IRRADIATION OF SOUND WAVES TO ASPERGILLUS KAWACHII AND THE CHARACTERIZATION OF ENZYME ACTIVITIES IN RICE-KOJI

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ABSTRACT

Conidiospores of *A. kawachii* were irradiated with sound waves at three different frequencies, 1.0, 6.3, and 16.0 kHz, with a constant sound pressure of 5.0 dB. Steamed rice was inoculated with the resulting conidiospores and incubated at 25°C for 30 h to make rice-*koji*. By irradiation with sound waves at 6.3 kHz to conidiospores of *A. kawachii*, the activities of glucoamylase and acid protease of rice-*koji* were increased by 1.33 and 1.37 times, respectively, over that of rice-*koji* prepared without sound wave irradiation. The amount of mycelium in rice-*koji* prepared with conidiospores treated by sound wave irradiation at 6.3 kHz was also 1.31 times higher than that prepared without sound wave irradiation. To confirm the usefulness of sound wave irradiation as a condition for making rice-*koji*, the effects of sound waves on enzyme activities and mycelium weight of rice-*koji* produced by sound-treated conidiospores of *A. kawachii* were investigated.

Keywords: *Aspergillus kawachii*, rice-*koji*, sound wave, glucoamylase, acid protease.

INTRODUCTION

In Asian countries, a microbial starter called *koji*, which is prepared with various microorganisms, is an important saccharifying agent in the production of fermented foods [1]. *A. kawachii*, a natural mutant of *A. luchuensis*, produces large amounts of hydrolases, such as α-amylase and glucoamylase. *A. kawachii* is used in the production of *shochu*, traditional Japanese distilled spirits, as a saccharifying agent [1]. Further, this *A. kawachii* produces large amounts of citric acid, which maintains the acidic condition of s*hochu* fermentation mash resistant to microbial contamination. Temperature, humidity, and oxygen are key factors affecting the quality of rice-*koji* [2]. However, sound waves have not been reported as an important factor. For bacteria and plants, sound waves have also been reported to affect the quality. For example, bacteria such as *Staphylococcus* species showed high resistance against antibiotics such as ampicillin by irradiation with low-frequency noise [3]. In addition, many reports that relate to sound wave have been published. Sound waves have been used in various processes, including the aging of spirits [4], particularly *shochu*, the cultivation of fruits and vegetables, and even the breeding of cattle. Although the relationship between sound waves and *Aspergillus* species has not been reported; however, in our previous study, we found that glucoamylase activity decreased at the irradiation frequency of 6.3 kHz, and the acid protease activity increased at the irradiation frequency of 1.0 kHz while making the rice-*koji* of *A. oryzae* [4]. That was the first report concerning the relationship between sound waves and *A. oryzae*. However, the mechanism of response to sound waves in *Aspergilli* was unknown. This is because the amount of rice is large, and it is difficult to irradiate the sound wave evenly.

In this study, the method of sound wave irradiation was improved. Before making rice-*koji*, conidiospores of *A. kawachii* were irradiated with sound waves. The conidiospores of *A. kawachii* were then used for comparing with the enzyme activities of *A. oryzae* rice-*koji*. A possibility that the sound waves irradiation could be an important condition for making *koji* was investigated.

EXPERIMENTAL PROCEDURES

Preparation of rice-*koji*

A microbial starter for brewing called *Tane-koji* of *A. kawachii* was purchased from Kawachi Genichiro Shoten Co., Ltd. (Kagoshima, Japan). Conidiospores that were used for rice-*koji* making were prepared to separate from *Tane-koji* using a wire sieve, and 1.0 g of conidiospores were aliquoted in a 50 ml flask with wet-filter paper on the bottom. Then the entire flask was covered with aluminum foil for shade. Audio generator MINIRATOR MR2 (NTi, Tokyo, Japan) was used to generate sound waves. The power level of the audio generator was kept at around 5.0 dB. An earphone for the output of sounds was connected to the head of the flask with vinyl tape. Sound waves at 1.0, 6.3, and 16.0 kHz were irradiated at 25°C for 24 h. Polished rice (*Oryza sativa* var. *Japonica* cv. *Hinohikari*) that was cultivated in Kumamoto Prefecture, Japan, was purchased. For the control experiment, conidiospores incubated at 25°C with 0 dB, that is no sound, were prepared.

One hundred grams of raw rice was aliquoted in a 300 ml flask containing 30 ml of distilled water and steamed at 120°C for 15 min using an autoclave (TOMY, Tokyo, Japan) to make steamed rice. After decreasing the temperature in the flask to 40°C, the steamed

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rice was transferred to a sterile steel box and mixed with 0.01 g aliquots of sound-irradiated conidiospores. The resultant mixture was divided into two glass Petri dishes with filter paper on the lids. The rice-*koji* was produced through incubation at 30°C for 30 h.

