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# Characteristics of the Amylase and its Related Enzymes Produced by Ectomycorrhizal Fungus *Tricholoma matsutake*

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**Extracellular amylase properties were examined with the mycelium of *Tricholoma matsutake* isolated from ectomycorrhizal roots of *Pinus densiflora*. The molecular weights of α-amylase and glucoamylase were estimated as 34.2 kD and 11.5 kD, respectively, after eluted through Superdex 75 column. The optimum pH of the purified enzyme was found in a range of pH 5.0~6.0, with a peak at pH 5.0. The activities of these enzymes were stable from 4°C to 30°C. The α-amylase of *T. matsutake* readily hydrolyzed soluble starch and amylose-B, while it weakly hydrolyzed glycogen, dextrin, amylose and amylose-A. The main products of hydrolysis were confirmed to be glucose, maltose and maltotriose on the basis of the similarities in the thin layer chromatographic mobility.**

**KEYWORDS:** **α-Amylase, Glucoamylase, *Tricholoma matsutake***

Pine mushroom (Songyi), *Tricholoma matsutake* is one of the most popular edible mycorrhizal mushrooms in the world, especially in Korea and Japan. Although pure culture of *T. matsutake* was first established by Hamada (1964), artificial cultivation of the mushroom has not been established in a stable condition up to now. But there has been several reports on successful cultivation of ectomycorrhizal mushrooms such as *Lyophillum shimeji* (Ohta, 1994), *Cantharellus cibarius* (Danell and Camacho, 1997), *Tuber melanosporum* (Hall and Wang, 1998) and so on. In the meantime, several reports had been published for primordium formation and fruit-body production of *T. matsutake in vitro* (Inaba *et al.*, 1994; Kawai and Ogawa, 1976; Ogawa and Hamada, 1975). However, the reports did not lead to the further information concerning the artificial cultivation. Since the mids of the 20th century, the annual production of pine-mushroom in Korea and Japan has dwindled down to less than 500 tons, while the demand of the mushroom in Japan exceeds more than 3,000 tons a year. So, Japan has been importing the mushroom from China, South Korea, North Korea and other countries. As a result, artificial cultivation of *T. matsutake* is strongly demanded in Japan.

At present, there are several reports showing the possibility of artificial cultivation for *T. matsutake*; mycorrhizal synthesis of *T. matsutake* on *Pinus desiflora* (Sieb. et Zucc.) roots *in vitro* was successful (Guerin-Laguette *et al.*, 2000; Yamada *et al.*, 1999). But, much works would be still needed for practical cultivation system of the mushroom. To accomplish the goal, we have to solve several problems such as the mechanism of nutrient exchange between *P. densiflora* and *T. matsutake,* that of fruit-body initiation and growth, the micro-environment of fairy-ring and so on. In practice, the most serious barrier for the artificial cultivation of the mushroom is that the mycelium of *T. matsutake* grows so slowly on the artificial media (about 2 cm/month) and has low ability to decompose polysaccharides or proteins. So, the fungus uses monosaccharides as a carbon source.

Meanwhile, Ohta (1994) reported that *L. shimeji* forms mature fruit-bodies on a artificial medium, including mainly barley grain without a host plant although the fungus is a kind of ectomycorrhizal mushroom. He also depicted that several strains of *T. matsutake* had an ability to utilize starch as a carbon source (Ohta, 1997). For the artificial cultivation of *T. matsutake*, this ability is very important since the fungus has been known not to use starch as polysaccharides in the past. In several researches about the amylase activity of *T. matsutake,* Kawai (1973) indicated *T. matsutake* had poor activity among the *Basidiomycetes*, while Lee *et al.* (1998) reported that the fungus had high amylase activity among the other extracellular enzyme activities. Terashita *et al.* (2000b) reported that the amylase activity of *T. matsutake* showed higher in starch originated from barley grain than that originated from other sources. Hur *et al.* (2000) also reported that the amylase activities were variable among *T. matsutake* isolates.

In this research, we investigated physicochemical and enzymatic properties (especially for amylase) of *T. matsutake* strain isolated from the ectomycorrhizal roots of *Pinus densiflora* on barley-starch liquid medium.

