that not only miso, but other fermented

soybean products have α-glucosidase inhibitory activity, and the substance involved with inhibitory effects is polyphenol present in the fermented soybean [109-111]. It was also reported that isoflavone profiles were changed to aglycone-form isoflavones in fermented soybean products [112], and contribute to the significant part of carbohydrate digestive Enzymes [113]. As another food material rich in melanoidin, there is a report on coffee that the darker the coffee degree of roast, the greater α-glucosidase inhibitory activity, melanoidin and polyphenol revealed mixed-type to competitive inhibition mechanisms against α-glucosidase [114]. Since the correlation coefficients between melanoidin content, polyphenol content and α-glucosidase inhibitory activity are approximately the same, and the melanoidin content was 6 folds higher than polyphenol content, we estimated that the melanoidin was mainly related to the α-glucosidase inhibitory activity contained in rice miso products, and a certain of polyphenol content has more inhibitory activity than melanoidin content.

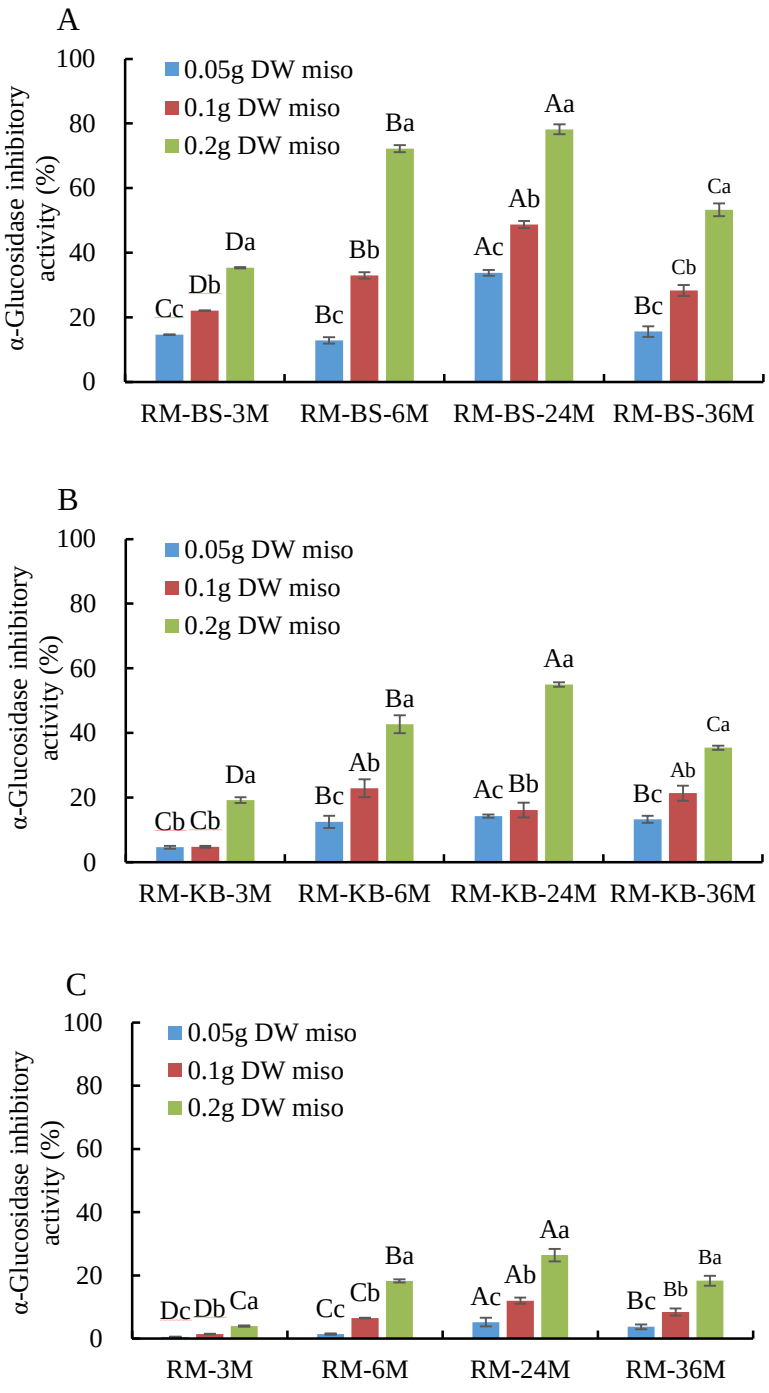

Figure 21. α-Glucosidase inhibitory activity in the Fra. Methanol of rice miso products with different weights and fermentation periods. A, RM-BS. B, RM-KB. C, RM. Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data

represent the mean ± SD from at least three independent studies. Values within a row followed by different small letters and values within a column followed by different capital letters are significant at $p < 0.05$.

