FULL PAPER

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Detection of β-glucosidase as saprotrophic ability from an ectomycorrhizal mushroom, *Tricholoma matsutake*

Abstract We investigated extracellular carbohydrase production in the medium of an ectomycorrhizal fungus, *Tricholoma matsutake*, to reveal its ability to utilize carbohydrates such as starch as a growth substrate and to survey the saprotrophic aspects. We found β-glucosidase activity in the static culture filtrate of this fungus. The β-glucosidase was purified and characterized. The purified enzyme was obtained from about 2.1 l static culture filtrate, with 9.0% recovery, and showed a single protein band on SDS-PAGE. Molecular mass was about 160 kDa. The enzyme was most active around 60°C and pH 5.0, and stable over a pH of 4.0–8.0 for 30 min at 37°C. The purified enzyme was activated by the presence of Ca^{2+} and Mn^{2+} ions (about 2–3 times that of the control). The enzyme readily hydrolyzed oligosaccharides having a β-1,4-glucosidic linkage such as cellobiose and cellotriose. However, it did not hydrolyze polysaccharides such as avicel and CM-cellulose or oligosaccharides having an α-glucosidic linkage. Moreover, cellotriose was hydrolyzed by the enzyme for various durations, and the resultant products were analyzed by TLC. We concluded that the enzyme from *T. matsutake* seems to be a β-glucosidase because cellotriose with a β-1,4-glucosidic linkage decomposed to glucose during the enzyme reaction.

Key words Amylase · β-Glucosidase · Edible mushroom · Mycorrhizal fungus · *Tricholoma matsutake*

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Introduction

The ectomycorrhizal mushroom *Tricholoma matsutake* (S. Ito et Imai) Singer is one of the most valuable edible mushrooms in Japan. However, artificial cultivation of the mushroom has not been established in a stable condition. This fungus can grow slowly on starch and inulin when a small amount of glucose is added as a starter (Norkrans 1950). The starch, which is used as a carbon source, is able to supply the conditions that allow a successful quantity of mycelial growth without raising osmotic pressure in the medium.

To reveal the ability to utilize starch and related substrates of *T. matsutake*, the production systems of extracellular amylase were studied using an artificial medium. As a result, it was shown that this fungus produced α-amylase and α-glucosidase in static culture filtrate. α-Amylase in these enzymes was already purified, and its enzymatic property was characterized from the aspect of substrate specificities. The purified α-amylase produced oligosaccharides from starch. However, α-glucosidase activity was only partially purified because the activity was very weak.

On the other hand, Vaario et al. (2002) reported that *T. matsutake* produced β-glucosidase when they used pine bark for cultivation of this fungus. In addition, they observed by transmission electron microscopy that *T. matsutake* decomposed sawdust cells of pine bark by the kind of strain and the fungus mycelia invaded the sawdust cell of pine bark (Vaario et al. 2003). We recently also detected a β-glucosidase having high activity from a static culture filtrate of this fungus. These results suggest that *T. matsutake* has saprotrophic abilities. However, no characterization of the highly purified enzyme from *T. matsutake* has been reported.

In this article, we purified and characterized β-glucosidase from this ectomycorrhizal mushroom and discuss possible roles of this extracellular enzyme of *T. matsutake*.

Material and methods

Organism

Tricholoma matsutake Z-1 strain was used in this experiment. This strain was isolated from the fruit-body of *T. matsutake* in 1985 by Inaba et al. (1995). The fungus was stored on partially modified matsutake agar medium (PMMA) (Terashita et al. 2000a) at a low temperature (4°C).