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## Materials and Methods

**Mycelial isolation of *T. matsutake* from ectomycorrhizal root of *P. densiflora*.** Ectomycorrhizal sample was collected from the edge of fairy-ring of *T. matsutake* in 70-year old *P. densiflora* stand located at Hongcheon, Korea. The detailed ecological description of the site was previously reported by Hur *et al.* (1997). For the collection of mycorrhizal roots of *P. densiflora*, about 1 of soil sample was collected from the edge of fairy-ring of *T. matsutake* on May. The sample was transferred into a polyvinyl bag and moved into the laboratory within an ice-box. Ectomycorrhizal roots separated from the soil sample was immediately washed with tap water, and then the washed ectomycorrhizas were cut down into the size of 5 mm, and put into 300 m*l* flask. After pouring 100 m*l* of tap water, the flask was vigorously shaked to remove soil particles from the ectomycorrhizas. This washing procedure was repeated three times with tap water. Washed ectomycorrhizas were rinsed two times with sterile distilled water. The ectomycorrhizas were surface sterilized with 1% calcium hypochloride solution for 3~4 minutes, then rinsed four times with sterile distilled water. The two pieces of the rinsed ectomycorrhizal tip was put on an agar plate for culture. The agar plate (8.7 cm in diameter) contained a modified Melin-Norklans medium (Marx, 1969). Streptomycin (100 $\mu$g/m*l*) and Kanamycin (10 $\mu$g/m*l*) were added to the melted MMN medium after autoclaving to keep from bacterial contamination.

The plates were incubated at 23°C, and the fungal isolates were separated 25 days after inoculation. Fungal isolates were confirmed as *T. matsutake* by morphological characteristics and PCR-RAPD. The isolates were transferred to Potato Dextrose agar (PDA; Difco) for the subculture of the strain.

**Culture and extraction of crude enzyme.** The subculture of *T. matsutake* (HEM) was grown in barley-starch liquid medium containing 20 g barley, 20 g glucose, 5 g Yeast Extract (Difco), 5 g sanparu-CP (product's name; separated sulphite pulp waste from softwood. Inaba *et al.*, 1994), and thiamin (100 $\mu$g/*l*) in 1,000 m*l* of distilled water. The medium was autoclaved at 121°C for 15 min., and the pH was adjusted to 5.2. The 100 m*l* Erlenmeyer flasks containing 20 m*l* of barley-starch liquid medium were incubated at 24±1°C in darkness for 80 days with standing culture condition. The inoculum of *T. matsutake* was an agar disk (5 mm diameter, about 2.5 mg of dry weight) that was cut from the leading edge of colonies grown on PDA (Difco lab., Detroit, USA).

After 80 days of incubation, the culture was filtered through a filter paper (Whatman No. 2) for mycelial removal. The filtrate and residual starch granules were removed by centrifugation at 8,000 g for 15 minute in 4°C. The supernatant was used as the crude enzyme for the assay of $\alpha$-amylase activity. The crude enzyme solution (5 m*l*) was dialyzed in 20 mM McIlvaine buffer with pH 5.0 at 4°C for 24 hours, and used for the assay of amylase and glucoamylase activity. Starch concentration was determined with colorimeter by the iodine color reaction (Terashita *et al.*, 2000a)

The mycelia harvested by filtering were washed three times with distilled water to determine the dry weight after drying at 105°C under vacuum for 3 hours.

**Assay for enzyme activities.** Amylase activity was assayed with the standard reaction mixture contained 100 mM McIlvaine buffer with pH 5.0, 0.4% (w/v) soluble starch and an appropriate amount of enzyme solution in a final volume of 0.22 m*l*, after incubating an hour at 50°C. The reaction was stopped by boiling for 10 minutes to measure the increase in the reducing sugar by adapting Somogyi-Nelson's method (Nelson, 1944; Somogyi, 1952). One unit of amylase activity was defined as the amount of enzyme that liberates 1 $\mu$mol of reducing sugar (as glucose) per minute under the standard assay condition.

The $\alpha$-amylase activity was assayed by Iodine-potassium iodide method and a unit of $\alpha$-amylase activity was defined as the amount of enzyme that decreases 0.01 absorbency degree at 690 nm per minute under the standard assay condition (Terashita *et al.*, 2000a). For all amylase reactions, 10 mM $CaCl_2$ was included in the reaction medium and soluble starch (Wako chem. Co., Japan) was used for the substrates.

