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FULL PAPER

Mizuho Kusuda · Masaru Nagai · Tae-Chul Hur
Mitsuhiro Ueda · Takao Terashita

Purification and some properties of α-amylase from an ectomycorrhizal fungus, *Tricholoma matsutake*

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Abstract α-Amylase from a still culture filtrate of *Tricholoma matsutake*, an ectomycorrhizal fungus, was isolated and characterized. The enzyme was purified to a homogeneous preparation with Toyopearl-DEAE, gel filtration, and Mono Q column chromatography. The α-amylase was highly purified (3580 fold) with a recovery of 10.5% and showed a single protein band by SDS-PAGE. The enzyme was most active at pH 5.0–6.0 toward soluble starch and stable within the broad pH range 4.0–10.0. This α-amylase was a relatively thermostable enzyme (optimum temperature, 60°C; thermal stability, 50°C). The molecular mass was 34 kDa by size-exclusion chromatography and 46 kDa by SDS-PAGE. This enzyme was not inhibited by the Hg^{2+} ion. Measurement of viscosity and TLC and HPLC analysis of the hydrolysates obtained from amylose showed that the amylase from *T. matsutake* is an endo-type (α-amylase). Substrate specificity was tested using amylose with different polysaccharides. This α-amylase readily hydrolyzed the α-1,4 glucoside bond in soluble starch and amylose A (MW, 2900), but did not hydrolyze the α-1,6 bond and cyclic polysaccharides such as α- and β-cyclodextrin.

Key words α-amylase · Mushroom · Mycorrhizal fungus · *Tricholoma matsutake*

Introduction

Ohta (1994) recently succeeded in artificial cultivation of the mycorrhizal fungus *Lyophyllum shimeji* (Kawam.) Hongo in bottle cultivation using barley grains without a host plant. The fruit-body formation of this fungus in artificial cultivation was reported by Watanabe et al. (1994) and Yoshida and Fujimoto (1994) around the same time. They mentioned that starch of sufficient quantity used as a carbon source was able to supply the factor that allow successful fruit-body formation without raising osmotic pressure in the medium.

Ohta (1997) examined the ability of ectomycorrhizal fungi (55 strains) to utilize starch and amylose, showing that mycelial growth was good in barley grain medium for strains in which mycelial growth was also good in amylose as a sole carbon source. Futhermore, he revealed that some mycorrhizal fungi have the ability to utilize polysaccharides. Terashita et al. (2000a) also showed that the amylases produced had relatively high values in the culture filtrate of *L. shimeji.* The enzymatic properties were clarified and have already been reported using the partially purified enzyme of this fungus.

Tricholoma matsutake (S. Ito et Imai) Sing. is one of the most popular edible mushrooms in the world. However, artificial cultivation of the mushroom has not been established in a stable condition. Norkrans (1950) reported that species of *Tricholoma* can grow on starch and inulin when a small amount of glucose is added as a starter. Kawai and Abe (1976) also reported that *T. matsutake can* grow slowly on a starch medium. Lee et al. (1998) reported that the fungus had relatively high amylase activity among the extracellular enzymes. Hur et al. (2001) also reported that amylase activities were variable among isolated *T. matsutake*.

Moreover, Terashita et al. (2000b) reported that the amylase activity of *T. matsutake* was higher in starch that originated from barley grains than that which originated from other sources. As a result, mycelial growth was remarkably promoted, and amylase production in the culture filtrate obviously increased, when yam and potato were added to the medium compared to that of the control without the addition of yam and potato. Furthermore, it was reported (Terashita et al. 2000b) that amylase activities in the culture filtrate of *T. matsutake* had relatively high values at 80 days after inoculation. Hur et al. (2001) characterized the amylases produced by *T. matsutake* and its related en-

M. Kusuda · M. Nagai · T.-C. Hur · T. Terashita (✉)
Laboratory of Food Microbiological Engineering, Faculty of Agriculture, Kinki University, 3327-204 Naka-machi, Nara 631-8505, Japan
Tel. +81-742-43-1511 (Ext. 3406); Fax +81-742-43-2252
e-mail: terasita@nara.kindai.ac.jp

M. Ueda
Laboratory of Biocycle Engineering, College of Agriculture, Osaka Prefecture University, Osaka, Japan

zymes. However, these enzymes were only partially purified enzymes.