Extraction of glucoamylases and acid proteases from rice-*koji*

Ten g of rice-*koji* was soaked in a 50 ml sodium chloride solution that included a 0.2 M acetic acid buffer (pH 5.5) at 5°C for 18 h. The resultant extract was filtered and dialyzed against 1 L of 0.01 M acetic acid buffer (pH 5.5) at 5°C for 24 h to remove the sugars or amino acids. This extract was diluted twofold with distilled water.

Measurement of the glucoamylase activity of rice-*koji*

Assays of glucoamylase and acid protease followed the methods described in the official methods of the National Tax Administration Agency of Japan for the Evaluation of Content [5]. One hundred µl of extracts was added to 1.0 ml of 2.0% soluble starch (Nacalai Tesque, Kyoto, Japan) and incubated at 40°C for 20 min. One hundred µl of the mixture was added to 3.0 ml of quantitative glucose reagent (FUJIFILM Wako, Osaka, Japan) and was incubated at 40°C for 30 min. The absorption was measured at 505 nm. A standard curve was created in advance with glucose solution, and its concentration was adjusted.

Measurement of acid-protease activity of rice-*koji*

Five hundred µl of extracts was added to 1.0 ml of 2.0% casein solution and incubated at 40°C for 20 min. The enzyme reaction was stopped by adding 3.0 ml of 0.4 M trichloroacetic acid. The mixture was filtered, and filtrates were recovered. One hundred µl of this filtrate was added to 5.0 ml of a 2.2 M sodium carbonate solution and 1.0 ml of Folin-Ciocalteu Reagent (Nacalai Tesque) and incubated at 40°C for 30 min. The absorption was measured at 660 nm. A standard curve was created in advance with tyrosine solution, and its concentration was adjusted.

Determination of the mycelium weight of *A. kawachii* cells in rice-*koji*

Procedures in the previous report [6] were followed. Five g of rice-*koji* was dried at 120°C for 15 min and ground in a mortar. Two g of ground rice-*koji* was weighed accurately and suspended with 10 ml of 50 mM phosphate buffer. This suspension was centrifuged at 3.000 rpm for 10 min. The supernatant was discarded, and 10 ml of 50 mM phosphate buffer (pH 7.0) was added again and centrifuged at 3.000 rpm for 10 min. These processes were repeated four times to elute out the ingredients. Ten mg of Yatalase (Takara Bio, Kusatsu, Japan), which catalyzes the degradation of the cell wall, was added to the suspension, and it was incubated at 37°C for 4 h with shaking. This mixture was centrifuged, and the resultant supernatant that contains degraded cell wall products were recovered. Five hundred µl of enzyme reaction solutions was added to 0.1 ml of 0.8 M boric acid buffer and was incubated in boiled water for 3 min. Three ml of 10%(v/v) of *p*-Dimethyl-amino-benzaldehyde was added and incubated at 37°C for 20 min. The N-acetyl-D-glucosamine that was produced by the degradation of the cell wall was measured at 585 nm. A standard curve was created in advance with an N-acetyl-D-glucosamine solution, and its concentration was adjusted. The growth level indicated the mycelium weight contained in 1.0 g of rice-*koji*, defined as (mg/g).

Determination of protein contents in extracts from rice-*koji*

The contents of protein and extracts of rice-*koji* were measured in accordance with the Folin-Lowry method [7]. Five hundred µl of extracts were added to 5.0 ml of alkaline copper reagent (Nacalai Tesque) and left for 40 min. Five hundred µl of Folin-Ciocalteu Reagent (Nacalai Tesque) was added and left for 30 min in the dark. The produced amino acid residues that corresponded to tyrosine and tryptophan were measured at 500 nm and 750 nm, respectively. A standard curve was created in advance with a solution of bovine serum albumin (Sigma Aldrich Co., MO, USA), and its concentration was adjusted.

Determination of amino acid contents in extracts from rice-*koji*

The contents of amino acids in the extracts of rice-*koji* were measured in accordance with amino acid colorimetric quantification [8]. Two ml of extracts were added to 2.0 ml of ninhydrin reagent, and it was incubated in boiling water for 15 min. Three ml of 50% ethanol was added, and it was incubated for 10 min. The produced amino acid residues that corresponded to the primary amine and proline were measured at 440 nm and 570 nm, respectively. A standard curve was created in advance with a solution of leucine (Nacalai Tesque), and its concentration was adjusted.

