Changes of trehalose content and trehalose-degrading activity during fruit-body formation and autolysis in Pleurotus sp.

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**Highlights**

- The trehalose content of whole fruit-bodies decreased sharply during autolysis process.

- Trehalose-degrading activity increased toward the inner region from the outer region of the pilei during autolysis process.

- Our results strongly suggested that the degradation of trehalose in the autolysis process does not occur at random in the fruit-body.

- The autolysis process may systematically take place toward the inner region from the outer region of pilei.
Note

Changes of trehalose content and trehalose-degrading activity during fruit-body formation and autolysis in *Pleurotus* sp.

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We studied how the content and degrading activity of trehalose changed during fruit-body development and autolysis. During the process of autolysis, the trehalose content of whole fruit-bodies decreased sharply whereas the trehalose-degrading activity increased toward the inner region (regions 5 and 4) from the outer region (region 6) of the pilei (during stage 5–9 of autolysis). Conversely, the trehalose content during autolysis decreased toward the inner region (regions 5 and 4) from the outer region (region 6) of the pilei, and further decreased toward the bottom region (region 1) from the top region (region 3) of the stipes.

Keywords: Autolysis, fruit-body formation, Pleurotus sp., trehalase, trehalose
Trehalose (α-D-glucopyranosyl-1, 1 α -D-glucopyranoside) consists of two molecules of glucose linked by an α -(1,1)-glycosidic bond. This compound is widely distributed in archaea, bacteria, fungi, plants, and animals. In fungi, trehalose is closely linked to important biological phenomena; for example, trehalose may be supplied as a carbon and energy source to germinating spores or resting cells (Francois and Parrou 2001, Jorge et al., 1997), and it may act as a stabilizer of cellular membranes and proteins (Singer and Lindquist 1998, Simola et al. 2000).

Mushrooms contain about 2%–20% trehalose. It was reported that trehalose, glycogen, sugar alcohols, and chitin are used as carbohydrate substrates in the growth of Agaricus bisporus (Lange) Imbach. (Wood and Goodenoug 1997), Flammulina veltipes (Curt. Ex Fr.) Sing. (Kitamoto and Gruen 1976), and Favolus arucularius (Fr.) Ames (Kitamoto et al., 1978). Trehalose is a very important substance for both mycelial growth and fruit-body formation and is degraded by two enzymes: trehalose phosphorylase and trehalse. Trehalose phosphorylase will produce either glucose and β-glucose-1-phosphate (β-G1P) or α-glucose-1-phosphate (α-G1P) from trehalose. In mushroom, the α-type trehalose phosphorylases were found and purified from Flammulina veltipes (Kitamoto et al., 1988), Schizophyllum commune Fr.:Fr (Eris and Nidetzky), A. bisporus (Lange) Imbach (Wannet et al., 1998), and Pleurotus ostreatus (Jacq.: Fr.) Kummer (Kitamoto et al., 2000). Nevertheless, the two trehalose-hydrolyzing enzymes, known as acid and neutral trehalases, have been found in several fungal species (Parrou et al., 2005), and the two differ in their subcellular localization, and various biochemical and regulatory properties.

Recently, Liu et al. (2016) reported that the trehalose contents and trehalase activities change during fruit-body formation in Flammulina veltipes (Liu et al. 2016). Earlier, the acid trehalase, from a culture filtrate of Lentinus edodes, was purified and characterized.
by Murata et al. (2001) (Murata et al. 2001); this enzyme was composed of two identical subunits (71–91 kDa) and contained carbohydrate molecules. However, for *Pleurotus* spp., there exist almost no published reports of how its trehalose content and degrading activity changes over the course of fruit-body formation and autolysis. To fill this knowledge gap, we investigated such changes in this paper.

*Pleurotus* sp. were supplied to us from Hokuto Co. (Nagano, Japan) (Ishikawa et al., 2016) and their fruit-bodies harvested according to the growth of fruit-bodies after mechanically scratching the exterior aerial mycelia (Fig. 1 A–I). Stages 1 and 2 entailed the primordium stage. Stages 3 and 4 is when the fruit-body developed. Stage 5 was the mature fruit-body stage. Stages 6 to 9 formed the autolysis stage. The fruit-bodies of stages 4 to 9 were divided into six regions (Fig. 2). Region 1 corresponds to the bottom region of the stipe (one-third region from bottom of the stipe). Region 2 corresponds to the middle region of the stipe (two-third region from the bottom of the stipe). Region 3 represents the top region of stipe. Region 4 is the circular inner region of the pileus (one-third region from the pileus center). Region 5 is the circular middle region of the pileus (two-third region from the pileus center). Finally, Region 6 represents the circular outer region of the pileus.

