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Application Note

No. 64

Foods

Visualization of Phospholipids and Glucose in Rice *Koji* Using Microscopic Mass Spectrometry Imaging

Shuichi Shimma *1, 2, Yoshihiro Tamada *3, Adinda Putri Wisman *1, Shuji Hirohata *3, Katsuya Gomi *4
Eiichiro Fukusaki *1, 2

Keywords: Rice *koji*, phospholipids, glucose, mass spectrometry imaging, sample preparation, *hazekomi*

Foods

Abstract

Rice *koji* is one of the most critical elements in sake brewing. Its main role in sake brewing is thought to be the supply of digestive enzymes which decompose starch and proteins. It is also well known that the composition of the finished rice *koji* has a large influence on sake quality (taste and aroma). Until now, however, evaluation of the quality of rice *koji* have often depended on the experience of the chief brewer. This suggests that scientific knowledge is insufficient, and there is still room for development in this area. When the chief brewer evaluates the quality of rice *koji*, the physical structure of the *koji*, that is, its external appearance and texture, seems to be an index of quality. In past research, the internal structure of rice *koji* was investigated using the scanning electron microscope, but until recent years, little progress has been made in research on evaluating the relationship between the structure and composition of rice *koji*. Because the Shimadzu iMScope imaging mass microscope enables simultaneous observation of the structure and component distribution of samples, in this Application Note, we applied the iMScope to the field of fermentation, and attempted to visualize the structure and component distribution of rice *koji*.

1. Introduction

In this research, we carried out technical development contributing to visualization of the spatial distribution of components in rice *koji* and evaluation of its quality properties. As expressed by the adage "First *koji*, second yeast, third brewing," *koji* is a critical element in sake brewing. The main role of rice *koji* in sake brewing is to provide the digestive enzymes that decompose starch and proteins, but it is also well known that components of rice *koji* have a large influence on sake quality (taste and aroma). Until now, evaluation of the quality of rice *koji* have often depended on the experience of the chief brewer, and it seems that scientific knowledge is not sufficiently obtained. When the chief brewer evaluates the quality of rice *koji*, its physical structure, that is, its external appearance and texture, seem to be an index of quality. In past research, the internal structure of rice *koji* was investigated using the scanning electron microscope, but until recent years, little progress has been achieved in research on evaluating the relationship between the structure and composition of rice *koji*.

*1 Osaka University, Graduate School of Engineering, Department of Biotechnology
*2 Osaka University Shimadzu Omics Innovation Research Laboratories
*3 Hakutsuru Sake Brewing Co., Ltd.
*4 Tohoku University, Graduate School of Agricultural Science, Division of Bioscience and Biotechnology for Future Bioindustries

The most suitable technique for observing the structure of rice *koji* and the distribution of the components involved in the determination of its quality is considered to be mass spectrometry imaging (MSI), as illustrated schematically in Fig. 1. As previous examples of application of MSI to foods, visualization of the distribution of the asparaptine in asparagus and curcumin in dried turmeric root has been reported [1], [2]. In this Application Note, we attempted to visualize the structure and component distribution in rice *koji* under the topic of "fermentation" as a new field in applied research in food science. In conducting an analysis by MSI, sectioning of rice *koji* without pretreatment is almost impossible, as *koji* is extremely fragile. Therefore, we examined sectioning methods and succeeded in visualizing the metabolites in the process of producing steamed rice and *koji* from raw rice.