Statistical significance test

The significant difference between the sound and no-sound conditions was examined by a statistical significance test (T-test) [9]. Each value is presented as the mean ± standard deviation of three independent experiments (n = 3). The error range was calculated from the standard deviation of three samples. A significant difference was recognized when the one-sided test was less than 0.05 ($P < 0.05$). In the table, the changing ratio was indicated as below, the HIGH indicated that a significant difference was observed ($P < 0.05$), and the changing rate was 1.1 higher than the control. The LOW indicates that a significant difference was observed, and the changing rate was 0.9 less than that of the control. + indicates that a significant difference was not observed, and the changing rate was 1.1 higher than that of the control. – indicates that a significant difference was not observed, and the changing rate was 0.9 less than that of the control. ± indicates that a significant difference was not observed, and the changing rate was 1.1 less than that of the control or more than 0.9 higher than that of the control. Then, the activities of each rice-*koji* enzyme were quoted from our previous report [4].

RESULTS AND DISCUSSION

In this study, experiments were performed to prove that sound waves are an important environmental factor influencing the enzyme activities of rice-*koji*. To identify the effects of three kinds of frequencies, 1.0, 6.3, and 16.0 kHz, that had been picked up as previously reported [4], rice-*koji* was produced with conidiospores *of A. kawachii* irradiated by sound waves.

Effects of sound waves on glucoamylase activity

The glucoamylase activity of rice-*koji* produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 204 ± 43.6, 306 ± 40.3, and 220 ± 32.1 U/g *koji*, respectively. In the absence of sounds, the activity was 230 ± 15.3 U/g *koji* (Figure 1). The frequency of 6.3 kHz was 1.33 times higher than that of control, and a significant difference was observed ($P < 0.05$). At frequencies of 1.0 and 16.0 kHz, its enzyme activities were just a little (0.89 and 0.96, respectively) less than that of the control. Therefore, a significant difference was not observed (Figure 1). As compared with the glucoamylase activity of *A. oryzae* [4], both glucoamylase activities were found to be affected by a frequency of 6.3 kHz. The glucoamylase activity of rice-*koji* from *A. kawachii* was increased, and that from *A. oryzae* was decreased. From these results, it was determined that even the same *Aspergilli* showed different responses for each species. It is noted that the conditions for making rice-*koji* described in Saigusa et al. [4] were different from those in our experiment. To make conditions the same, rice-*koji* was produced by conidiospores *of A. oryzae* in the same conditions, and its enzyme activities were compared with our results of *A. kawachii*.

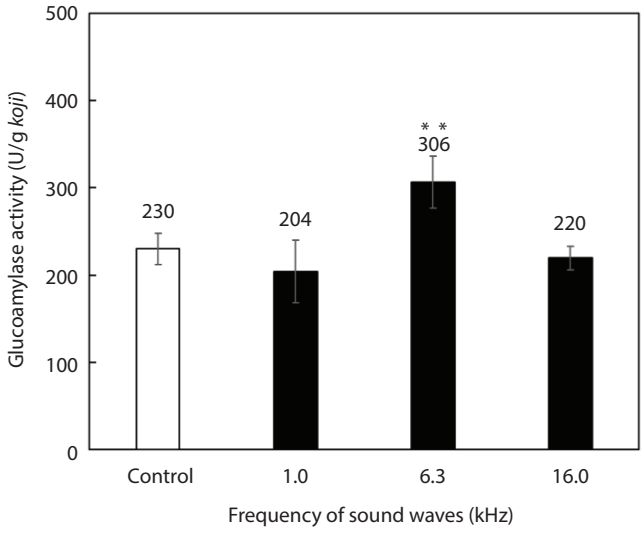

Figure 1 Effects of the sound wave frequency on the glucoamylase activity of rice-*koji*

Irradiating sound waves showed different results as compared with those of our previous report [4]. The glucoamylase activity of rice-*koji* of *A. oryzae* produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 459 ± 42.2, 423 ± 18.4, and 296 ± 16.5 U/g *koji*, respectively. In the absence of sound waves, the activity was 389 ± 26.5 U/g *koji*. At frequencies of 16.0 kHz, its enzyme activities were 0.68 less than that of the control, and a significant difference was observed ($P < 0.05$). At frequencies of 1.0 and 6.3 kHz, its enzyme activities were just a little (1.18 and 1.09 times, respectively) higher than the control. Therefore, a significant difference was not observed ($P < 0.05$). As compared with the results of *A. kawachii*, the frequencies at which activity was influenced easily were found to be different for each species. It has been reported that glucoamylase was produced well in rice-*koji* grown at a higher growth temperature (37.5°C) [2]. Even in growth conditions normal for making rice-*koji* (30°C), the glucoamylase activity with treatment at a frequency of 6.3 kHz differed significantly from that with no sound treatment (Figure 1). These results show that *A. kawachii* was affected by sound waves even if the temperature was constant. Also, these results are shown in Tables 1 and 2.

Table 1 The effect of frequencies on enzyme activities

	Rice-koji		Conidiospore of A. oryzae		Conidiospore of A. kawachii	
	Glucoamylase activity	Acid protease activity	Glucoamylase activity	Acid protease activity	Glucoamylase activity	Acid protease activity
1.0 kHz	-	+	+	±	-	+
6.3 kHz	LOW	+	±	±	HIGH	+
16.0 kHz	-	HIGH	LOW	+	±	HIGH

Note: Meaning of each symbol was described in section **Statistical significance test**. These results were quoted from Saigusa et al. [4].

