Research Article

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Analysis of soybean miso using koji mold isolated from the leaves of *Rhus javanica* Nurude

Abstract

The authors isolated koji mold from the leaves of Nurushi (*Rhus javanica*: hereafter referred to as "Nurude") and used this koji mold to produce soybean miso. The general composition of the soybean miso was made was measured and found to be as follows: moisture content 45.2 g/100g, protein content 16.0 g/100g, fat content 8.6 g/100g, ash content 19.0 g/100g, and carbohydrate content 11.2 g/100g.

Although this soybean miso had a short maturation period of only three months, it had a high protein content and a high glutamic acid content of approximately 230 mg/100g, and its color suggested that it was sufficiently matured. Compared to soybean miso listed in the Standard Tables of Food Composition in Japan, the mineral content of the Nurude soybean miso was higher in magnesium, zinc, and copper. When the titration acidity was measured, acidity I was 10.00 and acidity II was 11.00, suggesting high content of both organic acids and amino acids. The polyphenol content of the Nurude soybean miso was 595 mg/100 g, and its antioxidant activity by the DPPH radical scavenging method was 212 µmol Trolox/100 g.

Keywords: *Rhus javanica*, Nurude, soybean miso, koji mold of Aspergillus oryzae, fermentation, food, functionality

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Introduction

In Japan, a document describing the production of hishio (fermented soybean miso) from the leaves of urushi (*Rhus javanica*: hereafter referred to as "Nurude") was discovered in a temple.[1-3] This document dates of the Kamakura period and describes how to make hishio. Based on this, koji mold was isolated from Rhus javanica and soybean miso was produced.

Nurude is a member of the Rhus genus in the Anacardiaceae family, and is also known as Katsunoki, Kachinoki, and Fushinoki.[4] This Nurude is a deciduous shrub distributed throughout Japan. Its leaf axis has wings, and the galls that grow on the leaves contain a lot of tannin, so it was used as a medicinal herb and for dyeing. In some regions, it was used as a charm against evil in ancient societies and was revered as a tree with mysterious powers to protect against illness and disaster.[5]

Natural koji mold is attached to the leaves of Nurude, and it has been used in the production of miso and soy sauce since ancient times. Currently, miso and soy sauce are not produced using lacquer tree leaves. While the "Illustrated Guide to Wild Trees"[6] mentions that koji mold can adhere to Nurude leaves, this is the only document mentioning it; there are no other documents describing the production of miso or soy sauce using Nurude leaves.

The authors isolated koji mold using Nurude leaves and used it to produce soybean miso. Previously[7] only reported the general components of the produced miso; this time, we measured and report on the functional components.

Material and methods

Materials

The Nurude leaves used to isolate the koji mold were collected from Nurude trees wild on the Atsugi Campus of Tokyo University of Agriculture (Figure 1). The soybeans used as the material for the miso were Tsukui Zairai soybeans from Atsugi City, Kanagawa Prefecture.

Figure 1 *Rhus javanica* "Nurude".

Production of soybean miso

The koji mold was isolated from Nurude leaves. The Nurude leaves were lightly washed with water and soaked in Physiological saline solution (0.9%NaCl solution) for 20 hours (Figure 2). The leaves were placed on a standard agar medium (meat extract, peptone, sodium chloride, agar, pH and cultured at 35°C for 4 days (Figure 3). The Aspergillus oryzae was identified based on the morphology of the cells and sexual spores. The isolated Aspergillus oryzae were tested and confirmed not to produce aflatoxin.

Figure 2 *Rhus javanica* "Nurude"leaves.

(Soaked in physiological saline:0.9%NaCl solution)

Figure 3 Koji mold Aspergillus oryzae: isolated from Nurude.

Steamed soybeans (500g) were inoculated with isolated koji mold spores, mixed well, and cultured at 35°C for two days to produce soybean koji. 200g of salt was mixed with this soybean koji to make salt koji (Figure 4). This was minced, and 500g of minced steamed soybeans were added and mixed well to make a total of 1200g. The mixture was packed tightly into a container to prevent air from entering and aged at 35°C for three months to produce soybean miso (Figure 5).