These results suggested that a carbon source in which starch is important for the growth of *T. matsutake* is an indispensable substrate for research on fruit-body formation. Therefore, for artificial cultivation of this fungus, starch-hydrolyzing ability (amylases) is very important because the fungus has previously not been known to use many other polysaccharides except starch (Hur et al. 2001). However, no characterization of highly purified α-amylase from a mushroom origin involving a mycorrhizal fungus has been reported.

In the present study, we purified extracellular α-amylase from a still culture filtrate of *T. matsutake*, a mycorrhizal mushroom, and examined its enzymatic properties from the aspect of substrate specificities.

Materials and methods

Chemicals

Toyopearl-DEAE 650 M was obtained from Tosoh (Tokyo, Japan). Superdex 75, Superdex 200, and standard protein kits for the estimation of molecular mass and isoelectric point of the enzyme were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). Amylose was from Sigma-Aldrich Japan (Tokyo, Japan). All other chemicals were from Wako (Osaka, Japan), unless otherwise stated, and were of certified reagent grade.

Microorganism

Throughout this study, the microorganism *Tricholoma matsutake* Z-1 strain was used. This strain was isolated from the fruit body of *T. matsutake* in 1985 by Inaba et al. (1995).

Medium composition and culture conditions

As inoculum, a mycelial block (diameter, 10 mm) was cut from a plate culture that had grown on Matsutake modified agar medium (Terashita et al. 2000b) for 50 days at 24°C in a Petri dish (diameter, 90 mm). It was planted in a Erlenmeyer flask (100 ml) containing 20 ml Matsutake modified liquid medium (Terashita et al. 2000b) (the components were 22.7 g glucose, 5.0 g yeast extract, 77.0 g potato extract, 5.0 g Sunpearl CP per 1000 ml distilled water, initial pH 5.1). The medium was autoclaved at 121°C for 5 min before use. Then, it was cultured without shaking at 24°C for 80 days in a lighted area (about 200 lux).

Preparation of crude enzymes

Separation of culture broth (300 flasks) after 80 days of incubation was done by filter paper (no. 2). The culture filtrate was used for enzyme assay and amylase purification. Glucoamylase activity was measured after dialyzing against 20 mM McIlvaine buffer (pH 5.0).

Measurement of amylase activities

The reaction mixture of total amylase activity contained 100 μl 0.4% soluble starch solution as a substrate, 89 μl 0.1 M McIlvaine buffer (pH 5.0), 11 μl $CaCl_2$ solution, and 20 μl crude enzyme solution (culture broth); the reaction was carried out at 50°C for 60 min. After reaction, total amylase 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.

α-Amylase activity was assayed in a mixture containing 150 μl 0.4% soluble starch solution, 220 μl of the same buffer, 30 μl $CaCl_2$ solution, and 200 μl crude enzyme solution; the reaction took place at 50°C for 180 min. The enzyme reaction was stopped by adding acetate solution. Subsequently, by adding 200 μl iodine-potassium iodide solution (Terashita et al. 2000a) to the reaction, liquid color is created after 20 min by adding 3.4 ml distilled water to the reaction liquid. Absorbancy was read at 690 nm in a spectrophotometer. One unit of α-amylase activity was defined as the activity that decreased absorbancy for 0.01 of the reaction mixture (1.0 ml) at 50°C for 1 min.

Glucoamylase activity was measured in the mixture containing 100 μl 0.2% soluble starch solution, 20 μl 0.1 M McIlvaine buffer, 5 μl $CaCl_2$ solution, and 80 μl enzyme solution; enzyme reaction took place done at 50°C for 180 min. Then, the reaction was stopped by heating at 100°C for 10 min. Glucose, which was released by the reaction, was determined quantitatively with an F-kit glucose (Boehringer Mannheim, Germany). One unit of glucoamylase activity was defined as the activity that forms 1 μmol glucose in 1.0 ml of reaction mixture at 50°C for 1 min.