Table 2 The effect of frequencies on enzyme activities

	1.0 kHz		6.3 kHz		16.0 kHz	
	Glucoamylase activity	Acid protease activity	Glucoamylase activity	Acid protease activity	Glucoamylase activity	Acid protease activity
Rice-*koji*	-	+	LOW	+	-	HIGH
Conidiospore of *A. oryzae*	+	±	±	±	LOW	+
Conidiospore of *A. kawachii*	-	+	HIGH	+	±	HIGH

Note: Meaning of each symbol was described in section **Statistical significance test**. These results were quoted from Saigusa et al. [4].

The white bar indicates the activity of the control, and the black bar indicates the activity of the test that irradiated sound waves at frequencies of 1.0, 6.3, and 16.0 kHz for 24 h. Symbol (**) shows the significant difference ($P < 0.05$). The vertical axis indicates the activity showing the color reaction of 1 µg/ml of glucose equivalent at 40°C for 60 min from soluble starch, defined as (unit/g *koji*).

Effects of sound waves on acid-protease activity

The acid protease activity was increased at any frequency. The acid-protease activity of rice-*koji* produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 4.45 ± 0.25, 4.41 ± 0.17, and 5.23 ± 0.44 kU/g *koji,* respectively. In the absence of sound waves, it was 3.83 ± 0.14 kU/g *koji* (Figure 2). The frequency of 16.0 kHz was 1.37 times higher than that of the control, and a significant difference was observed ($P < 0.05$). At frequencies of 1.0 and 6.3 kHz, its enzyme activities were just a little (1.16 and 1.15 times, respectively) higher than that of the control. Therefore, a significant difference was not observed between the control and Figure 2. As compared with the acid-protease activity of *A. oryzae* [4], it was found that frequencies of 16.0 kHz increased both acid-protease activities. The measurement of rice-koji's acid protease activity produced by conidiospores *of A. oryzae* was determined as with the glucoamylase activity. The acid-protease activities of rice-*koji* of *A. oryzae* that was produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 6.54 ± 0.11, 6.82 ± 0.33, and 7.95 ± 0.11 kU/g *koji*, respectively. In the absence of sound waves, the activity was 6.96 ± 0.64 kU/g *koji.* At frequencies of 16.0 kHz, its enzyme activity was just a little (1.14 times) higher than that of the control, but a significant difference was observed ($P < 0.05$). At frequencies of 1.0 and 6.3 kHz, the activities were just a little (0.93 and 0.97 times, respectively) less than the control. Thus, a significant difference was not observed ($P < 0.05$). These results show the existence of a common mechanism of *Aspergillus* species that responds to sound waves.

Any acid-protease activities of each *Aspergillus* species were increased by an irradiating sound wave of 16.0 kHz. These results were expected to contribute to yielding more amino acid contents and increasing the concentration of umami contents. These results are shown in Tables 1 and 2.

Effects of sound waves on mycelium weight

Our results (Figures 1 and 2) indicated that the enzyme activities of rice-*koji* were increased at specific frequencies. The mycelium weight of rice-*koji* produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 7.06 ± 0.75, 9.14 ± 0.9, and 8.89 ± 2.26 mg/g, respectively. In the absence of sound waves, it was 6.96 ± 0.5 mg/g (Figure 3). At a frequency of 6.3 kHz, the activity was 1.28 times higher than that of the control, and a significant difference was observed ($P < 0.05$). At a frequency of 1.0 kHz, its enzyme activity was just a little (1.01 times) higher than that of the control. Therefore, a significant difference was not observed. At a frequency of 16.0 kHz, its enzyme activity was 1.28 times higher than that of the control, but a significant difference was not observed. In the presence of sound waves of 1.0, 6.3, and 16.0 kHz, the mycelium weights of *A. oryzae* were 7.80 ± 0.25, 12.7 ± 2.10, and 12.3 ± 0.75 mg/g, respectively. In the absence of sound, it was 10.4 ± 3.10 mg/g (Figure 3). At 6.3 and 16.0 kHz frequencies, the weights were 1.22 and 1.19 times higher than that of the control,