Medium compositions and culture conditions

A mycelial block (5 × 5 mm) was cut from a plate culture that had grown on PMMA (1.5% agar powder) medium (Terashita et al. 2000a) for 40 days at 24°C in a petri dish (diameter, 90 mm). It was inoculated in an Erlenmeyer flask (100 ml) containing 20 ml PMM liquid (PMML) medium after sterilization at 121°C for 5 min. The composition of the PMML medium was 22.7 g glucose, 5.0 g yeast extract, 77.0 g potato extract, and 5.0 g Sunpearl-CP (commercial name; sulfite pulp waste from softwood) per 1000 ml distilled water, with an initial pH of 5.1. After inoculation, it was static cultured at 24°C for 60 days in the light (about 200 lux).

Extraction of crude enzyme

For purification of the extracellular β-glucosidase, 60 days after incubation the culture medium was filtered through no. 2 filter paper (Advantec, Osaka, Japan) for mycelial removal. The culture filtrate was used as the crude enzyme for starting materials for purification.

Measurement of α- and β-glucosidase activity

α-Glucosidase and β-glucosidase activity was assayed using *p*-nitrophenyl-α- and β-glucopyranoside (*p*-NαG and *p*-NβG) as the substrates. The enzyme reaction was carried out at 37°C for 30 min. One unit of α- and β-glucosidase activity was defined as the activity that forms 1 μmol *p*-nitrophenol in 1.0 ml reaction mixture at 37°C in 1 min.

Purification procedures of extracellular β-glucosidase

Ammonium sulfate was added to make a 60% saturation of the culture filtrate (2096 ml). The precipitate by ammonium sulfate was then separated by centrifuge (9000 g, 20 min). The precipitate was dissolved in 20 mM Tris-HCl buffer containing 10 mM $CaCl_2$ (pH 7.0) and then was used as the starting material for the enzyme purification.

All purification steps were carried out at 4°C. The crude enzyme solution (520 ml) was dialyzed (in 20 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 7.0, overnight, 4°C) and put on a Toyopearl-DEAE 650M column (Tosoh, Tokyo, Japan) that was previously equilibrated with 20 mM Tris-HCl buffer containing 10 mM $CaCl_2$ (pH 7.0). The enzyme was eluted with a linear gradient of Tris-HCl buffer (pH 7.0) from 20 to 500 mM at a flow rate of 0.5 ml/min. The β-glucosidase activity of each fraction was measured.

The fractions containing β-glucosidase (fractions 25–50; total volume, 151.5 ml) from the 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 (pH 7.0) containing 20 mM $CaCl_2$, pH 7.0. Elution was performed with the same buffer at a flow rate of 250 μl/min, and 250-μl fractions were collected.

Enzyme homogeneity and estimation of molecular mass by SDS-PAGE

Protein homogeneity of the purified enzyme was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method detailed by Laemmli (1970), using 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, 200.0 kDa; β-galactosidase, 116.2 kDa; bovine serum albumin, 66.3 kDa; aldolase, 42.4 kDa; carbonic anhydrase, 30.0 kDa; myoglobin, 20.1 kDa). Protein bands on the gel were stained with Coomassie brilliant blue after SDS-PAGE.

Effect of pH and temperature

The temperature dependence of β-glucosidase activity was measured in a 0.1 M McIlvaine buffer, pH 5.0, for 30 min. The thermal stability of this enzyme was investigated by incubating it in a 0.1 M McIlvaine buffer, pH 5.0, for 30 min at various temperatures. The effect of pH on enzyme activity was measured using a 20 mM *p*-NβG solution as the substrate at 37°C for 30 min. The effect of pH on the stability of β-glucosidase 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.

Michaelis constant

p-Nitrophenyl-β-D-glucopyranoside and cellobiose were incubated at various concentrations with purified β-glucosidase; then, the initial rate of *p*-nitrophenol and glucose formation was measured.

Effect of metal ions

The β-glucosidase activity was assayed with various metal ions and ethylene diamnetetraacetic acid (EDTA) by the *p*-nitrophenol method (Kusuda et al. 2003).