The α-glucosidase activity was assayed in a mixture containing 90 μl 100 mM McIlvaine buffer with 20 mM $CaCl_2$ (pH 5.0) and 10 μl crude enzyme solution. Preincubation was done at 37°C for 10 min; after preincubation, the mixture of 100 μl 20 mM ρ-nitrophenyl α-D-glucopyranoside (ρ-N αG) was added. The enzyme reaction then was completed at 37°C for 60 min. The reaction was stopped by adding 100 μl Na_2CO_3 solution. One unit of α-glucosidase activity was defined as the activity that forms 1 μmol ρ-nitrophenol in 1.0 ml reaction mixture at 37°C for 1 min.

Purification procedures of amylase

Ammonium sulfate was added to make a 40% saturation of the crude enzyme solution (2000 ml culture filtrate), which was then centrifuged (5000 rpm, 10 min). The sediment precipitated by ammonium sulfate was then dissolved in 50 ml of 20 mM Tris-HCl buffer, pH 7.0. The crude enzyme solution was used as the starting material for purification of the enzyme. Unless otherwise specified, all purification steps were at 4°C. After being dialyzed against the 20 mM Tris-HCl buffer (pH 7.0), the crude enzyme solution was put on a Toyopearl-DEAE 650 M column (26 × 150 mm) equili-

brated with 20 mM Tris-HCl buffer, pH 7.0. After the column was washed thoroughly with the same buffer, the absorbed proteins were eluted by a linear gradient of NaCl (0–300 mM) in the same buffer at a flow rate of 2.0 ml/min, and 6.0-ml fractions were collected. The amylase activity of each fraction was then measured.

The active fractions (fractions 27–34; total volume, 38.5 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 (0–300 mM), 500 µl fractions were collected, and amylase activity was measured.

The fractions containing amylase activity (fractions 30–39; total volume, 4.79 ml) from a Toyopearl-DEAE column were concentrated at 4°C under reduced pressure to about 200 µl. The concentrated solution was charged on a column of Superdex 200 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 250-µl fractions were collected. The active fractions (fractions 56–60) were concentrated at 4°C under reduced pressure to about 200 µl and put on a column of Superdex 75 equilibrated with the same buffer. Elution was done with the same buffer at a flow rate of 250 µl/min, and 250-µl fractions were collected. The pooled enzyme solution was then chromatographed on a Mono Q column (5 × 50 mm) with Tris-HCl buffer (pH 7.0). The enzyme was eluted with a linear gradient of NaCl (0–500 mM) in the same buffer.

Enzyme homogeneity by SDS-PAGE

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

Estimation of molecular mass

The molecular mass of the enzyme was estimated by two methods: by size-exclusion chromatography on a Superdex 75 column equilibrated with 20 mM Tris-HCl buffer, and by pH 7.0 containing 100 mM NaCl with fast protein liquid chromatography (FPLC) apparatus (Amersham Pharmacia Biotech). Proteins were eluted with the same buffer at a flow rate of 250 µl/min. SDS-PAGE was done 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 (myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibiter, 20 kDa).

Effect of metal ions

The α-amylase activity was assayed with various metal ions by an iodine-potassium iodide method (Terashita et al. 2000a). To assay the effect of metal ions, 5 µl enzyme solution, 6 µl 0.1 M metal ion solution in McIlvaine buffer, 30 µl 0.2 M $CaCl_2$, and 0.01 M McIlvaine buffer, pH 5.0, in a final volume of 450 µl was preincubated at 37°C for 30 min. After preincubation, 150 µl 0.4% starch was added and the resulting mixture was held at 50°C for 30 min.

Viscosity and degradation of the substrate

Viscosity was measured in an Ostwald viscosimeter with 11.15 ml reaction mixture composed of 4.0% soluble starch solution, 0.1 M McIlvaine buffer, pH 5.0, $CaCl_2$ solution, and the enzyme. The changes in viscosity were followed together with the degradation rate of the substrate, which was measured by the increase in reducing groups by the method of Somogyi (1952) with glucose as the standard. The reactions were done at 37°C.

