Full Paper

A new method for determining the mycelial weight of the koji-mold *Aspergillus oryzae* by measuring its glycosylceramide content

(Received January 5, 2018; Accepted April 23, 2018; J-STAGE Advance publication date: June 21, 2018)

Jannatul Ferdouse,1,2 Miyuki Miyagawa,1 Mikako Hirano,1 Yuka Kitajima,1 Shigeki Inaba,1 and Hiroshi Kitagaki1,2,*

1 Department of Environmental Science, Faculty of Agriculture, Saga University, Saga city, Saga 840-8502, Japan
2 Department of Biochemistry and Applied Biosciences, United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890-8580, Japan

At present, the quantitation of the mycelial weight of the industrially important non-pathogenic fungus *Aspergillus oryzae*, which is used for manufacturing koji, is performed by quantitating N-acetylglucosamine. However, since N-acetylglucosamine is a cell wall component, the extraction procedure is costly and tedious, and its quantitative performance is poor. Here, we report a novel method for the quantitation of *A. oryzae* mycelial weight. The amount of glycosylceramide significantly correlated with both the mycelial weight of *A. oryzae* and the amount of N-acetylglucosamine, an established index of the mycelial weight of *A. oryzae* in koji. This new method is simple and efficient and can be used in the brewing and food industries to determine the mycelial weight of *A. oryzae*.

Key Words: *Aspergillus oryzae*; glycosylceramide; Koji; quantitation

Introduction

*Koji* is responsible for the saccharification of starch in crops used in the manufacture of most Japanese fermented foods, and corresponds to malt in beer (Kitagaki and Kitamoto, 2013). It is manufactured by proliferating the non-pathogenic fungus *Aspergillus oryzae* on steamed grains such as rice and barley. Because the mycelial weight of *A. oryzae* determines the quality of *koji*, methods for its quantitation have been intensively studied.

Most studies have adopted the quantitation of N-acetylglucosamine as an index of the mycelial weight of *A. oryzae*. Although this procedure has been applied successfully, it has several technical limitations. For example, N-acetylglucosamine is a component of the cell wall; hence, in order for it to be extracted, cell wall chitin must first be degraded with either acid (Arima and Uozumi, 1967; Sakurai et al., 1977, 1985) or by lytic enzymes (Fujii et al., 1992; Gomi et al., 1987), and the efficiency of this degradation may vary between analyses. Thus, the procedure becomes costly and complex, making quantitation difficult.

In earlier studies, we have shown that the concentration of glycosylceramide is significantly higher in *koji* than in crops such as rice and barley (Hirata et al., 2012; Sawada et al., 2015; Takahashi et al., 2014). Glycosylceramide is a component of sphingolipids, and that in *koji* is composed of a sphingoid base moiety, fatty acid and monohexose (glucose or galactose) (Hamajima et al., 2016). Sphingolipids are critical components of the cell membrane, where they perform various biological functions (Hannun and Obeid, 2017; Russo et al., 2013; Truman et al., 2014) and play important roles in fungal mycelial growth (Zhu et al., 2014).

In this report, we demonstrate that glycosylceramide levels correlate significantly with the mycelial weight of *A. oryzae*, and increase with incubation time during the koji manufacturing process in a manner similar to that observed with N-acetylglucosamine levels. We found that the amount of N-acetylglucosamine in *koji* correlates significantly with that of glycosylceramide. Our results dem-
onstrate that the glycosylceramide level is a good indicator of the mycelial weight of *A. oryzae*. This enables simplification of the extraction method from *koji* and quantitation of *A. oryzae* mycelial weight.

**Material and Methods**

**Materials.** *Koji* samples were purchased from Tokushima Seiko Co. Ltd. (Osaka, Japan). Conidia of *A. oryzae* were purchased from Higuchi Matsunosuke Shoten Co. Ltd. (Osaka, Japan). Cerebroside purchased from Matreva Inc. (USA) was used as a glycosylceramide standard. All chemicals were of analytical grade.

