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Metabolic function of glycogen phosphorylase and trehalose phosphorylase in fruit-body formation of *Flammulina velutipes*

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Glycogen phosphorylase in the vegetative mycelium of *Flammulina velutipes* converts glycogen to α-glucose 1-phosphate (G1P) in the colony during fruit-body development. Glycogen may contribute to the synthesis of trehalose as the starting material in the vegetative mycelium during the fruiting process of the colony, and the trehalose produced is translocated into the fruit-bodies as the main carbohydrate substrate for their development. Trehalose phosphorylase activity in the vegetative mycelium was at a relatively high level until fruit-body initiation, suggesting the turnover of this disaccharide during the vegetative stage of the colony development. Trehalose phosphorylase activity in the stipes showed a peak level at the early phase of fruit-body development, suggesting the continuing phosphorolysis of trehalose by this enzyme. The stipes also showed a high specific activity of phosphoglucomutase at a sufficient level to facilitate the conversion of G1P to α-glucose 6-phosphate (G6P). In the pilei a large amount of G1P remained until the growth of the fruit-bodies ceased. Trehalase activities in the stipes and pilei were at a very low level, and this enzyme may not contribute to the catabolism of trehalose in the fruit-body development.

Key Words—*Flammulina velutipes*; fruit-body; glycogen phosphorylase; phosphoglucomutase; trehalose phosphorylase.

The vegetative mycelium and fruit-bodies of basidiomycetes contain much trehalose (Kitamoto and Gruen, 1976; Hammond and Nicholas, 1976; Kitamoto et al., 1978; Hammond, 1979a, b; Yoshida et al., 1986). However, few reports have been published on the biochemical function of trehalose during the fruiting of basidiomycetes. Kitamoto and Gruen (1976) surveyed the distribution of cellular carbohydrates during the fruit-body development of *Flammulina velutipes* (Curt.: Fr.) Sing. Glycogen represented the largest accumulation in the vegetative mycelium as the major store of carbohydrates. The trehalose content in the vegetative mycelium of this mushroom was about 5%, but the endogenous glucose level was not over 0.5% (Kitamoto and Gruen, 1976). A large net loss of trehalose in the vegetative mycelium occurred as the fruit-bodies developed. In fruit-bodies, the trehalose content was about two times higher than that of glycogen. Trehalose increased in the fruit-bodies up to the middle phase of development, and thereafter decreased remarkably. ^{14}C-glucose labeling experiments indicated that the total incorporation of ^{14}C was highest in trehalose, next highest in glycogen, but a few percent in endogenous glucose. In the fruit-bodies, trehalose also contributed about 50% of the total radioactivity in the sugar pool, and the specific activity was much higher than that of endogenous glucose. Thus, trehalose seems to be the major carbohydrate translocated from mycelia into the fruit-bodies in *F. velutipes*.

In the present study, the metabolic regulation of trehalose was investigated by examining the activities of trehalose and glycogen phosphorylases and α-phosphoglucomutase (PgluM) during fruit-body development.

Materials and Methods

Culturing and collection of materials The stock culture of *F. velutipes* (721-B1) was used for all experiments. It was grown on a potato-glucose liquid medium, as described by Kitamoto and Gruen (1976), in 100-ml Erlenmeyer flasks containing 20 ml of the liquid medium. The inoculum was an agar block 5×5×5 mm that was cut from a culture grown on a PDA plate at 23°C for 10 d in the dark. Cultures were first grown in the dark at 23°C for 14 d, then transferred into a culture room at 18°C in the light at ca. 4.6 mW/cm^2 (about 200 lux) until the fruit-bodies appeared. The vegetative mycelium and fruit-bodies were collected at appropriate intervals, washed thoroughly with distilled water and stored in a deep freezer at −20°C. Larger fruit-bodies were dissected into stipes and pilei, but small fruit-bodies of less than 5 mm in height were stored without dissection,

since it was difficult to collect these stipes and pilei in sufficient amount to determine their dry weights and analyze the sugar phosphates and enzyme activities. Each datum of the dry weights of mycelium, small fruit-bodies, stipes and pilei of fruit-bodies is the average of five replicates. The deviation of the data among the same replicates was within 40% of the average.

