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## Short Communication

# Purification and characterization of an extracellular acid trehalase from *Lentinula edodes*

**Mika Murata[1], Masaru Nagai[1], Makoto Takao[1], Akira Suzuki[2], Takuo Sakai[1] and Takao Terashita[1]**

[1] Laboratory of Food Microbiology, Faculty of Agriculture, Kin-ki University, 3327–204 Nakamachi, Nara 631–8505, Japan

[2] Faculty of Education, Chiba University, 1–33 Yayoicho, Inageku, Chiba 263–8522, Japan

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**Trehalase from the culture filtrate of *Lentinula edodes* was purified and characterized. Molecular masses were estimated to be 158 kDa and 79–91 kDa by gel filtration and SDS-PAGE under the reduced condition, respectively. The enzyme was composed of two identical subunits and contained carbohydrate molecules. The optimum temperature and pH were obtained at around 40°C and pH 5.0, respectively. The enzyme was stable up to 40°C and in a range pH of 4–10 at 30°C. It cleaved α-1,1 linkages of trehalose, but not α-1,4, α-1,6 or β-1,4 glycosyl linkages, and was defined as an acid trehalase.**

Key Words——Basidiomycotina; fruit-body formation; *Lentinula edodes*; mushroom; trehalase.

Mushroom fungi usually form fruit-bodies after vegetative mycelium have grown sufficiently and accumulated nutrients needed for forming the fruit-bodies. The change of nutritive components used as substrates for vegetative growth and fruiting were studied in *Agaricus bisporus* (Lange) Imbach. (Wood et al., 1977), *Flammulina velutipes* (Curt. ex Fr.) Sing. (Kitamoto et al., 1976), *Favolus arcularius* (Fr.) Ames (Kitamoto et al., 1978). It was shown that trehalose, glycogen, sugar alcohols and chitin are used as carbohydrate substrates for the growth of these fungi (Kitamoto et al., 1978).

Trehalose (α-D-glucopyranosyl (1→1)-α-D-glucopyranoside) is a nonreducing disaccharide with an α,α-1,1 glycosidic linkage. It is widespread in nature and has been isolated from bacteria, algae, fungi, insects, invertebrates, and plants (Elbeln, 1974). The mushroom contains about 2–10% trehalose (dry weight) (Yoshida et al., 1996), but the biosynthetic pathway is not well known. Trehalose is degraded by two enzymes. One is trehalose phosphorylase, which has phosphorylating activity. The enzyme producing glucose and β-glucose-1-phosphate (β-G1P) from trehalose was reported for *Euglena gracilis* Klebs. (Maréchal et al., 1972) etc. In *F. velutipes*, α-type trehalose phosphorylase was found and partially purified (Kitamoto et al., 1988). The enzyme was purified from *Schizophyllum commune* Fr.: Fr. (Eis and Nidetzky, 1999), *A. bisporus* (Lange) Imbach (Wannet et al., 1998) and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Kitamoto et al., 2000). The second trehalose-degrading enzyme is trehalase, which has hydrolyzing activity and produces two glucoses. This enzyme has been purified from various fungi (Jorge et al., 1997), but there are few reports on its partial purification from basidiomycetes (Williams et al., 1968).

In the present report, we purified extracellular trehalase from a culture filtrate of *Lentinula edodes* (Berkeley) Pegler, and examined its enzymatic characteristics.

*Lentinula edodes* 'Mori 465' was obtained from Mori Co., for use in this study. As inoculum, a mycelial block (φ 1 cm) was cut from a plate culture that had grown on a potato-dextrose agar medium (PDA, Nissui Co.) for 10 d at 24°C in a Petri dish (φ 90 mm). It was planted in a tall Petri dish (φ 11 cm × 7 cm) containing 50 ml of glucose-peptone-yeast extract medium (Mohamed et al., 1992; with a modification replacing $FeCl_2$ with $FeSO_4$) and cultured at 24°C for 30 d. It was then placed at 15°C for 30 d to induce fruit-body formation. When the young fruit-bodies were observed, the extracellular trehalase activity reached its maximum level. Therefore, the culture filtrate was used as crude enzyme solution for purification.

After separation of mycelium by filtration, the culture filtrate was assayed for trehalase activity. The culture filtrate was dialyzed with 5 mM HEPES/5 mM MES buffer (pH 5.0) and used as the crude enzyme.