Table 1. Purification of extracellular β-glucosidase from *Tricholoma matsutake* Z-1

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture filtrate	23 456	133	0.006	1	100
60% (NH₄)₂SO₄-sat. ppt.	1 505	152	0.101	18	114
Toyopearl-DEAE	14.3	36	2.52	442	27
Superdex 200	0.2	12	63.89	11 200	9

Substrate specificities

The substrate specificity of β-glucosidase was tested using synthetic substrates (*p*-NβG, *p*-NαG, and *p*-nitrophenyl-β-D-cellobioside) and natural substrates (disaccharides; different degrees of oligosaccharides and polysaccharides: maltose, cellobiose, isomaltose, sucrose, lactose, trehalose, cellotriose, isomalto oligosaccharide, dextrin, CM-cellulose, avicel, curdlun). The enzyme activity was measured by the *p*-nitrophenol method for synthetic substrates and the F-kit D-glucose method (Kusuda et al. 2004) for natural substrates. Moreover, cellotriose was hydrolyzed by β-glucosidase at various durations, and the resultant products were analyzed by thin-layer chromatography (TLC).

Results

Purification procedure

The extracellular β-glucosidase from *T. matsutake* that shows activity at about pH 5.0 was purified using the crude enzyme solution prepared from a static culture filtrate (about 2100 ml, for 60 days cultivation) as the starting material. In the final purification step, using a Superdex 200 column chromatography (Fig. 1), β-glucosidase was recovered as a single and symmetrical protein peak (Fr. no. 45–52). However, a small peak (Fr. no. 53–55) having α-glucosidase activity appeared. The overall process for the purification of β-glucosidase is summarized in Table 1. The purified extracellular β-glucosidase represents about 11 200-fold purification over the original culture filtrate with about 9% recovery. The homogeneity of the purified enzyme was ascertained by SDS-PAGE. The purified β-glucosidase gave a single sharp protein band on SDS-PAGE (Fig. 2).

Molecular mass

The molecular mass of purified amylase was about 160 kDa by SDS-PAGE.

Effect of pH and temperature

The results are shown in Table 2. The optimal temperature of the enzyme activity toward *p*-NβG was 60°C; the thermal stability of this enzyme was at 40°C. The enzyme exhibited
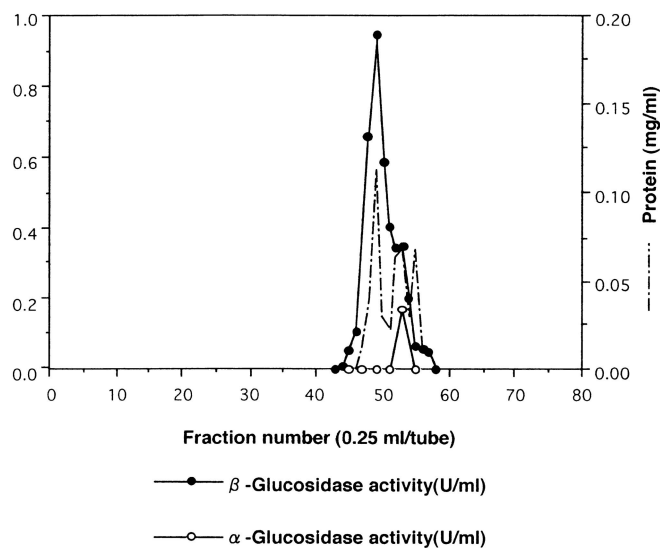
Fig. 1. Gel filtration profiles of β-glucosidase from *Tricholoma matsutake* Z-1 by Superdex 200 column chromatography. Column, Superdex 200 column (Pharmacia, 10 × 300 mm); flow rate, 0.25 ml/min; fraction size, 0.25 ml/tube; elution, 20 mM Tris-HCl buffer (pH 7.0) containing 20 mM CaCl₂

maximum activity at pH 5.0 and was stabilized at pH 4.0–8.0 after incubation at 37°C for 30 min.