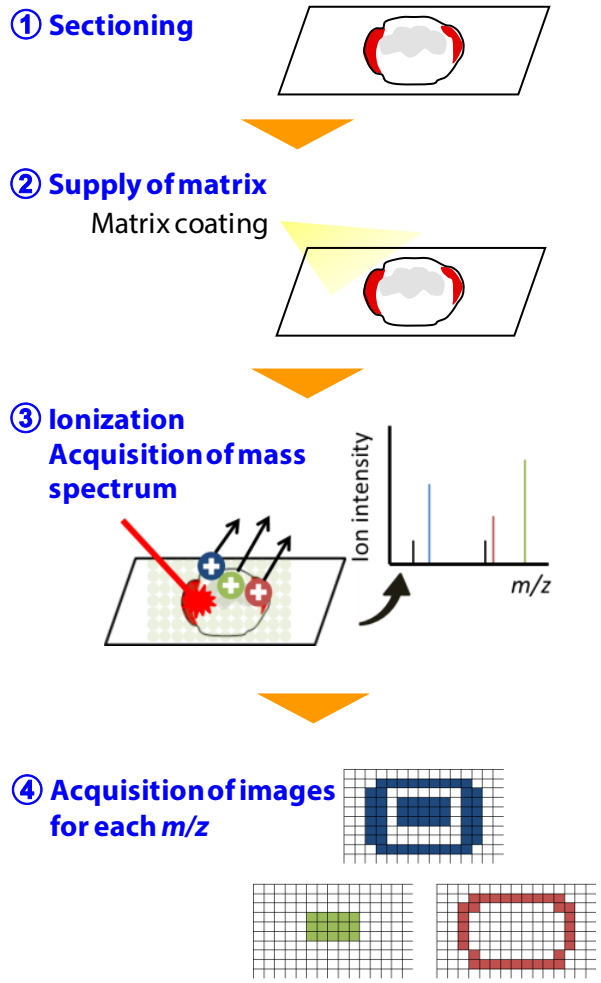


Fig. 1 Workflow in Mass Spectrometry Imaging (MSI) Method

2. Experiment

2-1. Reagents

A carboxymethyl cellulose embedding agent was purchased from FUJIFILM Wako Pure Chemical Corporation. Water was added to adjust the concentration to 4 %, and the solution was placed in a thermostatic chamber at 70 °C overnight to ensure complete dissolution. The matrix used in this experiment were *α*-cyano-4-hydroxycinnamic acid (CHCA) and *N*-(1-Naphthyl)ethylenediamine dihydrochloride (NEDC) which were purchased from Merck, and the acetonitrile, isopropanol, and methanol used as solvents were purchased from FUJIFILM Wako Pure Chemical Corporation. Ultrapure water produced with a Thermo Fisher Scientific Inc. GenPure™ UV-TOC xCAD Plus was used.

2-2. Sectioning

Steamed rice and rice *koji* made from Yamada Nishiki rice (sake brewer's rice) with polishing rate of 70 % provided by the Hakutsuru Sake Brewing Co., Ltd. was used. In visualization of raw rice, a commercially-available rice was used. As already noted, these sample materials are extremely fragile. Therefore, sections were prepared with a cryomicrotome, and the obtained sections were recovered using an adhesive cryofilm (cryo-lab). After embedding the rice grains in the above-mentioned 4 % carboxymethyl cellulose, the grains were frozen at −80 °C. The thickness of the sections was set at 20 µm, and the obtained films were fixed on ITO coated glass (without MAS coating, surface resistance: 100 Ω/m^2) manufactured by Matsunami Glass Ind., Ltd. using conductive double-sided adhesive tape purchased from 3M (Fig. 2).

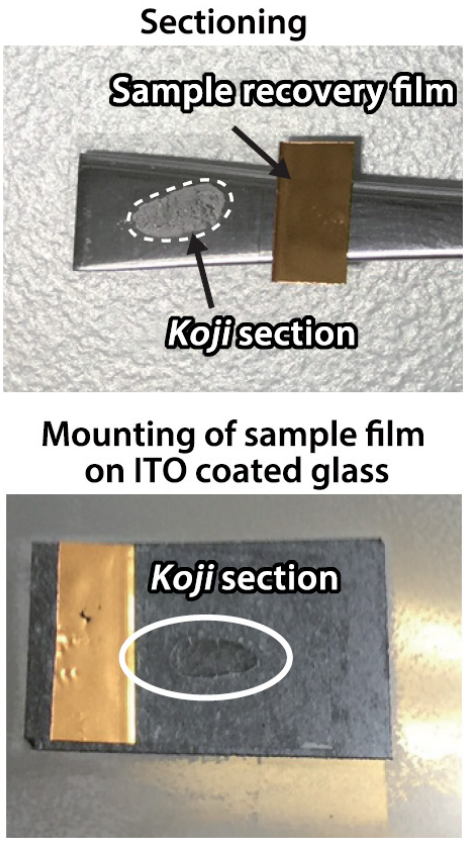


Fig. 2 Preparation of Rice *Koji* Section

2-3. Matrix Supply

In detecting phospholipids in the rice grain sections and rice *koji* sections, CHCA was vapor-deposited on the sample surface using a Shimadzu iMLayer™ matrix vapor deposition system, as shown in Fig. 3, followed by spraying of the CHCA solution [3]. The film thickness of the vapor-deposited matrix was set at 0.5 µm. The CHCA solution was adjusted to a concentration of 10 mg/mL by a mixed solvent consisting of acetonitrile : isopropanol : ultrapure water (3 : 1 : 6) including 0.1 % formic acid. NEDC, which is known to enable efficient ionization of glucose, was vapor-deposited using the iMLayer, which was set to a vapor deposition temperature of 220 °C and a time of 10 min. After vapor deposition of the NEDC matrix, it was further recrystallized using 5 % methanol.