Trehalase activity was assayed by measuring the glucose released from trehalose. The reaction mixture contained 10 mM trehalose in 25 mM HEPES/25 mM MES buffer (pH 5.0) and a suitable amount of the enzyme solution in a total volume of 200 μl. The reducing sugar produced was determined by the method of Somogyi

(1952). One unit of the activity was defined as the amount of the enzyme that catalyzed the hydrolysis of 1 $\mu$mol of trehalose $min^{-1}$. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

All procedures were carried out at 4°C, unless stated otherwise. The crude enzyme solution (8,600 ml) was concentrated 5-fold by ultrafiltration (Amicon, Diaflo Ultrafiltration membrane cut-off 10 kDa). Next, solid ammonium sulfate was added to the enzyme solution to make 60% saturation, and the resulting precipitate was removed by centrifugation at 27,200×*g* for 15 min. The supernatant was put on a phenyl Sepharose CL-4B column (bed volume 50 ml, 30×105 mm) (Pharmacia LKB) equilibrated with 5 mM HEPES/5 mM MES buffer (pH 5.0) containing 60% saturated ammonium sulfate. After washing the column with the same buffer, adsorbed proteins were eluted with a linear gradient of ammonium sulfate (400 ml, 60 to 0% saturation) in 5 mM HEPES/5 mM MES buffer (pH 5.0). The fractions containing trehalase activity were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). The pooled enzyme was applied on a DEAE-Toyopearl 650 M column (bed volume 20 ml, 15×120 mm) (Tosoh Co.) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The enzyme was eluted with a linear gradient of NaCl (160 ml, 0 to 500 mM) in the same buffer. The fractions containing trehalase activity were pooled and dialyzed against 20 mM sodium acetate buffer (pH 5.0).

The trehalose affinity column was prepared as described by Teunissen et al. (1992) with some modifications. The dialyzed enzyme was applied on a trehalose affinity column (bed volume 3.1 ml, 3×110 mm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with a linear gradient of NaCl (0 to 150 mM) in the same buffer. The enzyme solution was chromatographed again on the trehalose affinity column. The fractions containing trehalase activity were pooled and concentrated to about 250 $\mu$l.

The concentrated enzyme was applied on a Superdex 200 HR 10/30 (10×300 mm) (Pharmacia LKB) equilibrated with 20 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.0). The enzyme was eluted with the same buffer. The fractions containing trehalase activity were pooled and solid ammonium sulfate was added to 50% saturation. The enzyme was then applied on a phenyl Superose column (5×50 mm) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 50% saturated ammonium sulfate. The adsorbed proteins were eluted with a linear gradient of ammonium sulfate (50 to 0% saturation) in 20 mM Tris-HCl buffer (pH 7.0). The pooled enzyme was then chromatographed on a Mono Q HR column (5×50 mm) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The enzyme was eluted with a linear gradient of NaCl (0 to 500 mM) in the same buffer.

The molecular mass of the enzyme was estimated by gel filtration on Superdex 200 HR 10/30 by use of a Bio-Logic Automation system A (Bio-Rad Lab, Japan, Tokyo). The purified enzyme and the reference proteins (Pharmacia LKB), namely, thyroglobulin (bovine, molecular mass, 670 kDa), gamma globulin (bovine, 158 kDa), ovalbumin (chicken, 44 kDa), myoglobulin (horse, 17 kDa), and cyanocobalamin (1.35 kDa), were injected into the column equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 100 mM NaCl.

Protein homogeneity was examined by native polyacrylamide gel electrophoresis (PAGE) using a gradient gel (NPG-520L PAGEL®, ATTO Co.) according to the user's manual, and sodium dodecyl sulfate (SDS)-PAGE by the method of Laemmli (1970). The molecular mass of the purified enzyme was examined by SDS-PAGE using reduced samples: the enzyme (4 $\mu$g) was boiled in 2% of SDS and 5% of 2-mercaptoethanol and put on a gel. Proteins on the gel were stained with Coomassie Brilliant Blue R-250. Carbohydrate molecules in the enzyme were also detected by Endoglycosidase H (Roche Molecular Biochemicals, Tokyo, Japan) treatment. The enzyme (4 $\mu$g) was boiled with 1 $\mu$l of 2-mercaptoethanol, then incubated at 37°C for 12 h with 2 mU of Endoglycosidase H. After this treatment, the molecular mass was measured by SDS-PAGE.

The isoelectric point (p*I*) of the enzyme was determined by the chromatofocusing using a Mono P HR 5/20 column (Pharmacia LKB). The purified enzyme was applied on the column equilibrated with 25 mM Tris-HCl buffer (pH 7.4) and eluted with a pH linear gradient (pH 7.4–3.0) in Polybuffer (Pharmacia LKB). The pH of the fraction containing trehalase activity was taken as the isoelectric point of the enzyme.