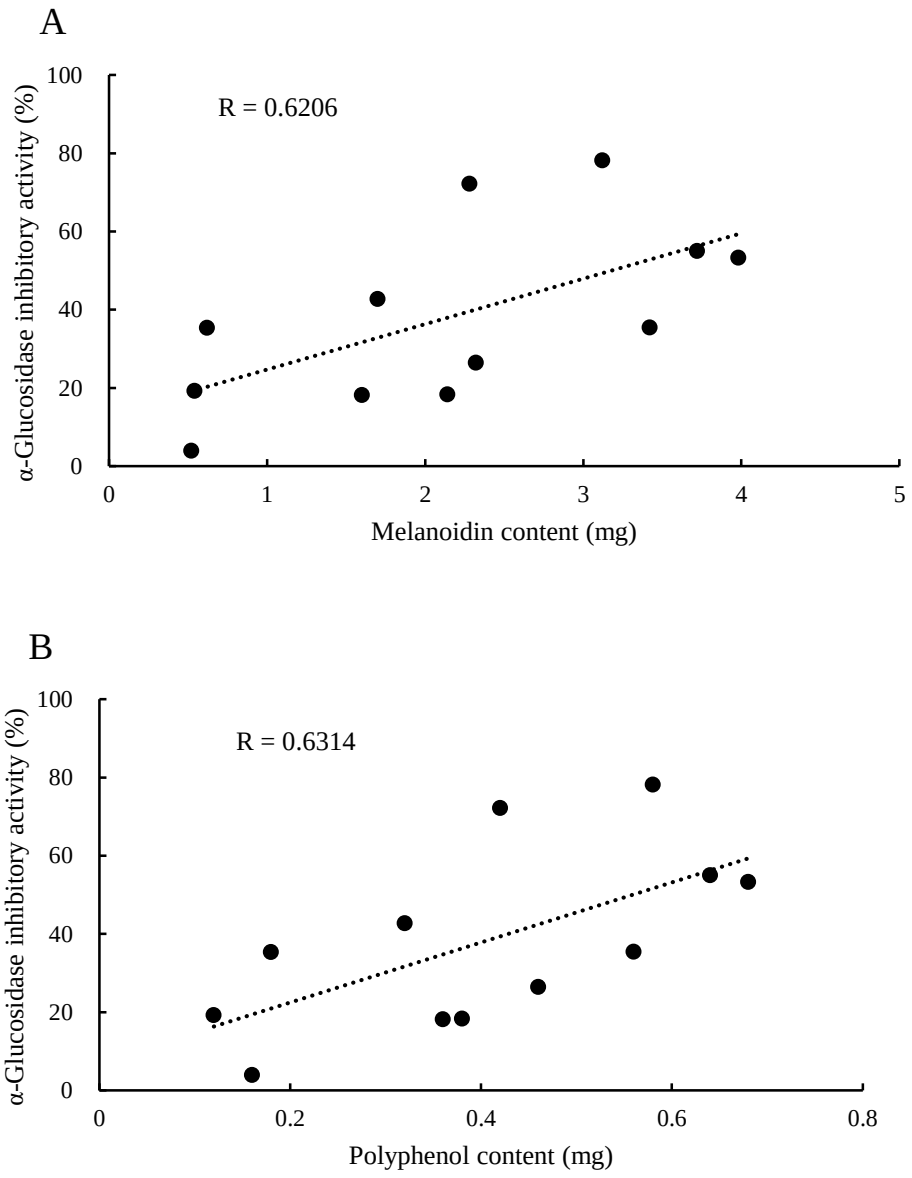

Figure 22. Relationship between melanoidin content, polyphenol content and α-glucosidase inhibitory activity contained in Fra. Methanol of 0.2 g DW rice miso products with different fermentation period. A, melanoidin content and α-glucosidase inhibitory activity. B, polyphenol content and α-glucosidase inhibitory activity.

General Discussions

In the present study, we fermented rice miso (RM) supplementary with black soybean (RM-BS), kidney bean (RM-KB), buckwheat (RM-BW) and adzuki bean (RM-AB) to improve the potential functionality of traditional rice miso products, quantified and clarified the peptide, reducing sugar, melanoidin and polyphenol content, DPPH radical scavenging activity, ABTS radical scavenging activity, lipase inhibitory activity and α-glucosidase inhibitory activity contained in rice miso products to compared with rice miso (RM) with different fermentation periods (fermented after 3 months, 6 months, 24 months and 36 months). We investigated the influence of these functional components in fermented rice miso products on the antioxidant activity, the effect on the anti-obesity action, and the effect on the inhibition of glucose metabolism.

The results showed that the melanoidin and polyphenol content was increased since the fermentation period of rice miso products were processed, and the ratio between the melanoidin and polyphenol content was increased from 3 folds to 6 folds. Moreover, since the protein and carbohydrate are decomposed by rice koji malt (*Aspergillus oryzae*), peptide and reducing sugar content were increased up, and then for the melanoidin is synthesized, peptide and reducing sugar content were sharply decreased. In addition, the peptide, reducing sugar, melanoidin, and polyphenol content contained in RM-BS, RM-KB, RM-AB, and RM-BW were significantly higher than that of RM (control) (p <0.05).