Michaelis constant

A Lineweaver–Burke plot of β-glucosidase activity with *p*-NβG and cellobiose as substrates was obtained. As shown in Table 2, the K_m values for *p*-NβG and cellobiose were calculated to be 0.22 mM and 0.99 mM for this β-glucosidase. Conversely, the V_{max} values for both were 2.89 mM/min (*p*-NβG) and 3.59 mM/min (cellobiose), respectively.

Effect of metal ions

The effects of metal ions are shown in Table 3. β-Glucosidase activity was remarkably activated by the presence of Ca^{2+} (287%) and Mn^{2+} (257%). Among the metal ions tested, Ba^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, Na^+, Cu^{2+}, Li^+, Co^{2+}, Fe^{2+}, Al^{3+}, and Hg^{2+} and EDTA were in some degree activators for β-glucosidase. In contrast, K^+ and Ag^+ weakly inhibited the activity.
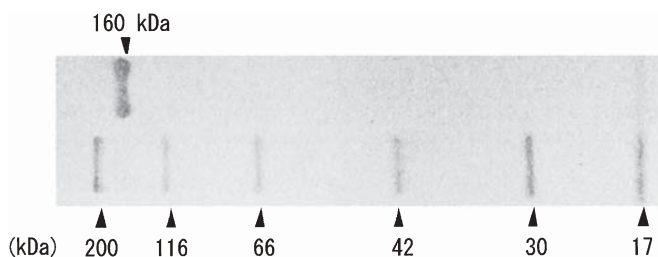
Fig. 2. Sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) of purified extracellular β-glucosidase from *T. matsutake* Z-1. Molecular weight markers: 200 kDa, myosin; 116 kDa, β-galactosidase; 66 kDa, bovine serum albumm (BSA); 42 kDa, ardolase; 30 kDa, carbonic anhydrase; 17 kDa, myoglobin; *arrow*, protein band of β-glucosidase

Table 2. Some properties of extracellular β-glucosidase from *T. matsutake* Z-1

Molecular weight (SDS-PAGE)	160 kDa
pH stability (37°C, 30 min)	4–8
Optimal pH (37°C)	5.0
Thermal stability (pH 5.0, 30 min)	~40°C
Optimal temperature (pH 5.0)	60°C
K_m[a]	0.22 mM (*p*-NβG)
	0.99 mM (cellobiose)
V_{max}[a]	2.89 mM/min (*p*-NβG)
	3.59 mM/min (cellobiose)
Final resolution for substrate	Glucose
	Cellobiose

p-NβG, *p*-nitrophenyl-β-D-glucopyranoside
[a] β-Glucosidase activity was assayed with various concentrations of *p*-NβG and cellobiose by the *p*-nitrophenol method and F-kit D-glucose method, respectively

Table 3. Effect of metal ions and chemical agents on β-glucosidase activity from *T. matsutake*

Chemical reagent	Relative activity (%)
Standard	100
$CaCl_2 \cdot 2H_2O$	287
$MnCl_2 \cdot 4H_2O$	257
$BaCl_2 \cdot 2H_2O$	186
$MgCl_2 \cdot 6H_2O$	180
$ZnCl_2$	177
$PbCl_2$	163
NaCl	163
$CuSO_4 \cdot 5H_2O$	160
LiCl	154
$CoCl_2$	151
$FeCl_2 \cdot 4H_2O$	149
$AlCl_3 \cdot 6H_2O$	143
$HgCl_2$	129
KCl	94
$AgNO_3$	71
Ethylenediaminetetraacetic acid (EDTA)	214

Enzyme activity was assayed in the presence of metal ions or chemical agents in the final concentration of 1 mM under standard conditions
Enzyme activity as a standard was measured in the absence of $CaCl_2 \cdot 2H_2O$ β-Glucosidase activity was assayed with various metal ions and EDTA by the *p*-nitrophenol method

Table 4. Substrate specificity of purified extracellular β-glucosidase from *T. matsutake* Z-1

