A study of Taka-amylose A of conidia from Aspergillus oryzae RIB40 was done. During the research, proteins from conidia and germinated conidia were analyzed using SDS–PAGE, 2-D gel electrophoresis, Western blot analysis, MALDI-TOF Mass spectrometry, and native-PAGE combined with activity staining of TAA. The results showed that TAA exists not only in germinated conidia but also in conidia. Some bands representing degraded products of TAA were detected. Conidia, which formed on starch (SCYA), glucose (DCYA), and glycerol (GCYA) plates, contained mature TAA. Only one active band of TAA was detected after native-PAGE activity staining. In addition, TAA activity was detected in cell extracts of conidia using 0.5M acetate buffer, pH 5.2, as extraction buffer, but was not detected in whole conidia or cell debris. The results indicate that TAA exists in conidia in active form even when starch, glucose, or glycerol is used as carbon source. TAA might belong to a set of basal proteins inside conidia, which helps in imbibition and germination of conidia.

Key words: Aspergillus oryzae; conidia; germinated conidia; soluble proteins; Taka-amylose A

Aspergillus oryzae is a very useful filamentous fungus for Japanese fermented foods such as sake (rice wine), miso (soybean paste), and shoyu (soy sauce). The strain is widely used for the production of various enzymes, homologous and heterologous proteins, in bio-technological industries since it has a great ability to secrete diverse proteins. In the life cycle of this species, there are many stages of development, spanning from germination of conidia to formation of mature conidiophore-bearing chains of conidia. Especially, the late mycelial growth stage is very important for industrial purposes. During this stage many of hydrolytic enzymes are produced, such as glucoamylase, Taka-amylose A, proteases, β-galactosidase, lipases, and cellulases which benefit industry with enormous amounts of industrial enzymes for fermented food products. Despite the industrial importance of germination, relatively little is known about them. The nutrition of the conidial germination stage depends on extracellular or-and intracellular hydrolytic enzymes, which help the germ tubes to penetrate into the substrate.

In a previous paper, we reported extracellular enzymes from the early stage of germination by the proteomic approach. Taka-amylose A (TAA), glucoamylase (GLAA), and aspergillopepsin A (PEPA) were the main products during germination. TAA is a useful secreted enzyme that can degrade starch into glucose to fulfill nutrient demands and help in the development of A. oryzae. TAA was detected in glucose liquid medium at the fourth hour of germination. On the other hand, EST of taa was not detected in the conidia. It is unclear why the conidia of the early germinated stage secreted TAA in the medium. There is no information at the molecular level regarding this. In this study, TAA from conidia of A. oryzae RIB40 was analyzed using Western blot analysis of one dimension (1-D), 2-dimension gel electrophoresis (2-DE), the peptide mass fingerprinting method with Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), and native-PAGE methods. The results show that conidia contained active TAA even when starch, glucose, or glycerol was used as carbon source.

Materials and Methods

Strain and preparation of purified conidia and germinated conidia. A. oryzae RIB40 (ATCC 42149) was maintained on potato dextrose agar (PDA) (Nissui, Tokyo, Japan). Conidiating culture was made as described in the previous paper. The conidiating culture medium contained 1% casamino acid, 1% yeast nitrogen base w/o amino acid, 1.5% agar, and 2% sugar. SCYA contained starch, DCYA contained glucose, and GCYA contained glycerol as carbon source. Conidia were inoculated on plates and incubated at 30°C for 4 d as preculture. Then the conidia were harvested and transferred to new PDA, SCYA, DCYA, and GCYA plates covered with autoclaved filter paper (No. 5C, 90 mm, Advantec). The plates were incubated again at 30°C for 7 d. The mature conidia were harvested separately and filtered through a P11G250 glass filter, and centrifuged...
at 11,000 g for 10 min to concentrate the conidia. Thereafter, the conidia were washed five times with distilled water and centrifuged at 13,000 g at 4 °C for 5 min. The washed conidia were used for further experiments. Germinated conidia were prepared as follows: 100 mg wet weight of washed conidia was inoculated into SP medium (3.5% starch, 2% peptone, 0.5% MgSO$_4$·7H$_2$O, 0.5% KH$_2$PO$_4$, pH 5.6), and incubated at 28 °C for 10 h. Germinated conidia were harvested and purified on ice with gauze (Hasegawa Men). The purified germinated conidia were harvested by centrifugation at 13,000 g, 4 °C for 5 min and stored at −80 °C. The purified conidia and germinated conidia were confirmed by microscopy (Fig. 1).