DPPH radical scavenging activity and ABTS radical scavenging activity contained in rice miso product were also increased with the prolongation of the fermentation period, and the RM-BS, RM-KB, RM-AB, and RM-BW were significantly higher than those of RM (p<0.05). Furthermore, the melanoidin content and the polyphenol content have a high positive correlation between DPPH radical scavenging activity and ABTS radical

scavenging activity, respectively, and since the melanoidin content was 6 folds higher than the polyphenol content, it was recognized that the substances contributed to antioxidant activity in rice miso products were mainly melanoidin.

The lipase inhibitory activity and α-glucosidase inhibitory activity of rice miso products were also increased with the prolongation of the fermentation period, and the lipase inhibitory activity and α-glucosidase inhibitory activity of other rice miso products were significantly higher than that of RM (p <0.05). There was also a correlation between melanoidin content, polyphenol content and lipase inhibitory activity with the correlation coefficient 0.7405 and 0.7754 at 0.2g DW miso, and a correlation between melanoidin content, polyphenol content and α-glucosidase inhibitory activity with the correlation coefficient 0.6206 and 0.6314 at 0.2g DW miso, respectively.

From the above results, we suggest traditional rice miso supplementary with black soybean, kidney bean, adzuki bean, and buckwheat had useful components may exert health beneficiary, in particular, potential applications for high antioxidant activity, and suppression of diabetes and body weight increase.

Abstract

In recent years, prevention of lifestyle-related diseases such as obesity, diabetes mellitus is noted, the development of new rice miso products for beneficial and healthy has been expected. As a processed soybean fermented product, rice miso is a traditional Japanese seasoning food which not only prolongs the consumption period but also has good sensory characteristics. Therefore, we processed rice miso products by supplementary with black soybean, kidney bean, red bean and buckwheat to improve the potential functionality, and aim to clarify the nutritional components, antioxidant activity and enzyme inhibitory activity of improved rice miso products with different fermentation period (fermented after 3 months, 6 months, 24 months and 36 months).

Black soybean, kidney bean, adzuki bean, and buckwheat were respectively mixed with soybean, and fermented by rice malt (*Aspergillus oryzae*) to prepare rice miso products. Rice miso supplementary with black soybean (RM-BS), rice miso supplementary with kidney bean (RM-KB), rice miso supplementary with adzuki bean (RM-AB), rice miso supplementary with buckwheat (RM-BW) and rice miso (RM; as a control) were fermented for 3 months, 6 months, 24 months and 36 months, and a part of the fermented products was cooled at -20°C freezer for chemical analysis. Subsequently, the peptide, reducing sugar, melanoidin, and polyphenol content were quantified and DPPH radical scavenging activity, ABTS radical scavenging activity, lipase inhibitory activity and α-glucosidase inhibitory activity of rice miso products with different fermentation period were evaluated. We investigated the influence of these functional components in fermented rice miso products on the antioxidant activity, the effect on the anti-obesity action, and the effect on the inhibition of glucose metabolism.

The results showed that the melanoidin and polyphenol content was increased since

the fermentation period of rice miso products were processed, and the ratio between the melanoidin and polyphenol content was increased from about 3 folds to 6 folds. Moreover, since the protein and carbohydrate are decomposed by rice koji malt (*Aspergillus oryzae*), peptide and reducing sugar content were increased up, and then for the melanoidin are synthesized, peptide and reducing sugar content were sharply decreased. In addition, the peptide, reducing sugar, melanoidin, and polyphenol content contained in rice miso products supplementary with black soybeans, kidney bean, adzuki bean, and buckwheat were significantly higher than that of rice miso (control) ($p < 0.05$).

DPPH radical scavenging activity and ABTS radical scavenging activity contained in rice miso product were also increased with the prolongation of the fermentation period, and the RM-BS, RM-KB, RM-AB, and RM-BW were significantly higher than those of rice miso (RM) ($p < 0.05$). Furthermore, the melanoidin content and the polyphenol content have a high positive correlation between DPPH radical scavenging activity and ABTS radical scavenging activity, respectively, and since the melanoidin content was about 6 folds higher than the polyphenol content, it was recognized that the substances contributed to antioxidant activity in rice miso products were mainly melanoidin.

The lipase inhibitory activity and α-glucosidase inhibitory activity of rice miso products were also increased with the prolongation of the fermentation period. however, and the maximum value of RM-BS was detected at fermented after 36 months, and the highest value of RM-KB, RM-AB, RM-BW, and RM were detected at fermented after 24 months. Moreover, the lipase inhibitory activity and α-glucosidase inhibitory activity of RM-BS, RM-KB, RM-AB, and RM-BW were significantly higher than that of RM ($p <0.05$). There was also a correlation between melanoidin content, polyphenol content and lipase inhibitory activity the correlation coefficient was 0.7405 and 0.7754 at 0.2g DW

miso and a correlation between melanoidin content, polyphenol content and α-glucosidase inhibitory activity with the correlation coefficient 0.6206 and 0.6314 at 0.2g DW miso, respectively.

