Mode of α-Amylase Production by the Shochu Koji Mold Aspergillus kawachii

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Aspergillus kawachii produces two kinds of α-amylase, one is an acid-unstable α-amylase and the other is an acid-stable α-amylase. Because the quality of the shochu depends strongly on the activities of the α-amylases, the culture conditions under which these α-amylases are produced were examined. In liquid culture, acid-unstable α-amylase was produced abundantly, but, acid-stable α-amylase was not produced. The acid-unstable α-amylase was produced significantly when glycerol or glucose was used as a carbon source, similarly to the use of inducers such as starch or maltose. In liquid culture, A. kawachii assimilated starch at pH 3.0, but no α-amylase activity was recognized in the medium. Instead, the α-amylase was found to be trapped in the cell wall. The trapped form was identified as acid-unstable α-amylase. Usually, acid-unstable α-amylase is unstable at pH 3.0, so its stability appeared to be due to its immobilization in the cell wall. In solid-state culture, both kinds of α-amylase were produced. The production of acid-stable α-amylase seems to be solid-state culture-specific and was affected by the moisture content in the solid medium.

Key words: Aspergillus kawachii; α-amylase; solid-state culture-specific

Shochu (a traditional distilled liquor in Japan) is made from some crops such as rice, barley, sweet potato and others. The saccharide in these crops is mainly starch, so saccharification of starch by amylase is needed prior to the fermentation by yeast. α-amylase as well as glucoamylase plays a crucial role in this process. These enzymes, along with other enzymes, are supplied as a koji (solid-state culture of fungi) in the fermentation process. White koji mold, usually Aspergillus kawachii, is used for shochu koji making, though A. oryzae is used for sake fermentation.

White koji mold is considered to be an albino mutant from the black koji mold A. awamori, and produces a lot of citric acid, so the environment is very acidic (about pH 3.0). Consequently, acid-stability is needed in the enzymes produced by these koji molds, and indeed this strain produces many kinds of acid-stable or acidophilic enzymes.¹³ Besides the use in the fermentation industry, this strain is considered to be useful for the production of industrial acid-stable enzymes.

It has been shown that both white⁴ and black⁵ koji mold produce two kinds of α-amylase. One is an acid-unstable α-amylase, which has almost the same characteristics as TAKA-amylase from A. oryzae,⁶,⁷ and another is an acid-stable α-amylase. The genes of the acid-unstable α-amylase have been cloned from A. shirousamii⁵⁰ and A. awamori,⁷ and it was shown that these genes, including the TAKA-amylase gene from A. oryzae, share extensive high homology (over 98% identity). The gene of an acid-stable α-amylase has been cloned from A. kawachii by Kaneko et al.⁵⁰ The acid-stable α-amylase contains a starch-binding domain and linker, and is stable at low pH.⁹ Because the pH in shochu mash is very low (about pH 3.0) as a result of the citric acid produced by white koji mold, most of the α-amylase activity in shochu mash has been thought to be due to the acid-stable form. To improve the shochu fermentation process and industrial enzyme production that rely on A. kawachii, the conditions under which the two amylases are produced need to be better understood.

Generally, expression of amylase is induced by the addition of inducers such as starch or maltose,¹⁰ and repressed by the addition of glucose,¹⁰ and it is also known that glucose and xylene have no effects on amylase expression. These phenomena were well analyzed in A. oryzae.¹⁵ However, in preliminary studies, we found that the conditions affecting α-amylase production by the shochu koji mold A. kawachii were very different from those affecting the α-amylase production of A. oryzae. We report on these differences here.