The glucoamylase activity was assayed by F-kit glucose method (Boehringer Mannheim Co.), and a unit of glucoamylase activity was defined as the amount of enzyme that liberates 1 $\mu$mol of glucose per minute under the standard assay condition.

## Purification Step

**Step 1: DEAE-Toyopearl column chromatography.** The dialyzed enzyme solution (20 mM Tris-HCl buffer pH 8.0, 24 h, 4°C) was applied to the 5 m*l* of DEAE-Toyopearl column (flow rate 60 m*l* $h^{-1}$, Tosoh Co., Japan) previously equilibrated with 20 mM Tris-HCl buffer with pH 8.0 and eluted with the same buffer containing a linear gradient of NaCl in the range of 0~400 mM at 4°C.

**Step 2: Superdex 200 column chromatography.** The active fractions from DEAE-Toyopearl column were pooled, concentrated and applied to a column of Superdex 200 (32.0×1.0 cm, Bio-Rad Co., USA) pre-equilibrated with 20 mM Tris-HCl buffer with pH 7.0 including 100 mM NaCl. The protein was eluted with the same buffer at a flow rate of 0.25 m*l*/min at 4°C.

**Step 3: Superdex 75 column chromatography.** The active peak fractions from Superdex 200 column were pooled, concentrated and applied to a column of Superdex 75 (32.0×1.0 cm, Bio-Rad Co., USA) pre-equilibrated with 20 mM Tris-HCl buffer with pH 7.0 including 0.1 M NaCl. The protein was eluted with the same buffer at a flow rate of 0.25 m*l*/min at 4°C. The purified enzyme was used for physicochemical analysis. For the assay of the effect of metal ions, enzyme solution, 0.05 m*l* of 1 mM metal ion solution, and 100 mM McIlvaine buffer with pH 5.0, in a final volume of 450 $\mu l$ were pre-incubated at 37°C for 30 minutes. After pre-incubation, 150 $\mu l$ of 0.4% (w/v) soluble starch was added, and then the resulting mixture was kept at 37°C for 15 minutes.

**Thin layer chromatography.** The production of starch decomposition were confirmed by TLC. Activated fractions achieved by superdex 75 were spotted on TLC and TLC was developed by the ascending method with solvent system of chloroform-acetic acid-distilled water (5 : 7 : 1). After drying at 120°C for 10 minutes, the products were detected by the sulfur-methane (1 : 4) method (Yoshioka and Endo, 1985).

## Results

**General properties.** The mycelial weight, concentration of starch, and pH variations were shown in Table 1. The mycelial weight was 242 mg after incubation, which was three times of the weight compared to the result by the Modified Matsutake Liquid medium used by Terashita *et al.* (2000b). The pH was changed from 5.2 to 6.2 through incubation period.

We presumed that the decomposed starch could be utilized as a substrate by *T. matsutake* (HEM). Starch concentration changed from 3.32% to 0.36% by the culture of *T. matsutake* (HEM), which indicated that almost 90% of starch was decomposed.

**Table 1.** The variaions of growth weight, starch concentration and pH of *Tricoloma matsutake* (HEM strain) on a partly barely modified matsutake liquid medium by stationary culture

| | before incubation | 80 days after inoculation |
|---|---|---|
| Dry weight (mg) | 2.5±0.02[a] | 242.1±13.2 |
| Starch (%) | 3.32±0.13 | 0.36±0.009 |
| pH | 5.2 | 5.9±0.3 |

[a]Average±standard deviation resulted from the five replicates.

Crude enzyme activities of $\alpha$-amylase and glucoamylase by *T. matsutake* (HEM) showed 1.93 U/mg and 0.015 U/mg, respectively (Table 2). In comparison to the result of Terashita *et al.* (2000b), the amylases activities were higher on poor starch medium. These results suggested that *T. matsutake* (HEM strain) had an ability of anabolism from barely starch as Ohta (1997) depicted that several ectomycorrhizal fungi had abilities to assimilate starch.