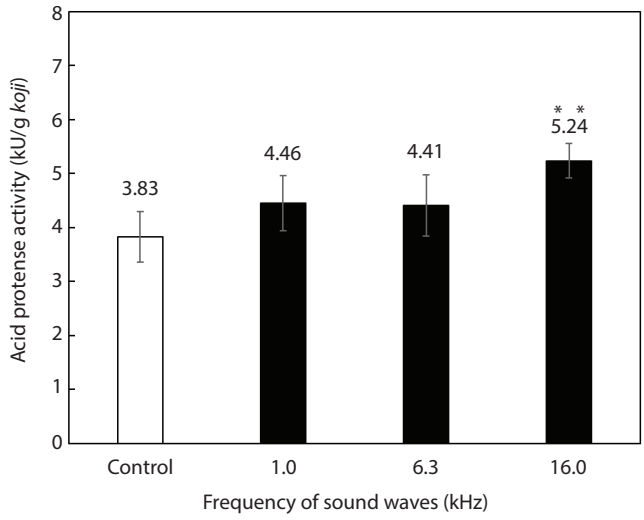

Figure 2 Effects of sound wave frequency on the acid protease activity of rice-*koji*

Note: The white bar indicates the activity of the control, and the black bar indicates the activity of the test that irradiated sound waves at frequencies of 1.0, 6.3, and 16.0 kHz for 24 h. Symbol (**) shows the significant difference ($P < 0.05$). The vertical axis indicates the activity showing the color reaction of 1 µg/ml of tyrosine equivalent at 40°C for 60 min from casein, defined as (unit/g *koji*).

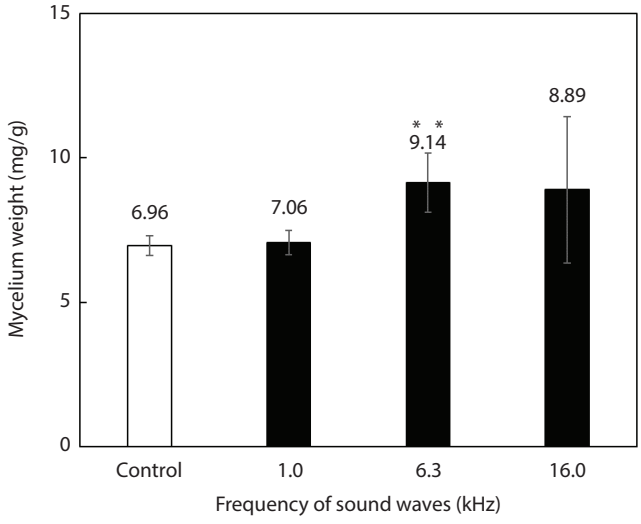

Figure 3 Effects of the sound wave frequency on the mycelium weight of rice-*koji*

Note: The white bar indicates the activity of the control, and the black bar indicates the activity of the test that irradiated sound waves at frequencies of 1.0, 6.3, and 16.0 kHz for 24 h. Symbol (**) shows the significant difference ($P < 0.05$). The vertical axis indicates the mycelium weight contained in 1.0 g of rice-koji, defined as (mg/g).

Table 3 The effect of frequencies on the contents of the rice-*koji* of *Aspergilli* produced by conidiospores that were treated with sound waves

	Conidiospore of A. oryzae			Conidiospore of A. kawachii		
	1.0 kHz	6.3 kHz	16.0 kHz	1.0 kHz	6.3 kHz	16.0 kHz
Mycelium weight	-	+	+	±	+	HIGH
Protein contents at 500 nm	-	+	±	+	HIGH	+
Protein contents at 750 nm	-	+	±	+	HIGH	+
Amino acid contents at 440 nm	+	±	+	+	HIGH	+
Amino acid contents at 570 nm	+	+	±	+	HIGH	+

but a significant difference was not observed. At a frequency of 1.0 kHz, the mycelium weight was 0.75 times less than the control, and a significant difference was not observed.

As compared with *Aspergillus* species, it was found that both mycelium weights were increased at frequencies of 6.3 and 16.0 kHz. At a frequency of 1.0 kHz, the weight decreased. Both mycelium weights and enzyme activities increased at frequencies of 6.3 or 16.0 kHz. These results indicated a correlation between the mycelium weight and each enzyme activity, and the activation of each enzyme activity was derived from the increasing mycelium weight (Figures 1 and 3). These results are shown in Table 3.

Effects of sound waves on protein content

The amounts of proteins and amino acids secreted from extracts as described in the experimental procedure were confirmed. Absorption was measured at 500 nm. The protein concentrations of extracts from rice-*koji* that was produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 37.5 ± 4.51, 42.7 ± 1.4, and 40.2 ± 13.2 µg/ml, respectively. In the absence of sound waves, the protein concentration was 31.3 ± 5.75 µg/ml (Figure 4A). As compared with the control, the concentration was increased at any frequency. In particular, the concentration at a frequency of 6.3 kHz was 1.37 times higher than that of the control, and a significant difference was observed ($P < 0.05$). At frequencies of 1.0 and 16.0 kHz, the protein concentrations were 1.2 and 1.28 times higher than that of the control, respectively, but a significant difference was not observed. In the case of absorption at 750 nm, a result similar to that at 500 nm was confirmed. In particular, at a frequency of 6.3 kHz, the absorption was 1.27 times higher than that of the control, and a significant difference was observed (Figure 4B). In the presence of sound waves of 1.0, 6.3, and 16.0 kHz, the protein concentrations of *A. oryzae* were 46.3 ± 15.1, 53.2 ± 6.31, and 50.4 ± 6.30 µg/ml, respectively. In the absence of sound waves, it was 50.0 ± 5.52 µg/ml. This absorption was measured at 500 nm. As compared with the control, a significant difference was not observed at any frequency. At a frequency of 16.0 kHz,