**Koji manufacture.** *Koji* was manufactured using a rice cultivar (Yamadanishiki) used for sake brewing (whose surface (30%) was polished). Rice was soaked in water until the weight increased by 30% (w/w). Soaked rice was steamed with vapor for 1 h, and then cooled to room temperature. Conidia of *A. oryzae* (10 mg) were inoculated into the steamed rice (29 g). The steamed rice grains were mixed well and incubated in a packed container at 37°C for 72 h.

**Quantitation of glycosylceramide of the mycelia of *A. oryzae*.** Conidia of *A. oryzae* were inoculated into potato dextrose broth (Difco Laboratories, Detroit, USA) and incubated at 30°C and 200 rpm for 3 days. The mycelia were collected by centrifugation and lyophilized. To 100 mg of mycelia, 1 ml methanol and 0.5 ml chloroform were added, and the mixture was vortexed for 3 min. Following this, 0.5 ml chloroform was added to the solution, which was vortexed again for 3 min. The solution was then sonicated for 10 min. Methanol (2 ml) containing 0.8 M KOH was added, and the solution was incubated at 42°C for 30 min. Chloroform (5 ml) and distilled water (2.25 ml) were then added, and the solution was vortexed until saponification. After centrifugation at 800 g for 10 min the lower phase was recovered, evaporated under vacuum, and dissolved in 200 μl chloroform/methanol (2:1). An aliquot of the resultant solution (60 μl) was spotted onto a thin layer chromatography (TLC) plate (Silica gel 60 plate, Merck Millipore Inc., Darmstadt, Germany). The dried plate was developed with saturated chloroform:methanol:acetic acid:water (20:3.5:2:3:0.7 v/v). As a standard, 4 μl of 10, 5, 2.5, and 1.25 mg/ml cerebroside (Matreya Inc., Pleasant Gap, USA) was spotted onto the TLC. To detect glycosylceramide, 2 mg/ml of orcinol (MP Biomedicals, Illkirch, France) in 70% sulfuric acid was sprayed onto the TLC plate and heated at 100°C for 40 min.

**Quantitation of glycosylceramide in *koji* samples.** *Koji* samples were lyophilized and pulverized with a mill. To 1.2 g of the samples, methanol (4 ml) and chloroform (2 ml) were added, and the mixture was vortexed for 1 min. More chloroform (2 ml) was added, and the sample was vortexed again for 1 min. The solution was then sonicated for 5 min; methanol (8 ml) containing 0.8 M KOH was added, and the solution was incubated at 42°C and 160 rpm in a water bath for 30 min. Chloroform (20 ml) and distilled water (9 ml) were then added, and the solution was vortexed until saponification. The tube was centrifuged at 800 g for 10 min, and the lower layer was recovered as a sphingolipid-containing fraction. Sphingolipid-
containing fractions obtained as described above were evaporated to dryness and dissolved in 120 μl of chloroform/methanol (2:1 [vol/vol]). A 10-, 20- or 30-μl aliquot of the solution was applied onto a silica gel TLC plate and visualized as described above.

Table 1. The coefficient of variation of glycosylceramide measurement.

| Time (h) | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Average |
|---------|--------|--------|--------|--------|---------|
| 0       | 0.810  | 0.722  | 0.812  | 0.430  | 0.693   |
| 24      | 0.244  | 0.243  | 0.403  | 0.387  | 0.319   |
| 48      | 0.102  | 0.207  | 0.044  | 0.061  | 0.104   |
| 72      | 0.058  | 0.037  | 0.201  | 0.096  | 0.098   |

Quantitation of N-acetylglucosamine in koji samples. Koji was manufactured using rice used for traditional sake brewing. Glycosylceramide levels in koji were plotted against N-acetylglucosamine levels. Correlation coefficients were calculated using Microsoft Excel Software. N-acetylglucosamine and glycosylceramide levels were quantitated using the method described in the text.