Materials and Methods

Strains, media, and cultivation. A. kawachii IFO 4308 was used. Two kinds of medium were used for liquid culture. One was a semi-synthetic basal medi-

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um consisting of 0.1% Bactotryptone, 0.5% yeast extract, 0.1% NaNO₃, 0.05% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄, and 2% carbon source. This medium is referred to by the name of its carbon source, e.g., glucose-medium. The other was wheat-bran extract medium, which was prepared by autoclaving 5% wheat bran in tap water for 10 minutes and squeezing the extract out with a cloth. The pH was adjusted with citric acid. The conidia of *A. kawachii* were inoculated into 150 ml of medium in 500-ml baffled flasks and cultivated with shaking at 30°C for 48 hours. In the solid-state culture, the substrate was wheat bran or rice granules. The appropriate amount of tap water was soaked up by the solid materials and autoclaved. After cooling, conidia of *A. kawachii* were inoculated and the culture was incubated at 30°C for 48 hours. The first 24 hours of cultivation was in a closed petri dish to prevent the evaporation of water, and the following 24 hours of cultivation was in a thermostat-hydropstat test chamber (Tokyo Rikakikai) in which the relative humidity was controlled at 92%.

**Preparation of enzyme solution.** In the solid-state culture, enzymes were extracted with 20 mM acetate buffer (pH 5.0) containing 0.5% NaCl and raw enzyme solution was prepared. In the liquid culture, cultivated medium was used as a raw enzyme solution. To the raw enzyme solution, ammonium sulfate was added up to 85% saturation, and centrifuged. The sediment was redissolved with 20 mM acetate buffer (pH 5.0), then desalted by gel filtration using an Econo-Pac P6 cartridge (BIO-RAD) with the same buffer used for redissolution to prepare the crude enzyme solution.

**Measurement of enzyme activity.** α-Amylase activity was measured with 2-chloro-4-nitrophenyl 6'-azido-6'-deoxy-β-maltosaccharides (N₃-G5-β-CNP) as a substrate according to the method of Shirokane *et al.*¹⁹ using a measuring kit (Kikkoman). In this method, 2-chloro-4-nitrophenyl β-maltosaccharides released from the substrate by the action of α-amylase was immediately cleaved to 2-chloro-4-nitrophenol (CNP) in a coupled reaction catalyzed by glucoamylase and β-glucosidase. The increment of CNP, measured by the absorbance at 400 nm, was proportional to the α-amylase activity. One unit of enzyme activity was defined as the activity releasing one μmol of CNP from N₃-G5-β-CNP per minute. Activity was measured at 37°C, pH 5.0. The glucose-forming activity was measured with 4-nitrophenyl O-α-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (G2-PNP) as a substrate according to the method of Imai *et al.*¹⁵ using a measuring kit (Kikkoman). This substrate can be hydrolyzed by both glucoamylase and α-glucosidase, so activity is shown as glucose-forming activity (that is, the sum of these two enzyme activities). In this method, 4-nitrophenyl β-D-glucopyranoside (G1-PNP) released from G2-PNP was immediately cleaved to 4-nitrophenol (PNP) in a coupled reaction catalyzed by β-glucosidase, and the increment of PNP was measured by the absorbance at 400 nm. One unit of enzyme activity was defined as the activity releasing one μmol of PNP from G1-PNP per minute. Activity was measured at 37°C, pH 4.0. The pH stability of enzyme was measured by the methods of Sudo *et al.*⁴

**Fractionation of α-amylases by HPLC and identification of enzymes.** The desalted crude enzyme solution was put on an anion exchange column DEAE-5PW (TOSOH) equilibrated with 20 mM acetate buffer (pH 5.0) and the column was eluted with a linear gradient of NaCl, from 0 M (0 minute) to 1 M (60 minute), using HPLC, and the α-amylase activity in each fraction was measured. The active fractions were pooled and purified further to identify the amino acid sequence for identification of the enzyme, by hydrophobic interaction chromatography, gel filtration, and reversed phase chromatography, referring to the methods in the literature.¹⁴,¹⁰ Details of this procedure are available on request. The N-terminal amino acid sequences of the purified enzymes after reversed phase chromatography were analyzed with a gas phase protein sequencer (model 491 Procise; Applied Biosystems). Activities of the different α-amylases were measured after separation using anion exchange chromatography (DEAE-HPLC).