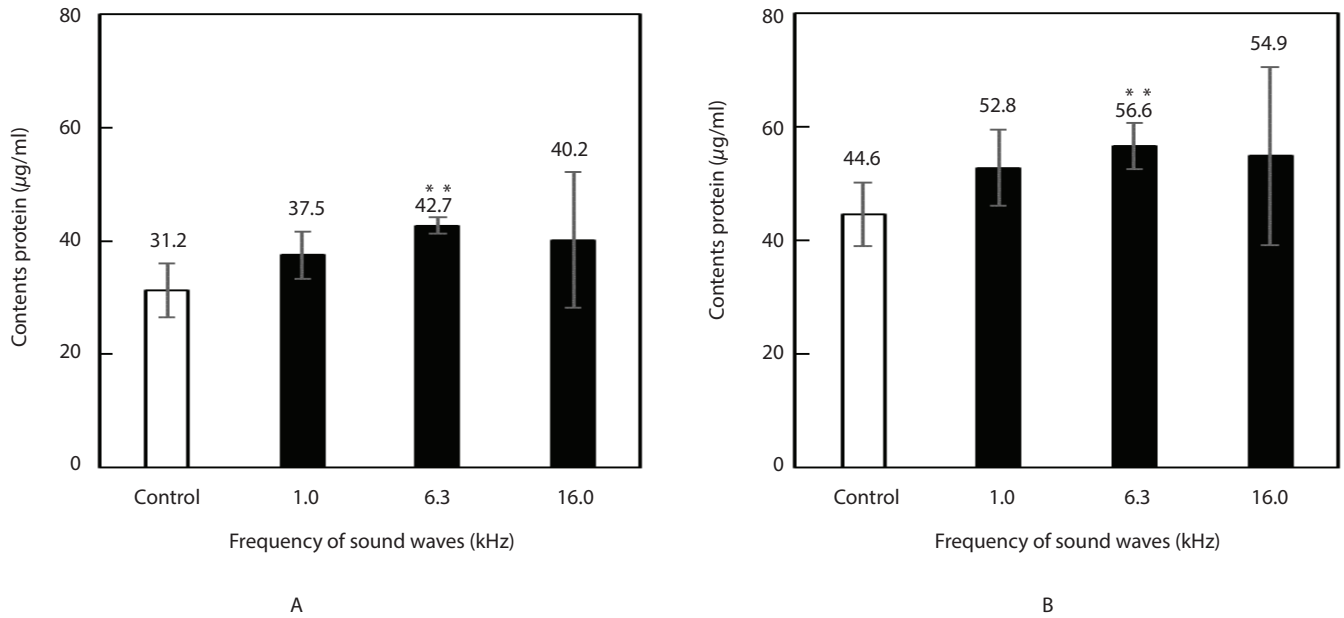

Figure 4 Effects of the sound wave frequency on the contents of rice-*koji* protein

Note: The white bar indicates the activity of the control, and the black bar indicates the activity of the test that irradiated sound waves at frequencies of 1.0, 6.3, and 16.0 kHz for 24 h. The produced amino acid residues of A and B were measured at 500 nm and 750 nm, respectively. Symbol (**) shows the significant difference ($P < 0.05$). The vertical axis indicates the protein contents contained in 1.0 ml of rice-*koji* extracts, defined as (µg/ml).

the protein concentrations were not changed. At a frequency of 6.3 kHz, it was just a little (1.06 times) higher than that of the control. At a frequency of 1.0 kHz, it was just a little (0.93 times) less than that of the control. In the case of absorption at 750 nm, the same tendency of absorption was confirmed at 500 nm, but a significant difference was not observed at any frequency.

As compared with *Aspergillus* species, the protein yields of *A. kawachii* were found to be more easily influenced than those of *A. oryzae*. In *A. kawachii*, the same tendency of mycelium weight was shown at a frequency of 6.3 kHz. Therefore, the relationship between the accumulation of proteins and cell growth was shown. These results are shown in Table 3.