From the above results, we suggest that adding high-performance raw materials, such as black soybean, kidney bean, adzuki bean, and buckwheat into traditional rice miso could increase useful components and improve the functionality, which may exert health beneficiary, in particular, potential applications for high antioxidant activity, and suppression of diabetes and body weight increase.

要約

近年、糖尿病、肥満など生活習慣病の予防に、健康機能性の高い食品の開発が期待されている。米味噌は、消費期限の長い大豆発酵品として人気があり、良好な感覚特性を有する日本の伝統的な調味食品で、栄養機能性が高い食品の1つである。本研究では、黒大豆、金時豆、小豆及び蕎麦を添加して従来より栄養機能性効果の高い発酵物を作り、それらの各熟成期間における機能性成分および抗酸化活性の変動について明らかにすることを目的とした。また、それらの味噌が酵素阻害活性を示すことも明らかにしようとした。

黄大豆に黒大豆、金時豆、小豆及び蕎麦をそれぞれ混合して、米麹菌（アスペルギルス・オリゼー）で発酵し、新しい米味噌の発酵加工品を調製した。黒大豆を添加した米味噌（RM-BS)、金時豆を添加した米味噌（RM-KB)、小豆を添加した米味噌（RM-AB)、蕎麦を添加した米味噌（RM-BW）及びコントロールとして黄大豆米味噌（RM）の5種類の米味噌発酵物を仕込み後3ヵ月、6ヵ月、24ヵ月および36ヵ月間発酵・熟成して分析試料とした。各発酵品は-20℃の冷凍庫に保存した。熟成期間の異なる各種の米味噌のペプチド、還元糖、メラノイジン及びポリフェノール含量を定量し、DPPHラジカル消去活性、ABTSラジカル消去活性、リパーゼ阻害活性およびα-グルコシダーゼ阻害活性の値から、発酵物の抗酸化作用、抗肥満作用、血糖値上昇抑制作用に及ぼす影響につ

いて検討した。

黒大豆、金時豆、小豆と蕎麦を添加した米味噌に含まれるメラノイジンとポリフェノールの含量は発酵熟成期間の増加に伴い上昇し、コントロールの米味噌のそれらよりも有意に高い値を示した($p < 0.05$)。熟成が進むに従い、メラノイジン含量とポリフェノール含量の比率は、熟成初期に比べ約 3 倍から 6 倍まで増加した。その理由として、豆類、蕎麦のタンパク質と糖質が米麹菌（アスペルギルス・オリゼー）により分解され、生じたペプチドと還元糖の含量が 6 ヵ月まで上昇し、それらがメラノイジン合成ために順次利用され、その後減少したと推定した。

黒大豆、金時豆、小豆と蕎麦を添加した米味噌の熟成期間の延長に伴い、DPPH ラジカル消去活性と ABTS ラジカル消去活性は徐々に増加し、コントロールの米味噌のそれらよりも有意に高いことが明らかとなった($p < 0.05$)。メラノイジン含量は DPPH ラジカル消去活性と ABTS ラジカル消去活性の両者においてそれぞれ高い正の相関が認められた。また、ポリフェノール含量と DPPH ラジカル消去活性および ABTS ラジカル消去活性の間にもそれぞれ高い正の相関が認められた。メラノイジン含量はポリフェノール含量よりも約 6 倍高く、メラノイジンとポリフェノールの重量当たりの抗酸化活性の効果はほぼ同じであることから、米味噌の抗酸化活性に寄与する物質は含量の多いメラノイジン

であると考えられる。

コントロールの米味噌に比べ、黒大豆、金時豆、小豆、蕎麦を添加した米味噌のリパーゼ阻害活性とα-グルコシダーゼ阻害活性は有意に高いことが認められた($p < 0.05$)。熟成期間の延長に伴い、5 種類の米味噌のリパーゼ阻害活性とα-グルコシダーゼ阻害活性は増加し、金時豆、小豆、蕎麦を添加した米味噌及びコントロールの米味噌の最大の阻害を示したのは 24 ヵ月熟成した味噌で、黒大豆を添加した米味噌で最大に阻害を示したのは 36 ヵ月熟成した味噌であった。リパーゼ阻害活性とメラノイジン含量及びポリフェノール含量との間にそれぞれ正の相関関係のあることが認められ、α-グルコシダーゼ阻害活性とメラノイジン含量及びポリフェノール含量との間にもそれぞれ正の相関関係のあることが認められた。

以上の結果より、新しく調製した黒大豆、金時豆、小豆、蕎麦を添加した米味噌の機能性成分含量はコントロールの米味噌に比べて多く、生体内の抗酸化作用や消化酵素の阻害活性が向上していることにより体内脂肪の蓄積を抑制することおよび血糖値上昇を抑制することが期待される。

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Supplementary Table 1. Melanoidin Content in the Hydrophilic Fraction of Rice Miso Products with Different Fermentation Period.