**Southern and Northern blot analysis.** The Southern blot analysis was done under high stringency conditions (65°C, three washes with 1×SSC/0.1% SDS). The screening of α-amylase genes was done under low stringency conditions (55°C, three washes with 1×SSC/0.1% SDS). Using the TAKA-amylase cDNA as a probe, the genomic DNA library which was constructed in λ-DASHII and the cDNA library (from mRNA of mycelia grown in xylan/xyllose medium) in λ-gt10 were screened. The methods of library construction and the general DNA manipulation are the same as reported previously.¹⁵ Total RNA was extracted from the grown mycelia by the hot phenol method¹⁰ and hybridization analysis was done by hybridization method. A 230-bp cDNA fragment of an acid-unstable α-amylase, which showed no homology with acid-stable α-amylase, was amplified by PCR using primers 5’-ggttcatattgactccggtag-3’ for sense and 5’-tggtattgcttgatccag-3’ for antisense, and then used as a probe for the detection of acid-unstable α-amylase mRNA.

**Dissolution of cell walls.** Crude amylase solution from cell wall was prepared as described previously,¹⁸,¹⁹ using the fungal cell wall-lyasing enzyme Yatalase (Takara) which is not contaminated with...
Amylase. Lysing solution consisted of 2% Yatalase in 10 mM Tris-HCl buffer (pH 7.0), 0.8 M NaCl. Briefly, 10 g wet mycelia were mixed with 100 ml lysing solution for 3 hour at 30°C with gentle shaking to prevent the bursting of newly formed protoplasts. The mixture was then centrifuged at 2000 rpm for five minutes, and the supernatant was centrifuged again at 15,000 rpm for 10 minutes. The supernatant was used for analysis of amylase activity.

Citric acid content. Citric acid was extracted from 20 g of the solid cultivated materials with 100 ml of distilled water and the pH of the extract was measured. Citric acid content was measured enzymatically using a kit (Roche Diagnostics).

Results

α-amylases genes of A. kawachii

We searched a genomic DNA library by Southern blot hybridization using TAKA-amylase cDNA as a probe, and three kinds of genomic α-amylase gene were found: an acid-unstable α-amylase gene (amyA, DDBJ accession number AB109452), an acid-stable α-amylase gene (asaA), and a new α-amylase gene candidate (amyX). This new α-amylase gene candidate was located between the regulator gene amyR and the α-glucosidase gene in the genome, however, this candidate gene did not appear to be expressed under any conditions tested, so we considered it to be a pseudo/silent gene (data not shown). The cDNA of amyA was obtained from cDNA library. The predicted amino acid sequence of acid-unstable α-amylase was almost the same as Taka-amylase (3 residues different), however, about 60 base pairs of deletion comparing with that of TAKA-amylase gene was found in the promoter. The characterization of this gene will be reported in another paper. From the genomic Southern analysis, the copy numbers of these genes appeared to be single (Fig. 1). From these results, it is considered that A. kawachii produces only two kinds of α-amylase.

Fractionation of α-amylases by HPLC

The activities of the two α-amylases were assayed separately. The crude enzyme prepared from solid-state wheat bran culture was put through DEAE-HPLC, and two fractions with α-amylase activity (F-I and F-II) were obtained (Fig. 2). These fractions were further purified by reversed phase HPLC (data not shown) and their N-terminal amino acid sequences were identified (ATPADWR- and

Fig. 1. Genomic Southern Analysis for α-Amylases Genes of A. kawachii.

Five μg of genomic DNA was digested with EcoRI, KpnI and HindIII. After electrophoresis, Southern blot analysis was done using DNA fragments encoding ORF (including introns, putative ORF in amyX) of each gene as a probe. amyA: acid-unstable α-amylase, amyX: unidentified new α-amylase, asaA: acid-stable α-amylase.

Fig. 2. Fractionation of α-Amylases by HPLC.

The crude enzyme from a solid-state wheat bran culture was separated by DEAE-HPLC and α-amylase activity in each fraction was measured.
\(\alpha\)-Amylase Production by Aspergillus kawachii

Fig. 3. Effects of Different Carbon Sources on \(\alpha\)-Amylase Production (A) and Glucose-forming Activity (B) in Liquid Culture after One Day (●) and Two Days (□).

