Formation of Cytoplasmic P-Bodies in Sake Yeast during Japanese Sake Brewing and Wine Making

Shingo IZAWA,1,1 Takeomi KITA,1 Kayo IKEDA,1 Takeo MIKI,2 and Yoshiharu INOUE1

1Laboratory of Molecular Microbiology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan
2The Institute of Enology and Viticulture, Yamanashi University, Kofu, Yamanashi 400-0005, Japan

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Recent studies have revealed that cytoplasmic processing bodies (P-bodies) play important roles in the control of eukaryotic gene expression in response to stress. Since the formation of P-bodies is in dynamic competition with translation, the status of translation is reflected in the assembly and disassembly of P-bodies in eukaryotic cells. During the brewing of Japanese sake and the making of wine, yeast cells are exposed to stress caused by increases in the concentration of ethanol. Here we found that ethanol stress enhances the formation of P-bodies in yeast cells in SD medium. In the wine-making process, P-body formation was also enhanced as alcoholic fermentation proceeded, but the formation of P-bodies was not simply affected by the ethanol concentration in the sake mash. These findings suggest differences in the rate of translation and the cytoplasmic mRNA flux during the sake brewing and wine making processes.

Key words: processing body; wine making; Japanese sake brewing; ethanol stress; sake yeast

Various post-transcriptional events play critical roles in the regulation of eukaryotic gene expression, and the flux of mRNA is ingeniously regulated not only in the nucleus but also in the cytoplasm. In yeast cells as well as mammalian cells, it has been reported that mRNAs in an untranslated state accumulate in discrete cytoplasmic foci called processing bodies (P-bodies).1,2) P-bodies contain various proteins implicated in mRNA degradation,1,3) mRNA storage,4,5) and general translational repression.6,7) Initially, P-bodies were identified as sites where mRNAs can be decapped and degraded by decapping enzymes and exonucleases.1,3) Moreover, recent studies have revealed that P-bodies are responsible for translational repression, serving as sites of untranslated mRNA storage under several stressed conditions,3,5,8) i.e., stresses that lead to decreases in translation rates enhance the formation of P-bodies, and mRNAs in an untranslated state can be sequestered into P-bodies.7–10) Additionally, mRNAs can exit P-bodies and return to translation when the stress is eliminated.4) In yeast cells, the number and size of P-bodies increase under conditions of glucose depletion, which decreases translation initiation.8,11) Conversely, restoration of glucose levels rapidly leads to the disassembly of P-bodies and a resumption of translation.9) These findings indicate that the formation of P-bodies is in dynamic competition with translation, and also suggest that eukaryotic cells can transiently arrest translation using P-bodies in response to stress and subsequently restore translation for recovery from the stress.5,8,9,12) Hence, one can estimate the status of translation based on changes in the formation of P-bodies.

During the brewing of Japanese sake and the making of wine, yeast cells are exposed to stress caused by increases in the concentration of ethanol. The adverse effects of ethanol on yeast cells are a key problem facing sake brewers and wine growers. Hence, the ethanol stress response of yeast cells has been investigated with great interest, but there is little information regarding the reality of gene expression in yeast cells during the brewing of alcoholic beverages. We have investigated changes in the nuclear export of bulk poly(A)+ mRNA with the progression of alcoholic fermentation in wine making and Japanese sake brewing, and have found that yeast cells show an unique response to ethanol during sake brewing.13,14) This finding suggests that the gene expression of yeast cells during the sake brewing process is much more complicated than has been supposed. To gain clues regarding the reality of gene expression as alcoholic fermentation proceeds, here we characterized the formation of P-bodies in yeast cells during the brewing of wine and Japanese sake. We found that yeast cells show unique responses to ethanol stress as to P-body formation during sake brewing.
suggesting differences in the rate of translation and the cytoplasmic mRNA flux during the wine making and sake brewing processes.

