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FULL PAPER

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Characterization of extracellular glucoamylase from the ectomycorrhizal mushroom *Lyophyllum shimeji*

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Abstract To investigate the function of amylases in the fruit-body formation of an ectomycorrhizal fungus, *Lyophyllum shimeji*, we purified the extracellular amylase in the medium of this fungus. The purified enzyme was obtained from 1.7 l stationary culture filtrate, with 4.2% recovery, and showed a single protein band on SDS-PAGE. The molecular mass was about 25 kDa. The enzyme was most active at around 40°C and pH 5.0 and stable over pH 4.5–6.5 for 30 min at 37°C. This amylase was remarkably activated by the presence of Ca^{2+} ion (7.7 times that of the control), but Ba^{2+} and Ag^{+} completely inhibited the activity. The amylase readily hydrolyzed the α-1,4 glucosidic linkage such as dextrin and amylose A (MW, 2900), converting into glucose, and hydrolyzed the α-1,6 glucosidic linkage of isomaltohexaose and amylopectin. However, the enzyme did not hydrolyze the cyclic polysaccharides. On the other hand, when a low molecular mass amylose A was hydrolyzed by this amylase, β-anomer glucose was produced. From these results, we concluded that the amylase from *L. shimeji* seems to be a glucoamylase.

Key words Amylase · Edible mushroom · Glucoamylase · *Lyophyllum shimeji* · Mycorrhizal fungus

Introduction

The ectomycorrhizal fungus *Lyophyllum shimeji* (Kawam.) Hongo is one of the most valuable edible mushrooms in Japan. The ectomycorrhizal fungi such as *L. shimeji* and *Tricholoma matsutake* (S. Ito et Imai) Sing. are difficult to cultivate artificially without the host plant. However, in 1994, Ohta (1994) succeeded in the artificial cultivation of *L. shimeji* using barley grain without the host plant. The fruit-body formation of this fungus in artificial cultivation was reported by Watanabe et al. (1994) and by Yoshida and Fujimoto (1994) about the same time. The fungus was cultivated using commercially available culture instruments, and the culture conditions for mass production of this mushroom were determined by Ohta (1998). Ohta mentioned that the quantity of sufficient starch used as a carbon source was able to supply the factor that allows successful fruit-body formation without raising osmotic pressure in the medium. These facts suggested that the amylases and starch-hydrolyzing ability are very important for fruit-body formation.

In 2000, Terashita et al. (2000a) had already reported that amylase production from *L. shimeji* varied considerably among the strains used, and that production in the fruit-body-forming strains showed a higher than those of the fruit-body-nonforming strains. Moreover, they clarified that the amylase activity was remarkably activated by the presence of Ca^{2+} ion. However, the enzymatic properties were studied with only partially purified enzyme at that time.

In the present study, we examined the amylase activities produced in the barley grain medium during fruit-body formation using plastic bottle cultivation. As a result, it was shown that glucoamylase activity in the medium increased markedly during fruit-body formation. Then, we purified the extracellular glucoamylase from *L. shimeji* and revealed its enzymatic properties from the aspect of starch utilization as a growth substrate.

Materials and methods

Organism

L. shimeji MH 01721 strain was used in this experiment. This strain, which formed fruit-bodies on barley grain

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medium, was provided by Hokuto (Nagano, Japan). The fungus was stored on potato dextrose agar (PDA) medium at a low temperature (4°C).

Medium compositions and culture conditions

For the measurement of amylase activity during the development of the fruit-body, the cultivation of *L. shimeji* was used in a barley grain medium containing 80 g barley, 8 g sawdust, and 140 ml of one of ten diluted synthetic media (Ohta 1994). A 800-ml polypropylene (PP) bottle [commercial cultivation for *Pholiota nameko* (T. Ito) S. Ito et Imai (diameter, 77 mm; height, 135 mm] containing about 228 g barley grain medium was used. The culture bottle was autoclaved at 121°C for 60 min before use. After inoculation (Kusuda et al. 2003), the vegetative mycelia were grown at 24°C under continuous illumination (about 200 lux) for 60 days. Then, the induction of fruit-body formation was carried out by adjusting the temperature to 15°C, and the medium surface was covered with peat moss (about 5 mm thick).