**Purification and characterization of amylases on *T. matsutake* (HEM strain).** The concentrated dialyzed enzyme solution from the crude enzyme was passed over a column of DEAE-Toyopearl. The amylase activity was achieved in one fraction by DEAE-Toyopearl column. After purification using Superdex 200, fraction No. 28, 29, 30, 31 were separated into previous DEAE-Toyopearl column having most activities of amylases (Fig. 1). The amylase activity was shown in two fraction peaks; the first fraction ($\alpha$-amylase peak) adsorbed on the Superdex 200 and eluted at 100 mM NaCl concentration in 20 mM Tris-HCl buffer, had specific activity 1071.94 U/mg, purification fold 555.41 and a yield of 113.0%, especially. The second fraction (glucoamylase peak) presented specific activity 9.289 U/mg, purification fold 629.15 and a yield of 71.55%. But the peaks were not being clearly purified. Therefore, active two peaks from Superdex 200 chromatography were used to be purified on Superdex 75 (Fig.

**Table 2.** Purification of amylase, glucoamylase and $\alpha$-amylase extracted from *Tricholoma matsutake* (HEM strain)

| | Enzymes | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| amylase | crude enzyme | 413.58 | 23.76 | 0.057 | 1 | 100 |
| | DEAE-Toyopearl column | 25.87 | 15.88 | 0.614 | 10.69 | 66.86 |
| | Superdex 200 column | 1.08 | 14.54 | 13.488 | 234.82 | 61.19 |
| | Superdex 75 column | 0.16 | 1.76 | 11.351 | 199.15 | 7.42 |
| glucoamylase | crude enzyme | 413.58 | 6.11 | 0.015 | 1 | 100 |
| | DEAE-Toyopearl column | 24.24 | 4.41 | 0.182 | 12.33 | 72.27 |
| | Superdex 200 column | 0.47 | 4.37 | 9.289 | 629.15 | 71.55 |
| | Superdex 75 column | 0.06 | 1.79 | 28.073 | 1871.51 | 29.24 |
| $\alpha$-amylase | crude enzyme | 482.51 | 933.33 | 1.93 | 1 | 100 |
| | DEAE-Toyopearl column | 19.21 | 908.18 | 47.28 | 24.44 | 97.30 |
| | Superdex 200 column | 0.97 | 1039.78 | 1071.94 | 555.41 | 113.00 |
| | Superdex 75 column | 0.09 | 406.58 | 4405.98 | 2282.89 | 43.56 |

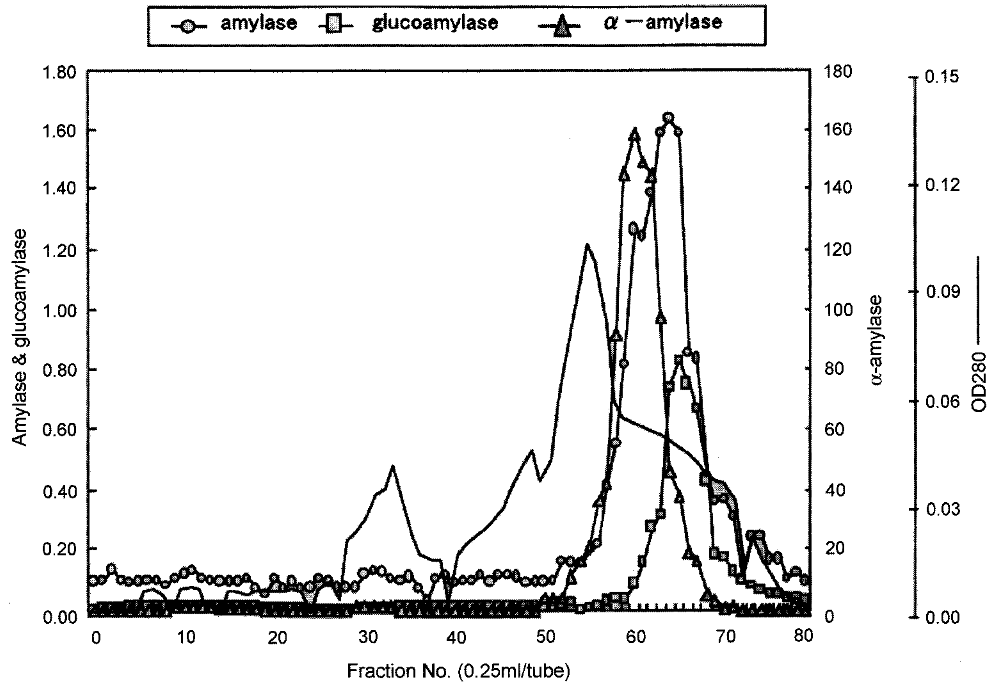


**Fig. 1.** Patterns of extracelluar amylases from *Tricholoma matsutake* (HEM strain) by Superdex 200 column chromatography. Flow rate: 0.25 m*l*/min. Enzyme unit: U/m*l* buffer: 100 mM Nacl in 20 mM Tris-HCl buffer (pH 7.0). The chromatography was done using the active fractions from DEAE-Toyopearl column chromatography.

