

Hypoallergenicity of Various Miso Pastes Manufactured in Japan

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(Received May 7, 2013)

Summary Miso paste (miso), a fermented soybean food, is popular in Japan and other Asian countries. However, the soybean is known to induce an allergenic response in some individuals. In the present study, we evaluated the allergenicity of various kinds of miso available in Japan. Total proteins were extracted from Amakuti-kome miso, Karakuti-kome miso, Mugi-miso and Mame-miso, and the protein profiles were analyzed. The major protein bands detected in the intact soybean extract were not present in any of the miso samples, which instead showed various low molecular weight protein bands of approximately 10–25 kDa. The existence levels of six major soybean allergens were determined by Western blotting using specific antibodies. We found that the allergen levels varied among miso and allergen types; however, allergen levels were consistently lower in miso than in the soybean extract. We obtained similar results for IgE-ELISA experiments using serum IgE from soybean allergy patients. Taken together, these results indicate that compared to soybean extract, various types of miso contain small quantities of intact soybean allergens. Additionally, several lines of evidence indicated that the allergen levels were exceptionally low in the dark-colored Karakuti-kome miso and Mame-miso, which are produced with relatively long fermentation periods, suggesting that the duration of fermentation might be a key factor in the hypoallergenicity of miso.

Key Words miso, soybean allergen, fermentation, hypoallergenicity

Miso (miso) is a fermented paste made from soybeans and salt (and sometimes cereal grains such as rice or barley) through a two-step fermentation process with molds, yeasts and bacteria (1, 2). The preparation of miso begins by inoculating steamed rice and/or barley with *Aspergillus oryzae* or other *Aspergillus* species, which results in the production of koji. Then, steamed soybeans and a yeast inoculum are mixed with the koji, and the mixture is fermented for anywhere from a month to several years to develop taste (3). Additionally, as a result of contamination during the manufacturing processes, *Bacillus subtilis* and *B. amyloliquefaciens* are usually present in miso (4).

In Japan, there are several kinds of miso pastes, which are categorized into four major categories: Karakuti-kome miso, Amakuti-kome miso, Mugi-miso, and Mame-miso (5). The most popular kind of miso in Japan is Karakuti-kome miso, a salt-rich miso made from soybeans and rice, which is produced in several different regions of the country. Amakuti-kome miso, popular in the Kansai region (particularly in Kyoto), is a mild miso

that is also made from soybeans and rice and is sweetened with syrup. Mugi-miso, which is made from barley and soybeans, is mainly produced in the Shikoku and Kyushu regions of western Japan. Mame-miso is made from soybeans only, and is produced mainly in the Tokai area.

Miso and its constituents are known to be beneficial for health by decreasing blood pressure, inhibiting osteoporosis, preventing cancer and heart disease, and reducing the risk of degenerative disease (6). Because of these health benefits, miso is a popular functional food in Japan and has gained recent popularity in other parts of the world. Indeed, Miso production continues to grow steadily in the United States and other Western countries (7). However, as miso is mainly made from soybeans, which can be allergenic, there may be soybean allergens also present in miso. Nevertheless, to our knowledge, relatively few studies have been conducted on the soybean allergen levels in various kinds of miso pastes (8, 9). Therefore, in this study we aimed to analyze the soybean allergen levels in the various miso pastes available in Japan. We determined that all of the misos tested had lower allergen levels than whole soybean extract, and identified Karakuti-kome miso and Mame-miso as particularly hypoallergenic varieties. On the basis of our data, we propose that the allergenicity of miso may be inversely related to fermentation time.

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Abbreviations: CBB, Coomassie brilliant blue; HRP, horseradish peroxidase; IgE, immunoglobulin E; IgG, immunoglobulin G; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Extraction of proteins from miso. We obtained several types of commercially available miso produced in various regions of Japan (Table 1). Five grams of each miso paste or soybean extract was homogenized with 25 mL of distilled cold water in a homogenizer, followed by the filtration through gauze. Protein concentrations of the miso extracts were then measured by Bradford assays (10) with a protein assay kit (Bio-Rad, Richmond, CA) using bovine γ -globulin as a standard.

SDS-PAGE and Western blotting. Equal quantities of proteins isolated from each miso or soybean extract were boiled for 5 min in SDS sample buffer (11). Proteins were separated by SDS-PAGE according to the method of Laemmli (11). For protein profile analysis, the SDS-PAGE gels were stained with Coomassie brilliant blue (CBB) R-350. For Western blotting, proteins separated by SDS-PAGE were transferred onto PVDF membranes (ImmobilonTM-P; Millipore, Billerica, MA). After incubation in blocking buffer (PBS containing 5% skim milk and 0.1% Tween20), the membrane was incubated with the indicated primary antibody at a dilution of 1 : 500–20,000 at room temperature for 1 h, and then washed 3 times with PBST (PBS containing 0.1% Tween20). The washed membrane was then incubated with HRP-conjugated secondary antibodies against mouse IgG or rabbit IgG for 1 h. All immunoreactive proteins were

detected by using ECLTM Western Blotting Detection Reagents (GE Healthcare, Chalfont St. Giles, UK). Western blotting experiments were carried out three times and band densities were determined using Alpha Ease software (Alpha Innotech, San Leandro, CA).