Substrate	Relative activity (%)
Synthetic substrate[a]	
p-Nitrophenyl-β-D-glucopyranoside	100
p-Nitrophenyl-α-D-glucopyranoside	0
p-Nitrophenyl-β-D-cellobioside	0
Natural substrate[b]	
Cellobiose (β1 → 4)	100
Cellobiose (β1 → 4)	63
Maltose (α1 → 4)	0
Isomaltose (α1 → 6)	0
Lactose (β1 → 4)	0
Treharose (α1 → 1)	0
Sucrose (α1 → 2)	0
Isomaltooligosaccharide (α1 → 6)	0
Dextrin (α1 → 4, α1 → 6)	0
CM-cellulose (β1 → 4)	0
Avicell (β1 → 4)	0
Curdlun (β1 → 3)	0

[a] Reaction mixture (200 μl) containing 3 μl purified enzyme solution, 100 μl each synthetic substrate (20 mM), and 20 mM $CaCl_2$ containing 0.1 M Mcllvaine buffer (97 μl) was incubated at pH 5.0 and 37°C for 30 min; reactant product (yield) was calculated from the decomposition of each substrate, and enzyme activity was expressed in values relative to *p*-nitrophenyl-β-D-glucopyranoside, 100, as standard; enzyme activity was measured by the method of *p*-nitrophenol
[b] Reaction mixture (200 μl) containing 5 μl enzyme, 100 μl each natural substrate (oligosaccharide, 20 mM; polysaccharide, 0.2%), and 20 mM $CaCl_2$ containing 0.1 M Mcllvaine buffer (95 μl) was incubated at pH 5.0 and 37°C for 120 min, and enzyme activity was expressed in values relative to cellobiose, 100, as standard; enzyme activity was measured by the method of F-kit D-glucose

Substrate specificities toward synthetic substrates and natural substrates

The substrate specificity of β-glucosidase was tested using different glycosidic bonds of saccharides for synthetic substrates (Table 4). The glucosidase readily activated *p*-NβG [relative activity (R.a.), 100%]. However, the glucosidase did not hydrolyze other synthetic substrates. Also, the substrate specificity of β-glucosidase was tested using carbohydrates with different glycosidic bonds of saccharides for natural substrates (Table 4). The β-glucosidase activity was measured by the F-kit D-glucose method. The glucosidase readily hydrolyzed cellobiose (R.a., 100%) and cellotriose (R.a., 62%) but did not hydrolyze other saccharides. As a result, it was indicated that β-glucosidase hydrolyzed only oligosaccharides having the β-1,4-glycosidic bond.

Also, the natural substrate cellotriose was hydrolyzed by β-glucosidase. The resultant products were analyzed by TLC (Fig. 3). It was observed that the cellotriose was decomposed to cellobiose and glucose. In the long-term reaction (24 h), the substrates were decomposed completely.

Discussion

When the fungi forms fruit bodies, large amounts of mycelia may be needed. However, it is very difficult to cultivate large amounts of mycelia using monosaccharides in a pure
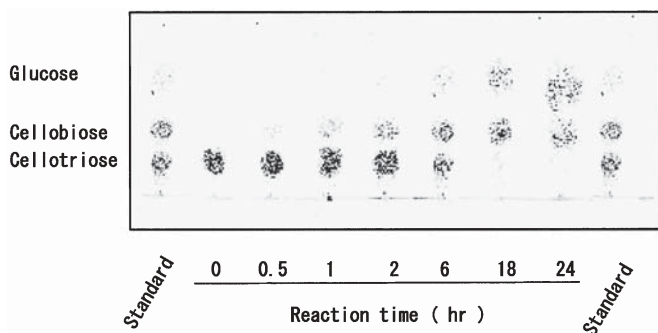
Fig. 3. Resultant products of cellotriose by β-glucosidase from *T. matsutake* Z-1. Reaction mixture was composed of 5μl enzyme solution, 100μl cellotriose (20mM), 95μl 0.1 M McIlvaine buffer, pH 5.0, in a final volume of 200μl. The degradation pattern of the cellotriose was analyzed by thin-layer chromatography (TLC). Reactions were done at 37°C for 0–24h. Developing solution, acetic acid∶chloroform∶distilled water, 7∶5∶1; developing time, about 4h; chromoagent, 20% sulfalic acid-methanol

culture because of the osmotic pressure in the medium (Ohta 1994). Kawai (1973) indicated that *T. matsutake* had poor amylase activity among the many mushroom fungi. Ohta (1997) reported that several strains of *T. matsutake* had the ability to utilize starch as a carbon source.