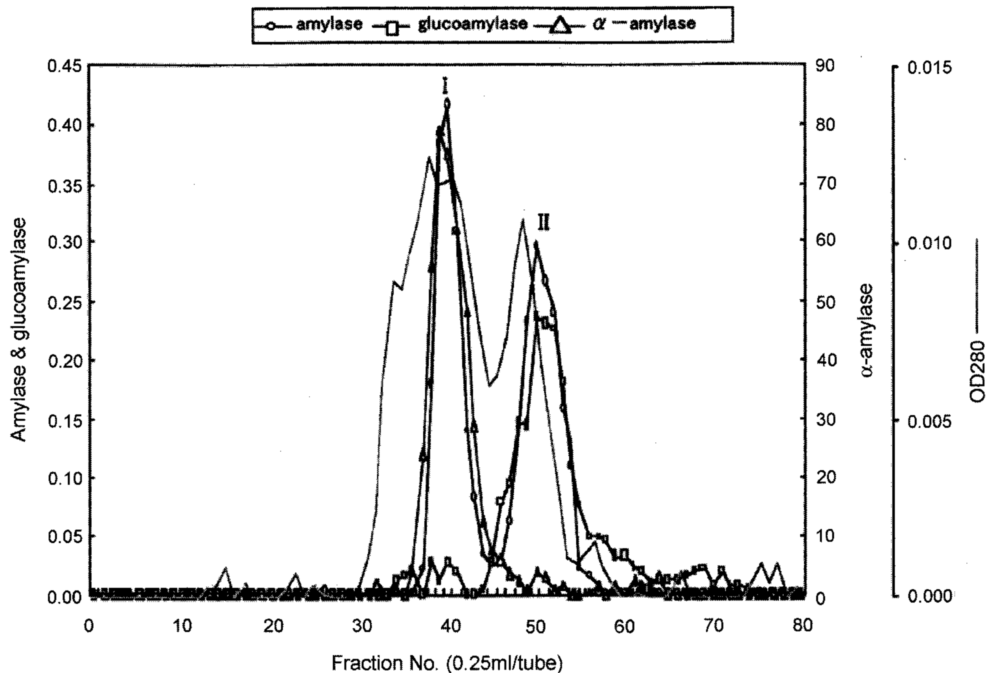


**Fig. 2.** Patterns of extracelluar amylases from *Tricholoma matsutake* (HEM strain) by Superdex 75 column chromatography (peak No. I, II). Flow rate: 0.25 m*l*/min. Enzyme unit: U/m*l* buffer: 100 mM Nacl in 20 mM Tris-HCl buffer (pH 7.0).

2); the first fraction, presumed α-amylase active peak, adsorbed on the Spuerdex 75 and eluted at 100 mM NaCl concentration in 20 mM Tris-HCl buffer, had specific activity 4405.98 U/mg, purification fold 2282.89, and a yield of 43.56%. The second fraction, presumed glucoamylase active peak, presented specific activity 28.073 U/mg, purification fold 1871.51 and a yield of 29.24%.