Fig. 3 iMLayer™ Matrix Vapor Deposition System

2-4. Mass Spectrometry Imaging

The MSI measurements were carried out using a Shimadzu iMScope. The laser irradiation count was 100 times per spot. The measurements were performed for phospholipid with spatial resolution of 25 µm by positive ion mode, and for glucose with spatial resolution of 50 µm by negative ion mode. The measured *m/z* ranges were from 400 to 800 with the positive ion mode and from 180 to 230 with the negative ion mode. In all measurements, the laser intensity was set at 45, and the detector voltage was set at 2.1 kV.

2-5. Constructing MS Images

The Shimadzu MSI analysis software Imaging MS Solution™ and IMAGEREVEAL™ MS were used in data analysis and construction of the MS images. Although not discussed in this paper, IMAGEREVEAL MS is a software which enables non-targeted analysis by statistical technique. It has excellent correction functions (image filter, pixel interpolation) for the obtained images and also includes a "similar image extraction" function. The glucose distribution presented in the latter part of this paper was visualized using IMAGEREVEAL MS.

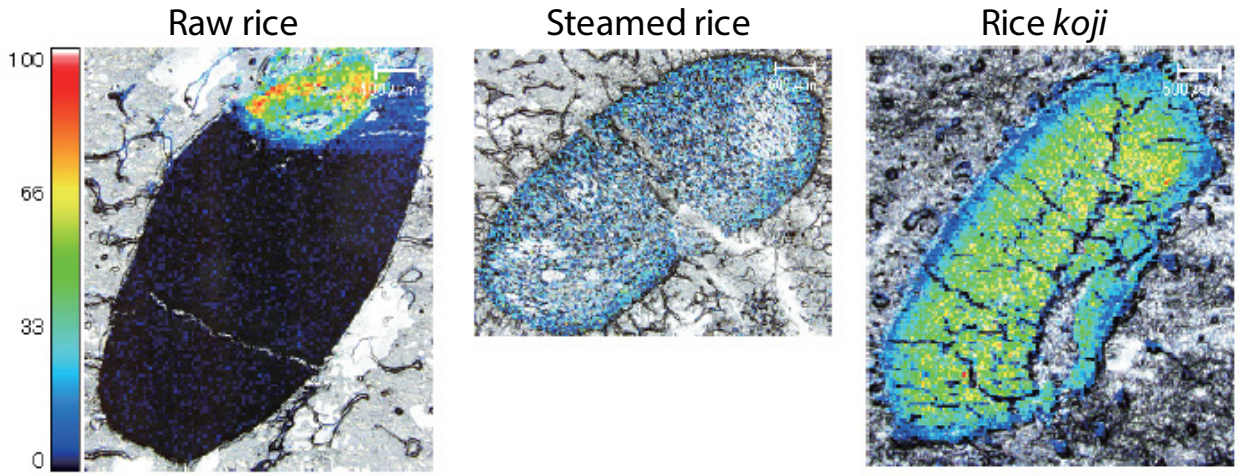


Fig. 4 Distribution of Choline in Raw Rice, Steamed Rice, and Rice *Koji*

3. Results

3-1. Distribution of Phospholipids in Raw Rice, Steamed Rice, and Rice Koji

Fig. 4 shows the distributions of choline in the sections of the raw rice, steamed rice, and rice *koji*. Choline is an example of a component that undergoes large changes in distribution and amount in the *koji*-making process. The result for the raw rice was measured before rice milling, and it shows that choline has accumulated in the rice germ. In the steamed rice after milling, the intensity of the peak originating from choline decreased sharply, but in the rice *koji* an extremely strong peak intensity was observed in the inner part. This suggests that choline is formed by the rice *koji* fermentation process (i.e., *koji*-making process). Thus, the fact that the quantity and spatial distribution of choline change dynamically in the *koji*-making process can be observed by using MSI.

Visualization of the accumulation of various phospholipids (including lysophospholipid) was also achieved in the inner part of the rice *koji* (Fig. 5). In particular, such a tendency was observed for *m/z* 496.34 and 520.34 which correspond to the lysophosphatidylcholines LPC (16:0) and LPC (18:2), respectively [4]. MS images for *m/z* 748.35 and 786.30, which correspond to phospholipids, show a heterogeneous distribution in the rice *koji*. This heterogeneity is thought to originate from the invasive growth of *haze* (hyphae) into the steamed rice from the *koji* mold (*Aspergillus oryzae*) in the rice *koji*, that is, the process called *hazekomi*. In the next section, we will introduce the development of a method visualizing the *hazekomi* process and the results of combining this method with MSI.