Fig. 4. Identification of \(\alpha\)-Amylase Produced in Liquid Culture.
A: The crude enzyme prepared from wheat bran extract culture was separated by DEAE-HPLC and \(\alpha\)-amylase activity in each fraction was measured. B: SDS-PAGE of the crude enzymes prepared from xylose-medium culture. The black arrow indicates acid-unstable \(\alpha\)-amylase and white arrow indicates the size of acid-stable \(\alpha\)-amylase (not produced in this culture).

**LSAAGWR**, respectively). These sequences corresponded with the sequences of acid-unstable \(\alpha\)-amylase and acid-stable \(\alpha\)-amylase, respectively. Consequently, F-I and F-II were identified as the acid-unstable \(\alpha\)-amylase and the acid stable \(\alpha\)-amylase, respectively.

**Effects of carbon sources on \(\alpha\)-amylase production in liquid culture**
\(\alpha\)-amylase was produced after one day of culture by starch, maltose, glycerol, xylose, and even glucose, which often has a repressive effect (Fig. 3, left panel, dark bars). The activities in glycerol, xylose, and glucose medium were much greater after two days culture (hatched bars). This production mode is quite different from that of \(A.\) oryzae. In contrast, glucose forming activity, which is due to the sum of the activities of glucoamylase and \(\alpha\)-glucosidase, was high only when inducers such as starch were used (Fig. 3, right panel). This production mode was almost the same as that of \(\alpha\)-amylase in \(A.\) oryzae.

**Identification of \(\alpha\)-amylase produced in liquid culture**
Crude enzyme prepared from wheat bran extract was separated by DEAE-HPLC and only one active peak corresponding to acid-unstable \(\alpha\)-amylase (Fig. 4A) was recognized. Acid-unstable \(\alpha\)-amylase
Fig. 5. Effects of pH on α-Amylase Production.
A. kawachii was cultured in glucose medium for one day to adjust the growth, and equal amounts of mycelia were transferred to the starch media adjusted pH and then cultivated for one further day. α-amylase in liquid media and in cell-wall fraction (2) were shown. The pHs after cultivation were written in parentheses.

was the major protein produced in liquid culture. The same results were obtained in other liquid media regardless of carbon sources (data not shown). This result was confirmed by SDS-PAGE of crude enzymes prepared from xylose medium (Fig. 4B). This suggests that acid-stable α-amylase is not produced in liquid culture. In the medium cultivated for a longer period according to Sudo et al., no acid-stable α-amylase was detected.

Effect of pH on α-amylase production
A. kawachii produces a large quantity of citric acid, which can reduce the environmental pH by up to several units, so the effects of pH on the α-amylase production were examined and the results are shown (Fig. 5, dark bars). In this experiment, A. kawachii was cultured in glucose medium for one day, and then equal amounts of mycelia were transferred to starch media adjusted to various pHs and cultivated for one more day. Little change in pH occurred in this period, except for pH 7. Production of acid-unstable α-amylase was high and almost at the same level from pH 5 to pH 7, and slightly low at pH 4. At pH 3.0, no α-amylase activity was observed in liquid medium, whereas digestion of starch and mycelial growth were observed. In this experiment, all of the α-amylases found in liquid media were acid-unstable α-amylase (data not shown). In Fig. 4-B, the band of acid-unstable α-amylase was not recognized at pH 3.0, in spite of the abundant amount at pH 6.0, also in xylose medium.

Northern blot analysis of the acid-unstable α-amylase expression
The absence of α-amylase activity in liquid medium at pH 3.0 could be due to either pH-dependent gene expression or deactivation of enzymes at low pH, so Northern blot analysis was done to confirm the gene expression at various pHs. With both maltose medium and glucose medium, expression of acid-unstable α-amylase at pH 3 was about the same as that at pH 7 (Fig. 6), indicating that the absence of activity in liquid medium at low pH was caused by deactivation at low pH. The same results were obtained with other carbon sources (data not shown). In this experiment, the expression of acid-unstable α-amylase in glucose medium was also confirmed.