For the purification of glucoamylase, a mycelial block (diameter, 10 mm) was cut from a plate culture that had been grown on modified matsutake agar (MMA, 1.5% agar powder) medium for 30 days at 24°C in a Petri dish (diameter, 90 mm). It was inoculated in an Erlenmeyer flask (100 ml) containing 20 ml MM liquid (MML) medium (Terashita et al. 2000a) after sterilization at 121°C for 5 min. The composition of MML medium was 22.7 g glucose, 5.0 g yeast extract, 77.0 g potato extract, 5.0 g Sunpearl-CP (commercial name; sulfite pulp waste from softwood) per 1000 ml distilled water, initial pH 5.1. After inoculation, it was stationary cultured at 24°C for 40 days in light condition (about 200 lux).

Extraction of crude enzyme

For the measurement of the changes in extracellular amylase activities during vegetative growth and development of fruit-bodies of *L. shimeji* in barley grain medium, 100 ml 0.1 M McIlvaine buffer solution (pH 5.0) was added to the medium (30 g) and mixed for 1 h at 4°C. Crude enzyme solution was collected by filtration with gauze, and then the filtrate was centrifuged (8000 *g*, 10 min).

For purification of the extracellular glucoamylase, after 40 days incubation, the culture medium was filtered through a filter paper (no. 2) for mycelial removal. The culture filtrate was used as the crude enzyme for purification start materials.

Measurement of amylase activities

The measurements of total amylase, α-amylase, and glucoamylase activities used a soluble starch solution as a substrate. The reaction for total amylase activity was carried out at 37°C for 60 min, and the activity was measured by the Somogyi–Nelson method (Somogyi 1952). The enzyme activity unit was calculated from the calibration curve of glucose as a standard.

The reaction for α-amylase activity took place at 37°C for 180 min. This activity was stained by iodine-potassium iodide solution (Terashita et al. 2000a) and was read at 690 nm in a spectrophotometer. One unit of α-amylase activity was defined as the activity that decreased absorbancy for 0.1 at 37°C for 1 min.

Glucoamylase activity took place at 37°C for 180 min. Then, glucose released by the reaction was determined with a F-kit glucose (Boehringer, Mannheim, Germany). One unit of the activity was defined as the activity that forms 1 μmol glucose at 37°C for 1 min.

The α-glucosidase activity was assayed using *p*-nitrophenyl α-D-glucopyranoside (*p*-NαG) as a substrate. The enzyme reaction was carried out at 37°C for 60 min. One unit of α-glucosidase activity was defined as the activity that forms 1 μmol *p*-nitrophenol in a 1.0-ml reaction mixture at 37°C for 1 min. More detailed procedures are described in our previous paper (Kusuda et al. 2003).

Purification procedures of extracellular glucoamylase

Ammonium sulfate was added to make a 55% saturation of the culture filtrate (1700 ml), from which the precipitate was recovered by centrifugation (9000 *g*, 20 min). The precipitate dissolved in a 20 mM Tris-HCl buffer (pH 7.0) and was dialyzed against the same buffer for 2 days.

The crude enzyme solution (520 ml) after dialyzing was concentrated by ultrafiltration (Asahi Kasei Microza pencil type module; cutoff 50 kDa). Unless otherwise specified, all purification steps were at 4°C. The crude enzyme solution was dialyzed (20 mM Tris-HCl buffer, pH 7.0, overnight at 4°C) and put on a Toyopearl-DEAE 650 M column (Tosoh, Tokyo, Japan, 26 × 150 mm, 40.0 ml) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0), and eluted with the same buffer containing a linear gradient of NaCl in the range of 0–1.0 M at a flow rate of 2.0 ml/min; a 6.0-ml fraction was collected. The amylase activity of each fraction was measured.

The active fractions (fractions no. 22–41; total volume, 120 ml) were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 7.0. The dialyzed solution was chromatographed again on a Toyopearl-DEAE column (10 × 50 mm, 5.0 ml) with a linear gradient of NaCl (0–0.5 M), a 3-ml fraction was collected, and the amylase activity was measured.