3-2. Visualization of *Hazekomi* and its Combination with MSI [5], [6]

Haze (pronounced *ha-zay*) refers to the white spots that represent the mycelia of *koji* mold spreading over the surface of the steamed rice, and is used as an indicator of the outcome of the rice *koji* in visual inspections by the chief brewer. In earlier work on visualization of *haze*, Yoshii et al. published a pioneering report based on observation with a scanning electron microscope (SEM), in which they successfully observed the characteristics of the growth of *A. oryzae* in rice *koji* by directly visualizing the *koji* mold propagation process, thereby contributing to improvement of *koji*-making [7].

In visualization of the *hazekomi* process by SEM, the ability to observe a microscopic region is an important feature. However, the authors believed that an approach for visualizing the *hazekomi* process of the entire rice *koji* and a technique which can obtain information on the distribution of components would also be useful. To solve this problem, we introduced a GUS reporter system using *β*-glucuronidase (GUS) as the marker gene for visualization of *hazekomi*. Specifically, we established a system that enables clear observation of the growth of the *koji* mold during *koji*-making by constructing a GUS expressing strain of *A. oryzae* and by producing rice *koji* (hereinafter, GUS rice *koji*), in which that strain was used. Use of the GUS rice *koji* makes it possible to visualize the location of the *koji* mold from the color reaction, and when this technique is used in combination with MSI, it also becomes possible to obtain information on the distribution of components. The integration of these two techniques achieves both visualization of *hazekomi* of the entire rice *koji* and visualization of the component distribution.

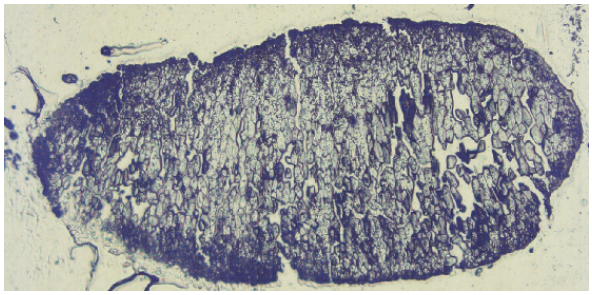
Here, we would like to describe the innovation that was conceived to enable application of the GUS reporter gene system to rice *koji*. The GUS reporter gene system was originally developed for visualization of mycelia in plant tissue. In plant tissues, the common practice is to immerse the sample in a solution of 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide (X-Gluc), which is a chromogenic substrate for coloration. Plant tissues with extremely hard cell walls can maintain the morphology necessary for sample observation without problems, even after extended immersion in X-Gluc solution.

However, as already mentioned, rice *koji* is very fragile, and its properties are completely different from those of plant tissues. This means it would be exceptionally difficult to use the existing coloration protocol. In fact, we confirmed that the morphology of the sample was greatly changed by water absorption when rice *koji* was immersed in X-Gluc for the time required to fix coloration. To avoid this problem, the way to add X-Gluc must be changed. Therefore, we conceived a technique for visualizing *hazekomi* by spraying the X-Gluc solution on sections of GUS rice *koji*.

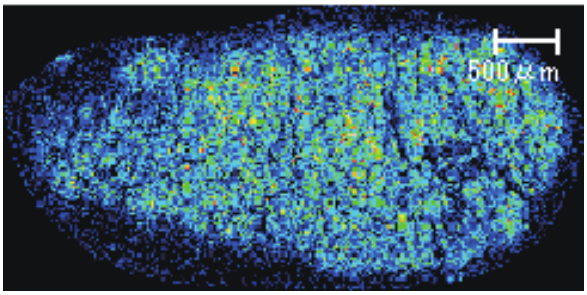
Fig. 6 shows the results obtained by this method. Here, *koji*-making was carried out using polished Hakutsuru Nishiki rice (proprietary sake brewing rice of Hakutsuru Sake Brewing Co., Ltd.) with a polishing rate of 70 %, and samples were taken 24 h, 31 h, and 43 h after the start of *koji*-making. As *koji*-making progressed, it was observed that an indigo color penetrated from the surface of the *koji* to the interior. In particular, after 43 h when *koji*-making was completed, a strong indigo color was detected not only at the *koji* surface but also in the interior, indicating that the *koji* mold had reached the interior of the rice.

One main role of *koji* is to supply various enzymes in order to form nutrients for the yeast in the brewing (fermentation) stage. The enzymes mainly observed are *α*-amylase or glucoamylase, which form glucose as a nutrient for yeast growth. In addition, the possibility that *α*-amylase is the most important enzyme affecting invasive growth by the mycelia of *koji* has also been reported.