Results

Purification procedure

The extracellular α-amylase from *T. matsutake* that shows activity at about pH 5.0 was purified using the crude enzyme solution prepared from a still culture filtrate (about 2000 ml, for 80 days incubation) as the starting material. The elution profiles of amylases of this fungus from Toyopearl-DEAE 650 M column chromatography are shown in Fig. 1. The results showed that the fungus has three types of amylases: α-amylase, glucoamylase, and α-glucosidase. α-Amylase activity showed the highest value among these activities.

Subsequently, the α-amylase fraction obtained from the Toyopearl-DEAE 650 M column was purified on the Superdex columns. In the final purification step, which involved Mono Q column chromatography (Fig. 2), α-amylase was recovered as a single and symmetrical protein peak. The overall process for the purification is summarized in Table 1. The purified extracellular α-amylase represented 3580-fold purification over the original culture filtrate with about 10.5% recovery. The homogeneity of the purified enzyme was ascertained by SDS-PAGE. The purified α-amylase gave a single sharp protein band on SDS-PAGE (Fig. 3).

Characterization of highly purified α-amylase

Molecular mass

The molecular mass of purified α-amylase was 34 kDa by size-exclusion chromatography on Superdex 75 and 46 kDa by SDS-PAGE.

Effect of pH and temperature

The effects of pH and temperature are shown in Fig. 4. The influence of pH on enzyme activity was measured using

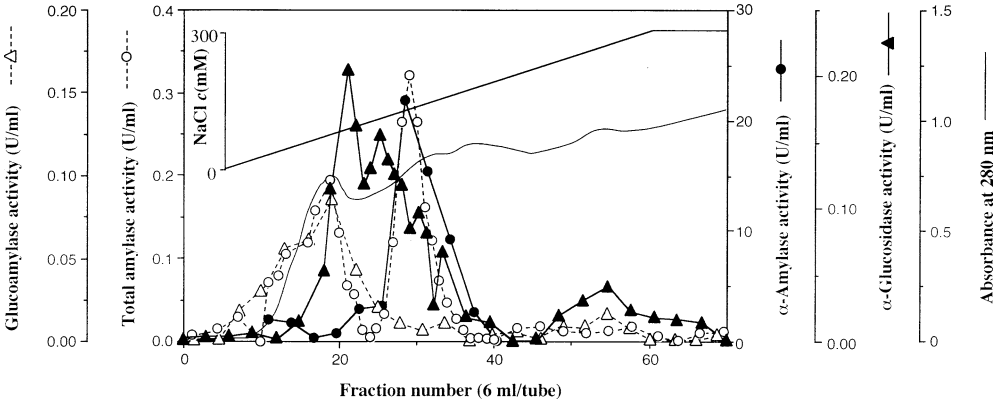

Fig. 1. Elution profiles of extracellular amylases of *Tricholoma matsutake* Z-1 from DEAE-Toyopearl column chromatography. Column, DEAE-Toyopearl 650M (Tosoh, 26 × 150 mm); flow rate, 2.0 ml/min; fraction size, 6 ml/tube; elution, linear gradient elution of sodium chloride from 0 to 300 mM in 20 mM Tris-HCl buffer (pH 7.0)

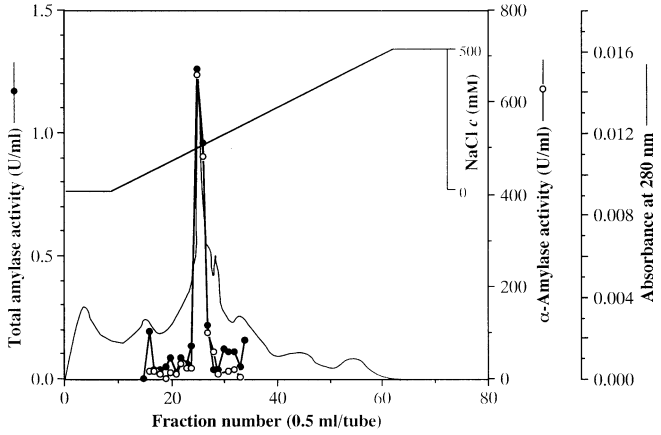

Fig. 2. Elution profiles of α-amylase from Mono Q column chromatography. Column, Mono Q (Pharmacia, 5 × 50 mm); flow rate, 0.5 ml/min; fraction size, 0.5 ml/tube; elution, a linear gradient elution of NaCl from 0 to 500 mM in 20 mM Tris-HCl buffer (pH 7.0) for 40 min