For the artificial cultivation of this fungus, the ability of starch utilization is very important because the fungus had previously not been known to use other polysaccharides except starch. From these facts, it is important to investigate the ability of amylase production in this fungus. Lee et al. (1998) reported that *T. matsutake* had relatively high amylase activity among its other extracellular enzymatic activities. Terashita et al. (2000b) have reported that the mycelial growth was remarkably promoted, and amylase production in the culture medium obviously increased, when starch from yam and potato was added to the medium as a growth substrate.

In our previous report, we studied the production of amylases and purified extracellular α-amylase from a static culture filtrate. Enzymatic properties of the highly purified α-amylase had already been reported from the aspect of starch utilization as a growth substrate (Kusuda et al. 2003). This α-amylase readily hydrolyzed the α-1,4-glucosidic bond in soluble starch and amylose but did not hydrolyze the α-1,4-glucosidic bond in oligosaccharides, the α-1,6-glucosidic bond, and cyclic polysaccharides such as α- and β-cyclodextrin.

In this article, we have reported that *T. matsutake* produce a β-glucosidase having high activity from static culture filtrate in the research of carbohydrase production to elucidate the utilization of carbohydrates as the growth substrate in ectomycorrhizal mushrooms. This finding suggests the saprotrophic abilities of this fungus.

Then, we tried purification procedures and characterization of β-glucosidase from *T. matsutake*. Until quite recently, it was reported that *T. matsutake* does not use substrates having a β-glucosidic bond such as cello-oligosaccharides and cellulose (Ogawa 1978). Conversely,

Nakazawa et al. (1974) reported that *T. matsutake* cellulolytic enzymes produced a very weak activity. Enokibara et al. (1993) reported the production of cellulolytic enzymes in mycorrhizal mushrooms and showed that *Hebeloma vinosophyllum* Hongo and *Laccaria bicolor* Maire P.D. Orton produced very weak activities of the subneutral cellulases active at pH 5.5–6.8. Also, *Lepista nuda* (Bull.: Fr.) Cooke produced strong activities of the acid cellulase active at pH 3.0–5.5. Furthermore, Terashita et al. (1995) also showed the production of exoglucosidases of mycorrhizal mushrooms such as *T. matsutake* and *Lyophyllum shimeji* (Kawam.) Hongo on potato dextrose liquid medium. As a result, they detected that *T. matsutake* produced weak activities of CM-cellulase and avicelase in all test strains.

Guerin-Laguette et al. (2001) recently reported the saprotrophic growth of *T. matsutake* in soil. They showed mycelial growth stimulation and increase in β-glucosidase excretion from the hyphae of *T. matsutake* using surfactants such as Tween 80 and Tween 40. The addition of the surfactants seemed to greatly improve the saprotrophic potential of this fungus on pine bark. Moreover, Vaario et al. (2003) reported *T. matsutake* produced β-glucosidase when pine bark was used as the substrate for cultivation of this fungus. They indicated that hyphae of *T. matsutake* were able to degrade and invade the xylem cell walls of sawdust (from a pine tree) by transmission electron microscope observations. However, no characterization of the highly purified β-glucosidase from a mycorrhizal mushroom has been reported up to the present.