Assay of sugar phosphates Cell-free extracts for the enzyme assay were prepared by homogenization of 1.0-2.0 g of the frozen mycelia and fruit-bodies. Samples were taken into a glass homogenizer (Digital Homogenizer, Iuchi Co.), mixed with 10 ml of 100 mM potassium phosphate buffer containing 25% glycerol and 2 mM EDTA (pH 7.0), and then homogenized at 3,000 rpm for 5 min. After removal of the cell debris by centrifugation at 12,000×*g* for 15 min, the supernatant solution was dialyzed by use of a Centriflo CF-25 apparatus (Amicon Corp., U.S.A.) at 500×*g* for several hours, and the dialyzed solution passed through the Centriflo was collected as the analytical sample. A 0.5-ml portion of the sample was mixed with the reaction mixture (2.5 ml) containing 120 μmol of potassium phosphate buffer (pH 7.0), 30 μmol of reduced glutathione (GSH), 4 μmol of $MgCl_2$, 0.2 μmol of α-glucose 1,6-diphosphate (GDP), 3 μmol of NADP, and the absorbance of the mixture at 340 nm was measured. Then, 0.2–0.4 unit of α-glucose 6-phosphate (G6P) dehydrogenase was added, and the mixture was incubated at 30°C until the increase in the absorbance ceased. The amount of G6P was calculated from the increase in absorbance after incubating the enzyme reaction mixture. After the G6P measurement, a further 0.2–0.4 unit of G6P dehydrogenase and 0.2–0.4 unit of α-phosphoglucomutase (PgluM), and incubated at 30°C until the increase in the absorbance ceased. The amount of α-glucose 1-phosphate (G1P) in the solution was also calculated from the increase in absorbance of the mixture after the second enzyme reaction. The increase in absorbance at 340 nm by the production of NADPH of the above reactions was followed at 30°C with a spectrophotometer (Shimazu, UV-2100S) fitted with a temperature controlled cell housing. Each datum of the sugar phosphates is the average of five replicates. Deviation of the data among the same replicates was within 35% of the average.

Preparation of crude enzymes Crude cell extracts were prepared by homogenization of the frozen mycelia and fruit-bodies in 100 mM potassium phosphate buffer containing 25% glycerol and 2 mM EDTA (pH 7.0), by using a glass homogenizer at 2,000 rpm for 5 min. After removal of the cell debris by centrifugation at 12,000×*g* for 15 min, the supernatant was concentrated with a Centriflo CF-25 apparatus and used as the crude enzyme.

Enzyme assay Trehalose phosphorylase activity was determined spectrophotometrically by a coupled reaction with PGluM and G6P dehydrogenase (Kitamoto et al., 1988). The reaction mixture (3 ml) contained 120 μmol of potassium phosphate buffer (pH 7.0), 600 μmol of trehalose, 30 μmol of GSH, 4 μmol of $MgCl_2$, 0.2 μmol of GDP, 3 μmol of NADP, 3 units of PgluM, 3 units of G6P dehydrogenase, and an appropriate amount of the crude enzyme. The assay was initiated by addition of the enzyme solution to the reaction mixture. The increase in absorbance at 340 nm by the production of NADPH was followed at 30°C with a spectrophotometer.

Glycogen phosphorylase activity was also determined spectrophotometrically by a similar coupled reaction. The reaction mixture (3 ml) contained 120 μmol of potassium phosphate buffer (pH 7.0), 3 mmol of glycogen, 4 μmol of $MgCl_2$, 30 μmol of GSH, 0.2 μmol of GDP, 3 μmol of NADP, 3 units of PgluM, 3 units of G6P dehydrogenase and an appropriate amount of the crude enzyme. The assay was initiated by the addition of the enzyme solution to the reaction mixture. The change in absorbance at 340 nm was followed at 30°C with a spectrophotometer.

Trehalase was assayed by the method of Williams and Niederpruem (1968). The reaction mixture (2 ml) contained 25 mmol of sodium acetate buffer (pH 4.5), 2 mmol of trehalose, 10 μmol of NaF, and an appropriate amount of the crude enzyme. The liberated glucose was measured by the method of Somogyi (1952).