Figure 4 A mixture of soybean koji, steamed soybeans and salt.

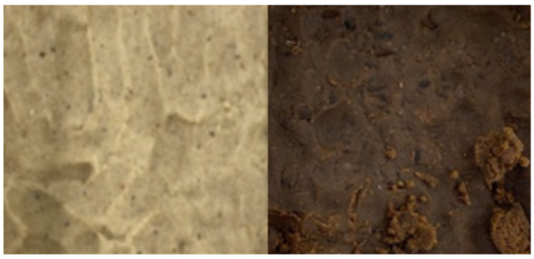

Figure 5 Nurude soybean miso.

Right: Immediately after production, Left: After 3 months of maturation (35℃).

General component analysis of soybean miso

The general analysis of soybean miso was performed in accordance with the measurements in the Standard Tables of Food Composition in Japan. Moisture[8] was measured using the atmospheric pressure heating and drying method, protein[9] using the Kjeldahl nitrogen method, lipid[10] using the Soxhlet extraction method, and ash[11] using the direct ash method at 550 °C. Each test used a precisely weighed 2g of miso. Carbohydrate[12] was calculated using the subtraction method, by subtracting moisture, protein, lipid, and ash from 100.

Measurement of pH and acidity

pH was measured using a pH meter (manufactured by Horiba Co., Ltd.),[13] and acidity was measured by titration[14] according to the standard miso analysis method included in the Miso Technology Handbook.

Measurement of mineral content

Potassium, calcium, magnesium, phosphorus, iron, and zinc in the sample were analyzed by atomic absorption spectrometry (Shimadzu AA-6300).[15] The sample was prepared by dissolving ash obtained by direct ash in a 0.1 M hydrochloric acid solution. Sodium (589.0 nm), calcium (422.7 nm), magnesium (285.2 nm), iron (248.3 nm), zinc (213.9 nm), and copper (324.8 nm) were measured at standard wavelengths, and a calibration curve was created for quantification.

Measurement of glutamic acid content

Glutamic acid content was measured using the L-glutamic acid measurement kit "Yamasa NEO".[16] For sample preparation, 5g of miso was added to 50 ml of water, stirred in a boiling bath for 60 minutes, then brought to a final volume in a 100 ml volumetric flask, and the supernatant was used after centrifugation at 10,000 r.p.m. for 10 minutes.

Measurement of polyphenol content

Polyphenol content was measured by the colorimetric quantitative method using the Forin-Dennis method.[17] Sample preparation was carried out in the same manner as for glutamic acid measurement, and the supernatant after centrifugation at 10,000 rpm for 10 minutes was used. 1ml of Forin-Dennis reagent was added to 1ml of sample and allowed to stand for 3 minutes. Then, 1 ml of 10% sodium carbonate solution was added and allowed to stand for 30 minutes. The absorbance of this solution was measured at 700 nm. Water was used as the blank instead of the sample. A calibration curve was created using gallic acid as the standard substance, and measurements were taken.

Measurement of antioxidant activity by 1-dipheny-2-picrylhydrazyl (DPPH) radical scavenging ability

Antioxidant activity was investigated by measuring the radical scavenging ability of DPPH.[18] Sample preparation was carried out in the same manner as for glutamic acid measurement, and the supernatant after centrifugation at 10,000 rpm for 10 minutes was used. A mixture of 12ml of 400 µM DPPH, 12 ml of 200 mM MES buffer (pH 6.0), and 12 ml of 20% (V/V) ethanol was prepared. 0.9 ml of this mixture was mixed with 0.3 ml of the sample solution diluted with 80% (V/V) ethanol, and after a reaction of 20 minutes, the reaction solution was measured at 520 nm. Sample solutions diluted with 80% ethanol were prepared at several dilutions, and the absorbance reduction rate was determined. A calibration curve was created using Trolox, and antioxidant activity was calculated using the Trolox conversion method.