Trapped α-amylase in cell wall
Even at pH values under 3.0, starch was digested and the turbidity decreased rapidly, suggesting that an α-amylase is present somewhere. Because no activity was detected in liquid medium, the α-amylase appeared to be present in the cell wall and appeared to act on starch in the same manner as β-glucosidase. To confirm this idea, the cell walls were solubilized and the recovery of α-amylase was examined. α-amylase activity in the liquid phase increased along with the solubilizing enzyme treatment (Fig. 7), indicating the existence of α-amylase in the cell wall. Using DEAE-HPLC, the trapped enzyme was identified as acid-unstable α-amylase (Fig. 8), though this enzyme is unstable at pH 3.0. The amounts of trapped enzyme in cell wall of mycelia grown at various pHs were measured and the results are shown (Fig. 5, hatched bars). The larger amounts of acid-unstable α-amylase existed in the cell wall from pH 4 to 7.

pH stability of trapped acid-unstable α-amylase
Because free acid-unstable α-amylase is soon deactivated at pH 3, it was considered that the acid-stability of this enzyme was increased by trapping, so the pH stability of trapped enzyme was measured using wet whole mycelia grown at pH 4. In this experimen-

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Fig. 6. Northern Blot Analysis of the Expression of Acid-unstable α-Amylase (AUA).
A. kawachii was cultured in glucose medium (pH 6.0) for one day to adjust the growth, and an equal amount of mycelia were transferred to the maltose (M) or glucose (G)-medium adjusted pH and then cultivated for five hours to induce amylase expression. The probe was an AUA-specific DNA fragment.
Fig. 7. Liberation of α-Amylase Activity from the Cell Wall of *A. kawachii.*
*A. kawachii* was cultivated in starch medium (pH 3.0) for two days and the harvested mycelia were treated with fungal cell wall-lysing enzyme.

tal condition, about 20% of released enzyme activity was estimated as activities of trapped enzyme in whole mycelia. The pH stability at lower pH increased by trapping and significant activity remained at pH 3 as shown in Fig. 9.

*α*-amylose production in solid-state culture
As shown by DEAE-HPLC, both kinds of α-amylose were observed in solid-state culture using wheat bran as the substrate, whereas only acid-stable α-amylose was observed when rice granules were used as the substrate (Fig. 10). The absence of acid-unstable α-amylose in the rice culture appeared to be due to the fact that the citric acid content in rice culture (12.4 mg/g) was higher and its pH (3.2) was lower than those in the wheat bran koji (4.1 mg/g and 4.3, respectively).

Fig. 8. Identification of Trapped α-Amylase by DEAE-HPLC.

Fig. 9. pH Stability of Trapped Acid-unstable α-Amylase.
The residual activities of trapped enzyme (●) and free enzyme (○) after one hour of pH treatment at various pHs were shown.

Fig. 10. α-Amylase Production in Solid-state Culture.
The α-amylases produced in solid-state culture using wheat bran (left side) or rice granules (right side) as substrates were analyzed by DEAE-HPLC.
Effects of moisture on acid-stable α-amylase production in solid-state culture

The above experiments suggested that acid-stable α-amylase production was specific to solid-state culture. Because the most conspicuous difference between the solid state and liquid culture is the lower water activity in the solid state culture, the effects of moisture on the production of the two enzymes were measured. Increasing the moisture content decreased the production of acid-stable α-amylase significantly but had less effect on the acid-unstable α-amylase production (Fig. 11).

Discussion

The mode of α-glycosidase gene expression has been well analyzed in some fungi such as A. nidulans or A. oryzae. The expression of these genes is induced via an activator protein called AmyR and in the presence of glucose is repressed via a protein called CreA. However, in A. kawachii, the mode of α-amylase expression was quite different from that of A. oryzae. The acid-unstable α-amylase is expressed in the presence of various carbon sources including glucose, though the mode of glucoamylase expression is the same as that of glycosyl hydrolase from A. oryzae. We consider that this phenomenon may be caused by the 60 bps of deletion in the promoter, so we are now analyzing the expression profile of this promoter.