Quantitation of the intensities of spots on TLC using imaging software. Quantitative image analysis was performed using ImageJ software (ij150-win-jre6) obtained from https://imagej.nih.gov/ij/download.html. TLC photo files were opened with ImageJ and converted to a binary format. Spots with an Rf value of 0.4 on TLC plates (indicated by arrows in the figures) were used for the quantitation of glycosylceramide, since other spots did not generate corresponding signals during the analysis using LC/MS (Sawada et al., 2015). Spots on each plate were surrounded, as was a blank space of equal size, and the mean intensity values of the compartments were calculated. The intensity of the compartment surrounding the blank space was subtracted from the average intensity of the spots (applied quantity: 10-, 20-, 30-μl) on each plate. The results for each spot across a range of glycosylceramide standards were used to calculate an equation. Detailed methods are deposited in protocols.io (dx.doi.org/10.17504/protocols.io.n8hdht6).

Quantitation of N-acetylglucosamine and glycosylceramide levels during koji production experiment. Koji was manufactured using rice used for traditional sake brewing. A. TLC analysis. Arrows indicate the position of glycosylceramide, which was used for quantitation. B. Time course of glycosylceramide levels during koji production. C. Time course of N-acetylglucosamine levels during koji production. N-acetylglucosamine and glycosylceramide levels were quantitated using the method described in the text. cb1, cb2, cb4 and cb8 indicates 20, 10, 5, and 2.5 μg glycosylceramide, respectively. Representative data of 4 trials is shown with high reproducibility.

Fig. 2. Correlation between N-acetylglucosamine and glycosylceramide levels during koji production experiment. Koji was manufactured using rice used for traditional sake brewing. A. TLC analysis. Arrows indicate the position of glycosylceramide, which was used for quantitation. B. Time course of glycosylceramide levels during koji production. C. Time course of N-acetylglucosamine levels during koji production. N-acetylglucosamine and glycosylceramide levels were quantitated using the method described in the text. cb1, cb2, cb4 and cb8 indicates 20, 10, 5, and 2.5 μg glycosylceramide, respectively. Representative data of 4 trials is shown with high reproducibility.

Fig. 3. Correlation between N-acetylglucosamine and glycosylceramide levels during four koji production experiments. Koji was manufactured using rice used for traditional sake brewing. Glycosylceramide levels in koji were plotted against N-acetylglucosamine levels. Correlation coefficients were calculated using Microsoft Excel Software. N-acetylglucosamine and glycosylceramide levels were quantitated using the method described in the text.

Quantitation of the intensities of spots on TLC using imaging software. Quantitative image analysis was performed using ImageJ software (ij150-win-jre6) obtained from https://imagej.nih.gov/ij/download.html. TLC photo files were opened with ImageJ and converted to a binary format. Spots with an Rf value of 0.4 on TLC plates (indicated by arrows in the figures) were used for the quantitation of glycosylceramide, since other spots did not generate corresponding signals during the analysis using LC/MS (Sawada et al., 2015). Spots on each plate were surrounded, as was a blank space of equal size, and the mean intensity values of the compartments were calculated. The intensity of the compartment surrounding the blank space was subtracted from the average intensity of the spots (applied quantity: 10-μl, 20-μl, 30-μl) on each plate. The results for each spot across a range of glycosylceramide standards were used to calculate an equation. Detailed methods are deposited in protocols.io (dx.doi.org/10.17504/protocols.io.n8hdht6).
was quantitated as described previously (Reissig et al., 1955) with minor modifications to the method. Briefly, 0.167 ml of the sample was put into a screw-capped glass tube, and 0.3 ml of saturated potassium tetraborate was added. The reaction mixture was heated at 100°C for 3 min and cooled with water. One milliliter of p-dimethylaminobenzaldehyde (DMAB, Nacalai Tesque Inc., Kyoto, Japan) solution (10 g of DMAB was dissoloved in 100 ml acetic acid containing 12.5% v/v of 10 N HCl and diluted with water 9-fold) was added to the tube, and incubated at 37°C for 20 min. The absorbance/OD of the final solution was measured at 544 nm.