Period	Melanoidin content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	6.1 ± 0.2Ad	5.9 ± 0.4Bd	5.5 ± 0.1Cc	5.4 ± 0.1Cc	5.6 ± 0.1Cc
6M	19.6 ± 0.9Ac	16.5 ± 0.4Bc	13.5 ± 0.3Db	14.4 ± 0.3Cb	14.6 ± 0.1Cb
24M	23.7 ± 0.6Bb	25.9 ± 0.3Ab	20.2 ± 0.4Ca	23.7 ± 0.3Ba	16.8 ± 0.2Da
36M	29.7 ± 0.2Aa	26.5 ± 0.2Ba	19.9 ± 0.5Da	23.7 ± 0.8Ca	16.6 ± 0.2Ea

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 2. Peptide Content in the Hydrophilic Fraction of Rice Miso Products with Different Fermentation Period.

Period	Peptide content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	86.6 ± 2.7Dd	121.5 ± 0.7Ab	97.7 ± 1.3Cc	99.8 ± 2.3Cd	118.4 ± 4.3Bb
6M	188.7 ± 8.3Ba	177.5 ± 1.7Ca	155.2 ± 2.7Ea	171.1 ± 0.6Da	177.1 ± 6.3Aa
24M	130.5 ± 2.3Bb	123.8 ± 4.9Cb	109.3 ± 3.0Db	140.8 ± 0.6Ab	96.8 ± 2.3Ec
36M	101.7 ± 6.5Cc	108.9 ± 1.6Bc	105.2 ± 1.8Bb	127.1 ± 0.6Ac	93.8 ± 2.0Dd

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 3. Reducing Sugar Content in the Hydrophilic Fraction of Rice Miso Products with Different Fermentation Period.

Period	Reducing sugar content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	389.7 ± 1.5Bb	425.0 ± 2.6Ab	260.3 ± 0.8Eb	274.7 ± 2.2Db	289.1 ± 2.3Cb
6M	443.3 ± 5.3Ba	481.7 ± 5.9Aa	317.0 ± 4.8Ea	354.8 ± 6.4Da	349.2 ± 8.7Ca
24M	146.3 ± 0.5Bc	121.4 ± 2.7Dc	132.0 ± 0.8Cc	201.1 ± 2.3Ac	93.6 ± 2.2Ec
36M	125.8 ± 0.7Bd	108.9 ± 2.9Dd	121.8 ± 0.7Cd	142.0 ± 0.8Ad	79.9 ± 3.2Ed

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 4a. Melanoidin Content in Fra. Water of Rice Miso Products with Different Fermentation Period.

Period	Melanoidin content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	1.0 ± 0.1Bc	1.4 ± 0.1Ac	1.0 ± 0.1Bd	0.9 ± 0.1Cd	0.7 ± 0.1Dd
6M	4.7 ± 0.1Ab	4.0 ± 0.1Bb	3.1 ± 0.1Cc	2.5 ± 0.1Dc	2.0 ± 0.1Ec
24M	4.6 ± 0.1Ab	4.7 ± 0.1Aa	3.4 ± 0.1Bb	2.8 ± 0.1Cb	2.7 ± 0.1Cb
36M	5.5 ± 0.1Aa	4.8 ± 0.1Ba	4.7 ± 0.1Ba	4.2 ± 0.1Ca	3.4 ± 0.1Da

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 4b. Melanoidin Content in Fra. Water of Rice Miso Products with Different Fermentation Period.

Period	Melanoidin content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	3.1 ± 0.1Ad	2.7 ± 0.1Bd	2.5 ± 0.1Cc	2.1 ± 0.1Dc	2.6 ± 0.1Bd
6M	11.4 ± 0.5Ac	8.5 ± 0.1Bc	6.5 ± 0.1Db	5.2 ± 0.2Eb	8.0 ± 0.1Cc
24M	15.6 ± 0.3Bb	18.6 ± 0.2Aa	10.6 ± 0.4Ea	12.7 ± 0.1Ca	11.6 ± 0.2Da
36M	19.9 ± 0.8Aa	17.1 ± 0.1Bb	10.5 ± 0.4Da	12.7 ± 0.6Ca	10.7 ± 0.1Db

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 5. Melanoidin Content of RM-BS with Different Fermentation Period in Fractions after LH-20 Column.