Molecular weight distribution by electrophoresis (SDS-PAGE)

Electrophoresis was performed using a compact PEGE (AF-7300, ATTO Corporation). Electrophoresis was performed by adding electrophoresis buffer (tris (2-carboxyethyl) phosphine hydrochloride) to the electrophoresis apparatus, setting a 10% gel, adding the sample with marker dye (BPB), and performing electrophoresis.[19] After electrophoresis was completed, the gel was removed and immersed in Coomassie Brilliant Blue staining solution for 30 minutes, and then destained by immersion in a destaining solution.

Color measurement

After manufacturing the Nurude soybean miso, the color tone was measured. The color tone was measured using a colorimeter (Konica Minolta CR-400 spectrophotometer) for L* (lightness), a* (green < red), and b* (blue < yellow).

Results and discussion

General food components of nurude soybean miso

The results of the analysis of the general food components of Nurude soybean miso were reported in the previous report,[7] but here again we present the results of the general food analysis of the Nurude soybean miso was manufactured (Table 1).

Table 1 General food components of Nurude soybean miso (g/100g miso)

	Water	Protein	Fat	Carbohydrate	Ash
Nurude soybean miso	45.2	16.0	8.6	11.2	19.0
Soybean miso*	44.9	17.2	10.5	14.5	12.9

*Standard Tables of Food Composition in Japan, 2020 (Eight Revised Edition).

The moisture content of Nurude soybean miso was 45.2 g/100 g, which is higher than the average value of 43.0 g/100 g for miso at the National Miso Competition and the value from the Standard Tables of Food Composition in Japan of 44.9g/100g. Generally, soybean miso is hard and difficult to dissolve, but this miso was high in moisture and easily dissolved.

The protein content was 16.0 g/100 g, which is about 1g less than the average value of 17.0g/100 g at the National Miso Competition[20,21] and the value from the Standard Tables of Food Composition in Japan[22] of 17.2 g/100 g. This was thought to be due to the nitrogen content of the soybeans used as raw material.

The fat content was 8.6g/100g, which was lower than the average value at the National Miso Tasting Competition[20,21] and the Standard Tables of Food Composition in Japan[22] of 10.5 g/100 g. The low-fat content suggests that a low-calorie miso was produced.

The ash content was 19.0 g/100 g, which was approximately 1.5 times higher than the average value at the National Miso Tasting Competition and the Standard Tables of Food Composition in Japan[22] of 12.9 g/100g. This result suggests that a mineral-rich miso was produced.

The carbohydrate content was 11.2 g/100g, which was approximately 0.7 to 0.8 times lower than the average value at the National Miso Tasting Competition of16.6g/100g and the Standard Tables of Food Composition in Japan of 14.5 g/100g.

While miso is generally aged for six months to a year, this miso was aged for a very short period of three months. Despite this, its composition was not significantly different from that of ordinary miso, confirming that the koji mold had effectively broken down the raw materials.

pH and acidity of nurude soybean miso

The pH of the Nurude soybean miso was 5.32. The pH immediately after preparation was 6.31, and the pH decreased with maturation (Table 2). This suggests that the production of glutamic acid and organic acids during maturation led to the decrease in pH.

The Nurude miso produced had a higher pH value compared to the soybean miso from the National Miso Competition, which had a pH of 4.93. The maturation period in this experiment was three months, but it was inferred that longer maturation would lead to a shift towards acidity.

Acidity I includes organic acids and some acidic amino acids, while acidity II includes neutral and basic amino acids, peptides, and some phosphates. Acidity I immediately after preparation was 3.10, but after three months of maturation it increased to 10.00, approximately 3.2 times, suggesting an increase in organic acids, etc. Acidity II also increased from 8.1 to 11.0, approximately 1.4 times, suggesting an increase in amino acids and peptides (Table 2).

Table 2 pH and acidity of Nurude soybean miso

	pH	Acidity I	Acidity II
Immediately after preparation	6.31	3.10	8.10
After 3 months of maturation	5.32	10.00	11.00

Based on pH data from commercially available miso, Nurude soybean miso is classified as a sweet miso, and based on the acidity levels I and II, it is classified as a red spicy miso.[23]

Mineral content of nurude soybean miso

The mineral content of Nurude soybean miso was measured, and the results showed that sodium was 4,700 mg/100 g, potassium was 640 mg/100 g, calcium was 140 mg/100 g, magnesium was 287 mg/100 g, iron was 4.8 mg/100 g, zinc was 2.4 mg/100 g, and copper was 0.90 mg/100 g (Table 3). Compared to the values for soybean miso in the Standard Tables of Food Composition in Japan, the content of magnesium, zinc, and copper was higher.