Antibodies. In this study, we used the following primary antibodies against the major soybean allergens listed in Table 2: anti-7S globulin (rabbit polyclonal antibody) (12), anti-Gly m Bd 30K (mouse monoclonal antibody) (13), anti-Gly m Bd 28K (mouse monoclonal antibody) (14), anti-Gly m 4 and anti-Gly m 3 (rabbit polyclonal antibodies), and anti-soybean trypsin inhibitor (rabbit polyclonal antibody; Chemicon, Temecula, CA). All antibodies used in this study except for anti-soybean trypsin inhibitor were prepared by us or other researchers. Anti-Gly m Bd 30K and Anti-Gly m Bd 28K antibodies were kindly donated by Dr. Tadashi Ogawa (emeritus professor of Kyoto University).

Anti-Gly m 4 and anti-Gly m 3 antibodies were raised in rabbits using recombinant Gly m 4 and Gly m 3 as antigens. The Gly m 4 and Gly m 3 recombinant proteins were expressed in *Escherichia coli* from cDNAs cloned from the soybean leaf cDNA library via PCR.

IgE-ELISA analysis. Miso or soybean extracts (0.1 mL) resolved in PBS (0.1 mg protein/mL) were coated onto the wells of ELISA plates (Iwaki, Tokyo, Japan), and then soybean allergy sera (diluted 50× with PBS) were added to the wells. After incubation for 3 h at room temperature, the sera were removed and the wells were washed 4 times with PBST. Bound IgE was detected with HRP-labeled anti-human IgE secondary antibody and TMB reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Once the desired color was achieved, the reaction was stopped by adding 1 M phosphoric acid, and absorbance at 450 nm was measured for each well using a plate reader (Wallac ARVO multi-label counter; PerkinElmer, Waltham, MA). The relative IgE binding capacities of miso or soybean extract were evaluated on the basis of the absorbance values at 450 nm. Soybean allergy sera were purchased from Kokusai Bio (Tokyo, Japan) and Cosmo Bio (Tokyo, Japan). All experimental procedures using human sera conformed to the guidelines of the Declaration of Helsinki in biomedical research involving human subjects. Using the commercial allergy sera was approved by the local ethics committee.

Table 1. Miso samples used in this study.

Miso sample	Ingredients	Sample No.
Amakuti-kome miso	rice, soybeans, salt, starch syrup, ethanol	1
Karakuti-kome miso	A rice, soybeans, salt, ethanol B soybeans, rice, salt C soybeans, rice, salt, ethanol D soybeans, rice, salt E soybeans, rice, salt, ethanol F soybeans, rice, salt, ethanol G soybeans, rice, salt, ethanol	2 3 4 5 6 7 8
Mugi-miso	barley, soybeans, salt, ethanol	9
Mame-miso	soybeans, salt	10

Table 2. Major soybean allergens detected in this study.

Allergen name	Molecular mass (kDa)	Features	References
Gly m Bd 30K	34 (30)*	Homologous to papain	13, 15
Gly m Bd 28K	28	Vicilin-like storage protein	14, 15, 16
7S globulin (β -conglycinin)	75, 70, 50	Major storage protein, glycoproteins	18, 19, 20
Trypsin inhibitor	18	Trypsin inhibitor	21
Gly m 4	17	Betv1 homolog, PR-10 family	22, 23
Gly m 3	14	Profilin (actin-binding protein)	24

*The theoretical molecular mass of Gly m Bd 30K from cDNA is approximately 30 kDa, but the position determined via SDS-PAGE is reported to be 34 kDa.