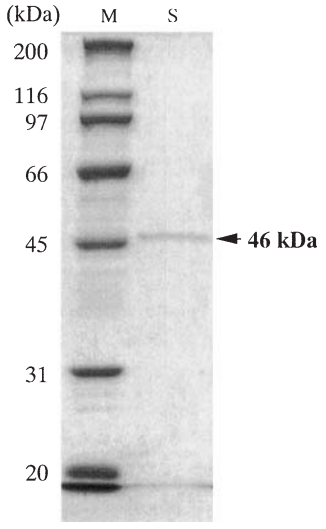

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified extracellular α-amylase. *M*, molecular weight markers; *S*, purified α-amylase; *arrow*, protein band of α-amylase

0.4% soluble starch solution as the substrate at 50°C for 30 min. The enzyme showed maximum activity at pH 5.0–6.0. 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 at pH 1.0–12.0. The enzyme was widely crossed and has been stabilized at pH 4.0–10.0.

Temperature dependence of α-amylase activity was measured in a 0.1 M McIlvaine buffer, pH 5.0, for 30 min, and the optimum temperature of enzyme activity toward soluble starch was observed to be 60°C. 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. Incubating the enzyme at 50°C in this condition resulted in only 5% loss of activity. Thus, α-amylase is a relatively thermo-stable enzyme.

Effect of metal ions

Among the metal ions tested, Co^{2+}, Fe^{2+}, and Na^{+} were in some degree inhibitors for the amylase (Table 2). In contrast, Al^{3+} and Hg^{2+} did not inhibit amylase activity in this fungus.

Amylolytic patterns and substrate specificities

For identification of the mode of sugar chain degradation, the relationship between decrease in viscosity and the rate of reducing group formation was examined during the enzyme reaction (Fig. 5). When relative viscosity of the substrate solution decreased 50%, about 2% of the total glucoside bonds were split in the enzyme reaction (α-1,4 bond). Viscosity rapidly decreases with decomposition of the substrate, such that it actually stops the reaction in a short time. From the results, the enzyme was classified as an endo-type enzyme (α-amylase). Short-chain amylose (MW, 2900) was hydrolyzed by α-amylase for various lengths of time, and the resultant products were analyzed by thin-layer chromatography (TLC) (Fig. 6). The sugars produced by the 2-h reaction were G2 (maltose), G3 (maltotriose), G4 (maltotetraose), G5 (maltopeptaose), and larger maltooligosaccharides. The products of the 24-h reaction were mainly G2 and G3. Therefore, the resultant products were analyzed by high performance liquid chromatography (HPLC), but G1 (glucose) was not observed. These results

Table 1. Purification of extracellular α-amylase from *Tricholoma matsutake* Z-1

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture filtrate	3770	5430	1.44	–	100
30%$(NH_4)_2SO_4$-sat. ppt.	863	3260	3.78	2.63	60.0
First DEAE-Toyopearl	7.85	2550	325	226	47.0
Second DEAE-Toyopearl	3.21	1420	442	307	26.2
Superdex 200	0.196	935	4770	3310	17.2
Superdex 75	0.171	841	4920	3420	15.5
Mono Q	0.111	572	5150	3580	10.5

Table 2. Effect of metal ions and chemical agents on α-amylase activity

Chemical agent	Relative activity (%)
Standard ($CaCl_2$)[a]	100
$AgCl_2$	95.4
$AlCl_3$	111
$BaCl_3$	91.9
$CoCl_2$	75.4
$FeCl_2$	59.0
NaCl	59.3
$MnCl_2$	98.0
KCl	87.1
$MgCl_2$	88.9
$ZnCl_2$	86.7
$CuSO_4$	115
LiCl	121
$HgCl_2$	123
Ethylenediaminetetraacetic acid (EDTA)	82.5

Enzyme activity was assayed in the presence of metal ions or chemical agents in the final concentration of 1 mM under standard conditions
[a] Enzyme activity as a standard was measured in the presence of $CaCl_2$

Table 3. Substrate specificity of purified α-amylase

Substrate	Relative activity (%)
Soluble starch	100
Amylose A (MW = 2900)	86.5
Amylose	84.0
Amylose B (MW = 16000)	42.3
Glycogen	10.5
Pullulan	n.d.
α-Cyclodextrin	n.d.
β-Cyclodextrin	n.d.

n.d., not detected
Enzyme activity was measured by the method of Somogyi

indicated that the amylase from *T. matsutake* is an endo-type amylase (α-amylase).