**Materials and Methods**

The yeast strains used in this study were UT-1 as a sake yeast strain\(^{15}\) and W303-1A\(^{16}\) as a laboratory strain. yRP1724 (carrying Dhh1p-GFP) and yRP1727 (carrying Dcp2p-GFP) were donated by Dr. Parker of University of Arizona.\(^{11}\) GFP plasmids were constructed as follows: For Ylp-DHH1-GFP, a 1.9-kbp fragment encoding the open reading frame of \(DHH1\) was amplified using 5'-TGAGCTCTGAGCAGATTGATCCCTCTA-3' and 5'-CTTATTCTTGCTAGCCCTATGCAAATGCT-3'. The amplicon was digested with XhoI and cloned into the XhoI site of pPS1630\(^{17}\) to construct Ylp-DHH1-GFP. To integrate the \(DHH1\)-GFP gene at the chromosomal \(DHH1\) locus, Ylp-DHH1-GFP was linearized by EcoRI and introduced into the yeast cells. For Ylp-DCP2-GFP, a 2.1-kbp fragment encoding the open reading frame of \(DCP2\) was amplified using 5'-GAGCAGATTGATCCCTCTA-GAGATGTCCTG-3' and 5'-CTTATTCTTGCTAGCCCTATGCAAATGCT-3'. The amplicon was digested with XbaI and cloned into the XbaI sites of pPS1630 to construct Ylp-DCP2-GFP. To integrate the \(DCP2\)-GFP gene at the chromosomal \(DCP2\) locus, Ylp-DCP2-GFP was linearized by EcoRI and introduced into the yeast cells. For laboratory scale experiments, UT-1 cells were cultured in SD minimal medium (2% glucose and 0.67% yeast nitrogen base without amino acids) with tryptophan (40 \(\mu\)g/ml) at 28 °C with reciprocal shaking (120 rpm) in 300-ml Erlenmeyer flasks. For the cultivation of strain W303-1A, histidine (20 \(\mu\)g/ml), leucine (60 \(\mu\)g/ml), and adenine (40 \(\mu\)g/ml) were also added to the medium.

Laboratory-scale Japanese sake breeding (kojikomi test) and wine making were independently carried out more than three times, as previously described.\(^{13,14,18}\) The brewer’s rice and koji (rice malt) used in this study were prepared using 70% milled Gohyakuman-goku (a breed of rice). Filtrated water from an underground spring (in the Fushimi area, Kyoto), known as Fushimiizu, was used for sake brewing. Brewage ingredients were mixed with the yeast mash (moto) three different times (soe, naka, and tome feeding procedures).\(^{18}\) The moto was prepared with 8 g \(koji\), 27 mg \(KH_2PO_4\), 4.5 mg \(NaCl\), 158 mg lactic acid, 4 \(\times\) 10\(^5\) UT-1 cells, and 45 ml of water at 15 °C. The soe procedure (day 2 of fermentation, 22 g of brewer’s rice was added) was carried out at 15 °C. Both the naka (day 4, 9 g of \(koji\), 47 g of brewer’s rice, and 66 ml of water were added) and the tome (day 5, 17 g of \(koji\), 67 g of brewer’s rice, and 98 ml of water were added) procedures were carried out at 10 °C. After the tome procedure, alcoholic fermentation was carried out at 11.5 °C on day 6, at 13 °C on day 7, and at 15 °C after day 8.

For wine making, grape must was obtained from Chardonnay grapes grown in Yamanashi Prefecture, Japan, in 2006. Grapes were crushed in a stainless steel tub after being de-stemmed and compressed to yield grape must. The must was stored at ~20 °C prior to use. Sulfur dioxide and glucose were added to the must at 50 mg/liter and 86 g/liter as the final concentration, respectively. The indices of the must were pH 3.3, 24.0 Brix, and 7.5 g of total acid/liter. The must (250 ml) was filtered through a 0.22-μm-pore size membrane filter and inoculated with a 3% starter culture of UT-1 cells (approximately 10\(^6\) cells/ml), and then fermented at 15 °C. The sugar content of the must and sake mash (sake moromi) was measured using a refractometer (Master-T, Atago Co., Ltd.).

**Results**

**Ethanol caused the formation of P-bodies in the SD medium**

First we investigated the effects of ethanol on the formation of P-bodies in a sake yeast strain (UT-1) and a laboratory strain (W303-1A) at mid-logarithmic growth phase (OD\(\text{\textlesseq}10\) = 0.3) in SD medium. We used Dcp2p-GFP as a P-body marker, since Dcp2p plays a critical role in the formation of P-bodies and functions as a decapping enzyme in P-bodies.\(^{8,10}\) More than 6% ethanol as well as glucose depletion enhanced the formation of P-bodies, i.e., the number and size of P-bodies increased under ethanol-stressed conditions (Fig. 1A). The formation of P-bodies under ethanol stress occurred rapidly (within 5 min). On the other hand, heat shock at 42 °C did not enhance the formation of P-bodies (data not shown). We confirmed that the formation of P-bodies was enhanced by ethanol stress using Dhh1p-GFP as another P-body marker (data not shown). Dhh1p is an RNA helicase that interacts with both the decapping and deadenylase complexes in P-bodies.\(^{11}\)

We also examined whether the formation of P-bodies in response to ethanol is reversible. Elimination of 10% ethanol stress led to the disassembly of P-bodies within 5 min (Fig. 1B). The rapid formation and disassembly of P-bodies in response to ethanol indicates that components of P-bodies, such as Dcp2p and Dhh1p, assemble in response to ethanol stress in a reversible manner.