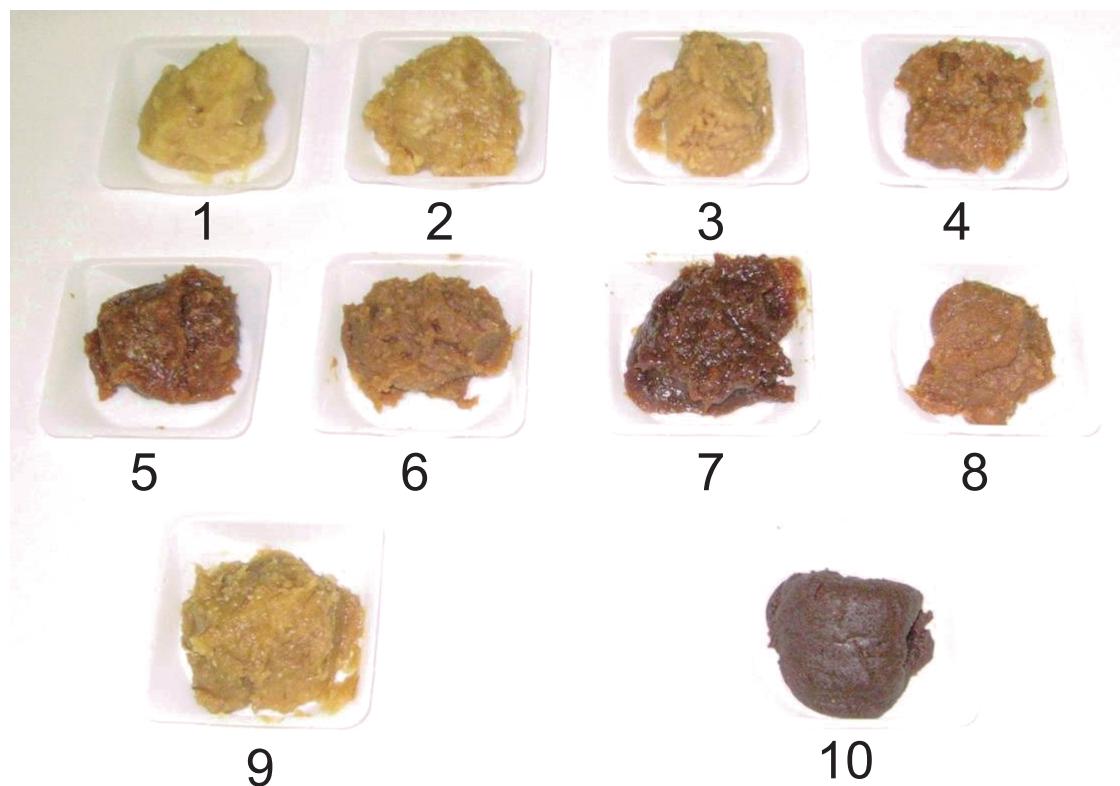


Fig. 1. Appearance of the miso samples tested in this study. The commercially available miso samples tested in this study were photographed. 1, Amakuti-kome miso; 2–8, Karakuti-kome miso A–G; 9, Mugi-miso; 10, Mame-miso.