One unit of enzyme activity is defined as the amount of enzyme required for the formation of 1 μmol of product per min. Specific activity is expressed as units of enzyme activity per mg of protein. Protein was determined by the method of Lowry et al. (1951). Each datum of enzyme activity is the average of five replicates. Deviation of the data among the same replicates was within 35% of the average.

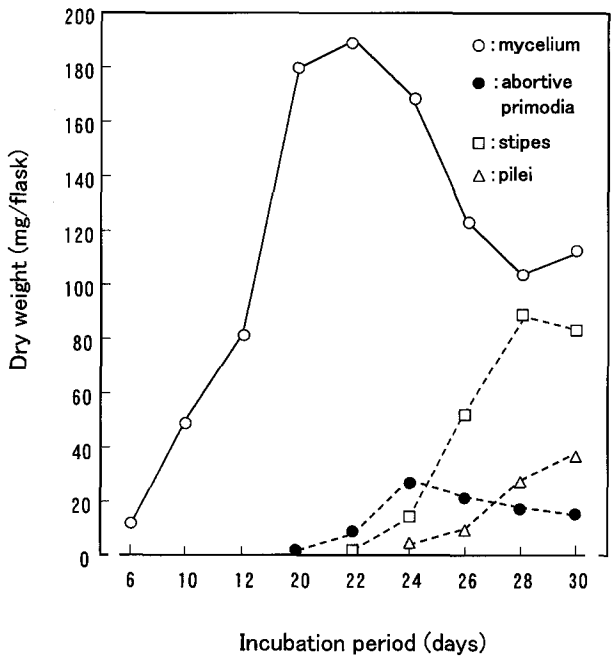

Fig. 1. Changes in the dry weights of vegetative mycelium, the abortive primordia, and the stipes and pilei of fruit-bodies during development of *F. velutipes*.

Results

Changes in dry weights of the mycelium and fruit-bodies during development When vegetative mycelium grown at 23°C for 14 d in the dark was transferred into a culture room at 18°C in the light, a cluster of fruit-body primordia appeared within 4–6 d after the transfer to low temperature room. Only two or three of these primordial developed into fruit-bodies of over 5 cm in length, and they matured by about 30 d after inoculation. However, most primordia did not grow up in this culture system.

Figure 1 shows the time course of fruit-body development and the changes in the dry weights of vegetative mycelium and fruit-bodies during colony development. Mycelial weight began to increase rapidly 1 wk after inoculation and attained its maximum between 20 and 22 d after inoculation, when a number of primordia were growing actively. The dry weights of stipes increased rapidly up to 28 d after inoculation but decreased slightly thereafter. The pilei of fruit-bodies increased until their maturation. On the other hand, the maximum dry weight of the abortive primordia reached only one-fifth of the maximum value of the fruit-body fraction.

Changes in G1P and G6P levels in the mycelium and fruit-bodies during development The levels of sugar and sugar phosphates in the colony of mushrooms are the results of the metabolic conversion from storage to translocated carbohydrates in each part of the colony. Glycogen may serve as a storage carbohydrate in the vegetative mycelium, and the phosphorolysis of this polysaccharide produces G1P. This sugar phosphate ester may also be utilized as a precursor of the biosynthesis of trehalose and/or glycogen in the colony. The involvement of trehalose phosphorylase may also contribute to the production of G1P by this enzyme. G1P is reversibly converted to G6P by the action of PgluM, and the resulting G6P may enter the glycolytic metabolism in mushrooms. Therefore, the changes in the amounts of G1P and G6P may be applied as criteria for estimating the metabolic functions of both glycogen and trehalose phosphorylases and also for the biosynthesis of the polysaccharide and the disaccharide.

