Solid-state culture encourages high-level enzyme secretion by *Aspergillus oryzae*. Using the real-time quantitative reverse transcriptase-polymerase chain reaction, we confirmed that expression of the glucoamylase-encoding gene in *A. oryzae* cultured in solid-state culture depends on the water content of the culture.

**Key words:** *Aspergillus oryzae*; real-time PCR; solid-state culture; water content

*Aspergillus oryzae* is predominantly used in Japanese fermentation industries, such as sake, shoyu, and miso manufacturing. *A. oryzae* is grown in solid-state culture on, for example, steamed rice or wheat, resulting in high levels of enzyme secretion. *A. oryzae* grown in solid-state culture have been found to secrete more proteins than samples grown in submerged culture. Moreover, especially in sake brewing, experience shows that enzymes produced in solid-state culture change with the water content of the culture.

Glucoamylase (α-1,4-glucan glucohydrolase, EC 3.2.1.3) is produced by *A. oryzae* during solid-state culture, and plays an important role as a hydrolytic enzyme during koji-making. *A. oryzae* has two different glucoamylase-encoding genes, *glaA* and *glaB*. *glaA* is expressed in submerged and solid-state culture, but its promoter activity is lower in solid-state culture than in submerged culture. *glaB* is highly expressed in solid-state culture. Ishida *et al.* found that expression of *glaB* is induced by starch, high temperature, and low water activity (Aw) using a β-glucuronidase promoter assay. Similar results have been reported to the effect that GlaB-glucoamylase production was increased by decreasing the moisture content in cellulose agar cube culture for the model system of fungal solid-state culture. Accordingly, during the process of koji-making, depletion of water and maintenance of a high temperature in the later stages of incubation are important for increasing glucoamylase activity. However, te Biesebeke *et al.* demonstrated that transcriptional induction of *glaB* is due to an effect of the maltose used for adjustment of the Aw by Ishida *et al.*, rather than to induction of low Aw. Hence, the relationship between *glaB* expression and water activity remains unclear.

We are interested in enzyme groups the expression of which depends on the water content of the culture, because water is one of the most important factors affecting various gene expressions during solid-state culture. Therefore, in this study, we examined the effect of the culture water content on expression of *glaB* and other genes using real-time PCR, not β-glucuronidase promoter assay.

*A. oryzae* strain RIB40 was used throughout this study. It was grown on 5 g of wheat bran containing various amounts of water (2, 3, 4, 6, and 8 ml) at 30 °C for 48 h. Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA) by adding 5 ml of TRIzol to 0.5 g of culture. Next, 1 ml of chloroform was added, followed by centrifugation at 8,000 rpm for 10 min at 4 °C. The supernatant was then moved to another tube, to which 2.5 ml of isopropylalcohol was added. After mixing, the sample was centrifuged at 8,000 rpm for 10 min at 4 °C. The RNA pellet was washed with 30% ethanol, centrifuged at 8,000 rpm for 10 min at 4 °C. The supernatant was then centrifuged at 8,000 rpm for 10 min at 4 °C. Following this, 2.5 ml of isoproplalcohol was added to the supernatant, which was centrifuged at 8,000 rpm for 10 min at 4 °C. The RNA pellet was then dissolved to 200 μl of nuclease-free water.

mRNA was purified using an OligoEx™-dT30(Super) mRNA Purification Kit (TaKaRa, Kyoto) according to the manufacturer’s protocol. Reverse transcription and
real-time PCR were performed with a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green® (Invitrogen), using 8 μl of mRNA as recommended by the manufacturer. qRT-PCR primers were designed using Primer Express® software (PE Applied Biosystems, Foster City, CA). Sequences of qRT-PCR primers are described in Table 1. Thermal cycling was performed with an ABI 7500 sequence detection system (PE Applied Biosystems). Reaction conditions were: 50°C for 2 min, then 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ technique (ABI User Bulletin 2). Relative quantification provides accurate comparison between the initial levels of the template in the samples, without requiring the exact copy number of the template. Further, the relative levels of the template in the samples can be determined without the use of standard curves. Each sample was analyzed in triplicate, and data points were used to calculate the average of the three. Non-specific bands in the PCR product were confirmed by polyacrylamide gel electrophoresis.

Glucoamylase activity was measured using a glucoamylase and α-glucosidase assay kit (Kikkoman, Tokyo) according to the manufacturer’s protocol. The mycelium content of the culture was determined by measuring the N-acetylglucosamine content with an Aspergillus assay kit (Kikkoman).

The relative expression levels of glaB (DDBJ accession no. AB007825), glaA (D10698), melB (BD165761), and pepA (D13894) were compared using real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Fig. 1). melB and pepA encode tyrosinase and acid protease, respectively, and are specifically expressed in solid-state culture (rice-koji). Histone H1 was used as an endogenous control. The level of glaB expression was increased by reducing the water content of the wheat bran culture. This result agrees with that of Ishida et al., who showed that β-glucuronidase activity of the glaB promoter increased with low water activity. On the contrary, glaA was expressed at a high water content of the solid-state culture, but the level of glaA expression decreased when the water content of the wheat bran culture was reduced. It was found that gene expressions of melB and pepA reached a maximum at water contents of 0.8 and 0.6 ml/g respectively. At present, we do not know why such variance was observed, but we intend to analyze this further in the future.

Glucoamylase activity was measured as relative protein level to confirm that enzyme activity increases with increasing gene expression. First we measured the mycelium weight to determine whether water content affects the growth of A. oryzae, and found that the growth rate increased with increasing water content (data not shown). Then, we calculated glucoamylase activity based on mycelium weight to eliminate the effect of growth rate. Glucoamylase activity was 30.3 U/g wet mycelium at higher water content (1.6 ml/g), and 655 U/g at lower water content (0.4 ml/g). Glucoamylase activity was measured using a glucoamylase and α-glucosidase assay kit (Kikkoman).
ase activity showed an increase of about 20-fold when the water content was reduced from 1.6 to 0.4 ml/g. This change in enzyme activity with decreasing water content is in line with the change in glaB expression with decreasing water content. From these data, it was found that the water content of the culture is closely related to glucoamylase activity, while the growth rate of A. oryzae is not important. Hence we concluded that the increase in glaB expression and enzyme activity observed by Ishida et al. were the result of the low water content of the solid-state culture, not of the maltose used to adjust the Aw.

The glaB gene is thought to be expressed in response to stresses such as low Aw, high temperature, and physical barriers. Here, we confirmed that the water content of solid-state culture plays an important role in glaB expression. This phenomenon suggests that a very low water content acts as a stressor (e.g., drought stress), causing A. oryzae to acquire nutrients for survival by producing large amounts of glucoamylase.

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References