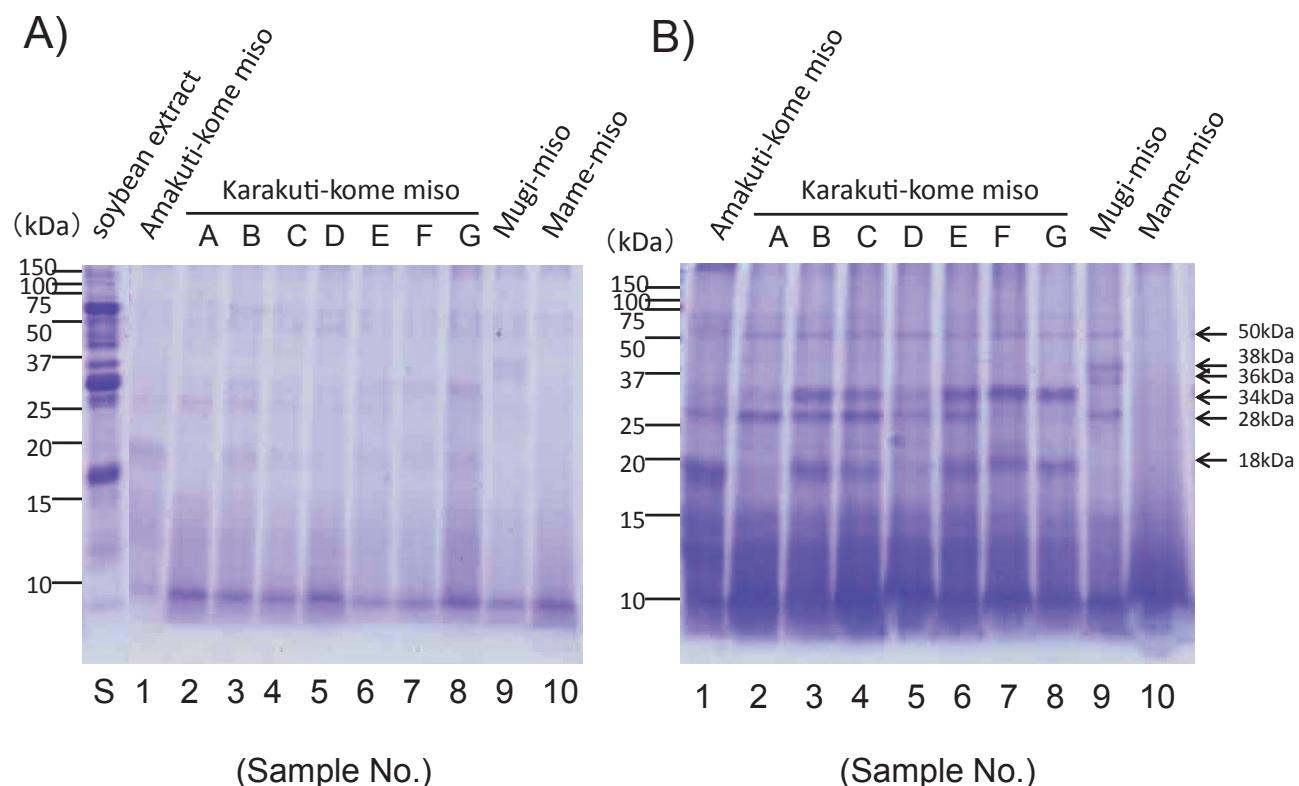


Fig. 2. Protein profiles of soybean extract and miso samples on SDS-PAGE gels stained with CBB. Soybean and miso samples (10 µg (A) or 100 µg (B) protein/lane) were separated via SDS-PAGE and the gels were stained with CBB R-350. Lane S, soybean extract; lane 1, Amakuti-kome miso; lanes 2–8, Karakuti-kome miso A–G; lane 9, Mugi-miso; lane 10, Mame-miso.

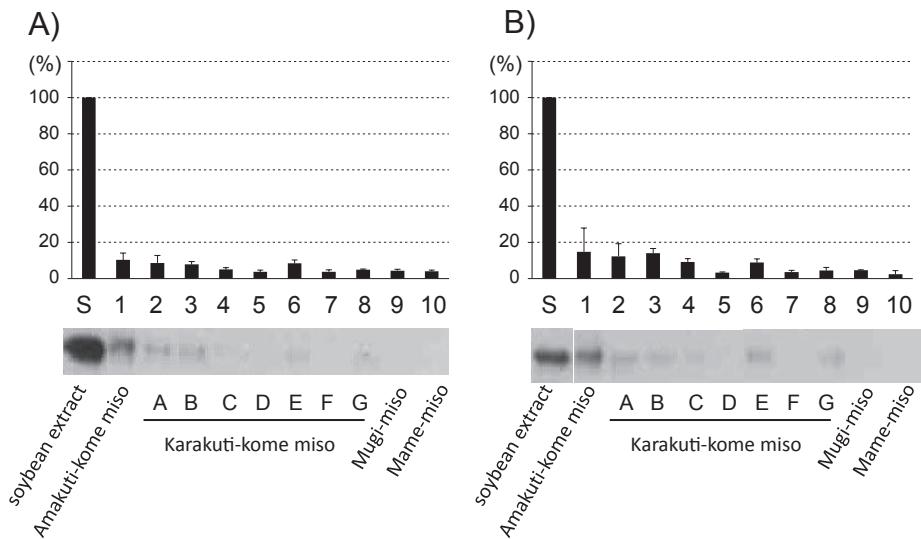


Fig. 3. Relative levels of Gly m Bd 30K and Gly m 28K allergens in soybean extract and miso samples. The soybean extract and indicated miso samples were subjected to SDS-PAGE and Western blot analysis using anti-Gly m Bd 30K (A) or anti-Gly m Bd 28K (B) antibodies. Signal intensities were measured as described in "Materials and Methods." Experiments were performed three times and the data are presented graphically, with bars representing means and error bars representing standard deviation (SD). S, soybean extract; 1–8, Karakuti-kome miso A–G; 9, Mugi-miso; 10, Mame-miso. The mean value of each miso was significantly different from the control (soybean extract) value ($p<0.001$).