The substrate specificity of α-amylase was tested using amylose with different polysaccharides (Table 3). The α-amylase readily hydrolyzed soluble starch [relative activity (R.a.) 100%; K_m value, 0.069%], amylose (R.a., 84.0%), amylose A (MW, 2900; R.a., 86.5%; K_m value, 1.30 mM), and weak amylose B (MW, 16000; R.a., 42.3%), and very slightly, glycogen (R.a., 10.5%). The α-amylase was not hydrolyzed by the α-1,6 bond and cyclic polysaccharides such as α- and β-cyclodextrin. Moreover, Purulan, with α-1,4, α-1,6, and sometimes the α-1,3 bond, also was not hydrolyzed.

Discussion

When a mushroom fungus forms fruit bodies, large amounts of mycelia may be needed either to store the nutrients or to transport the nutrients to the fruit bodies (Hirato and Kitamoto 1995). However, in practice it is very difficult to cultivate large amounts of mycelia using monosaccharides in a pure culture because of the osmotic pressure in the medium (Ohta 1997). In particular, the most serious barrier for the artificial cultivation of the mushroom is that the mycelium of *T. matsutake* grows so slowly on the artificial

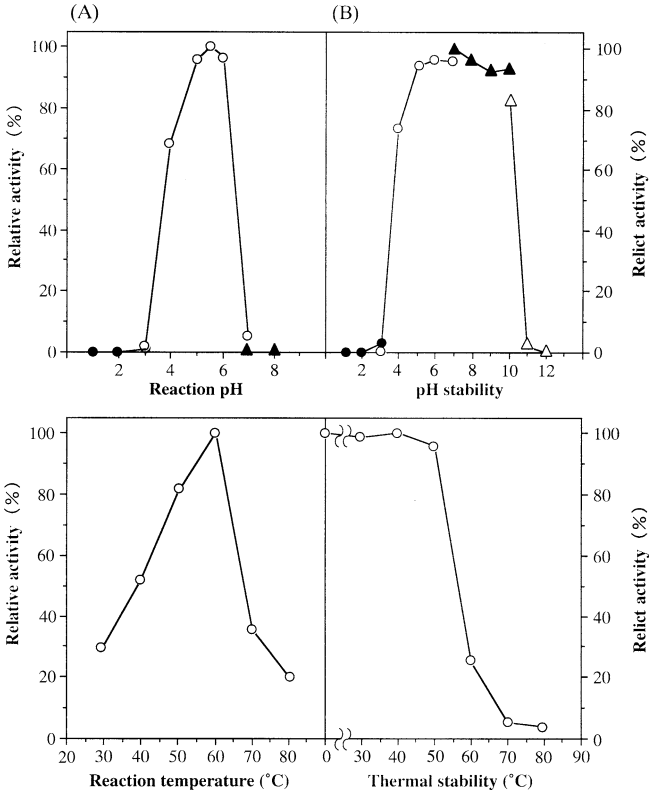

Fig. 4. Effect of pH and temperature on α-amylase activity. **A** Enzyme activity was measured in each buffer. **B** Remaining activity was measured after being held at various pHs at 30°C for 30 min. —●—, 100 mM acetate buffer; —○—, 100 mM McIlvaine buffer; —▲—, 100 mM Tris-HCL buffer; —△—, 100 mM CAPS buffer