When starch is used as a carbon source, the α-amylase of A. oryzae is expressed efficiently at the early stage but is soon repressed by the glucose formed from starch by the action of amylase. However, the α-amylase of A. kawachii is expressed constantly without repression by glucose. This expression mode of acid-unstable α-amylase, that is, its expression in the presence of various carbon sources and no repression by glucose, may have industrial applications. For example, this amylase may be cheaply produced by using various hexoses as a carbon source, or may be highly expressed in starch medium throughout the course of cultivation without repression by glucose, or its promoter may be used for heterologous gene expression.

The acid-stable α-amylase was produced in solid-state culture but not in liquid culture, and the production in solid-state culture was affected by moisture. The liquid culture medium using wheat bran extract and the solid-state culture medium using wheat bran had almost the same chemical composition and differed mainly in moisture content. Thus, the acid-stable α-amylase expression is considered to be solid-state specific. Expression of the glucoamylase gene (glaB) of A. oryzae is also reported to be solid-state-specific and to be controlled at the transcriptional level. However, in acid-stable α-amylase expression, a significant amount of mRNA was detected also in liquid culture, suggesting a different regulation manner. We are now analyzing the gene expression of A. kawachii α-amylase genes using reporter gene techniques and other biochemical methods. The results of these studies will be reported in another paper.

A. kawachii digested starch at pH 3.0 in liquid culture even though there was no α-amylase activity in the liquid medium. Because the turbidity decreased in a short time as the result of the digestion of starch, the digestion did not appear to be due to the activities of only glucoamylase or α-glucosidase. Rather, it was probable that an endo-type amylase, that is α-amylase, was involved. Under low pH conditions, a significant amount of mRNA of acid-unstable α-amylase was observed, which suggests that acid-unstable α-amylase protein was produced and secreted normally but soon was denatured by low pH. The denatured protein seems to be digested by protease and to soon disappear because no α-amylase protein was observed in SDS-PAGE. Because an α-amylase was present in the cell wall, it appeared to be the enzyme that was acting on starch. At first, we thought that this trapped enzyme was acid-stable α-amylase, because acid-unstable α-amylase was not known to be active at this pH. Unexpectedly, however, the trapped enzyme was found to be an acid-unstable α-amylase. To explain how an acid-unstable enzyme could be active at low pH, we hypothesize that the trapped enzyme was stabilized in the same manner that artificially immobilized enzymes are stabilized. Indeed, it was confirmed that the pH stability of trapped enzyme increased. In addition to this
mechanism, the trapped enzymes may be stabilized by polysaccharides in the cell wall, as is the case with the β-glucosidases of A. kawachii\(^3\) and Trichoderma reesei.\(^3\) It was considered that trapped acid-unstable α-amylase also existed in mycelia grown in solid-state culture, though the amount was less than that of liquid culture, as the same manner of β-glucosidases.\(^3\) The apparent activity of trapped enzyme was very low, so we consider that the contribution of this enzyme in shoucho fermentation is negligible but it may be enough for the use of 2% gelatinized starch in liquid culture.

In the shoucho fermentation industry, A. kawachii is cultivated on solid-state materials (rice or barley koji) and the environmental pH is very acidic because of the citric acid produced. Under these conditions, the only remaining α-amylase is acid-stable α-amylase\(^3\) except for the slight amount of trapped acid-unstable α-amylase. This acid-stable α-amylase is stable at low pH, so it can efficiently under the acidic conditions of fermentation mash. Though the productivity and specific activity of acid-stable α-amylase is low, it is considered that the activity of this enzyme in ordinary shoucho koji is enough for shoucho fermentation, because the necessity of α-amylase activity for shoucho fermentation is not very high.\(^3\) However, if the acid-stable α-amylase production is hampered by some factors, e.g. excess water in the koji, hindrance of the materials use will occur. A detailed analysis of gene expressions will be reported in another paper.

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