Table 3 Mineral content of Nurude soybean miso

	Na	K	Ca	Mg	Fe	Zn	Cu
Nurude soybean miso	4700	640	140	287	4.8	2.4	0.90
Soybean miso*	4300	930	150	130	6.8	2.0	0.66

**Standard tables of food composition in Japan, 2020 (Eight Revised Edition).

In particular, the magnesium value was more than twice as high, suggesting that Nurude soybean miso have various functions, such as being involved in cellular energy metabolism and protein synthesis, when used in food.

Glutamic acid content of nurude soybean miso

The glutamic acid content of the Nurude soybean miso was approximately 230 mg/100g. The glutamic acid content of miso varies depending on the manufacturing conditions, and the glutamic acid content of general bean miso ranges from 87 mg to 329 mg.[24] Compared to 83.96 mg/100 g immediately after preparation, it increased by approximately 2.7 times after 3 months of maturation, suggesting an increase in umami components (Table 4).

Table 4 Glutamic acid content of Nurude soybean miso (mg/100g)

Immediately after preparation	83.96
After 3 months of maturation	230.18

Polyphenol content of nurude soybean miso

The polyphenol content was 189 mg/100g immediately after preparation but increased to 595 mg/100 g after 3 months of maturation, approximately three times the original amount (Table 5). It is thought that the Nurude soybean miso, being made from soybeans, contains flavonoids including isoflavones, and that the polyphenol content increased due to reactions between substances produced during maturation.

Table 5 Polyphenol Content of Nurude soybean miso (mg/100g)

Immediately after preparation	189
After 3 months of maturation	595

Antioxidant activity to DPPH radical scavenging ability of nurude soybean miso

Measurements of antioxidant activity due to DPPH radical scavenging ability showed that immediately after preparation, the value was 105 µmol Trolox/100 g, but after 3 months of maturation, it increased to 212 µmol Trolox/100g, approximate doubling (Table 6). The authors had previously manufactured rice miso using commercially available koji mold and measured its antioxidant activity using the same method, and the result was 158 µmol Trolox/100 g, indicating that the original miso had higher antioxidant activity.[25] There is a correlation between antioxidant activity and polyphenol content, and it is thought that the increase in polyphenols generated during maturation led to an increase in antioxidant activity.

Table 6 Antioxidant Activity t of Nurude soybean miso* (µmol Trolox/100g)

Immediately after preparation	105
After 3 months of maturation	212

*DPPH Radical Scavenging Ability

The miso has been confirmed to have antioxidant activity in other reports, and there is a positive correlation between myocardial damage and tissue reactive oxygen species concentration in salt-sensitive hypertension, and it is known that the reactive oxygen species scavenging ability of miso is related to the protection of blood vessels and organ function.

Molecular weight distribution of nurude soybean miso

In the water-soluble fraction of Nurude soybean miso, the molecular weight distribution was examined, and the main electrophoretic bands were observed around 40,000, 32,000, 24,000, and 14,000. Commercially available bean miso showed main bands around 66,000, 45,000, 38,000, 30,000, 24,000, and 14,000, differing from Nurude soybean miso and showing many more bands (Figure 6). Furthermore, a concentrated band was observed around 24,000 in commercially available bean miso, and numerous bands were observed below 20,000, suggesting a high content of low-molecular-weight peptides. Additionally, the differences in the molecular weight distribution of the bands suggest that the cleavage sites of the Aspergillus oryzae enzymes differ.

Since the Nurude soybean miso was aged for a short period of three months, it is thought that there were few low-molecular-weight peptide bands, and it is possible that more peptides could be obtained by extending the maturation period. From these results, it can be inferred that Nurude miso has less umami flavor compared to commercially available miso.