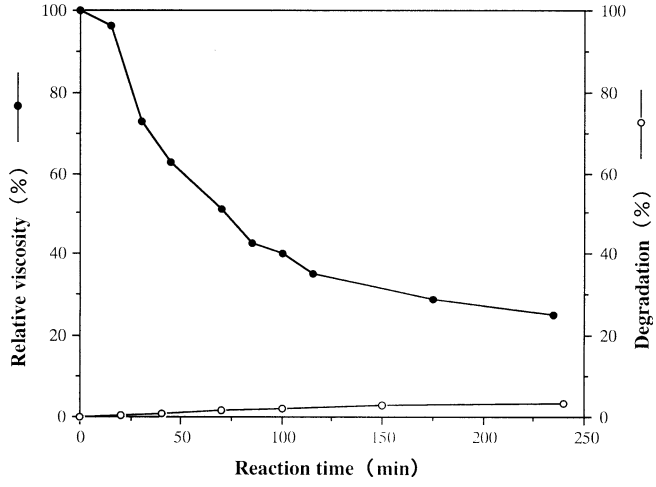

Fig. 5. Effect of extracellular α-amylase on viscosity of soluble starch. Relative viscosity was measured by on Ostwald viscosimeter with reaction mixture composed of 4.0% soluble starch solution. Viscosity values followed the degradation rate of the substrate. Changes in reducing groups were measured by the Somogyi method

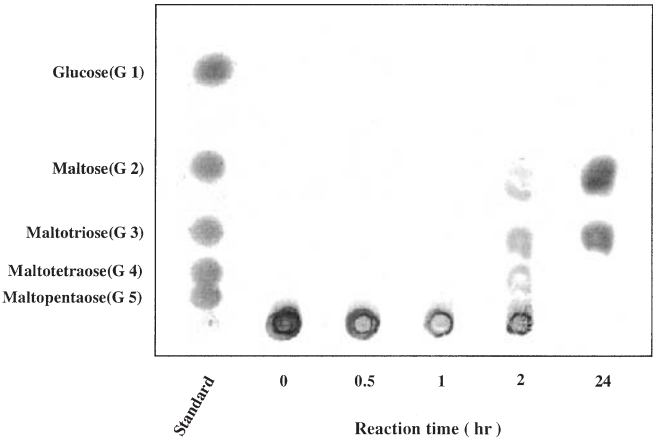

Fig. 6. Thin-layer chromatograms of hydrolyzates from amylose (MW = 2900). The developing solution used was acetic acid:chloroform:distilled water, 7:5:1. Developing time was about 4 h; chromoagent was 20% sulfalic acid–methanol

media (about 2 cm/month) and has low capability to decompose polysaccharides (Hur et al. 2001).

Little research has been conducted on hydrolytic enzymes of mycorrhizal mushrooms. Nakazawa et al. (1974) reported that *T. matsutake* produced very weak cellulolytic enzyme activity; however, the detailed profile of the enzymes was unclear. Terashita and Kono (1987) investigated the proteinases of *T. matsutake* in a still culture and three carboxyl proteinases purified as a single protein band with SDS-PAGE and also characterized some properties. Moreover, Terashita and Kono (1989) examined the enzymatic properties of proteases from mycelia of 17 strains of mycorrhizal mushrooms including *T. matsutake* and related species. As a result, these stocks were divided into two groups by the optimum temperature of purified carboxyl proteinase. In all stocks of *T. matsutake*, the optimum temperature was about 70°C; for most of the related species, it was about 40°C.

The diversity of the pH spectra of cellulolytic enzymes in Basidiomycotina was reported by Enokibara et al. (1993). They examined the productivity of cellulolytic enzymes in mycorrhizal mushrooms and showed that *Hebeloma vinosophyllum* Hongo and *Laccaria bicolor* (Maire) P.D. Orton produced very weak activity of subneutral cellulases at pH 5.5–6.8. In contrast, *Lepista nuda* (Bull.: Fr.) Cooke produced strong acid cellulase activity at pH 3.0–5.5. They suggested that the enzymatic properties of cellulases, such as optimum pH, were different among the mushrooms depending on pH habitat.