Sample preparation of protein. 100 mg wet weight of freshly harvested mature conidia and 160 mg wet weight of germinated conidia were disrupted separately with liquid nitrogen. Proteins were extracted with lysis buffer (9M urea, 2% triton X-100, 2% IPG buffer, 8 mM PMSF, 2% DTT). After centrifugation at 13,000 g at 4 °C for 5 min, extracts were collected and stored at −80 °C as sample stock sources. These samples were used for SDS–PAGE and Western analysis experiments. For the activity staining and enzyme activity assay, 100 mg wet weight of conidia from SCYA, DCYA, or GCYA plates were disrupted with liquid nitrogen, and then protein was extracted with 1.5 ml of 0.5M acetate buffer, pH 5.2. The concentration of the protein was calculated by the Lowry method using lyophilized bovine plasma γ-globulin as a standard.

Assay of amylase activity. After centrifugation, the supernatant was used as soluble protein. The pellet was re-suspended into 1 ml of 0.5 M acetate buffer, pH 5.2, and the suspension was used as cell debris. 100 mg wet weight of conidia was also suspended into 1 ml of 0.5 M acetate buffer, pH 5.2, to verify the trapping of TAA on the surface of the conidia. To measure enzyme activity, 30 μl of enzyme sample was allowed to react with 120 μl of 0.5% soluble starch in acetate buffer at 30 °C for 15 min. Then reactions in the debris and the conidia were stopped and it was centrifuged immediately. Reducing sugar in the reaction mixture was determined by the Nelson–Somogyi method. One unit of substrate hydrolyzing activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of glucosidic linkages per min under optimal conditions.

1-D gel electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 12% polyacrylamide gel. 20 μl/well (about 200 μg protein/well) of each sample was loaded according to the previous report.

Native-PAGE and activity staining. All the constituents of native-PAGE are the same as SDS–PAGE, except that native-PAGE does not include SDS or 2-mercaptoethanol. Freshly prepared soluble proteins of conidia, cultivated on various carbon sources, were collected separately and mixed with sample buffer (50 mM tris–HCl pH 6.8, 10% (v/v) glycerol, 1% BPB) in a ratio of 3:1 just before use. Forty μl/well was loaded into acrylamide gel. After PAGE, the gel was incubated for 1 h at 30 °C in 3% soluble starch, and then stained with Lugol solution. In order to compare TAA in conidia with mature TAA secreted by germinated conidia, A. oryzae from the PDA plate was inoculated in 100 ml of SP medium and incubated overnight at 28 °C. Mycelia were removed from the cultivated medium by centrifugation at 11,000 g for 5 min. The supernatant was used as a secreted protein sample, and diluted by the sample buffer (constituents described above) in a ratio of 1:1, and the 40 μl/well was loaded into the acrylamide gel.

Fig. 1. Microscope Pictures of Purified Conidia and Germinated Conidia of Aspergillus oryzae RIB40.

Both conidia and germinated conidia were observed under the microscope at the same intervals. Scale bar, 10 μm. A, Conidia (PDA); B, Germinated conidia (Starch) 10 h.
2-D gel electrophoresis. Immobiline DryStrip (Amer- sham Biosciences, Uppsala, Sweden) pH 4–7 was rehydrated. Protein sample and buffer (9 M urea, 2% triton X-100, 2% IPG buffer, 65 mM DTT) was mixed in a ratio of 1:1, and 100 μl of sample was applied into a cup (1 mg/gel). First-dimensional electrophoresis was done according to the maker’s manual. After completing the first dimension run, the gel was equilibrated according to the instruction manual for 15 min (Amer- sham Biosciences). The equilibrated gel was put on a 2-DE cassette containing 12% SDS–PAGE with the surface sealed by 1 ml of 1% agar. Second-dimensional electrophoresis was started at 600 V and 10 mA/gel of current for the first 15 min, and then the current was increased to 20 mA/gel until the run was completed.

Western blot analysis. Separated proteins in the gels were transferred to PVDF membrane in buffer containing 20 mM tris-base, 150 mM glycine, and 20% (v/v) methanol for 1 h at 600 V, 90 mA. TAA antiserum was diluted with phosphate saline buffer (PBS) in a ratio of 1:500 and allowed to react with protein on the membrane for 1 h at 25 °C. Subsequently, the second antibody (Alkaline Phosphatase-Conjugated Goat Affinity Purified Antibody to Rabbit IgG; ICN-Cappel, Aurora, OH), diluted in a ratio of 1:5,000 with PBS, was allowed to react with TAA antiserum on the membrane for 1 h on orbital shaking at 25 °C. The membrane was then treated with detection solution, which included 4 ml buffer III (0.1 M tris–HCl pH 9.5, 0.1 M NaCl, 50 mM MgCl₂), 9 μl nitro blue tetrazolium, and 7 μl 5-bromo-4 chloro-3 indolyl-phosphate.