Optical view



m/z 452.24



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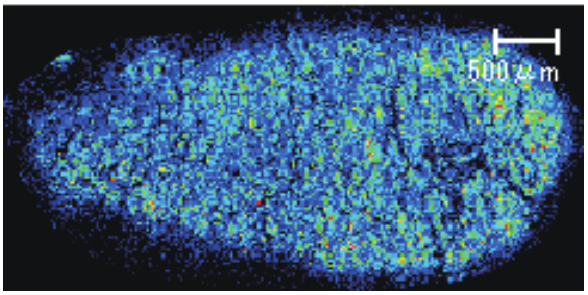
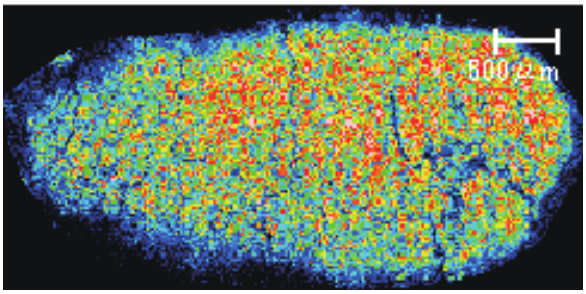
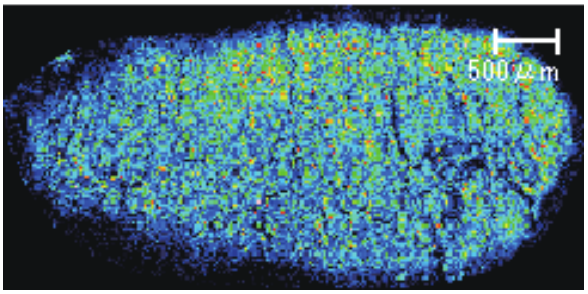


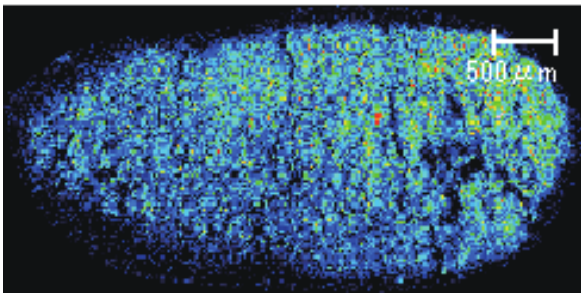
m/z 496.34



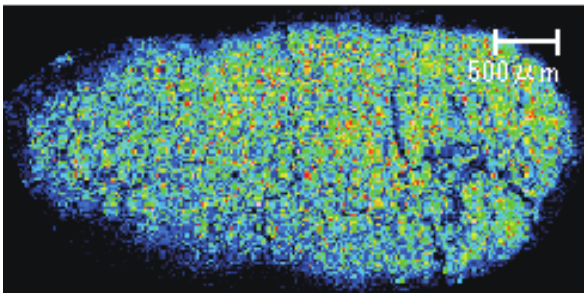
m/z 520.34



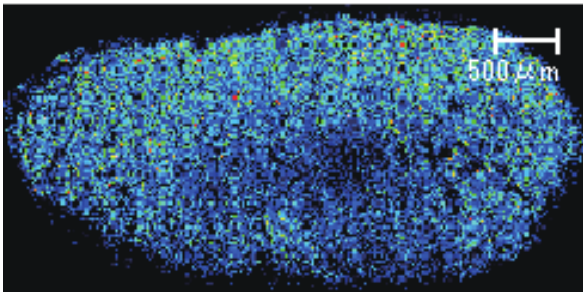
m/z 534.30



m/z 632.36



m/z 748.35



m/z 786.30

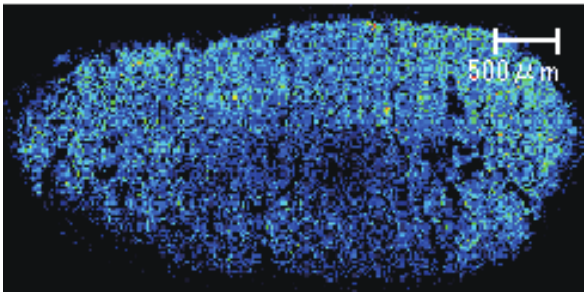


Fig. 5 Distributions of Lysophospholipids and Phospholipids in Rice *Koji* (Yamada Nishiki, rice polishing rate: 70 %)