Sample	Melanoidin (mg/g DW miso)		
	Fra. I	Fra. II	Fra. III
RM-BS-3M	0.2 ± 0.1Cb	0.3 ± 0.1Ca	0.2 ± 0.1Dc
RM-BS-6M	0.2 ± 0.1Bc	2.3 ± 0.1Bb	3.6 ± 0.1Ca
RM-BS-24M	0.4 ± 0.1Ac	2.7 ± 0.1Ab	5.1 ± 0.1Ba
RM-BS-36M	0.5 ± 0.1Ac	2.4 ± 0.1Ab	6.2 ± 0.1Aa

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 6. Polyphenol Content in the Hydrophilic Fraction of Rice Miso Products with Different Fermentation Period.

Period	Polyphenol content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	4.3 ± 0.1Bd	5.4 ± 0.1Ac	3.0 ± 0.1Cd	2.6 ± 0.1Dd	3.1 ± 0.1Cd
6M	12.2 ± 0.2Ab	10.4 ± 0.4Bb	8.4 ± 0.2Dc	8.7 ± 0.3Cc	8.7 ± 0.1Cb
24M	11.0 ± 0.2Ac	10.1 ± 0.1Bb	9.3 ± 0.3Cb	10.0 ± 0.2Bb	9.2 ± 0.2Ca
36M	12.9 ± 0.3Aa	11.5 ± 0.2Ba	10.5 ± 0.3Ca	11.8 ± 0.3Ba	8.2 ± 0.2Dc

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 7a. Polyphenol Content in the Fra. Water of Rice Miso Products with Different Fermentation Period.

Period	Polyphenol content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	0.9 ± 0.1Ad	0.6 ± 0.1Cd	0.6 ± 0.1Cd	0.5 ± 0.1Dc	0.8 ± 0.1Bc
6M	2.1 ± 0.5Ac	1.6 ± 0.1Cc	1.5 ± 0.1Cc	1.3 ± 0.1Db	1.8 ± 0.2Bb
24M	2.9 ± 0.3Bb	3.2 ± 0.1Aa	2.5 ± 0.1Ca	2.8 ± 0.1Ba	2.3 ± 0.1Da
36M	3.4 ± 0.8Aa	2.8 ± 0.1Bb	2.3 ± 0.1Cb	2.9 ± 0.1Ba	1.9 ± 0.2Db

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 7b. Polyphenol Content in the Fra. Methanol of Rice Miso Products with Different Fermentation Period.

Period	Polyphenol content (mg/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	3.5 ± 0.1Bd	4.2 ± 0.1Ac	2.5 ± 0.1Dd	2.3 ± 0.1Ed	2.8 ± 0.1Cb
6M	8.6 ± 0.1Aa	7.8 ± 0.1Ba	6.1 ± 0.1Ea	6.4 ± 0.1Da	5.9 ± 0.4Ca
24M	6.8 ± 0.1Ac	6.1 ± 0.1Bb	5.6 ± 0.1Cc	5.6 ± 0.1Cb	3.6 ± 0.1Db
36M	8.2 ± 0.1Ab	7.8 ± 0.1Ba	6.3 ± 0.1Cb	5.5 ± 0.1Dc	3.9 ± 0.1Eb

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 8. Polyphenol Content of RM-BS with Different Fermentation Period in Fractions after LH-20 Column.

Sample	Polyphenol(mg/g DW miso)		
	Fra. I	Fra. II	Fra. III
RM-BS-3M	0.12 ± 0.01Aa	0.02 ± 0.01Cb	<0.01
RM-BS-6M	<0.01	0.14 ± 0.02Aa	0.13 ± 0.01Ca
RM-BS-24M	<0.01	0.08 ± 0.01Bb	0.21 ± 0.01Ba
RM-BS-36M	<0.01	0.01 ± 0.01Cb	0.28 ± 0.01Aa

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 9. DPPH Radical Scavenging Activity in the Hydrophilic Fraction of Rice Miso Products with Different Fermentation Period.

Period	DPPH radical scavenging activity (μmol/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	3.2 ± 0.1Bd	3.6 ± 0.1Ac	3.1 ± 0.1Cd	2.3 ± 0.1Dd	2.8 ± 0.1Cd
6M	5.6 ± 0.1Ab	5.3 ± 0.1Bb	5.1 ± 0.1Dc	5.2 ± 0.1Cc	5.4 ± 0.1Cb
24M	8.8 ± 0.2Ac	8.6 ± 0.1Bb	8.1 ± 0.1Cb	8.2 ± 0.1Bb	8.4 ± 0.1Ca
36M	9.1 ± 0.1Aa	7.8 ± 0.1Ba	8.5 ± 0.1Ca	8.5 ± 0.1Ba	6.9 ± 0.1Dc

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 10a. DPPH Radical Scavenging Activity in the Fra. Water of Rice Miso Products with Different Fermentation Period.