The fractions containing glucoamylase (fractions no. 16–20; total volume, 11.7 ml) from a Toyopearl-DEAE column were concentrated by ultrafiltration at 4°C under reduced pressure to about 200 μl. The concentrated solution was charged on a column of Superdex 200 (10 × 300 mm) equilibrated with 20 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.0. Elution was carried out with the same buffer at a flow rate of 250 μl/min, and a 250-μl fraction was collected.

Enzyme homogeneity by SDS-PAGE

Protein homogeneity of the purified enzyme was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). Protein bands on the gel were stained with Coomassie brilliant blue.

Estimation of molecular mass

The molecular mass of the enzyme was estimated by the following two methods: by size-exclusion chromatography on a Superdex 200 column equilibrated with 20 mM Tris-HCl buffer, and by pH 7.0 buffer containing 100 mM NaCl with fast protein liquid chromatography (FPLC) apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with the same buffer at a flow rate of 250 µl/min; SDS-PAGE was performed in a mixture of 0.1% SDS and 100 mM Tris-glycine buffer, pH 8.3, at 15 mA for 5 h. The apparent molecular mass was calculated from the mobility versus molecular mass plots of the marker proteins (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.3 kDa; ovalbmin, 42.4 kDa; carbonic anhydrase, 30.0 kDa; soybean trypsin inhibitor, 20.1 kDa).

Effect of pH and temperature

The temperature dependence of amylase activity was measured in 0.1 M McIlvaine buffer, pH 5.0, for 30 min. The thermal stability of this enzyme was investigated by incubating it in 0.1 M McIlvaine buffer, pH 5.0, for 30 min at various temperatures. The effect of pH on the enzyme activity was measured using 0.4% soluble starch solution as the substrate at 37°C for 30 min. The effect of pH on the stability of amylase activity was investigated by measurement of the remaining activity after incubation for 30 min at 37°C in a buffer solution with pH values from 3.0 to 8.0.

Effect of metal ions

Glucoamylase activity was assayed with various metal ions and ethylenediaminetetraacetic acid (EDTA) by an iodine-potassium iodide method (Terashita et al. 2000a).

Substrate specificities

The substrate specificity of amylase was tested using carbohydrates with different degrees of polysaccharides (isomaltohexaose, amylose A and B, dextrin, amylopectin, pullulan, soluble starch, and cyclodextrin). Amylase activity was measured by the Somogyi–Nelson method (Somogyi 1952). Substrate specificity toward oligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose) was investigated, and the F-kit D-glucose method (Terashita et al. 2000a) was used for these substrates. Short-chain amylose A (MW, 2900; Nakalai Tesque) was hydrolyzed by the glucoamylase for various durations, and the resultant products were analyzed by thin-layer chromatography (TLC).

Anomer analysis by HPLC

Amylose A (MW, 2900) was dissolved in 0.1 M McIlvaine buffer, pH 5.0, and the purified enzyme solution that dissolved in the same buffer was added to the substrate solution. For determining the anomeric form of the hydrolytic products, the enzymatic reaction was conducted at a lower temperature (25°C) to suppress the mutarotation. For determination of time-courses of the substrate degradation and product formation, the reaction mixture was incubated at 37°C for an appropriate period. A portion of the reaction mixture was withdrawn. The resultant solution was applied on to a gel filtration column of Shodex Suger SC-1211 (6 mm ID × 250 ml). The elution was conducted with distilled water at a flow rate of 0.7 ml/min by HPLC, and the substrate and the products were monitored by RI.

Results

Changes in extracellular amylase activities during development of fruit-body on barley grain medium by bottle cultivation

During 150 days of culture of *L. shimeji*, the primordia of the fruit-bodies appeared, and the matured ones were formed about 180 days after inoculation. Table 1 shows the changes in amylase activities during the vegetative mycelial growth stage (60 days after inoculation) and the fruit-body growth stage (160 days after inoculation). All the amylase activities assayed (four kinds of assays) increased during cultivation. In particular, glucoamylase activity by the F-kit-D-glucose method remarkably increased compared with those of other amylase activities. Glucoamylase activity rose 4.6 fold at the fruit-body growth stage compared to the mycelial growth stage.