Regarding the formation of P-bodies upon ethanol stress, no obvious difference was observed between W303-1A cells (the laboratory strain) and UT-1 cells in SD medium (Fig. 1A and B). Additionally, other laboratory strains, yRP1724 and yRP1727, showed almost the same response to ethanol as UT-1 and W303-1A (data not shown). These results indicate that ethanol in SD medium has almost the same effect on the formation of P-bodies in sake yeast cells (UT-1) as it does in laboratory strain cells (W303-1A, yRP1724, and yPR1727).
Next we investigated the formation of P-bodies in UT-1 cells during wine making. After sampling from the fermenting must every day, the intracellular distribution of Dcp2p-GFP was immediately monitored. The changes in the ethanol concentration and sugar content of the fermenting must are shown in Fig. 2A. At the early stage of wine making (days 1 to 7, less than 6% ethanol concentration), one or two foci of P-bodies were rarely detected (Fig. 2B). However, the number and size of P-bodies began to increase when the ethanol concentration of the must reached about 6% (after day 8), and finally each
Fig. 2. P-Body Formation in Sake Yeast Cells during the Wine Making Process.
A. Changes in the ethanol concentration and sugar content of wine must during wine making. The results are representative of three independent wine making sessions. B, Formation of P-bodies (Dcp2p-GFP) in UT-1 cells during the wine making process. Ethanol concentrations of the wine must and days of fermentation are indicated under the panels. C, Cells from wine must containing 9.45% ethanol were transferred into ethanol-removed must (containing 1.60% ethanol) and incubated at 15 °C for 0–10 min, and the diffusion of Dcp2p-GFP was monitored.
cell showed 4–6 conspicuous foci of P-bodies when the ethanol concentration of the must reached about 9% (day 12) (Fig. 2B).

We further investigated whether this enhancement of P-body formation was caused by the increase in the concentration of ethanol in the must or by other factors, such as a decreased level of sugar. The wine must of day 13 (average ethanol concentration and sugar content, approximately 9.5% and 12.0 Brix) was collected and divided in two, and one half (10 ml) was centrifuged to eliminate yeast cells. The centrifuged must was subsequently bubbled with nitrogen gas, which in 5 h reduced the concentration of ethanol in the must to 1.6% (ethanol-removed must). Yeast cells were prepared from the other half by centrifugation and added to the ethanol-removed must. After incubation at 15°C, the distribution of P-bodies was monitored. As shown in Fig. 2C, cells of day 13 initially contained multiple conspicuous foci of P-bodies. However, the P-bodies were rapidly disassembled when the cells were transferred into the ethanol-removed must, and no clear P-body was detected after 10 min (Fig. 2C). This result indicates that the increase in the concentration of ethanol in the must during the process of wine making is probably the primary factor enhancing the formation of P-bodies, and that decreased levels of sugar in the must and other factors have little effect on the formation of P-bodies.

**P-Body formation during the sake brewing process**

We also investigated the formation of P-bodies in UT-1 cells during laboratory-scale sake brewing (kojikomi test). The change in the ethanol concentration of the fermenting sake mash (sake moromi) is shown in Fig. 3A. At the early stage of sake brewing (days 1 to 5, less than 4% ethanol), each cell contained 2 to 5 P-bodies (Fig. 3B). After the final addition of rice and water (day 5, tome procedure), the number of P-bodies in each cell gradually decreased during days 5–7. Surprisingly, the formation of P-bodies was not enhanced in the sake moromi when the concentration of ethanol exceeded 6% (after day 7). Compared with the cells in the wine must at the same concentration of ethanol (6–10%), the cells in the sake moromi contained fewer and smaller P-bodies (Figs. 2B, 3B). When the ethanol concentration of the sake moromi exceeded about 13%, the number and size of P-bodies began to increase gradually, and finally each cell showed multiple conspicuous foci of P-bodies when the ethanol concentration reached about 15% (Fig. 3B). No obvious cell death was observed in the sake moromi during the brewing process (days 1–23). These results clearly indicate that the yeast cells in the sake moromi showed unique responses to ethanol, and it is likely that sake yeast cells acquired tolerance and adapted to ethanol stress regarding the formation of P-bodies through the progress of alcoholic fermentation in the sake moromi.