Period	DPPH radical scavenging activity (μmol/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	3.2 ± 0.1Bd	3.6 ± 0.1Ac	3.1 ± 0.1Cd	2.3 ± 0.1Dd	2.8 ± 0.1Cd
6M	5.6 ± 0.1Ab	5.3 ± 0.1Bb	5.1 ± 0.1Dc	5.2 ± 0.1Cc	5.4 ± 0.1Cb
24M	8.8 ± 0.2Ac	8.6 ± 0.1Bb	8.1 ± 0.1Cb	8.2 ± 0.1Bb	8.4 ± 0.1Ca
36M	9.1 ± 0.1Aa	7.8 ± 0.1Ba	8.5 ± 0.1Ca	8.5 ± 0.1Ba	6.9 ± 0.1Dc

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 10b. DPPH Radical Scavenging Activity in the Fra. Methanol of Rice Miso Products with Different Fermentation Period.

Period	DPPH radical scavenging activity (μmol/g DW miso)				
	RM-BS	RM-KB	RM-AB	RM-BW	RM
3M	1.0 ± 0.1Ad	1.4 ± 0.1Cd	1.8 ± 0.1Cd	0.9 ± 0.1Dc	0.9 ± 0.1Bc
6M	4.0 ± 0.1Ac	3.5 ± 0.1Cc	3.0 ± 0.1Cc	2.6 ± 0.1Db	2.9 ± 0.1Bb
24M	6.0 ± 0.1Bb	7.1 ± 0.1Aa	5.7 ± 0.1Ca	6.1 ± 0.1Ba	6.6 ± 0.2Da
36M	6.9 ± 0.1Aa	5.7 ± 0.1Bb	5.3 ± 0.1Cb	6.6 ± 0.1Ba	5.4 ± 0.1Db

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 11. DPPH Radical Scavenging Activity of RM-BS with Different Fermentation Period in the Fractions after LH-20 Column.

Sample	DPPH radical scavenging activity(μmol/g DW miso)		
	Fra. I	Fra. II	Fra.III
RM-BS-3M	0.31 ± 0.03Ab	0.40 ± 0.04Ca	<0.01
RM-BS-6M	0.21 ± 0.18Ac	1.64 ± 0.11Ba	0.90 ± 0.11Cb
RM-BS-24M	<0.01	3.70 ± 0.29Aa	1.92 ± 0.20Bb
RM-BS-36M	<0.01	3.62 ± 0.39Aa	2.62 ± 0.23Ab

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 12. ABTS Radical Scavenging Activity in the Hydrophilic Fraction of Rice Miso Products with Different Fermentation Period.

Period	ABTS radical scavenging activity (μmol/g DW miso)		
	RM-BS	RM-KB	RM
3M	4.6 ± 0.8Ad	4.5 ± 0.4Ac	4.5 ± 0.1Ac
6M	11.0 ± 0.7Ac	9.6 ± 0.4Bc	8.6 ± 0.3Cb
24M	17.2 ± 0.4Aa	14.7 ± 0.7Ba	11.2 ± 0.3Ca
36M	15.3 ± 0.4Ab	12.2 ± 0.3Bb	8.4 ± 0.3Cb

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 13a. ABTS Radical Scavenging Activity in the Fra. Water of Rice Miso Products with Different Fermentation Period.

Period	ABTS radical scavenging activity (μmol/g DW miso)		
	RM-BS	RM-KB	RM
3M	1.7 ± 0.1Cd	2.3 ± 0.1Ad	1.9 ± 0.1Bd
6M	4.0 ± 0.1Ac	3.5 ± 0.1Bc	3.0 ± 0.1Cb
24M	5.1 ± 0.1Ab	4.5 ± 0.1Bb	4.7 ± 0.1Ca
36M	5.3 ± 0.1Aa	4.8 ± 0.1Ba	2.6 ± 0.1Cc

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 13b. ABTS Radical Scavenging Activity in the Fra. Methanol of Rice Miso Products with Different Fermentation Period.

Period	ABTS radical scavenging activity (μmol/g DW miso)		
	RM-BS	RM-KB	RM
3M	1.4 ± 0.1Bd	1.8 ± 0.1Ad	1.3 ± 0.1Bd
6M	5.0 ± 0.1Ac	4.5 ± 0.1Bc	3.6 ± 0.1Cc
24M	9.1 ± 0.1Aa	7.8 ± 0.1Ba	6.8 ± 0.2Ca
36M	7.0 ± 0.1Ab	6.7 ± 0.1Bb	5.2 ± 0.2Cb

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 14a. Lipase Inhibitory Activity in the Fra. Methanol of 0.05g Rice Miso Products with Different Fermentation Period.

Period	Lipase inhibitory activity (%/0.05g miso)					
	RM-BS	RM-KB	RM-AB	RM-BW	RM	
3M	6.3 ± 0.3Bc	9.7 ± 0.6Ad	4.8 ± 0.3Cc	9.4 ± 0.1Ad	6.1 ± 0.5Bb	
6M	29.8 ± 3.2Ab	11.6 ± 0.6Cc	7.9 ± 0.7Db	12.7 ± 0.3Bc	5.3 ± 1.3Ec	
24M	41.4 ± 4.4Aa	14.8 ± 2.3Cb	14.2 ± 0.4Ca	16.3 ± 1.9Ba	7.0 ± 2.6Da	
36M	41.4 ± 3.8Aa	18.8 ± 0.7Ba	8.0 ± 0.8Db	14.4 ± 0.7Cb	7.8 ± 0.9Da	

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 14b. Lipase Inhibitory Activity in the Fra. Methanol of 0.1g Rice Miso Products with Different Fermentation Period.