Effects of sound waves on the amino acid content

Changes in the accumulation of amino acids in the resulting extracts were measured. This absorption was measured at 440 nm. The amino acid concentrations of extracts from rice-*koji* that was produced in the presence of sound waves of 1.0, 6.3, and 16.0 kHz were 54.0 ± 1.20, 64.9 ± 0.25, and 58.0 ± 8.12 µg/ml, respectively. In the absence of sound waves, it was 48.9 ± 0.70 µg/ml (Figure 5A). As compared with the control, the concentration was increased at any frequency. In particular, at a frequency of 6.3 kHz, the concentration was 1.33 times higher than that of the control, and a significant difference was observed ($P < 0.05$). At frequencies of 1.0 and 16.0 kHz, the amino acid concentrations were 1.1 and 1.19 times higher than the control, respectively, but a significant difference was not observed. A result similar to that at 440 nm was confirmed in the absorption at 570 nm. In particular, at a frequency of 6.3 kHz, the absorption was 1.39 times higher than that of the control, and a significant difference was observed (Figure 5B). In the presence of sound waves of 1.0, 6.3, and 16.0 kHz, the amino acid concentrations of *A. oryzae* were 95.4 ± 2.42, 65.7 ± 14.8, and 99.3 ± 7.51 µg/ml, respectively. In the absence of sound waves, it was 72.5 ± 7.50 µg/ml. This absorption was measured at 440 nm. As compared with the control, significant differences were observed at frequencies of 1.0 and 16.0 kHz, and the amino acid concentrations were 1.31 and 1.37 times higher than that of the control, respectively. At a frequency of 6.3 kHz, it was just a little (0.91 times) less than that of the control, but a significant difference was not observed. The absorption tendency at 570 nm was not confirmed to be the same as that at 440 nm. Only at a frequency of 1.0 kHz, the amino acid concentration increased more than that of the control in the case of absorption at 570 nm, but a significant difference was not observed at any frequency.

As compared with *Aspergillus* species, the same tendency of change in amino acid concentration was shown only in the protein concentration of *A. kawachii*. Therefore, the relationship between the accumulation of proteins and amino acids was shown. The production of amino acids from acid protease activity was shown to be affected by sound wave irradiation, and these results are shown in Table 3.

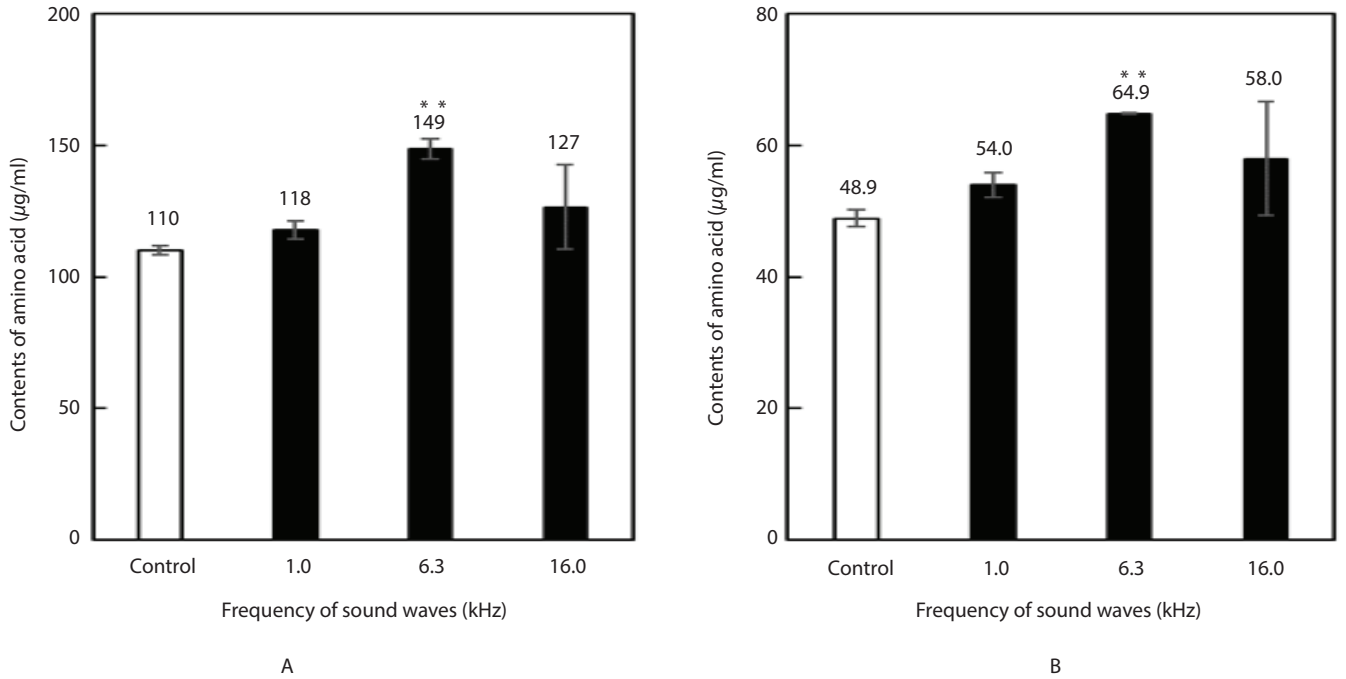

Figure 5 Effects of the sound wave frequency on the amino acid contents of rice-*koji*

Note: The white bar indicates the activity of the control, and the black bar indicates the activity of the test that irradiated sound waves at frequencies of 1.0, 6.3, and 16.0 kHz for 24 h. The produced amino acid residues of A and B were measured at 440 nm and 570 nm, respectively. Symbol (**) shows the significant difference ($P < 0.05$). The vertical axis indicates the amino acid contents contained in 1.0 ml of rice-*koji* extracts, defined as (µg/ml).