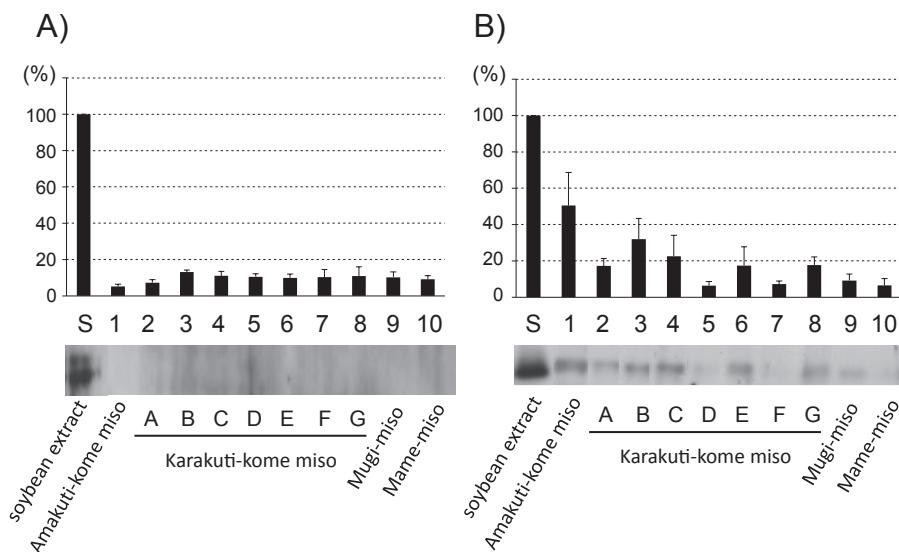


Fig. 4. Relative levels of 7S globulin and trypsin inhibitor allergens in soybean extract and miso samples. The soybean extract and indicated miso samples were subjected to SDS-PAGE and Western blot analysis using anti-7S globulin (A) or anti-trypsin inhibitor (B) antibodies. Experiments were performed three times and the data are presented graphically, with bars representing means and error bars representing SD. S, soybean extract; 1, Amakuti-kome miso; 2–8, Karakuti-kome miso A–G; 9, Mugi-miso; 10, Mame-miso. The mean value of each miso was significantly different from the control (soybean extract) value ($p<0.001$).

Statistical analysis. Data are presented as mean \pm standard deviation (SD) from three separate experiments. Statistical analysis of data was performed using Student's *t*-test. A difference was considered significant when $p<0.05$.

RESULTS

Appearance of miso paste samples

Several different kinds of miso paste were obtained from various manufacturers around Japan, including

an Amakuti-kome miso, a Mugi-miso, a Mame-miso, and 7 varieties of Karakuti-kome miso (A–G) (Table 1). The appearance of the miso pastes varied significantly (Fig. 1). The Amakuti-kome miso, Karakuti-kome miso A and B, and Mugi-miso, known to be fermented for relatively short periods (2 wk–3 mo), were light brown. In contrast, the Karakuti-kome miso D and F and Mame-miso, which are fermented for relatively long periods (3 mo–2 y), were dark brown (Fig. 1).

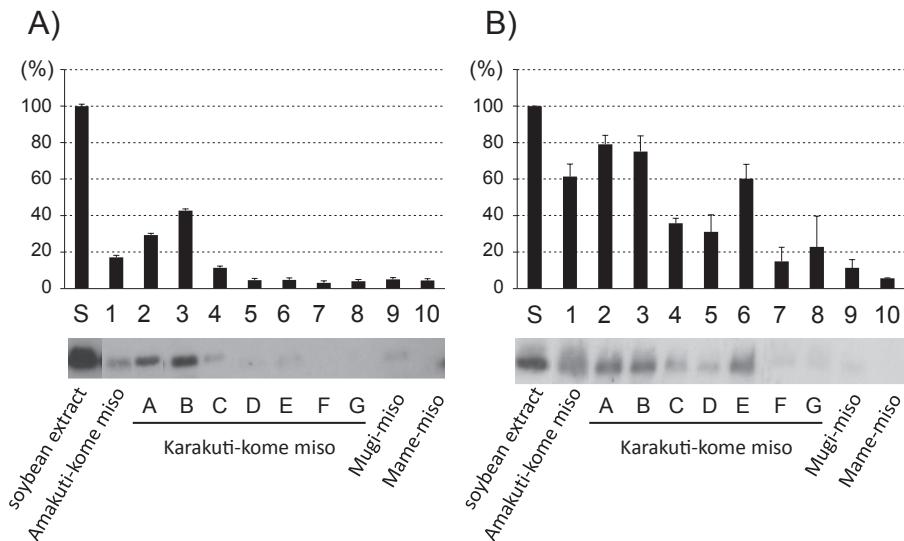


Fig. 5. Relative levels of Gly m 4 and Gly m 3 allergens in soybean extract and miso samples. The soybean extract and indicated miso samples were subjected to SDS-PAGE and Western blot analysis using anti-Gly m 4 (A) or anti-Gly m 3 (B) antibodies. Experiments were performed three times and the data are presented graphically, with bars representing means and error bars representing SD. S, soybean extract; 1, Amakuti-kome miso; 2–8, Karakuti-kome miso A–G; 9, Mugi-miso; 10, Mame-miso. The mean value of each miso was significantly different from the control (soybean extract) value ($p < 0.001$ for all samples in panel A and samples 4, 5, and 7–10 in panel B; $p < 0.05$ for samples 1, 2, 3, and 6 in panel B).