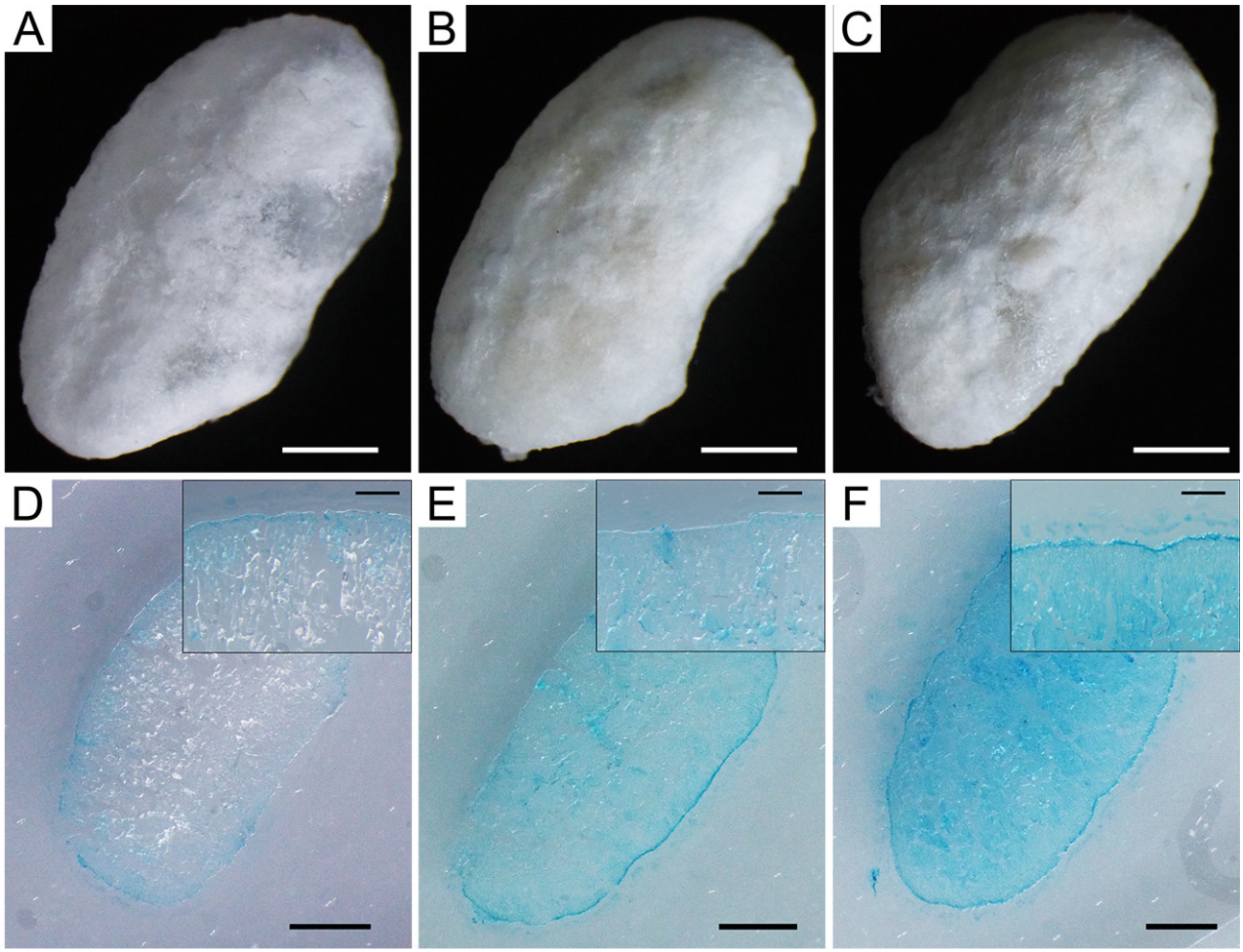


Fig. 6 Visualization of *Hazekomi* Process in GUS Rice *Koji* (Scale bars: 1 mm (inserted images: 200 µm))

Although an increase in glucose after *koji*-making has been reported in previous research, the relationship between *hazekomi* and the distribution of glucose has not been confirmed yet. In the mass spectra of rice *koji* sampled at each stage in the *koji*-making process, a rise in the peak intensity of glucose was actually observed (Fig. 7). Here, it may be noted that $[M + Cl]^-$ = *m/z* 215.02 was detected as a negative ion when NEDC was used as the matrix for MSI of glucose. NEDC was used here because it has been reported that NEDC increases the sensitivity for glucose detection in cancer tissue [8].

In order to investigate the relationship between *hazekomi* with GUS rice *koji* and the distribution of glucose, MSI was carried out using an adjacent section to a GUS-stained section, in order to compare the distribution of the obtained glucose ion intensity and the GUS-stained image. Fig. 8 shows the results.