Relationship between sound-wave treatment and making rice-*koji*

These results indicated that irradiation by sound waves was possibly one important environmental factor for making rice-*koji*. This study found that irradiating conidiospores with sound waves affects their enzyme activity, as does irradiating mycelium cells throughout the making of rice-*koji* [4]. These results were expected to contribute to optimizing the method using sound waves for brewing. It is important to irradiate conidiospores of rice-*koji* with sound waves because it is very difficult to irradiate sound waves evenly throughout the making of rice-*koji*. Although the mechanism of the effects of the waves is still unknown, the underlying mechanism of frequency on enzyme production needs to be conclusively determined.

Possible effects of sound waves on the gene expression of *A. kawachii*

Considering from our results that the increase in the amounts of secreted enzymes was associated with the increased mycelium weight, it was supposed that the levels of gene expression involved in specific enzymes were increased by irradiation with specific sound waves. It has been reported that the gene expressions related to the metabolisms of glycerol, trehalose, and pentose phosphate are dependent on the growth conditions of *A. kawachii* [1]. It was observed that *E. coli* K-12 could respond rapidly to sound stress at both the transcriptional and posttranscriptional levels by promoting the synthesis of intracellular RNA and total protein. It has been reported that some potential mechanisms were involved in the responses of bacterial cells to sound stress [10]. Thus, some of the regulatory mechanisms of the glycolytic and proteolytic genes would also relate to the response to irradiation by sound waves. In order to investigate the precise relationship between fungal enzyme activities and sound waves, an experiment designed to reveal the effects of sound waves on the gene expression of *A. kawachii* is being planned. It is expected that this research will help improve the quality of beverage brewing.

REFERENCES

[1] K. Kitahara & M. Yoshida, "On the so-called Awamori white mold part III. (1) Morphological and several physiological characteristics," *J. Ferment. Technol.*, 27, pp. 162–166, 1949.

[2] N. Okazaki, K. Takeuchi, & S. Sugama, "Effects of *koji*-making conditions on cell growth and enzyme production," *J. Brew. Soc. Jpn.,* 74, pp. 683–686, 1979. [Online]. Retrieved from https://doi.org/10.6013/jbrewsocjapan1915.74.683

[3] H.W. Kim, "The effects of low frequency noise on the growth and resistance to antibiotics of soil bacteria and *E. coli*.," *APEC Youth Scientist J.*, 8, pp. 1–8, 2016. [Online]. Retrieved from http://www.sigs.or.kr.

[4] N. Saigusa, S. Imayama, & Y. Teramoto, "Effects of sound waves on the enzyme activity of rice-*koji*," *African. J. Biochem. Res.*, pp. 35–39, 2015. [Online]. Retrieved from http://www.academicjournals.org/AJBR

[5] N. Taniguchi, "Official methods of the National Tax Administration Agency of Japan for the evaluation of content, solid *koji," Brewing. Soc. Jpn.,* pp. 211–228, 1993.

[6] S. Masuda, K. Kikuchi, Y. Matsumoto, T. Sugimoto, H. Shoji, & M. Tanabe, "Analysis of enzyme production by submerged culture of *Aspergillus oryzae* using whole barley," *Biosci. Biotechnol. Biochem,* 73, pp. 2190–2195, 2009. [Online]. Retrieved from https://doi.org/10.1271/bbb.90270.

[7] O.H. Lowry, N.J. Rosebrough, A.L. Farr, & R.J. "Randall, Protein measurement with the Folin phenol reagent," *J. Biol. Chem*, 193, pp. 265–275, 1951.

[8] H. Meyer, "The ninhydrin reaction and its analytical applications," *Biochem. J.*, 67, pp. 333–340, 1957. [Online]. Retrieved from https://doi.org/10.1042/bj0670333.

[9] J.M. Sakoda, B.H. Cohen, & G. Beall, "Test of significance for a series of statistical tests," *Psychol. Bull,* 51, pp. 172–175, 1954.

[10] G. Shaobin, Z. Yongzhu, & W. Ying, "Effects of sound exposure on the growth and intracellular macromolecular synthesis of *E. coli* k-12," *Peer J.*, 4, e1920, 2016. [Online]. Retrieved from https://doi.org/10.7717/peerj.1920.