Purification procedure

To elucidate the hydrolyzing mechanism of starch as the main substrate for fruit-body growth of this mushroom, the extracellular glucoamylase from *L. shimeji* that shows activity at about pH 5.0 was purified using the crude enzyme solution prepared from a stationary culture filtrate (about 1700 ml, for 40 days incubation) as the starting materials. After column chromatography twice with Toyopearl-DEAE 650 M, Superdex 200 column chromatography was done as a final purification step. The amylase was shown as a single and symmetrical protein peak (Fig. 1). The overall process for the purification is summarized in Table 2. The purified extracellular amylase represents about 788-fold purification over the original culture filtrate with about 4.2% recovery. The homogeneity of the purified enzyme was ascertained by SDS-PAGE. The purified amylase gave a single sharp protein band on an SDS-PAGE (Fig. 2).

Table 1. Changes in amylase activities in culture medium during growth of *Lyophyllum shimeji* on barley grain medium

Assay method (amylases)	Mycelial growth stage (M) (60 days after inoculation) (unit/ml)	Fruit-body growth stage (F) (160 days after inoculation) (unit/ml)	Magnification (F/M)
Somogyi–Nelson (total amylase)	7.7	15.5	2.0
F-kit D-glucose (glucoamylase)	1.7	7.8	4.6
Iodine-potassuim iodine (α-amylase)	29.0	62.9	2.2
p-Nitrophenyl α-Glu(α-glucosidase)	4.5	10.3	2.3

Total amylase was assayed by the Somogyi–Nelson method with soluble starch as a substrate; glucoamylase was assayed by the F-kit-D-glucose method with soluble starch as a substrate; α-amylase was assayed by the iodine-potassium iodide method with soluble starch as a substrate; α-glucosidase was assayed by the *p*-nitrophenol method using *p*-N-αGlu (*p*-nitrophenyl α-D-glucopyranoside) as a substrate
After inoculation, the vegetative mycelia were grown at 24°C for 60 days; then, the induction of fruit-body formation was carried out by adjusting the temperature to 15°C and covering the medium surface with peat moss (about 5 mm thick) as casing soil
The time required for mature fruit-body formation of this mushroom was about 180 days after inoculation