We further investigated to determine whether the acquired tolerance to ethanol regarding P-body formation is retained even outside the sake moromi. To address this issue, UT-1 cells from the sake moromi of day 9 (ethanol concentration, 10.25%), in which one or two small P-bodies per cell were detected, were collected and transferred into SD medium or wine must containing almost the same concentration of ethanol. On transfer of the cells into SD medium containing 10.00% ethanol, the number and size of P-bodies rapidly increased within 10 min (Fig. 3C, upper panels). Such a rapid enhancement of P-body formation was also observed on transfer into wine must containing 10.05% ethanol (Fig. 3C, upper panels). These results indicate that the acquired tolerance of sake yeast cells to ethanol regarding P-body formation during sake brewing was retained only in the sake moromi, while it was rapidly lost on transfer into SD medium or wine must. On the other hand, the number and size of P-bodies did not decrease on transfer of cells from the SD medium containing 10.00% ethanol to the moromi containing...
9.50% ethanol (Fig. 3C, lower panels), indicating that simply transferring cells into sake moromi is not sufficient to establish tolerance to ethanol regarding P-body formation. It is more likely that fermentation of the sake moromi for days is critical to establish ethanol tolerance regarding P-body formation.

**Discussion**

Since decreases in translation rates enhance the formation of P-bodies and untranslated mRNAs can be sequestered in P-bodies, P-body formation reflects translational repression. Additionally, it has been
reported that P-bodies play important roles in the control of mRNA translation and degradation in response to various stresses.\textsuperscript{1,2}\textsuperscript{1,2} Here we found that ethanol stress also enhanced the formation of P-bodies in sake yeast as well as laboratory strains in SD medium (Fig. 1A), and that elimination of the ethanol rapidly caused the disassembly of P-bodies (Fig. 1B). These findings suggest that ethanol stress depresses the rate of translation and affects the flux of mRNA in the cytoplasm. It is likely that yeast cells can change their translation efficiency according to the concentration of ethanol in SD medium, and that P-bodies play a role in the flexible regulation of translation.

We also found that sake yeast cells show distinct responses to ethanol regarding P-body formation during sake brewing and wine making (Figs. 2B and 3B). During the process of wine making, the formation of P-bodies was enhanced as alcoholic fermentation progressed. As it was in SD medium, the concentration of ethanol in the wine must was the primary factor enhancing the formation of P-bodies (Fig. 2B and C). The number and size of P-bodies increased when the ethanol concentration in the wine must reached about 6\% (after day 8, Fig. 2B). These results indicate that translational repression was induced in the late stages of wine making. Indeed, we have previously reported that the cells barely synthesized any proteins late in the wine making process.\textsuperscript{1,3} We reported that the blocking of the nuclear export of bulk poly(A)$^+$ mRNA by ethanol was one of reasons for the repression of protein synthesis in the late stages of wine making. The findings presented here suggest that formation of P-bodies also participates in the repression of protein synthesis late in the wine making process.

In the case of Japanese sake brewing, P-body formation was not merely affected by the increased concentration of ethanol in the sake moromi. P-body formation was not enhanced when the concentration of ethanol in the sake moromi exceeded 6\% (Fig. 3B). In contrast with the process of wine-making, most of cells contained only one or two P-bodies under conditions of 6–13\% ethanol during the process of sake brewing. The size and number of P-bodies eventually increased when the concentration of ethanol rose above 13\% in the final stage of sake brewing. These results suggest that yeast cells can sustain translation efficiency under conditions of 6–13\% ethanol during the process of Japanese sake brewing. It is interesting that simply transferring cells into the sake moromi did not confer tolerance to ethanol regarding P-body formation (Fig. 3C). Therefore, it is likely that the unique responses of P-body formation to ethanol are specific phenomena caused by the progress of alcoholic fermentation in sake moromi for days.

In our previous study, we also found unique responses to ethanol regarding the nuclear export of bulk poly(A)$^+$ mRNA during the sake brewing process.\textsuperscript{1,4} Intriguingly, the nuclear export of bulk poly(A)$^+$ mRNA was not blocked by more than 6\% ethanol in the sake moromi during the brewing process, whereas it was mainly affected by the concentration of ethanol in SD medium and wine must.\textsuperscript{1,3,14,21} Such unique responses to ethanol regarding P-body formation and mRNA export in sake moromi may support the idea that sake moromi is a stressless environment of alcoholic fermentation for yeast cells as compared to wine must.\textsuperscript{1,3} So far at least, the mechanism by which yeast cells show such unique responses to ethanol in sake moromi is completely unknown. Compared with wine must, sake moromi is rich in lipids, fibers, kojic acid, amino acids, and peptides.\textsuperscript{22–25} One possibility is that various ingredients of sake moromi function as protectants against the adverse effects of ethanol on yeast cells. Further study to uncover novel physiological functions of sake moromi should lead to progress and innovation in the brewing industry.

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