Period	Lipase inhibitory activity (%/0.1g miso)					
	RM-BS	RM-KB	RM-AB	RM-BW	RM	
3M	10.3 ± 0.5Cd	20.6 ± 1.0Ac	7.8 ± 0.5Dd	16.3 ± 0.1Bd	10.3 ± 0.9Cb	
6M	50.2 ± 3.3Ac	12.2 ± 0.4Dd	16.3 ± 0.6Cc	23.1 ± 0.4Bc	10.3 ± 1.7Eb	
24M	62.4 ± 5.4Ab	33.5 ± 3.2Ca	26.3 ± 0.7Da	39.1 ± 2.0Ba	13.2 ± 2.6Ea	
36M	66.2 ± 6.5Aa	23.2 ± 6.1Cb	19.6 ± 1.3Db	29.9 ± 0.5Bb	9.8 ± 1.5Ec	

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 14c. Lipase Inhibitory Activity in the Fra. Methanol of 0.2g Rice Miso Products with Different Fermentation Period.

Period	Lipase inhibitory activity (%/0.2g miso)					
	RM-BS	RM-KB	RM-AB	RM-BW	RM	
3M	13.7 ± 0.6Dd	27.0 ± 1.7Bd	13.3 ± 0.9Dd	28.5 ± 0.1Ad	17.6 ± 1.4Cc	
6M	69.5 ± 3.4Ac	31.8 ± 1.1Cc	32.7 ± 0.9Cc	43.7 ± 0.6Bc	22.5 ± 3.1Db	
24M	82.4 ± 6.2Bb	56.5 ± 8.6Ca	49.9 ± 1.3Da	84.1 ± 2.1Aa	28.1 ± 2.9Ea	
36M	90.1 ± 5.4Aa	44.4 ± 7.0Cb	42.3 ± 2.3Cb	60.7 ± 1.3Bb	14.8 ± 2.4Dd	

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM-AB, rice miso supplementary with adzuki bean; RM-BW, rice miso supplementary with buckwheat; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 15a. α-Glucosidase Inhibitory Activity in the Fra. Methanol of 0.05g Rice Miso Products with Different Fermentation Period.

Period	α-Glucosidase inhibitory activity (%/0.05g miso)		
	RM-BS	RM-KB	RM
3M	14.7 ± 0.1Ac	4.6 ± 0.4Bd	0.5 ± 0.1Cd
6M	12.9 ± 1.0Ad	12.5 ± 1.9Ac	1.4 ± 0.2Bc
24M	33.8 ± 0.9Aa	14.3 ± 0.5Ba	5.2 ± 1.4Ca
36M	15.6 ± 1.6Ab	13.3 ± 1.1Bb	3.7 ± 0.8Cb

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 15b. α-Glucosidase Inhibitory Activity in the Fra. Methanol of 0.1g Rice Miso Products with Different Fermentation Period.

Period	α-Glucosidase inhibitory activity (%/0.1g miso)		
	RM-BS	RM-KB	RM
3M	22.1 ± 0.1Ad	4.8 ± 0.3Bd	1.4 ± 0.1Cd
6M	33.0 ± 1.0Ab	22.9 ± 2.8Ba	6.5 ± 0.1Cc
24M	48.7 ± 1.1Aa	16.2 ± 2.3Bc	12.0 ± 1.0Ca
36M	28.3 ± 1.7Ac	21.4 ± 2.3Bb	8.4 ± 1.1Cb

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.

Supplementary Table 15c. α-Glucosidase Inhibitory Activity in the Fra. Methanol of 0.2g Rice Miso Products with Different Fermentation Period.

Period	α-Glucosidase inhibitory activity (%/0.2g miso)		
	RM-BS	RM-KB	RM
3M	35.4 ± 0.2Ad	19.2 ± 0.9Bd	3.9 ± 0.2Cc
6M	72.2 ± 1.1Ab	42.7 ± 2.8Bb	18.2 ± 0.5Cb
24M	78.2 ± 1.5Aa	55.0 ± 0.7Ba	26.4 ± 2.0Ca
36M	53.3 ± 2.0Ac	35.4 ± 0.6Bc	18.3 ± 1.6Cb

Abbreviations: M, month; DW, dry weight; RM-BS, rice miso supplementary with black soybean; RM-KB, rice miso supplementary with kidney bean; RM, rice miso. Data represent the mean ± SD from at least three independent studies. Values within a row followed by different capital letters and values within a column followed by different small letters are significant at $p < 0.05$.