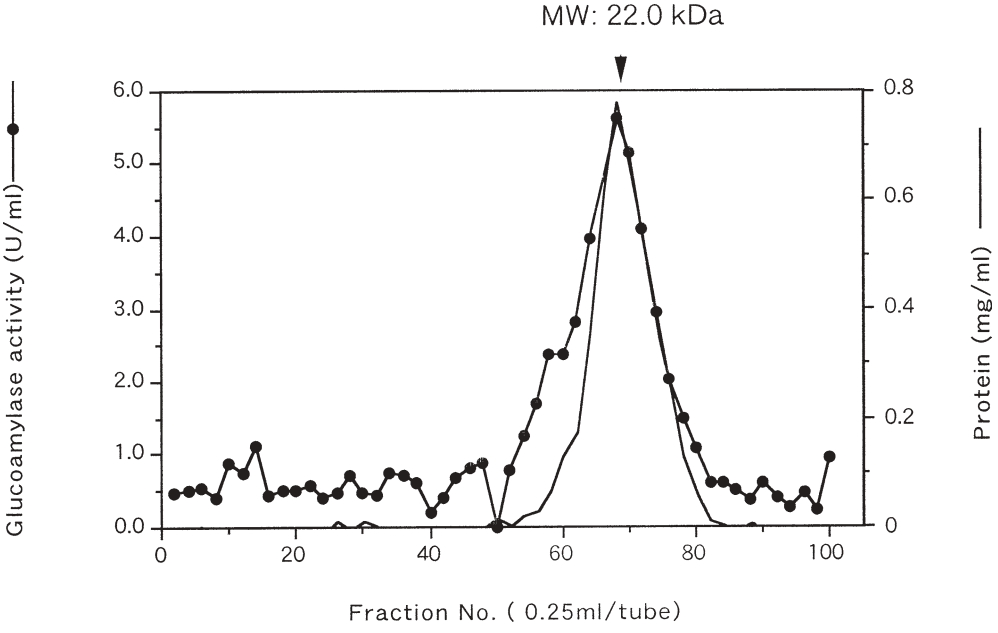

Fig. 1. Elution patterns of glucoamylase from *Lyophyllum shimeji* by Superdex 200 column chromatography. Column: Superdex 200 (Pharmacia, 10 × 300 mm); flow rate, 0.25 ml/min; fraction size, 0.25 ml/tube; elution, 20 mM Tris-HCl buffer (pH 7.0) containing 100 mM NaCl

Table 2. Overall process for the purification of glucoamylase from *Lyophyllum shimeji* MH 01721

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture filtrate	13639.1	249.1	0.018	–	100
55% $(NH_4)_2SO_4$-sat. ppt.	974.9	256.2	0.263	14.6	102.9
Ultrafiltration (>50 kDa)	740.5	213.0	0.282	15.7	85.5
First Toyopearl-DEAE	20.9	40.9	1.957	108.7	16.4
Second Toyopearl-DEAE	0.8	15.2	19.250	1069.4	6.1
Superdex 200 column	0.7	10.4	14.180	787.8	4.2

Characterization of highly purified amylase

Molecular mass

The molecular mass of purified amylase was about 22.0 kDa by size-exclusion chromatography on Superdex 200 and 28.9 kDa by SDS-PAGE.

Effect of pH and temperature

The results are shown in Fig. 3. The optimum temperature of the enzyme activity toward soluble starch was found to be 40°C. The thermal stability of this enzyme was shown from 20° to 35°C. The enzyme reached maximum activity at pH 5.0. This enzyme has been stabilized at pH 4.5–6.5 after inoculation at 37°C for 30 min.

Effect of metal ions

These results are shown in Table 3. The glucoamylase activity was remarkably activated by the presence of Ca^{2+} ion, but Pb^{2+} did not inhibit glucoamylase activity in this fungus. Among the metal ions tested, Al^{3+}, K^{+}, Li^{+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, and EDTA were in some degree

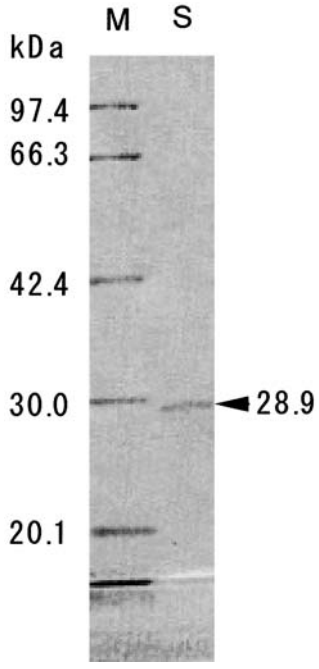

Fig. 2. SDS-PAGE of purified extracellular glucoamylase. *M*, molecular weight markers (97.4 kDa, phosphorylase b; 66.3 kDa, bovine serum albumin; 42.4 kDa, ovalbumin; 30.0 kDa, carbonic anhydrase; 20.1 kDa, soybean trypsin inhibitor); *S*, purified glucoamylase; *arrow*, protein band of glucoamylase