In this report, we showed good purification procedures and the enzymatic properties of highly purified β-glucosidase from *T. matsutake* for the first time. The molecular mass of the β-glucosidase (160kDa) was large compared to enzymes from other glucosidases of mushroom origin, such as β-glucosidase (110kDa) from *Termitomyces clypeatus* Heim (Sengupta et al. 1991), CMCase IIIa (24.3kDa) from *Polyporus arcularius* (Fr.) Ames, 69B (ATCC 24461) (Ishihara et al. 2005), and α-glucosidase (51kDa) from *Lentinula edodes* (Berk.) Sing. (Yamazaki and Suzuki 1978). The β-glucosidase from *T. matsutake* has a wide range of pH stability (pH 4.0–8.0) as compared with β-glucosidases from other fungi. This enzyme activity showed optimum temperature at 60°C. We studied the properties of extracellular α-amylase from *T. matsutake* (Kusuda et al. 2003), and the optimum temperature was about 60°C.

Among the metal ions tested for β-glucosidase activity, the enzyme from *T. matsutake* was activated remarkably by the presence of Ca^{2+} (2.87 times that of the control), Mn^{2+} ion (2.57 times), and EDTA (2.14 times). Ag^+ weakly inhibited glucosidase activity.

In the present study, a β-glucosidase from *T. matsutake* hydrolyzed disaccharides (cellobiose and *p*-NβG) and cellotriose having the β-1,4-glucosidic bond. However, the enzyme did not act upon disaccharides and polysaccharides having α-1,4-, α-1,1-, and α-1,6 bonds. Moreover, we examined the activity of the enzyme toward cellotriose by TLC analysis of hydrolyzates from the enzyme. From this result, it was decided that the enzyme β-glucosidase acted on oligosaccharides such as cellobiose. This β-glucosidase seem to

be similar to CMCase IIIa from a saprophytic mushroom, *Polyporus arcularius*, which had been reported by Ishihara et al. (2005) as to substrate specificity. The β-glucosidase from *T. matsutake* can be considered as acting on oligosaccharides in the range di–hepta. It is thought that the high concentration of the substrate or feedback inhibition is a cause for the results in our experiments. More detailed information is needed to reveal the substrate specificities of the β-glucosidase from *T. matsutake.*

To reveal the ability to utilize starch as a growth substrate and investigate the saprotrophic aspects of this fungus, we purified and characterized the α-amylase (Kusuda et al. 2003). Moreover, we identified a β-glucosidase from this mushroom. This enzyme hydrolyzed the β-1,4-glucosidic bond in oligosaccharides having a β-1,4 bond. Our finding suggests that *T. matsutake* is able to utilize oligosaccharides released from cellulose and its related compounds in nature such as "shiro soil." However, more detailed information is needed about amylase and β-glucosidase production from *T. matsutake* and other microbes, including litter inhabited by microbes, plants, and animals in "shiro soil." Ectomycorrhizal fungi are believed to obtain much of the carbon necessary for growth from the host plants through ectomycorrhizae. Recently, Hatakeyama and Ohmasa (2004) have showed that many strains of the genus *Suillus* and *Boletinus* can grow well at relatively high glucose concentrations (33.3–100 g/l). Conversely, Vaario et al. (2002) discussed that carbon transfer may be low via the mycorrhizae compared to their experimental results. From these results, quantification of carbon source flow from the host plant to the mycobiont *T. matsutake* is also important.

References

Enokibara S, Suzuki A, Fujita C, Kashiwagi M, Mori N, Kitamoto Y (1993) Diversity of pH spectra of cellulolytic enzymes in Basidiomycetes (in Japanese). Trans Mycol Soc Jpn 34:221–228

Guerin-Laguette A, Vaario L-M, Matsusita N, Suzuki K, Lapeyrie F (2001) Growth stimulation of a Shiro-like mycelium of *Tricholoma matutake* on artificial substrates by addition of non-ionic surfactants or vegetable oils. In: Abstracts, 2nd international workshop on edible ectomycorrhizal mushrooms, 3–6 July, 2001, Christchurch, New Zealand, p 24