Color tone of nurude soybean miso

The color of the Nurude soybean miso showed a decrease in the brightness (L* value) and an increase in the red (a* value) after three months of maturation. The L* value was less than half of what it was immediately after preparation, while the a* value was more than three times higher. In addition, the yellow (b* value) decreased, becoming approximately half after three months of maturation, confirming that the yellow color of the soybeans changed due to maturation (Table 7).

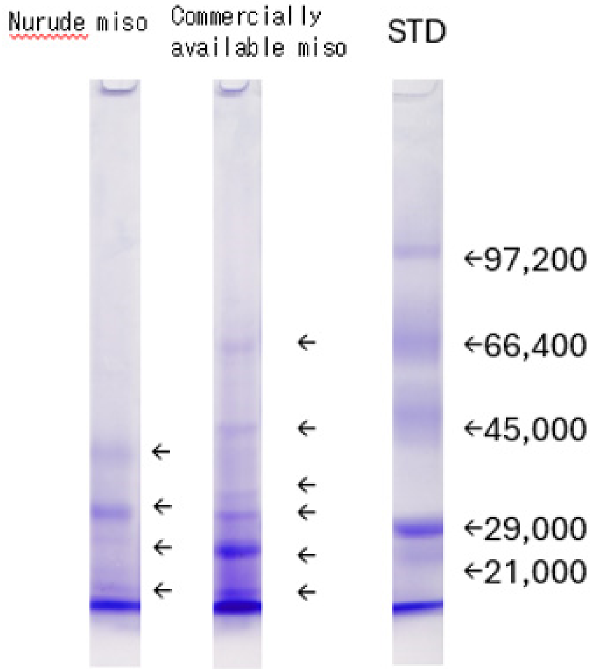

Figure 6 SDS-PAGE of the water-soluble fraction of Nurude soybean misoq

Table 7 Color Tone of Nurude soybean miso

	L*	a*	b*	470nm
Immediately after preparation	65.6	4.0	25.1	0.23
After 3 months of maturation	29.1	12.4	12.4	0.34

From these results, it was concluded that Nurude miso turned reddish-brown after 3 months, indicating that browning had progressed and maturation had advanced.

Conclusion

We were able to isolate the koji mold Aspergillus oryzae from Nurude leaves and produce soybean paste.

A general component analysis of the produced soybean miso revealed its moisture content of 45.2g/100 g, protein content of 16.0 g/100g, the fat content of 8.6 g/100g, the ash content of 19.0g/100g, and the carbohydrate content of 11.2 g/100g. This soybean miso was sufficiently matured, and despite a very short fermentation period of only three months, the raw materials had matured considerably.

The glutamic acid content was approximately 230 mg/100g, and the color indicated sufficient maturation. The mineral content of the Nurude soybean miso was higher than that of soybean miso listed in the Standard Tables of Food Composition in Japan, with higher levels of magnesium, zinc, and copper, confirming its functional properties.

Acidity I was 10.00 and acidity II was 11.00, indicating the presence of both organic acids and amino acids, like those of red spicy miso. The Nurude soybean miso contained 595 mg of polyphenols per 100 g, and its antioxidant activity, measured by the DPPH radical scavenging method, was 212 µmol Trolox per 100g, demonstrating its high functionality.

The koji mold isolated from Nurude decomposed and fermented the soybeans, confirming that Nurude leaves can be used in miso production. In the future, we plan to investigate the properties of the koji mold isolated from Nurude leaves, including its enzyme activity.

Furthermore, we intend to produce miso using the same raw materials with several commercially available koji molds for soybean miso, and compare the components of the miso made with the koji mold isolated from Nurude leaves to further clarify the characteristics of the miso.

Currently, miso production uses koji mold spores that are under controlled conditions, but we believe that miso can be produced overseas if Nurude plants are available. Japanese food is gaining global attention, and miso has been used in traditional Japanese cuisine for centuries. While miso is used in popular Japanese dishes such as miso soup and miso ramen, producing miso overseas is extremely difficult because koji mold cannot be exported. Since the Nurude plant grows wild not only in Japan but also overseas, we believe there is potential to produce miso using Nurude. In the future, we also plan to produce rice miso using Nurude leaves.

Acknowledgments

None.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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