Looking at the overlay of the glucose distribution and the GUS-stained image, it can be understood that glucose has increased from the outer side toward the inner side from the initial stage to the latter stages of *koji*-making. In other words, the results show that a correlation exists between *hazekomi* and the distribution of glucose. On the other hand, there are areas where the blue coloration due to X-Gluc is dark and the intensity of glucose is high, as shown by the black arrow, while other parts where the glucose intensity was low, although *hazekomi* was observed, could also be seen in this experiment, for example, in the region indicated by the black circle. These results suggested that diversity exists in the amount of glucose produced by *hazekomi*, depending on the location. In the future, it is thought that a better understanding of the phenomenon of *hazekomi* from the chemical point of view will be possible by a discussion which also includes analyses for various metabolites (e.g., amino acids, sugars, sugar alcohol).

Although the present discussion has focused on glucose and explained the changes in the distribution of glucose accompanying *hazekomi*, it is conceivable that the diffusion range and activity of the formed enzymes are also affected by other factors, such as the characteristics of the rice grains. This new visualization technique (fusion of GUS rice *koji* and MSI) can be expected to contribute to improvement of the *koji*-making processes for rice *koji* and other *koji*-derived products.

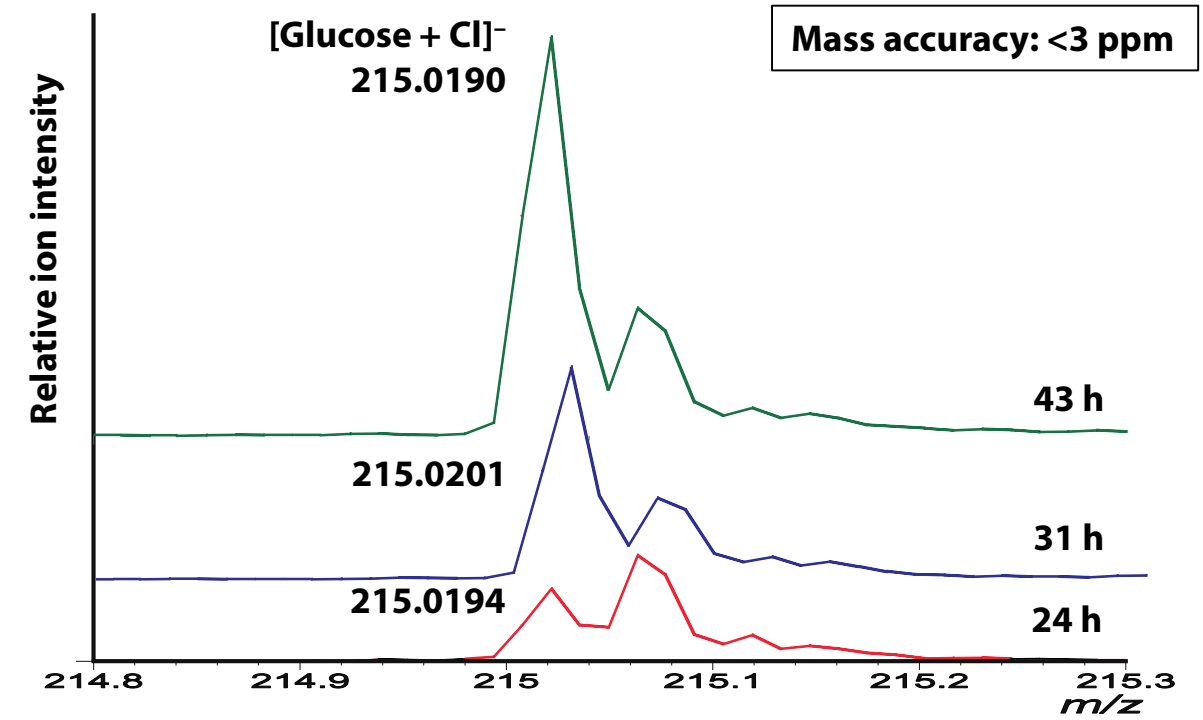


Fig. 7 Time-Dependent Change of Glucose Peaks Obtained Using NEDC Matrix

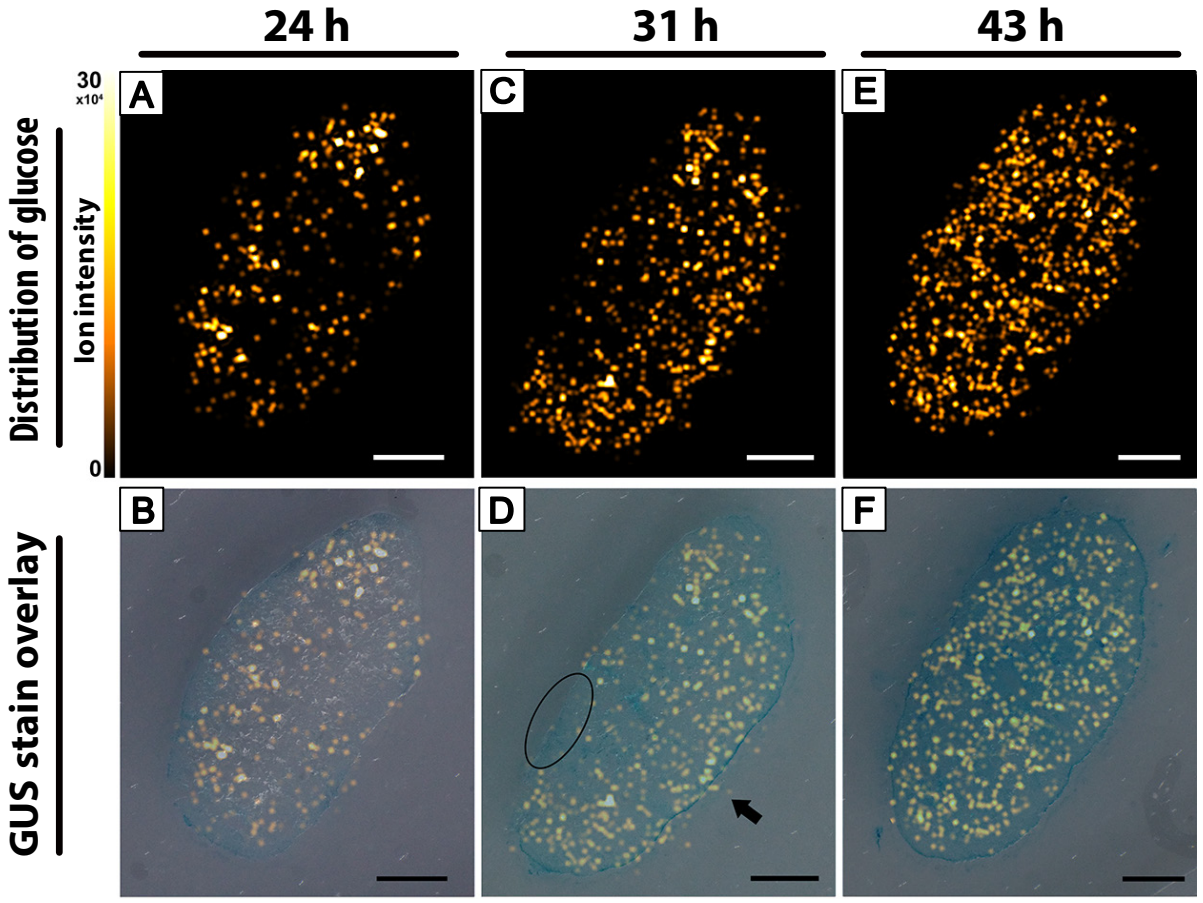


Fig. 8 Visualization of Glucose ([M + Cl]⁻) in GUS Rice *Koji* (Scale bars: 1 mm)

4. Conclusion