Fresh whole fruit-bodies, pilei, and stipes in each stage were crushed in a mortar and pestle with 20 mM of a potassium phosphate buffer (pH 6.0). The cell debris were removed by centrifugation at 12,000 g for 30 min. From each centrifuged sample, the supernatant was used as a crude enzyme solution.

Trehalose-degrading (trehalase) activity was assayed by measuring the glucose released from trehalose. The reaction mixture contained 20 mM of trehalose in 20 mM of an acetate buffer (pH 5.0) with a suitable amount of the enzyme solution, in a total volume
of 110 μl. The reducing sugars produced were determined following the method of Somogyi-Nelson (Somogyi, 1952). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol reducing sugar min⁻¹.

The fresh whole fruit-bodies, pilei, and stipes were dried in a freeze-drying machine (Freeze Dryer FD-1000, EYELA, Tokyo, Japan). Dried mushroom samples (10 mg) were crushed with Milli-Q ultrapure water (5 mL) by using a mortar and pestle. Each sample solution was centrifuged at 12,000 g for 5 min, and the ensuing supernatant used for HPAEC with a CarboPac PA-1 column (4 × 250 mm, Thermo Fisher Scientific, Waltham, USA). Elution was conducted with 0.1 M NaOH (0–10 min), 0.1 M NaOH, 0.8 M sodium acetate (10–15 min), and 0.1 M NaOH (15–30 min) at a flow rate of 1.0 mL/min, and the trehalose was monitored with a pulsed amperometric detector.

To ascertain the presence of the trehalose-degrading enzyme during the fruit-body development of Pleurotus sp., at each stage we assayed the trehalose-degrading activity. The activity of whole fruit-bodies significantly increased from stages 1 through 8, whereas it decreased in stage 9 (Fig. 3). At this end stage of autolysis, the enzyme might have been hydrolyzed by protease. The enzyme’s minimal activity was detected in stage 1 (0.06 U/g fresh weight), and its maximal activity observed at stage 8 (0.6 U/g fresh weight) in the tested Pleurotus sp. Notably, a significant increase in enzyme activity occurred in the transition from stage 4 to 5 (mature stage), and this activity gradually increased in stages 5 through 8 (autolysis stage). These results suggest that the trehalose-degrading enzyme is related to fruit-body formation and the autolysis process in the Pleurotus sp.

To analyze the localization of the trehalose-degrading enzymes during the autolysis of the fruiting body, the activities of each pileus and stipe (regions 1 to 6) at the
different stages were assayed. It was evident that the trehalose-degrading activities in
regions 5 and 6 of stage 5 are significantly higher than those of stage 4 (Fig. 4A, B).
Region 6 had higher activity than did region 5 in stage 5, while the latter was higher than
that of region 6 in stage 6 (Fig. 4B, C). The high activity in pilei persisted until stage 8
(Fig. 4B–E), after which the activity in all regions was decreased in stage 9 (Fig. 4F).
These results suggest that tehalose degradation shifted toward the inner region from the
outer region of the pilei during autolysis. Murata et al. (2001), who purified and
characterized the acid trehalase from a culture filtrate of *Lentinus edodes* (Murata et al.
2001), reported that the optimum pH of the acid trehalase was 5.0. We also identified that
the optimal pH of the trehalose-degrading (trehalase) activity of *Pleurotus* sp. is pH 5.0
(data not shown). This suggests that the activity found in the fruit-bodies of *Pleurotus* sp.
studied may be driven by acid trehalase.

In mushrooms, glucan and chitin are the main cell wall components. Glucanase and
chitinase activity also increased during fruit-body development in the *Pleurotus* sp. (data
not shown). Earlier studies showed that glucanase and chitinase activity increase during
the fruit-body development and autolysis process in *Coprinus comatus* (Bush, 1974),
*Coprinus macrorhizus* (Kamada et al., 1982), and *Lentinus edodes* (Minato et al., 1999).
It was also suggested that several carbohydrate hydrolases—namely trehalase, chitinase,
and glucanase—are associated with autolysis in these mushrooms.