**Amylase properties of *Tricholoma matsutake*.** The molecular weight of the enzyme was determined by

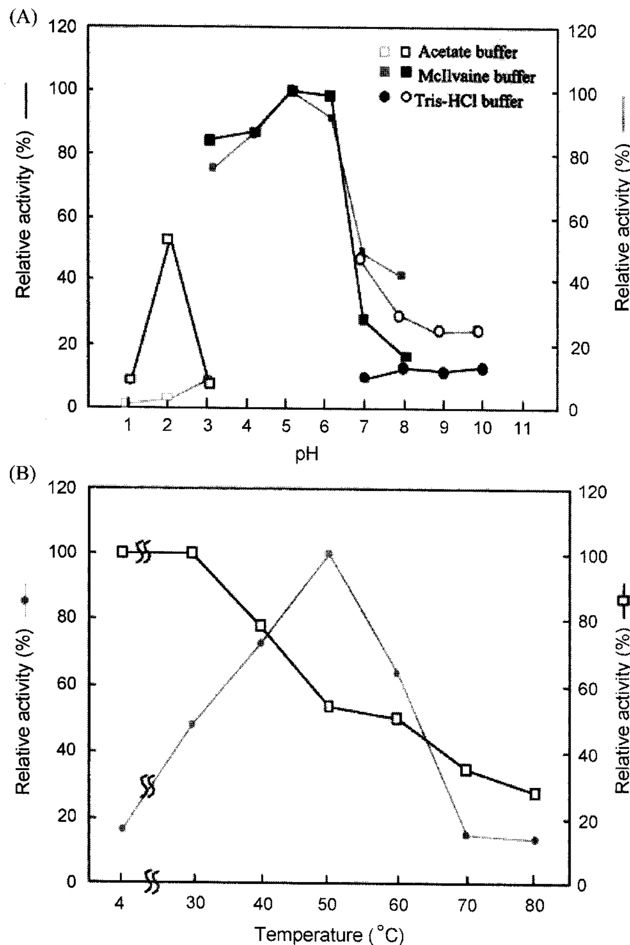


**Fig. 3.** The pH optimum, pH stability, temperature optimum and stability of *Tricholoma matsutake* amylase. Enzyme solution was used $\alpha$-amylase peak. (A) For pH stability, enzyme was pre-incubated for 30 min at 37°C with 100 mM McIlvaine buffer at various pH. (B) For temperature stability, enzyme was pre-incubated for 30 min at various temperature. * Soluble starch was used as substrate.

Superdex 75 gel filtration through a column (32.0×1.0 cm) equilibrated with 20 mM McIlvaine buffer pH 7.0. Thyroglobulin 670 kD; Gamma globulin 158 kD; Ovalbumin 44 kD; Myoglobin 17 kD; Cyanocobalamin 1.35 kD were used as the standard. The molecular weights of $\alpha$-amylase and glucoamylase were calculated to be 34.2 kD and 11.5 kD, respectively. The elution profile on Superdex 75 column confirmed the enzyme's purity and homogenity.

The optimum pH of the purified enzyme was found in the range of pH 5.0~6.0, with maximum at pH 5.0 (Fig. 3). The enzyme had 24.8% and 51.2% loss of the maximal activity at pH 3.0 and pH 7.0, respectively, with complete inhibition at pH 1.0.

The $\alpha$-amylase activity was estimated in aliquots of pure enzyme pre-incubated at different temperatures (30~70°C) for 30 min. The activity increased as temperature increased up to 50°C, followed by sharp decline till 70°C, which indicated an inhibition in enzyme activity. Temperature

**Table 3.** Substrate specificity of extracellular amylase from *Tricholoma matsutake* (HEM strain)

| Substrate | Relative activity (%)[a] |
|---|---|
| Starch[1] | 100 |
| Amylose-A[1] (MW=2,900) | 69.88 |
| Glycogen[1] | 47.29 |
| Dextrin[2] | 38.92 |
| Amylose[1] | 33.27 |
| Amylose-B[1] (MW=16,000) | 27.83 |
| $\beta$-cyclodextrin[2] | N.D. |
| $\alpha$-cyclodextrin[2] | N.D. |
| pullulan[2] | N.D. |

[a]The reaction mixture (0.22 m*l*) containing enzyme solution, 0.1 m*l* of 0.4% substrate, and 0.1 M McIlvaine buffer, pH 5.0, was incubated at 37°C for 15 min. Enzyme solution used was -amylase (fraction No. 40). N.D. means not detected. 1. Wako produced reagents (Japan). 2. Sigma produced reagents (USA).

stability was shown from 4°C to 30°C.

The $\alpha$-amylase of *T. matsutake* readily hydrolyzed soluble starch and amylose-B (MW=16,000), and weakly for glycogen, dextrin, amylose and amylose-A (MW=2,900), while it failed to hydrolyze $\alpha$-cyclodextrin, $\beta$-cyclodextrin or pullulan (Table 3). Although *T. matsutake* could hydrolyze low molecular substrates, the rate of the hydrolysis was quite lower than that for soluble starch.