Figure 2 shows the changes of G1P and G6P in the vegetative mycelium, and the stipes and pilei of the fruit-bodies during development. The G6P content in the vegetative mycelium was 2–8 times higher than that of G1P. The G6P in the mycelium began to increase at the start of the fruit-body formation, but it decreased at the time of maturation of the fruit-bodies. The level of G1P in the mycelium was very low throughout the colony development. The content of G1P in the stipes was about 10 times higher than that of G6P at the early stage of fruit-body development, although the equilibrium constant (=G6P/G1P) of PgluM of the microorganisms was reported to be over 10 (Ray and Peck, 1972). G1P content decreased rapidly during further development of the fruit-bodies. On the other hand, the G6P level in the stipes showed a peak at the middle phase of the development. The ratio of G6P/G1P in the stipes was less than 1.0 at the early growth phase, but it increased as the fruit-bodies approached maturity. The level of G1P in the pilei was much higher than the G6P level up to the maturation of fruit-bodies. A peak in the G1P level of pilei appeared at the middle phase, but the G6P level gradually increased until the fruit-bodies achieved maturity.

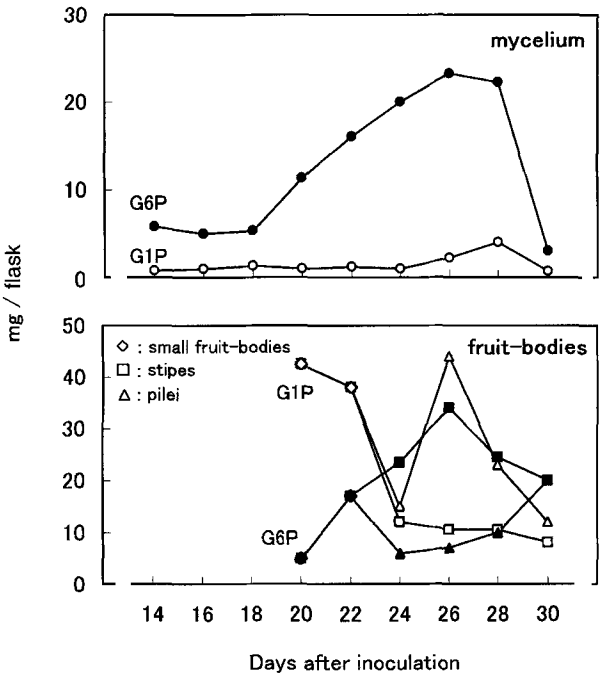

Fig. 2. Changes in G1P and G6P levels in the vegetative mycelium, and the stipes and pilei during fruit-body development of *F. velutipes*.

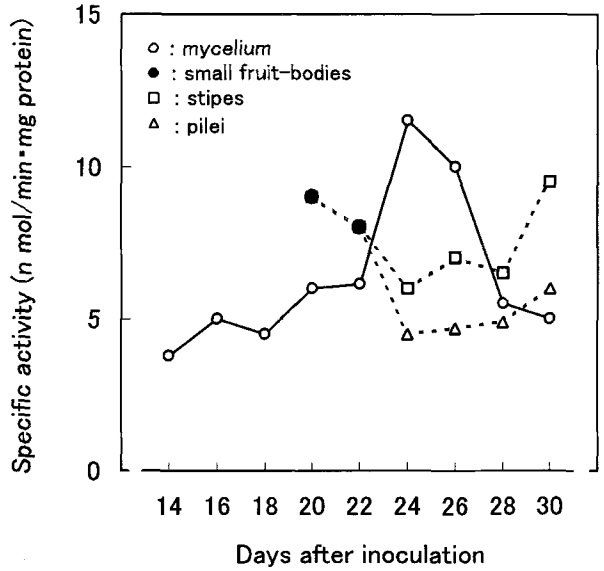

Fig. 3. Changes in glycogen phosphorylase activities in the vegetative mycelium, and the stipes and pilei during fruit-body development of *F. velutipes*.

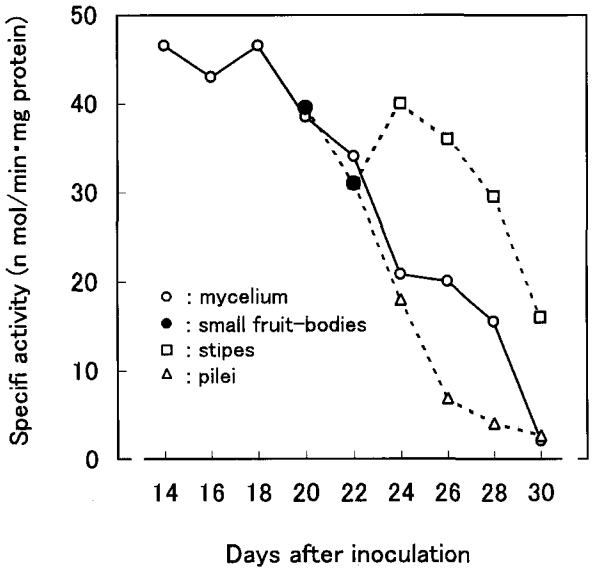

Fig. 4. Changes in trehalose phosphorylase activities in the vegetative mycelium, stipes and pilei during fruit-body development of *F. velutipes*.