Table 1 summarizes typical results of the purification protocols for *L. edodes* trehalase. After the final chromatographic step, the specific activity of trehalase was 3.50 U/mg protein, and 1,510-fold purification was achieved.

One protein band appeared after migration of the enzyme on the native polyacrylamide gel. Molecular mass of the purified enzyme was determined to be 158 kDa and 79–91 kDa by the gel filtration and SDS-PAGE, respectively. These results suggest that the enzyme is composed of two identical subunits. The molecular mass of the enzyme after treatment with Endoglycosidase H was determined to be 59 kDa by SDS-PAGE (Fig. 1). Many fungal trehalases have been reported to be glycoproteins (Jorge et al., 1997), and the carbohydrate contents of an extracellular enzyme from *Scytalidium thermophilum* (Cooney & Emerson) Austwick was estimated to be about 51% (Kadowaki et al. 1996). Trehalase from *L. edodes* was also a glycoprotein that was about 30% glycosylated. This carbohydrate could not be digested with O-glycosidase (Roche Molecular Biochemicals, Tokyo, Japan), but could be with Endoglycosidase H. Therefore, the enzyme might be N-glycosylated.

The characterization of the purified enzyme is shown in Table 2. The enzyme had an optimum activity at around 40°C and pH 5.0. The enzyme was stable up to 40°C at pH 5.0 for 30 min, and in the pH range between 4 and 10 at 30°C for 30 min.

Fungal trehalases are grouped into two categories (Jorge et al., 1997). Neutral trehalases are regulated as

Table 1. Purification of trehalase from cultured supernatant of *Lentinula edodes*.

| | Total protein (mg) | Total activity (U) | Specific activity (U/mg Protein) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Culture filtrate | 15,700 | 36.4 | 0.00232 | 1.00 | 100 |
| Amicon ultrafiltration | 5,090 | 22.3 | 0.00438 | 1.89 | 61.3 |
| 60% $(NH_4)_2SO_4$ | 3,560 | 19.8 | 0.00556 | 2.40 | 54.4 |
| Phenyl Sepharose CL-4B | 225 | 15.1 | 0.0671 | 28.9 | 41.5 |
| DEAE-Toyopearl 650M | 45.4 | 12.5 | 0.275 | 119 | 34.3 |
| 1st Trehalose affinity column | 6.91 | 7.53 | 1.09 | 470 | 20.7 |
| 2nd Trehalose affinity column | 5.38 | 7.23 | 1.34 | 578 | 19.9 |
| Superdex 200 | 2.89 | 6.63 | 2.29 | 987 | 18.2 |
| Phenyl Superose | 0.920 | 2.19 | 2.38 | 1,030 | 6.02 |
| Mono Q | 0.551 | 1.93 | 3.50 | 1,510 | 5.30 |