Hatakeyama T, Ohmasa M (2004) Mycelial growth characteristics in a split-plate culture of four strains of the genus *Suillus*. Mycoscience 45:188–199

Inaba K, Yoshida T, Takano Y, Mayuzumi Y, Mitsunaga T, Koshizima T (1995) An instance of fruiting-body formation of *Tricholoma matsutake*. Environ. Control Biol 33:59–64

Ishihara H, Imamura K, Kita M, Aimi T, Kitamoto Y (2005) Enhancement of the viscometric endocellulase activity of *Polyporus arcularius* CMCase IIIa by cellobiose and cellooligosaccharides. Mycoscience 46:148–153

Kawai M (1973) Productivity of amylolytic, cellulolytic and xylolytic enzymes among the Basidiomycetes (in Japanese). Nippon Nougei Kagaku Kaishi 47:529–534

Kusuda M, Nagai M, Hur T, Ueda M, Terashita T (2003) Purification and some properties of α-amylase from an ectomycorrhizal fungus, *Tricholoma matsutake*. Mycoscience 44:311–317

Kusuda M, Ueda M, Konishi Y, Matsuzawa K, Shirasaka N, Nakazawa M, Miyatake K, Terashita T (2004) Characterization of extracellular glucoamylase from the ectomycorrhizal mushroom *Lyophyllum shimeji*. Mycoscience 45:383–389

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685

Lee CY, Hong OP, Jung MJ, Han YH (1998) The extracellular enzyme activities in culture broth of *T. matsutake* (in Korean). Korean J Mycol 26:496–501

Nakazawa K, Jinguji I, Akiyama H, Akiyama R, Akiyama I, Kato A (1974) Studies on the cellulolytic enzyme in higher fungi. Mushroom Sci 4(pt 1):859–865

Norkrans B (1950) Studies in growth and cellulolytic enzymes of *Tricholoma*. Symb Bot Ups 11:1–126

Ogawa M (1978) The biology of Matsutake (in Japanese). Tukiji Shokan, Tokyo, pp 192–194

Ohta A (1994) Production of fruit-bodies of a mycorrhizal fungus, *Lyophyllum shimeji*, in a pure culture. Mycoscience 35:147–151

Ohta A (1997) Ability of ectomycorrhizal fungi to utilize starch and related substrates. Mycoscience 38:403–408

Sengupta S, Ghosh AK, Sengupta S (1991) Purification and characterisation of β-glucosidase (cellobiase) from a mushroom *Termitomyces clypeatus*. Biochim Biophys Acta 1076(2):215–220

Terashita T, Kono M, Yoshikawa K, Shishiyama J (1995) Productivity of hydrolytic enzymes by mycorrhizal mushrooms. Mycoscience 36:221–225

Terashita T, Kitao T, Nagai M, Yoshikawa K, Sakai T (2000a) Amylase productions during the vegetative mycelial growth of *Lyophyllum shimeji* (in Japanese). Mushroom Soc Biotechnol 8:61–69

Terashita T, Kusuda M, Matsukawa S, Nagai M, Yoshikawa K, Sakai T (2000b) Production of extracellular amylase from *Tricholoma matsutake* and its properties on starch hydrolysis (in Japanese). Mushroom Soc Biotechnol 8:115–120

Vaario L-M, Guerin-Laguette A, Matsushita N, Suzuki K, Lapeyrie F (2002) Saprobic potential of *Tricholoma matsutake*: growth over pine bark treated with surfactants. Mycorrhiza 12:1–5

Vaario L-M, Gill MW, Samejima M, Suzuki K (2003) Detection of the ability of *Tricholoma matsutake* to utilize sawdust in aseptic culture. Symbiosis 34:43–52

Yamasaki Y, Suzuki Y (1978) Purification and properties of α-glucosidase and glucoamylase from *Lentinus edodes* (Berk.) Sing. Agric Biol Chem 42:971–980