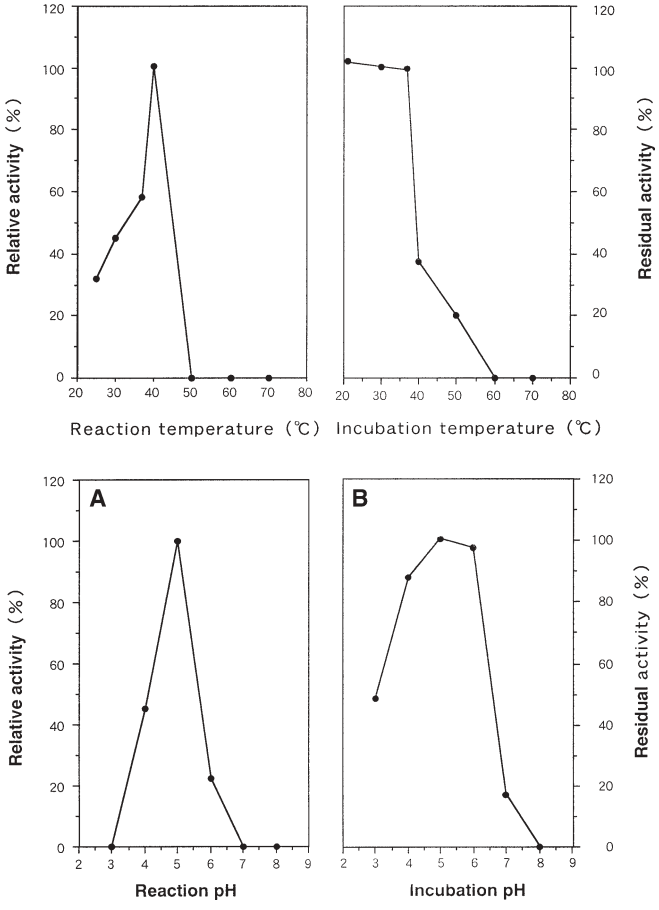

Fig. 3. Effect of temperature and pH on glucoamylase activity from *Lyophyllum shimeji*. **A** Enzyme activity was measured in each buffer. **B** Remaining activity was measured after being held at various pHs at 37°C for 30 min

inhibitors for the glucoamylase. In contrast, Ba^{2+} and Ag^{+} completely inhibited the activity.

Substrate specificities toward polysaccharides and oligosaccharides

The substrate specificity of amylase was tested using carbohydrates with different degrees of polysaccharide (Table 4). The amylase readily hydrolyzed isomaltohexaose [relative activity (R. a.), 753%], amylose A (MW, 2900; R. a., 728%), and dextrin (R. a., 591%) and showed weak activities against amylose B (MW, 16000; R. a., 59%), amylopectin (R. a., 44%), and pullulan (R.a., 44%) and dehydrolyzed cyclodextrin. Also, the substrate specificity of amylase was tested using different degrees of oligosaccharide (data not shown). Amylase activity was measured by the F-kit D-glucose method. The amylase readily hydrolyzed maltohexaose (R. a., 133%) and maltopentaose (R. a., 89%) and showed weak activity against maltotetraose (R. a., 47%) and maltotriose (R. a., 45%). As a result, it was indicated that amylase hydrolyzed not only the α-1,4 bond but also the α-1,6 bond of glycosyl polysaccharide.

Also, short-chain amylose A (MW, 2900) was hydrolyzed by glucoamylase. The resultant products were analyzed by TLC (Fig. 4). In a short time reaction, glucoamylase produced only glucose. However, in a long time reaction (24 h), glucoamylase produced glucose and maltose.

Table 3. Effect of metal ions and chemical reagent on glucoamylase activity from *L. shimeji*

Chemical reagent	Relative activity (%)
Standard (none)	100
$CaCl_2$	774
$PbCl_2$	99
$AlCl_3 \cdot 6H_2O$	67
KCl	63
LiCl	62
$HgCl_2$	52
$FeCl_2 \cdot 4H_2O$	51
$MnCl_2 \cdot 4H_2O$	51
$ZnCl_2$	50
$MgCl_2 \cdot 6H_2O$	49
$CuSO_4 \cdot 5H_2O$	37
$BaCl_2 \cdot 2H_2O$	5
$AgNO_3$	0
EDTA	50

EDTA, ethylenediaminetetraacetic acid
To assay the effect of metal ions, 5 μl enzyme solution, 6 μl 0.1 M metal ion solution in McIlvaine buffer, and 0.1 M McIlvaine buffer, pH 5.0, in a final volume of 420 μl, was preincubated at 37°C for 30 min; then, 150 μl 0.4% soluble starch was added and the resulting mixture was held at 37°C for 30 min
Amylase activity was assayed by an iodine-potassium iodide method