To survey the potential for production of extracellular hydrolytic enzymes by mycorrhizal mushrooms such as *T. matsutake* and *L. shimeji*, Terashita et al. (1995) examined the productivities of these exo-enzymes on a potato dextrose liquid medium. *T. matsutake* produced relatively high levels of CM cellulase and avicelase activities in all test strains. Moreover, comparison of the productivity of hydrolytic enzymes between the group of wood-rotting mushrooms and that of mycorrhizal mushrooms showed considerably higher xylanase activity in the former. Ohga (1992) reported that the most important enzyme for the fruit-body growth of mushrooms is xylanase, a nutrient decomposer, among the hydrolytic enzymes.

Ohta (1994) reported that *L. shimeji* forms mature fruit bodies on an artificial medium, including mainly barley grain without a host plant, although the fungus is a kind of ectomycorrhizal mushroom. He also showed that several strains of *T. matsutake* had the ability to utilize starch as a carbon source. For the artificial cultivation of *T. matsutake,* this ability is also very important because the fungus has previously not been known to use other polysaccharides except starch. In several studies of the amylase activity of *T. matsutake*, Kawai (1973) indicated that *T. matsutake* had poor amylase activity among the mushrooms, whereas Lee et al. (1998) reported that the fungus had high amylase activity among its other extracellular enzyme activities. Terashita et al. (2000b) reported that the amylase activity of *T. matsutake* was higher in starch that originated from barley grains than that which originated from other sources. Then, Hur et al. (2001) reported amylase production and the enzymatic properties of partially purified amylases from *T. matsutake*. There are several reports about the purification procedures of the enzymes and some properties of glucoamylase and α-glucosidase from wood-rotting mushrooms such as *Schizophyllum commune* Fr.: Fr. (Shimazaki et al. 1984) and *Lentinula edodes* (Berkeley) Pegler (Yamasaki and Suzuki 1978; Zhao et al. 2000). However, no characterization of highly purified α-amylase from a mushroom origin involving a mycorrhizal fungus has been reported to date.

In this article, we have showed the perfect purification procedures and enzymatic properties of highly purified α-amylase from *T. matsutake* for the first time. The molecular mass of this α-amylase was small compared to the enzymes from mushroom origins such as α-glucosidase (51 kDa) and glucoamylase (55 kDa) from *Lentinus edodes* (Yamasaki

and Suzuki 1978) and glucoamylase from *Schizophyllum commune* (Shimazaki et al. 1984).

On the other hand, there are reports of an α-amylase from *Aspergillus oryzae* having a stable pH, 4.0–5.0 (Chang et al. 1995), pH 5.0–8.5 from *A. flavus* Link: Fries (Khoo et al. 1994), and pH 4.5–7.5 from *Schwanniomyces alluvis* (Wilson and Ingledew 1982). As compared to α-amylase from other fungi, the α-amylase has a wide range of pH stability (pH 4.0–10.0). Consequently, α-amylase from *T. matsutake* seems to be a relatively thermostable and pH-stable enzyme. Moreover, α-amylase activity was inhibited by the addition of Hg^{2+} ion. This result suggested that the α-amylase did not have an SH group in its protein structure.

We have showed that *T. matsutake* has three types of amylases (α-amylase, glucoamylase, and α-glucosidase). We considered that these enzymes are associated with nutritional decomposition and feed the mycelial growth of *T. matsutake*. In the present study, an α-amylase from *T. matsutake* hydrolyzed a α-1,4 glucosidic linkage. However, the enzyme did not hydrolyze the α-1,6 glucosidic linkage and cyclic polysaccharides. More detailed information about glucoamylase and α-glucosidase is needed to elucidate the function of the starch degradation process in this mushroom.

Several reports had been published for primordium formation and fruit-body production of *T. matsutake* in vitro (Ogawa and Hamada 1975; Kawai and Ogawa 1976; Inaba et al. 1995). However, these reports did not lead to further information concerning artificial cultivation. On the other hand, many problems exist such as the mechanism of nutrient exchange between *Pinus densiflora* as a host plant and *T. matsutake,* the micro environment of a fairy-ring, and so on for the artificial cultivation of *T. matsutake* in nature.

To reveal the ability to utilize starch as a growth substrate for the artificial cultivation of *T. matsutake*, research on the production system of amylases in laboratories and in nature seems to be vital. The characterization of α-glucosidase such as substrate specificity, which was shown in our experiment, is especially needed.

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