When the enzyme solutions were pre-incubated with 0.4% soluble starch for 48 hours with fraction No. 40 ($\alpha$-amylase peak), No. 51 (glucoamylase peaks) from Superdex 75 chromatography (Fig. 4). As shown in Fig. 4, the main products were thought to be glucose, maltose and maltotriose on the basis of the similarities in the thin layer chromatographic mobility.

Among the metal ions tested, $Fe^{2+}$, $Na^{+}$ and $Co^{2+}$ were in some degree inhibitors for the amylase (Table 4). But

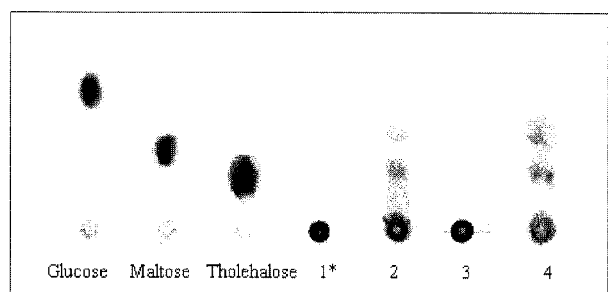


**Fig. 4.** Patterns of thin layer chromatography of $\alpha$-amylase and glucoamylase from *Tricholoma matsutake* (HEM strain) The reaction time 24 h, reaction temperature, 50°C, 50 minute. The reaction mixture (0.6 m*l*) containing enzyme solution, 0.1 m*l* of 0.4% starch substrate, and 0.1 M McIlvaine buffer, pH 5.0, was incubated at 50°C for 24 h. The extension time was 50 minutes. Enzyme solution used was $\alpha$-amylase (fraction No. 40) and glucoamylase (fraction No. 51) by Superdex 75 column chromatography.

**Table 4.** Effect of metal ions in extracellular amylase activity[a] of *Tricholoma matsutake* (HEM strain)

| Metal ion | Relative activity (%) |
|---|---|
| Control($CaCl_2$) | 100 |
| $AlCl_3$ | 109 |
| $BaCl_3$ | 93.9 |
| $CoCl_2$ | 80.2 |
| $Cu(CH_3COO)2$ | 82.0 |
| $CuSO_4$ | 120 |
| $FeCl_2$ | 56.4 |
| $HgCl_2$ | 119 |
| KCl | 89.4 |
| LiCl | 123 |
| $MgCl_2$ | 89.2 |
| $MnCl_2$ | 97.0 |
| NaCl | 57.5 |
| EDTA | 86.2 |

[a]The $\alpha$-amylase activity was assayed with various metal ion by Iodine-potassium iodide method. For the assay of the effect of metal ions, 5 $\mu l$ of enzyme solution, 6 $\mu l$ of 0.1 M metal ion solution in McIlvaine buffer, 30 $\mu l$ of 0.2 M $CaCl_2$ and 0.01 M McIlvaine buffer with pH 5.0, in a final volume of 450 $\mu l$ were pre-incubated at 37°C for 30 minute. After pre-incubation, 150 $\mu l$ of 0.4% starch was added and resulting mixture was kept at 50°C for 30 minute. Enzyme solution used was $\alpha$-amylase peak from Superdex 75 column.

Tris, $Pb^{2+}$, $Al^{3+}$ and $Hg^{2+}$ did not inhibited the amylase of *T. matsutake*, while those inhibited $\alpha$-glucosidase and glucoamylase in the *L. edodes* (Yamasaki and Suzuki; 1978).

## Discussion

When the fungi form fruit-bodies, large amounts of mycelia may be needed either to store the nutrients or to transport the nutrients to the fruit-bodies, or both. However, it is very difficult in practice to cultivate large amounts of mycelia using monosacaaharides in a pure culture. From these facts, We suppose the ability of *T. matsutake* to utilize starch as a substrate and amylase activity are very important.

A few research has been conducted on enzymes of ectomycorrhizal fungi such as *T. matsutake.* Terashita *et al.* (2000a) reported that *L. shimeji* fruit-body forming strains had higher amylases activity level than those of the fruit-body non-forming strains. Hur *et al.* (2000) also reported that the amylase activities were variable among *T. matsutake* strains. These reports supposed amylase activity concerned with fruit-body formation in ectomycorrhzal fungi.