Miso protein profiles

To evaluate the protein profiles of the various miso pastes, protein patterns were analyzed by CBB staining after SDS-PAGE with 10 μ g of protein per lane. The miso protein patterns were observed to be quite different from those of the intact soybean protein extract. In particular, most of the protein bands observed in the soybean extract were absent in the miso profiles, which instead had low molecular weight smears at approximately 10–25 kDa (Fig. 2A). However, when a larger quantity of miso protein was loaded onto the gels (100 μ g of protein per lane), several intense protein bands were detected (Fig. 2B). The visible protein band patterns of the Karakuti-kome miso pastes (A–G) were similar to each other (Fig. 2B). For each of these misos, four major protein bands (approximately 18, 28, 34 and 50 kDa) were detected. In the Mugi-miso, four major protein bands (approximately 28, 36, 38 and 50 kDa) were observed that were not present in the Karakuti-kome lanes, and two of the Karakuti-kome protein bands (34 and 18 kDa) were not detected (Fig. 2B). In the Mame-miso, no obvious protein bands were observed, even when 100 μ g of protein was analyzed (Fig. 2B).

Allergen levels in each miso paste

The various soybean allergens were detected via Western blotting using specific antibodies in order to compare the levels of each allergen in the miso pastes and the intact soybean extract. For each allergen, the densities of the detected bands were semi-quantitatively determined and compared to the level in the soybean extract, which was set to 100%.

We first analyzed the levels of the Gly m Bd 30K allergen (Fig. 3A). Gly m Bd 30K is one of the major soybean allergens, and was identified by Ogawa et al. (15) as the major causative allergen of soybean allergy in infants.

The structure of Gly m Bd 30K is similar to that of cysteine protease, which belongs to a papain super family; however, because its active center cysteine is replaced with serine, this allergen does not possess enzyme activity. Interestingly, we found that the Gly m Bd 30K levels in all misos tested were lower than 10% of that in the soybean extract (Fig. 3A). We also assessed the levels of the Gly m Bd 28K soybean allergen, which was first reported by Tsuji et al. (14), and was later identified as a vicilin-like storage protein (16). Gly m Bd 28K levels were markedly reduced in the miso samples compared to the intact soybean extract, at less than 20% in all samples and less than 10% in Karakuti-kome miso D, E, F, G, Mugi-miso, and Mame-miso (Fig. 3B). Gly m Bd 28K was previously reported to be undetectable in miso (17).

Next, the levels of the 7S globulin (β -conglycinin) allergen in the various misos were analyzed (Fig. 4A). 7S globulin is known as a major storage protein in soybean seeds, and is composed of three subunits: α (~68 kDa), α' (~72 kDa), and β (~50 kDa). The 7S globulin α subunit was first identified as the allergen (18); however, the structural homology among these three subunits is relatively high (about 70–75%), and the α' and β subunits were later found to also be allergenic (19). It was important to include this protein in our assessment of the allergen content of miso, as this protein was recently reported to be responsible for FDEIA (Food-Dependent Exercise-Induced Anaphylaxis) caused by the processed soybean food tofu (20). Immunoblotting revealed that the soybean 7S globulin levels were similar across all miso types tested, and were notably reduced to less than 20% of the level in soybean extract (Fig. 4A). In contrast, soybean trypsin inhibitor, which was first reported as a soybean allergen in 1980 (21), was detected at relatively high levels in the various miso samples. In the