Changes in glycogen phosphorylase activities during fruit-body development Figure 3 shows the changes of glycogen phosphorylase activities in the colony. Glycogen phosphorylase activity in the vegetative mycelium increased sharply during the early phase of the fruit-body development. This timing was consistent with the rapid disappearance of glycogen in the mycelium (Kitamoto and Gruen, 1976). The increase in the enzyme activity also coincided with the rapid accumulation of G6P in the mycelium. On the other hand, glycogen phosphorylase

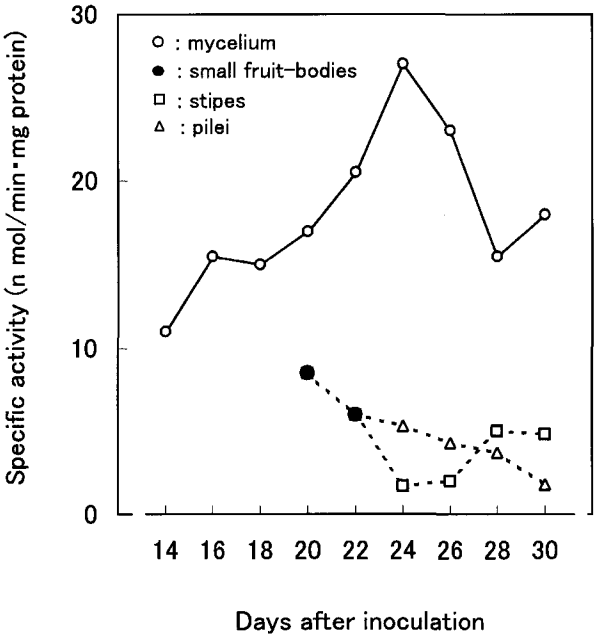

Fig. 5. Changes in trehalase activities in the vegetative mycelium, and the stipes and pilei during fruit-body development of *F. velutipes*.

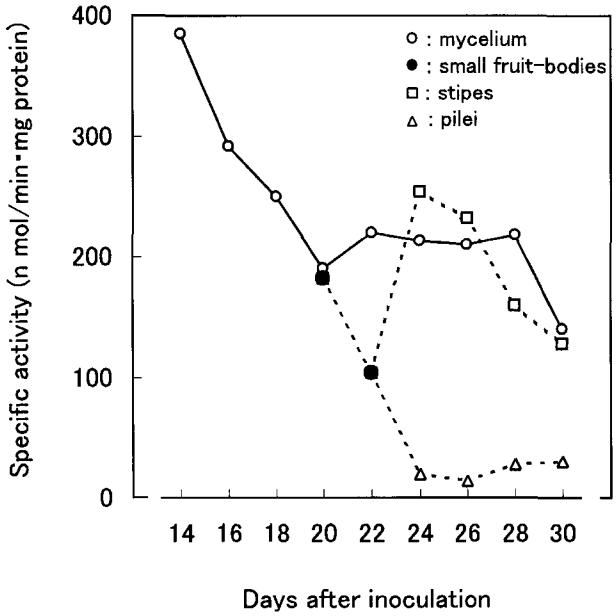

Fig. 6. Changes in phosphoglucomutase activities in the vegetative mycelium, stipes and pilei during fruit-body development of *F. velutipes*.

activities in the stipes and pilei were as low during development but increased as the fruit-bodies approached maturity.

Changes in trehalose phosphorylase and trehalase activities during fruit-body development Figure 4 shows the changes in trehalose phosphorylase activities in the colony. Trehalose phosphorylase activity in the vegetative mycelium remained high during the vegetative growth stage but gradually decreased following the fruit-body development. In the stipes, the activity showed a peak at the early phase of fruit-body development and gradually decreased thereafter. The activity in the pilei rapidly decreased from the early phase of fruit-body development through the maturation.