In the case of *T. matsutake*, it have a slight ability to decompose polymer substrates, such as starch, amlylose-B, amylose and have relatively saprophytic properties because it grew on artificial medium, barley-starch liquid medium. If these properties would developed in *T. matsutake*, it can be concluded that these properties of *T. matsutake* may offer a possibility of artificial cultivation.

However, more detailed information will be needed to elucidate the function of amylases in the fruit-body formation of *T. matsutake* in nature.

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## Reference

Danell, E. and Camacho, F. J. 1997. Successful cultivation of the golden chantharelle. *Nature* **385**: 303.

Guerin-Laguette, A., Vaario, L.-M., Gill, W. M., Lapeyrie, F., Matsushita, N. and Suzuki, K. 2000. Rapid *in vitro* ectomycorrhizal infection on *Pinus densiflora* roots by *Tricholoma matsutake. Mycoscience* **41**: 389-393.

Hall, I. R. and Wang, Y. 1998. Commercial production of *Tuber melanosporum* in New Zealand. Abstracts of the second international conference on mycorrhiza. Uppsala, Sweden. pp. 77-78.

Hamada, M. 1964. Method of pure culture of the mycelium of *Armillaria matsutake* and of its allies. In: Matsutake (*Tricholoma matsutake* Singer)-Its fundamental studies and economic production of the fruit-body, (ed. by The Matsutake Research Association), Nakanishi printing, Kyoto. pp. 97-100. In Japanese.

Hur, T. C., Park., H., Chung, J. H. and Joo, S. H. 1998. Changes in soil physicochemical properties and dehydrogenase activity by the formation of fairy ring of *Tricholoma matsutake. Journal of Korean Forestry Society* **87**(2): 270-275.

_____, Joo, S. H. and Terashita, T. 2000. Compare with intra and extracellular amylase activities of *Tricholoma matsutake.* Proceedings of the annual meeting of Japanese mushroom science and biotechnology 4th symposium. pp In Japanese.

Inaba, K., Yoshida, T., Takano, Y., Mayuzumi, Y., Mitsunaga, T. and Koshizima, T. 1995. An instance of the fruiting body formation of *Tricholoma matsutake. Environment Control In Biology.* **33**: 59-64.

Kawai, M. 1973. Productivity of amylolytic, cellulolytic and xylolytic enzymes among *Basidiomycetes. J. Agri. Chem. Japan* **47**: 529-534. In Japanese.

_____ and Ogawa, M. 1976. Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. IV. Studies on a seed culture and trial for the cultivation on solid media. *Trans. Mycol. Soc. Japan* **17**: 499-505. In Japanese.

Lee, C. Y., Hong, O. P., Jung, M. J. and Han, Y. H. 1998. The extracellular enzyme activities in culture broth of *T. matsutake. Kor. J. Mycol.* **26**: 496-501. In Korean.

Marx, D. H. 1969. The influence of ectotropic, mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **59**: 153-163.

Nelson, M. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**: 375.

Ogawa, M. and Hamada, M. 1975. Primordia formation of *Tricholoma matsutake* (Ito et Imai)Sing. in pure culture. *Trans. Mycol. Soc. Japan* **16**: 406-415. In Japanese.

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

_____. 1997. Ability of ectomycorrhizal fungi to utilize starch and related substrates. *Mycoscience* **38**: 403-408.

Somogyi, M. 1952. Note on sugar determination. *J. Biol. Chem.* **195**: 19.

Terashita, T. and Kono, M. 1989. Purification and some properties of carboxyl proteinase from *Tricholoma matsutake. Trans. Mycol. Soc. Japan* **28**: 245-256.

_____, Kitao, T., Nagai, M., Yoshikawa, K. and Sakai, T. 2000a. Amylase productions during the vegetative mycelial growth of *Lyophyllum shimeji. Mushroom Science and Biotechnology* **8**: 61-69. In Japanese.

_____, Kusuda, M., Matsukawa, S., Nagai, M., Yoshikawa, K. and Sakai, T. 2000b. Production of extracellular amylase from *Tricholoma matsutake* and its properties on starch hydrolysis. *Mushroom Science and Biotechnology* **8**: 115-120. In Japanese.

Yamada, A., Maeda, K. and Ohmasa, M. 1999. Ectomycorrhiza formation of *Tricholoma matsutake* isolates on the seedling of *Pinus densiflora in vitro. Mycoscience* **40**: 455-463.

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