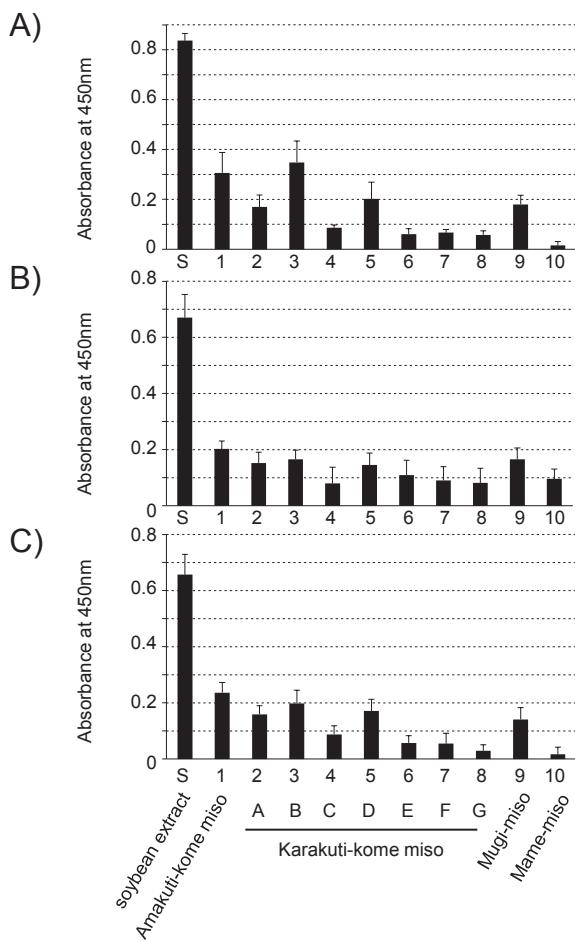


Fig. 6. IgE-ELISA using sera from soybean allergy patients. The binding capacities of serum IgE from three soybean allergy patients ((A) patient 1; (B) patient 2; (C) patient 3) were determined by IgE-ELISA as described in "Materials and Methods." The data represent absorbance at 450 nm resulting from the HRP-catalyzed enzyme reaction. Experiments were performed six times and the data are presented graphically, with bars representing means and error bars representing SD. The mean value of each miso was significantly different from the control (soybean extract) value ($p < 0.001$). S, soybean extract; 1, Amakuti-kome miso; 2–8, Karakuti-kome miso A–G; 9, Mugi-miso; 10, Mame-miso.

Amakuti-kome miso and Karakuti-kome miso A, B, C, E, and G, the levels were approximately 20–50% of that in soybean extract (Fig. 4B). However, in the Mugi-miso, Mame-miso, and Karakuti-kome miso D and F, the trypsin inhibitor levels were less than 10% of that in the soybean extract (Fig. 4B).

We also tested the miso levels of Gly m 4, a pollen-related soybean allergen described by Kleine-Tebbe et al. (22). This molecule is homologous to the Bet v1 protein, the major allergen of birch pollen, and has been indicated as a cause of pollinosis in patients who developed a soybean allergy (23). As shown in Fig. 5A, Gly m 4 allergen levels were considerably reduced in some of the Karakuti-kome misos (D, E, F, and G), and the levels in Mugi-miso and Mame-miso were less than 10% of that in the soybean extract. However, in the Karakuti-kome

miso A and B samples, Gly m 4 was present at relatively high levels (20–40%) (Fig. 5A). Somewhat similar results were obtained for Gly m 3, another pollen-related soybean allergen which is a homolog of the Bet v2 birch pollen allergen (23, 24) and belongs to the profilin pan-allergen family. This allergen remained at relatively high levels (60–80%) in Amakuti-kome miso and Karakuti-kome miso A, B and E. On the other hand, in Karakuti-kome miso F, Mugi-miso and Mame-miso, Gly m 3 levels were lower than 20% of that in the soybean extract (Fig. 5B).

IgE-ELISA analysis of miso samples using sera from soybean allergy patients

Although it is known that IgE reactivities might not reflect precise clinically relevant allergenicity, the in vitro reactivities of patients' IgE levels might be simple and valuable significant criteria for the evaluation of allergenicity. Therefore, the allergenicity of various kinds of miso was also evaluated by IgE-ELISA analysis using sera from 3 soybean allergy patients. The relative amounts of IgE that were bound to the soybean extract or miso samples were estimated by comparing absorbance at 450 nm (Fig. 6). We observed that the serum IgE from all soybean allergy patients bound to the soybean extract, whereas the absorbance values for the miso samples were all reduced compared to the soybean extract. For serum from patient 1 (Fig. 6A), the absorbance values for Amakuti-kome miso and Karakuti-kome miso B were relatively high (approximately 50% compared to the soybean extract). In contrast, those of Karakuti-kome miso C, E, F, G, and Mame-miso were less than 10% of soybean extract absorbance. Similar results were obtained using serum from patients 2 and 3 (Fig. 6B and C, respectively).

DISCUSSION

In this study, we prepared protein extracts from various kinds of miso manufactured in different regions of Japan. Miso is usually produced via traditional methods using ingredients suitable for the specific regional environment. In general, miso is made from soybeans and rice. However, some kinds of miso are made from soybeans and barley (Mugi-miso), or soybeans only (Mame-miso) (5). The most popular type of miso in Japan is Karakuti-kome miso, which is produced in various regions of Japan (5). Therefore, in the present study, we obtained 7 kinds of Karakuti-kome miso from distinct areas in Japan. The objective of the present study was to analyze and compare the presence of the major soybean allergens in miso, and to evaluate the hypoallergenicity of various kinds of miso paste manufactured in Japan.