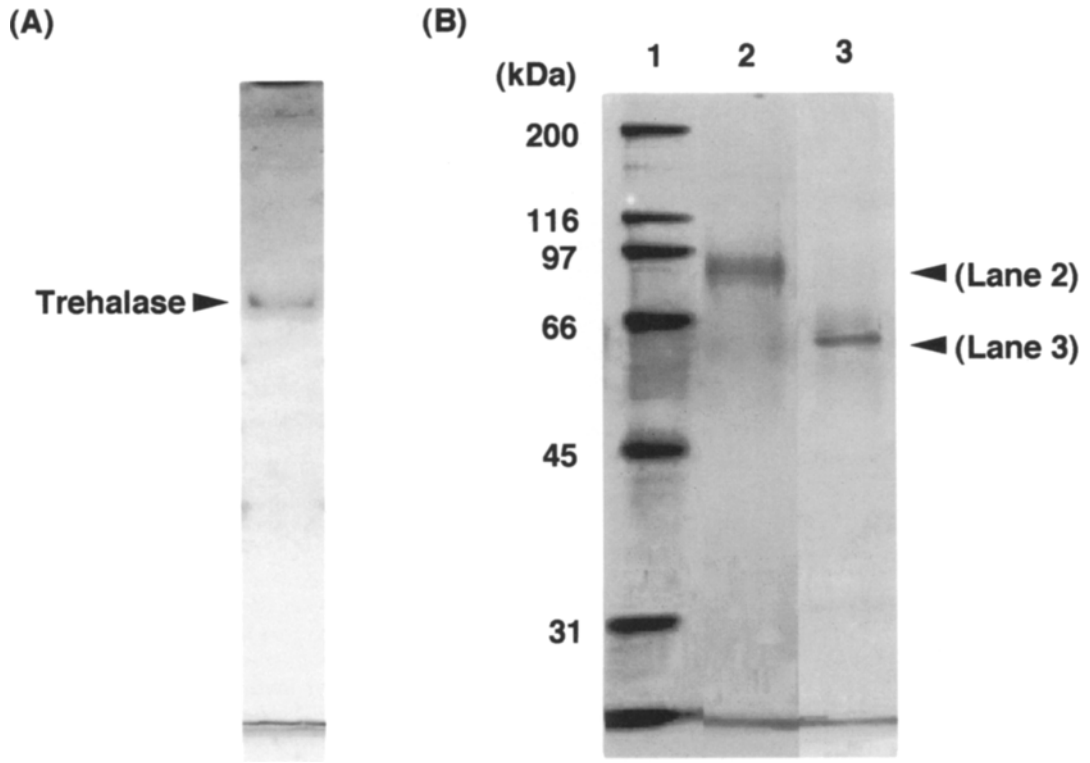


Fig. 1. Native-PAGE (A) and SDS-PAGE (B) of the purified trehalase samples.
(A) The purified trehalase fraction from *Lentinula edodes* (4 μg) was put on a 5–20% gradient polyacrylamide gel.
(B) The purified trehalase (4 μg, lane 2) and its Endoglycosidase H treated sample (4 μg, lane 3) were applied on a 10% polyacrylamide gel. Lane 1, protein standards.

cytosolic enzymes involved in the catabolism of internal trehalose. In contrast, acid trehalases with an acidic pH optimum are extracellular or vacuolar glycoproteins and considered to be non-regulated. Therefore, the enzyme from *L. edodes* is an acid trehalase with the optimum pH of 5.0.

The purified *L. edodes* trehalase hydrolyzed trehalose into two molecules of glucose, but did not hydrolyze sucrose, maltose, cellobiose, raffinose (10 mM), *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-β-D-glucoside (1 mM), soluble starch and glycogen (0.1%). The *K*m value for trehalose was 2.14 mM. Although this *K*m value was similar to that of trehalase from *Humicola grisea* var. *thermoidea* Cooney & Emerson (2.3 mM) (Sumida et al., 1989), it was lower than that of trehalase from *Chaetomium aureum* Chivers (0.29 mM) (Zimmermann et al., 1990). The effects of various reagents and metal ions on the enzyme activity are shown in Table 2. The trehalase activity was completely (100%) inhibited by 1 mM $AlCl_3$, $MnCl_2$ and $FeCl_3$. When 1 mM $CuSO_4$ or 1 mM ATP was added to the assay mixture, the trehalase activity decreased to 15% or 22%, respectively. The addition of $HgCl_2$ or the trehalose analog, 1-deoxynojirimycin (1 mM), resulted in the inhibition of 90%.

Table 2. Physicochemical and molecular properties of trehalase from *Lentinula edodes*.

| Property | Trehalase |
|---|---|
| pH optimum | 5.0 |
| Temperature optimum | 40°C |
| pH stability | 4.0–10.0 |
| Thermal stability | ~40°C |
| *K*m | 2.14 mM |
| *V*max | 46.2 mM/min/mg |
| Substrate specificity | (hydrolyze) trehalose |
| | (not hydrolyze) |
| | sucrose, maltose, cellobiose, |
| | *p*-nitrophenyl-α-D-glucoside |
| | *p*-nirtophenyl-β-D-glucoside |
| | raffinose, soluble starch, glycogen |
| Inhibitor | $AlCl_3$, $FeCl_3$, $MnCl_2$, |
| | $HgCl_2$, 1-deoxynojirimycin |
| Molecular mass | |
| by SDS-PAGE | 79~91 kDa |
| by gel filtration | 158 kDa |
| p*I* | 3.8 |

pH stability was assayed by incubating the enzyme at 30°C for 30 min. Thermal stability was assayed by incubating the enzyme at pH 5.0 for 30 min. The enzyme was considered stable if the remaining activity exceeded 80%. Substrate specificity was measured with substrates concentrations of 1 mM (*p*-nitrophenyl-α-D-glucoside and *p*-nitrophenyl-β-D-glucoside), 0.1% (soluble starch and glycogen), or 10 mM (others). Concentration of inhibitors was 1 mM. *K*m value was assayed using trehalose as a substrate.

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