Figure 5 shows the changes of trehalase activities in the colony. The activity in the vegetative mycelium showed the maximum level in the colony at the early phase of the fruit-body development and decreased thereafter. The levels of trehalase in the stipes and pilei were very low during the fruit-body development. However, the activities of trehalase in the stipes and pilei of the fruit-bodies were less than 1/4-1/5 of phosphorylase activity during the colony development.

Changes in PGluM activities during fruit-body development Figure 6 shows the changes of PGluM activities in the colony. The activity of PGluM in the vegetative mycelium decreased until the early phase of fruit-body development, then remained at the same levels until the colonies matured. The enzyme activity in the stipes peaked in the middle phase of the fruit-body development, then gradually decreased as the fruit-bodies matured. On the other hand, the activity in the pilei decreased to the minimum level in the early phase, then increased slightly during the maturation of the colony.

However, the PGluM levels detected in the stipes and pilei were higher than that of trehalose phosphorylase during fruit-body development.

Discussion

In the vegetative mycelium of *F. velutipes*, glycogen phosphorylase activity increased notably in the colony during the early phase of the fruit-body development (Fig. 3). This timing was consistent with the rapid disappearance of glycogen in the mycelium (Kitamoto and Gruen, 1976). Trehalose synthesis in the cells of vegetative mycelium increased at this phase, but the content of this sugar declined as the development of fruit-bodies proceeded in the colony. A transient increase in the amounts of G6P occurred in the mycelium of the colony from the onset up to the middle phase of the fruit-body development (Fig. 2). The accumulation of hexose phosphates in the mycelium suggests that the metabolic outflows to both the hexose monophosphate pathway (HMP) and the Embden-Meyerhoff-Parnas (EMP) routes may be interrupted considerably at this stage. Further, it is postulated that most of the G1P liberated by the phosphorolysis of glycogen may be utilized for the production of trehalose, and this disaccharide may be translocated into the fruit-bodies from the vegetative mycelium.

The trehalose content in the fruit-bodies of *F. velutipes* was about two times higher than that of glycogen (Kitamoto and Gruen, 1976). Many microorganisms contain trehalase for the breakdown of trehalose to two molecules of glucose by hydrolysis (Elbein, 1974). We found a novel enzyme, trehalose phosphorylase, which catalyzes the phospholysis of trehalose into α-G1P and glucose (Kitamoto et al., 1988). This enzyme commonly occurs in the vegetative mycelium and in the fruit-bodies of basidiomycetous mushrooms (Kitamoto et al., 1998). Trehalose phosphorylase activity in the vegetative mycelium was relatively high until fruit-body initiation, suggesting the turnover of this disaccharide during the vegetative stage of the colony development (Fig. 4). On the other hand, changes in the sugar phosphate pool in the fruit-bodies during the development of *F. velutipes* may reflect the function of trehalose phosphorylase. Trehalose phosphorylase activity in the stipes increased during early development, but it gradually decreased thereafter. The activity in the pilei almost disappeared by the middle phase of the fruit-body development. Trehalase activity in both stipes and pilei reached only 1/4–1/5 of the level of the phosphorylase (Fig. 5). Trehalase seems to have no physiological function in the fruit-body formation process of *F. velutipes*.

The stipes showed a high specific activity of PgluM, enough to facilitate the conversion of G1P to G6P, but this enzyme was actually inert in the pilei (Fig. 6). The G1P contents in both stipes and pilei were higher than the G6P contents during the early phase of fruit-body development, although the equilibrium constant of PgluM favors toward the formation of G6P (Ray and Peck, 1972). The ratio of G6P/G1P in the stipes increased as the fruit-bodies developed. The above results suggested that G1P is converted to G6P by PgluM. A high activity of glucokinase was observed in the developing stipes (Kitamoto et al., 1981). The G6P thus formed seems to enter into the known pathways of sugar metabolism. In the pilei, a large amount of G1P remained until the fruit-bodies were fully mature. It is possible that glucose might be more readily assimilated than G1P formed by the phosphorylase in pilei.

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