Peptide mass fingerprinting method. The target spots were excised and treated by the in-gel digestion method. Subsequently, peptide fragments were purified by Ziptip C18 pipette tip (Millipore, Bedford, MA) and measured with a MALDI-TOF mass spectrometer (Voyager DE-STR mass spectrometer; PerSeptive Bio- systems, Framingham, MA) as described in the previous paper. Internal calibrations were done with trypsin autolysis generated ions. All peak numbers were used to search in MS-Fit with Swiss-Prot database at 50 ppm tolerance for protein identification (http://prospector. ucsf.edu).

Results

Western analysis with TAA antiserum

In this study, purified conidia and germinated conidia were used. There were no mycelia in the purified conidia and no conidium in the purified germinated conidia (Fig. 1). In Fig. 2, lanes 1 to 6 are conidia soluble proteins, which were extracted from conidia cultivated on SCYA, DCYA, and GCYA plates separately and stained by CBB. Lanes 7 to 12 represent Western blot analysis with TAA antiserum corresponding to lanes 1 to 6. The results show that conidia, which formed on starch, glucose, or glycerol plates, contained mature TAA of about 54.5 kDa. Besides the main TAA band (54.5 kDa), there were other bands also corresponding to 42, 37, and 31 kDa, viz., bands b, c, and d, which reacted with TAA antiserum in germinated conidia (lanes 13, 14) as well as conidia. Soluble proteins from conidia in the PDA plate and from germinated conidia in SP medium were also analyzed by 2-D Western blot with TAA antiserum (Fig. 3) to reconfirm the results in Fig. 2. About 10 spots from conidia sample A were detected, while more than 10 spots from germinated conidia sample B were detected that reacted with TAA antiserum. Among them, eight spots in B were similar to that of A. The spot number 1 (Fig. 3B) was analyzed by the peptide mass fingerprinting method using MS-Fit with the Swiss-Prot database. This spot was identified as...
TAA, with 10 peptides matching with the database at high probability (Fig. 4). Other spots were also identified as TAA fragments by MALDI-TOF MS. These results reconfirm the presence of the main TAA (54.5 kDa) in both conidia and germinated conidia. Conidia contained TAA even when starch, glucose, or glycerol was used as carbon source. Several spots on 2-D gel having similar molecular weights but different pIs were also detected.

Activity staining of TAA in the conidia

A native-PAGE experiment was performed in order to determine whether TAA was in its active form. Figure 5A (lanes 1 to 4) is CBB staining of conidial samples, which were extracted with 0.5M acetate buffer, pH 5.2, as a mild condition. Figure 5B is active staining corresponding to Fig. 5A. The results indicate that there was one active TAA band detected from the conidial sample, not only from starch but also from the glucose and glycerol plates. The intensity of active TAA band was high enough even when low concentrations of protein samples were extracted with acetate buffer (Fig. 5). The tendency of TAA activity to decrease from soluble proteins in conidia, which were collected from SCYA, DCYA, to GCYA plates, was also detected (Fig. 5B, lanes 5, 6, and 7). We also tried to extract proteins of conidia from these plates by modified lysis buffer, which contained urea and triton X-100 but no DTT. Higher concentrations of proteins were extracted using modified lysis buffer than that extracted from 0.5M acetate buffer, pH 5.2. But the results of activity staining were similar under both buffer conditions after 1 h of reaction using 3% starch as substrate (data not shown).

Assay of amylase activity of conidia

Enzyme activities were measured from three kinds of samples: (i) the supernatant, (ii) the debris, and (iii) the whole conidia, which were prepared on various carbon sources (as described in “Materials and Methods”). The results in Table 1 show that the maximum TAA activities were detected from the supernatant samples. The extract of conidia from SCYA had TAA activity of 2.1 units/total proteins, whereas the extract from the GCYA sample had activity of 0.6 units/total proteins. The TAA activities in the debris and whole conidia from SCYA, DCYA, and GCYA were negligible.

Discussion

The soluble proteins used for the experiment were extracted from purified conidia (Fig. 1) cultivated on three kinds of plates. 1-D Western results (Fig. 2) indicate that TAA existed in the conidia even when starch, glucose, or glycerol was used as a carbon source. Those bands were then analyzed by 2-D Western blot. As shown in Fig. 3, there were many TAA spots on both membranes. The main TAA (54 kDa) was identified in both conidia (A) and germinated conidia (B). The TAA detected at the tenth hour of germination might be a
newly synthesized enzyme. We also analyzed other putative TAA spots, which had molecular weights lower or higher than 54.5 kDa (Fig. 3B) by the peptide mass fingerprinting method. The spectrum of these spots contained TAA fragment peaks. Hanzawa et al.\textsuperscript{12}) reported that α-amylase from \textit{A. oryzae} firmly combines with protease, and also indicated the degradation products of this enzyme corresponding to 42, 37, and 28 kDa. This suggests that the band at 54.5 kDa was the main mature TAA, while the bands of low molecular weight might correspond to the intermediate degradation products (Fig. 2). These intermediates were probably

\textbf{Fig. 4.} Identified Peptide Fragments in TAA Amino Acid Sequence of \textit{Aspergillus oryzae}.