By protein profile analysis, we determined that the protein band patterns of the various miso types were quite different from that of soybean extract. The major intact soybean protein bands were absent from the miso samples, and a smear of low molecular weight protein bands of approximately 10–25 kDa was enhanced (Fig. 2A). These proteins might be fragments of soybean proteins that are degraded during the fermentation process. Interestingly, some more distinct protein bands were

revealed after increasing the amount of total protein to 100 µg per lane during SDS-PAGE analysis (Fig. 2B). The band patterns did not differ considerably between different types of Karakuti-kome miso, whereas the patterns of Mugi-miso and Mame-miso protein bands differed significantly (Fig. 2B). It is possible that the protein bands specific to the kome-miso (34, 18 kDa bands) might be derived from rice, since rice is not used as an ingredient of Mugi-miso or Mame-miso (Table 1). Alternatively, the Mugi-miso specific protein bands (36, 38 kDa) might originate from its barley ingredient. Since soybeans are a common ingredient in all types of miso, it is likely that the commonly observed protein bands (28, 50 kDa) are soybean-derived protein fragments. However, the identification of these characteristic protein bands will require further study.

It is interesting that no distinct protein bands were observed for the Mame-miso (Fig. 2B). This may be due to the degradation of soybean proteins to low molecular weight protein fragments, peptides, and amino acids during the long fermentation periods used to produce Mame-miso (generally 6 mo–2 y). The color of miso results from amino carbonyl reactions (termed Maillard reactions) that occur when amino acids and sugars are combined during the fermentation and aging processes. Thus, a longer fermentation period generally produces a darker miso. Our results indicate that longer fermentation also results in extensive degradation of soybean proteins, suggesting that the darker misos may contain less intact soybean proteins.

The soybean allergen levels of various kinds of miso were evaluated using specific antibodies for major soybean allergens. In the case of Gly m Bd 30K, Gly m Bd 28K and 7S globulin (Fig. 3A, B and Fig. 4A), the allergen levels were less than 20% of those in soybean extract in all miso types tested, suggesting that these allergens were relatively easily degraded during fermentation. Tsuji et al. reported the fate of Gly m Bd 30K allergen in various kinds of miso. They found that Gly m Bd 30K allergen in three types of miso (Kome-miso, Mugi-miso and Mame-miso) was rapidly digested as fermentation proceeded (9). Our results supported their observations.

However, in the cases of trypsin inhibitors, Gly m 4, and Gly m 3, the allergen levels were relatively high, particularly in the Amakuti-kome miso and some of the light-colored types of Karakuti-kome miso (A and B). The molecular weights of trypsin inhibitor, Gly m 4, and Gly m 3 are relatively low, at 18, 17, and 14 kDa, respectively (Table 2). These low molecular weight soybean allergens might be resistant to proteolytic digestion during fermentation, particularly for short-fermentation types of miso. Trypsin inhibitor contains an especially high amount of S-S bondages. These conformational properties of allergens may influence the digestive potencies (25). Shibata et al. also reported that trypsin inhibitor still remained in the final products of Edo-ama miso, a kind of the Amakuti-kome miso (8).

The allergen levels in the Mame-miso were very low (lower than 10% of those in soybean extract for all allergens tested). This result is consistent with the low levels

of intact proteins observed for Mame-miso in the protein profiling experiments, and might also be due to the long fermentation periods used in Mame-miso production.

Interestingly, the results obtained from IgE-ELISA using patient sera were quite similar to the results obtained from Western blotting using specific antibodies to the major soybean allergens (Fig. 6). The sera from the three different patients produced similar results, and the IgE-binding allergenicity of Mame-miso was consistently low. It was also observed that the allergenicity of the dark-brown colored misos tended to be lower than that of light-yellow colored misos (Figs. 1 and 6). Although the causative soybean allergens of these patients were not identified, these similar results suggest that the combined use of specific antibodies against major soybean allergens might be useful for the evaluation of clinically relevant hypoallergenicity. In this study, we used three patients' sera, these sera were obtained from IgE-RAST positive-patients towards soybean as suppliers tested. For further studies, more sera from soybean allergy patients should be tested.

It has been reported that the addition of exogenous proteolytic enzymes might help the reduction of allergens in miso (8, 26). Therefore, the combination of addition of exogenous enzymes and elongation of fermentation periods might be useful methods for production of hypoallergenic miso.

In conclusion, this study demonstrated that the allergenicity of soybeans might be reduced by the fermentation process of miso production. We found that Mame-miso and certain types of Karakuti-kome miso were highly hypoallergenic compared to the Amakuti-kome miso and Mugi-miso. The fermentation periods of these hypoallergenic misos are probably relatively long, suggesting that the length of fermentation period may influence the allergenicity of miso. Further allergenic risk assessments of the various types of miso are required to clarify the effects of fermentation time on miso allergenicity.

Finally the hypoallergenicity of these miso pastes should be confirmed by a double-blind, placebo-controlled clinical challenge test in future studies. These studies may help to determine the production conditions needed to produce more hypoallergenic varieties of miso.

Acknowledgments

This work was supported by grants from the Central Miso Research Institute and in part supported by the Strategic Project to Support the Formation of Research Bases at Private Universities: Matching Fund Subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2011–2015 (S1101035).

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