The trehalose contents in the whole fruit-bodies increased from stages 1 to 3, but then
slightly decreased from stages 3 to 5 (Fig. 3). Importantly, the trehalose content showed
a markedly decrease from stage 5 to 6, and it continued to decrease until stage 9, the end
stage of autolysis (Fig. 3). The maximum amount of trehalose detected was 0.19 g/g dry
weight, measured in stage 3, while the minimum amount was 0.0079 g/g dry weight in
stage 9. Hence, we consider that the decreased trehalose content from stage 5 to 6 is due
to the increased trehalase activity seen in stage 5 (Fig. 3). Together, these results suggest
that trehalose was used as a carbohydrate nutrient for autolysis and then reused as other
constituents via trehalose degradation.

At stages 4–7, the trehalose content in region 3 exceeded those in the other regions
(Fig. 4A, F). The regions 5 and 6 contents of trehalose in stages 5 and 6 decreased more
sharply than those in the other regions (Fig. 4B, C). At stage 9, any trehalose was almost
undetectable in all regions of the fruit-body (Fig. 4F). In Flammulina velutipes, trehalose
in the stipes attained its maximum during fruit-body development but then decreased by
approximately 60% (Kitamoto and Gruen 1976). However, the trehalose contents in the
pilei continued to increase until the final growth stage. Therefore it seems plausible that
the trehalose degradation process differs between Flammulina velutipes and Pleurotus sp.

Our results strongly suggested that the degradation of trehalose in the autolysis process
does not occur at random in the fruit-body. The autolysis process may systematically take
place toward the inner region from the outer region of pilei and further shift to the stipes.

In this report, we first demonstrated that the trehalose-degrading activity of whole
fruit-bodies increased during the autolysis process, while the trehalose content sharply
decreased. In addition, trehalose was degraded toward the inner region from the outer
region of pilei during the process of autolysis. This trehalose degradation continued
toward the stipes from the pilei. Taken together, our results suggest that trehalose is used
as an energy source and is systematically degraded to translocate glucose from the fruit-
body to the vegetative mycelium during autolysis. To further elucidate the mechanism of
the autolysis process in Pleurotus sp., we intend to purify and characterize the acid
trehalase from Pleurotus sp.
REFERENCES


**Legends**

Fig. 1 Development of the fruit-bodies after scratching the exterior aerial mycelium on the surface of the culture medium in *Pleurotus* sp.

The surface of the culture medium was mechanically scratched to remove the exterior aerial mycelium. After scratching the mycelium, we separated the fruit-bodies from stages 1 to 9 during the growth of the fruit-body.

Fig. 2 Division into the pilei and stipe of the fruit-body in *Pleurotus* sp.

The fruit-body was divided into six regions. Region 1 corresponds to the bottom region of the stipe (one-third region from bottom of the stipe). Region 2 corresponds to the middle region of the stipe (two-third region from the bottom of the stipe). Region 3 represents the top region of stipe. Region 4 is the circular inner region of the pileus (one-third region from the pileus center). Region 5 is the circular middle region of the pileus (two-third region from the pileus center). Finally, Region 6 represents the circular outer region of the pileus.
Fig. 3  Change in the trehalose content and trehalose-degrading activity of the whole fruit-body during fruit-body formation and the process of autolysis (stages 4–9). Each bar or symbol represents the mean of triplicate measurements. The error bars show standard deviation of the measurements. Blue bars: trehalose-degrading activity, closed circle line: trehalose content.

Fig. 4  Changes in the trehalose content and trehalose-degrading activity of each region of the fruit-body during fruit-body formation and the process of autolysis (stages 4–9). Each bar or symbol represents the mean of triplicate measurements. The error bars show standard deviation of the measurements. Blue bars: trehalose-degrading activity, closed circle line: trehalose content.
After scratching:

Fig. 1 Alireza et al.
Fig. 2 Alireza et al.
Fig. 3  Alireza et al.
Fig. 4 Alireza et al.