Based on searches in Ms-fit software with Swiss-Prot database, 50 ppm tolerance; access code P10529; M.W 54811; pI 4.5. Underlining shows matched peptides.

\textbf{Fig. 5.} Native-PAGE and Activity Staining of TAA.

A, CBB staining; B, active staining corresponding to A. Lanes 1 to 3 are conidial protein from SCYA, DCYA, and GCYA, extracted from 0.5 M acetate buffer, pH 5.2. Lane 4 is total secreted protein from germinated conidia, cultivated in SP medium (control). Lanes 5, 6, 7, and 8 are active staining corresponding to lanes 1, 2, 3, and 4.
produced by the protease, which had restricted specificity. The main TAA was confirmed by native-PAGE through activity staining, and just one band was detected in its active form from conidia (Fig. 5, lanes 5 to 7). The band was similar to that of secreted TAA collected after cultivation in SP medium (Fig. 5, lanes 4, 8). These results suggest that the conidia contained active TAA, which was similar to secreted TAA, and that bands b, c, and d in Fig. 2 represent the intermediate degradation products of TAA. The degradation products might have different mobility in the gel. 12) It is possible that the intermediate degradation products have no activity.

TAA was found in conidia not only from SCYA but also from DCYA and GCYA. As shown in Fig. 2, the mature TAA concentration in conidia from the starch, glucose, and glycerol supplemented plates decreased in the order starch to glucose to glycerol. The conidia from GCYA also contained TAA that might have been synthesized constitutively. Probably, the expression of taa genes in A. oryzae is regulated by various transcription factors depending on particular cultivation conditions. For example, the taaG2 gene, which was cloned and sequenced at the genomic DNA13) as well as the cDNA14) level, is known as the main taa in A. oryzae. It is induced during mycelial growth by AmyR15) and enhanced by AoCP/AoCF transcription factors, 16,17) when the medium contains starch or maltose. It is repressed by CreA transcription factor when the medium contains glucose. 16) In A. nidulans, taa is repressed in a medium containing glycerol. 18) Conidia and germinated conidia of A. oryzae from the glycerol plate also had active TAA. In the case of A. oryzae, the low level expression of taa probably occurs constitutively, and the TAA produced is stored in the conidia.

The maximum TAA activity was found in the supernatant samples (soluble proteins) (Table 1). The activities in the debris were negligible, and no trace of TAA activity was found on the surface of the conidia after washing five times with distilled water. These results suggest that the TAA activity of conidia was not caused by the TAA, which was bound loosely on the surface of the conidia. It can be concluded that the conidia contain an active form of TAA. It has been reported that extracellular TAA was detected at the fourth hour of germination. 8) But the mechanism behind TAA secretion in the medium during the short time of germination is still not well understood. The conidia represents dormant cells and the results of more than 500 EST analysis on conidia indicated that there were no taa EST. Therefore, the ability of expression of taa in conidia might be poor. TAA activity was detected in the soluble fraction of disrupted conidia. It is suggested that TAA found in the conidia was produced in the mycelia during conidiation and transported to the conidia through secretory vesicles as the intermediate of secreted protein. Since a little TAA activity was also detected in the debris, a little TAA might also be secreted and trapped inside the cell wall of conidia. Conidia of early germination stage restore the dormant secretion system in conidia and continue secretion of TAA. TAA might belong to a set of basal proteins inside conidia, which helps in the imbibition and the early germination of conidia.

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References


9) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the folin

Table 1. Activity of Taka-Amylase A of Conidia

<table>
<thead>
<tr>
<th>Medium</th>
<th>Wet weight (mg)</th>
<th>Enzyme activity (units/mg total protein)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Supernatant</td>
</tr>
<tr>
<td>SCYA</td>
<td>100</td>
<td>2.1</td>
</tr>
<tr>
<td>DCYA</td>
<td>100</td>
<td>1.3</td>
</tr>
<tr>
<td>GCYA</td>
<td>100</td>
<td>0.6</td>
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The enzyme extraction and assay of enzyme activity methods are described in "Materials and Methods".