Anomer analysis

Enzymatic products from short-chain amylose A (MW, 2900) were analyzed by HPLC using a partition column, which can separate glucose of α- and β-anomers. The purified enzyme hydrolyzed the amylose A, producing β-anomer glucose (data not shown). All the glucoamylases reported to date yield predominantly β-anomer glucose (Chiba 1997). Only the glucose peak was detected at 48 h after the reaction. The peak of the β-anomer was predominantly increased by HPLC analysis.

Discussion

The ectomycorrhizal fungi such as *L. shimeji* and *T. matsutake* are difficult to cultivate artificially without the host plant. However, Ohta (1994) succeeded in the artificial cultivation of *L. shimeji* using barley grain medium as a growth substrate in bottle culture. He mentioned that a sufficient quantity of starch used as a carbon source could supply the factors that allow successful fruit-body formation without raising osmotic pressure in the medium.

These facts showed that the amylase production of this mushroom had high values, and it suggested that the starch-hydrolyzing ability is very important for fruit-body formation. However, no characterization of the highly purified amylase from *L. shimeji* has been reported.

Table 4. Substrate specificities of purified extracellular glucoamylase from *L. shimeji*

Substrate	Relative activity (%)
Soluble starch	100
Isomaltohexaose	753
Amylose A (MW, 2900)	728
Dextrin	591
Amylose B (MW, 16000)	59
Amylopectin	44
Pullulan	44
Cyclodextrin	0

Enzyme activity was measured by the Somogyi–Nelson method (Somogyi 1952)

Terashita et al. (2000a) have reported amylase production from *L. shimeji* in a partly modified matsutake liquid medium. The amylase activity varied considerably among the strains tested, and enzyme production of fruit-body-forming strains showed a higher level than those of the fruit-body-nonforming strains. A glucoamylase and an endo-type amylase (α-amylase) have been detected by Toyopearl-DEAE column chromatography from the amylase fractions in the culture medium of *L. shimeji.*

To show the ability of this fungus to utilize starch as a growth substrate and to investigate the saprotrophic aspect of this fungus, we assayed the amylase production system of *L. shimeji* in this report (see Table 1). During development from primordia to mature fruit-bodies, four assay methods for amylase activity showed apparent higher values (2.0–4.6 fold) compared to that of the vegetative growth period. In particular, glucoamylase activity increased remarkably compared with that of other amylase activity. These results suggest that glucoamylase plays an important role in the formation of the fruit-body of this mushroom.

Little research has been conducted on the amylase of mycorrhizal mushrooms. It has been reported the amylase that *Lyophyllum fumosum* (Fr.) Orton (Yoshida et al. 1994a,b) produces is a glucoamylase. Ohta (1997) showed that several strains of *T. matsutake*, an ectomycorrhizal mushroom, had the ability to utilize starch as a carbon source. For the artificial cultivation of *T. matsutake*, this ability is very important because the fungus previously has been known to only use starch among the polysaccharides. Lee et al. (1998) have reported that *T. matsutake* had high amylase activity among its other extracellular enzyme activities. Terashita et al. (2000b) have reported that the amylase activity of this mushroom was higher in starch from barley grain than that from other sources. Hur et al. (2001) have reported the amylase production and the enzymatic properties of partially purified amylase from *T. matsutake.* Kusuda et al. (2003) have shown the enzymatic properties including substrate specificity of highly purified α-amylase from the *T. matsutake* Z-1 strain for the first time.