Statistical analysis. Regression analysis was performed using Excel (Microsoft Office Professional Plus 365). Test for no correlation was judged using the p value in the t-distribution (t = r × \sqrt{\frac{n-2}{1-r^2}}).

Results

Correlation of glycosylceramide levels of A. oryzae with its mycelial weight

To test whether A. oryzae mycelial glycosylceramide levels are indicative of mycelial weight, we cultured A. oryzae in potato dextrose broth, collected the mycelia by centrifugation, lyophilized the mycelia, and measured the weight. We then quantitated glycosylceramide levels within the mycelia (Fig. 1A) and examined the correlation between mycelial weight and glycosylceramide levels (Fig. 1B), finding them to be highly correlated (R² = 0.9735, p < 0.01).

Increase in the glycosylceramide and N-acetylglucosamine contents with the growth of A. oryzae on rice

To confirm that increases in A. oryzae mycelial weight in koji can be reliably quantitated by monitoring glycosylceramide levels, we measured a time course of glycosylceramide levels during koji production. To ascertain the reproducibility of the experiment, 4 independent trials of koji production were performed. We found that glycosylceramide concentrations increased with incubation time (Figs. 2A and B). The coefficient of variation decreased with incubation time (Table 1), suggesting that the reliability of quantitation using this method is high for koji incubated for more than 48 h. N-acetylglucosamine, which is a measure of the mycelial weight of A. oryzae, also increased with incubation time (Fig. 2C) as reported previously (Arima and Uozumi, 1967).

Correlation of glycosylceramide levels with N-acetylglucosamine content of koji

The relationship between the glycosylceramide and N-acetylglucosamine content in koji samples manufactured from rice was investigated. The quantitation results of 4 independent trials were analyzed. These showed that N-acetylglucosamine and glycosylceramide levels are significantly correlated (Fig. 3, R² = 0.8942, p = 0.00150), indicating that glycosylceramide reliably reflects the amount of N-acetylglucosamine.

Discussion

Standard methods that use N-acetylglucosamine to determine the content of A. oryzae in koji involve complex sample preparation processes, acid or enzymatic treatment, and filtration (Arima and Uozumi, 1967; Fuji et al., 1992; Gomi et al., 1987; Sakurai et al., 1977). These methods are costly and tedious, and their quantitative performance is low. In contrast, the process developed in this study involves direct extraction of glycosylceramide, an electrophilic lipid, with chloroform and methanol, and with no need for additional treatment steps, thereby simplifying sample preparation. The extraction method does not require enzymatic treatment, which has the potential to interfere with quantitation. This method is cost-effective, rapid, highly quantitative, and reproducible.

As koji contains components from both rice and A. oryzae, discrimination of these components was key in this technique. Glycosylceramide is produced mainly by A. oryzae and only a small amount (below 10%) is present in rice (Sawada et al., 2015). Therefore, glycosylceramide content can serve as a measure of the mycelial weight of A. oryzae.

Under certain conditions, there is a degree of variability with our method. For example, the residual error of the correlation of N-acetylglucosamine with glycosylceramide was large when koji contained more than 100 µg or less than 10 µg of glycosylceramide (Fig. 3). Therefore, this technique is most appropriate for koji manufactured for 24 h to 62 h. When measuring the content of glycosylceramide of koji manufactured for less than 24 h or more than 62 h, it should be noted that the measured content contains some errors and then there is a need to increase the sampling number and check the statistical significance level.

Recent technical advances have radically decreased the cost of imaging photos, and many imaging programs are now freely available (e.g., NIH ImageJ); thus, quantitation of TLC spot intensities is cost-effective. Therefore, the method developed in this study has several advantages over previous techniques, including cost-effectiveness, simplicity, and ready availability of the equipment required. In conclusion, we have developed a new method for determining the mycelial weight of A. oryzae which involves quantitation of glycosylceramide contained in koji. As this method employs a simple extraction procedure, it is a cost-effective quantitative method that can be used in the brewing and food industries.

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