In this research, the distribution of phospholipids was visualized using Yamada Nishiki (sake brewer's variety of rice), and the relationship between the *hazekomi* process and the distribution of glucose was visualized using Hakutsuru Nishiki, which is a proprietary sake rice of the Hakutsuru Sake Brewing Co., Ltd. As a result, the change in phospholipids in the *koji*-making process was visualized. A strain of rice *koji* which expresses GUS was also produced from Hakutsuru Nishiki, and was used to reveal the relationship between the *hazekomi* process and the distribution of glucose. This new visualization technique using a combination of the GUS rice *koji* and MSI can be expected to contribute to a better understanding of the *koji*-making processes of rice *koji* and other *koji*-derived products and to improvement of *koji*-making methods. Because the Shimadzu iMScope imaging mass microscope used in this experiment enables optical microscope observation of microscopic areas together with mass spectrometric analysis under the microscope, it is thought that new scientific knowledge in the field of fermentation can be obtained by applying the iMScope to the analysis of various types of *koji* and other malts.

The iMScope QT (Fig. 9), which is the successor to the iMScope *TRIO*, was released in June of 2020. While continuing to provide the microscopic observation function and spatial resolution that were outstanding features of the iMScope *TRIO*, easier analysis is now possible with the new iMScope QT, which offers improved mass resolution, detection sensitivity, and analysis speed. As analyses of wider mas range become possible, heightened expectations will be placed on the possibilities of further applications of MSI.

Fig. 9 iMScope QT

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