In this article, we have shown the perfect purification procedures and enzymatic properties of highly purified amylase from *L. shimeji*. This enzyme was identified as a glucoamylase from the substrate specificities using oligosac-

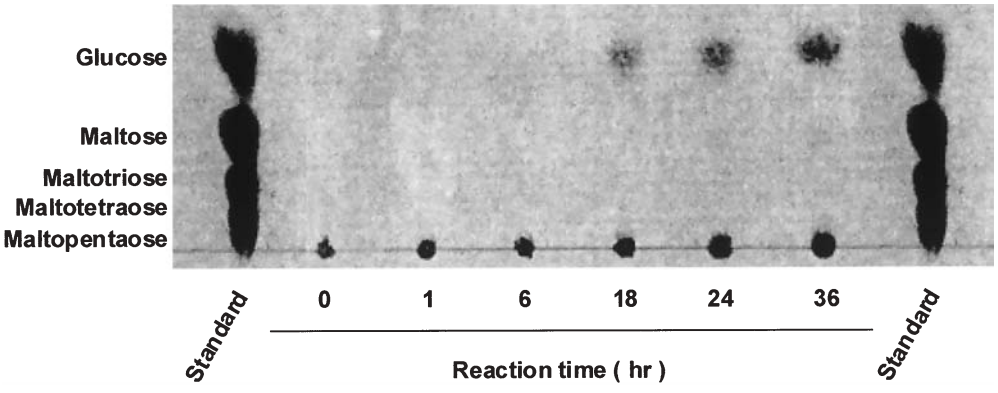

Fig. 4. Thin-layer chromatograms of hydrolysates from amylose A (MW, 2900). The developing solution used was acetic acid: chloroform: distilled water, 7:5:1; developing time was about 4 h; chromoreagent was 20% sulfalic acid–methanol

charides and the anomer analysis of producing glucose by HPLC.

The molecular mass (22–28.9 kDa) of this glucoamylase was smaller than those of the enzymes from mushroom origins such as glucoamylases from *Lentinula edodes* (Berk.) Sing. (55 kDa) (Yamasaki and Suzuki 1978) and *Schizophyllum commune* Fr. (66 kDa) (Shimazaki et al. 1984). On the other hand, the optimum pH (pH 5.0) and temperature (about 40°C) of this glucoamylase from *L. shimeji* had similar properties to those of wood-rotting fungi such as *L. edodes* and *S. commune*. However, this enzyme was relatively unstable against pH and temperature.

Among the metal ions tested on the glucoamylase activity, the enzyme from *L. shimeji* was remarkably activated by the presence of Ca^{2+} ion (7.74 times that of the control), but Ba^{2+} and Ag^{+} completely inhibited amylase activity. In contrast, Pb^{2+} did not inhibit glucoamylase activity of this fungus. Pb^{2+} ion was reported to be a potent inhibitor for the glucoamylase from *L. edodes* (Yamasaki and Suzuki 1978).

In substrate specificity toward polysaccharides, the glucoamylase from *L. shimeji* readily hydrolyzed the α-1,4-glucosidic linkage such as amylose A and dextrin. The hydrolysis of isomaltohexaose and amylopectin suggests that this enzyme has the ability to cleave the α-1, 6-glucosidic linkage of higher molecular substrates. However, the enzyme did not hydrolyze the cyclic polysaccharide cyclodextrin. On the other hand, maltohexaose was the most strongly hydrolyzed among oligosaccarides as a substrate, being converted almost into glucose. However, in a long time reaction, the glucoamylase produced glucose and maltose.

In this experiment, the molar concentration of the oligosaccharides decreased as the degree of substrate became higher. As a result, relative activities against maltotriose and maltotetraose were shown to be considerably lower than that of the maltose. More detailed investigation is needed to elucidate the substrate specificities for oligosaccharides based on the molar concentration.

Terashita et al. (2000a) have shown that *L. shimeji* has two types of extracellular amylases (glucoamylase and the endo-type amylase, α-amylase). It was considered that these two enzymes are associated with nutritional decomposition and fed the fruit-body growth of *L. shimeji*. Barley starch, which is highly utilized is composed of amylose 25% and amylopectin 75%, and it seemed to be suitable as the substrate of the glucoamylase from *L. shimeji.* It seemed that the combination of glucoamylase and the endo-type amylase (α-amylase) produced in the medium from *L. shimeji* is important for growth of the mycelia. To elucidate the starch utilization mechanism for mycelial growth, further research is needed, focusing on the substrate specificity of the endo-type amylase (α-amylase).

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