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SUMMARY OF DOCTORAL THESIS

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Title: Regulation of the starch degradation enzymes during fruiting in edible mushroom *Pholiota microspora*
(食用きのこナメコにおける子実体形成過程におけるデンプン分解酵素の発現制御に関する研究)

Starch, which is a polymer of glucose units linked by glycosidic bonds, is the second most abundant carbohydrate in nature. Because of the complex structure of starch, combinations of enzymes are required to degrade starch. These enzymes can be commonly divided into two groups, endoamylases and exoamylases. Endoamylases such as α-amylases (EC 3.2.1.1) belong to the glycoside hydrolase (GH) 13 family, which randomly acts on α-1, 4-glycosidic linkages in starch and related polysaccharides to produce shorter maltooligosaccharides and α-limit dextrins. Exoamylases include glucoamylase (EC 3.2.1.3), α-glucosidase (EC 3.2.1.20) and β-amylase (EC 3.2.1.2). β-Amylases are typically of plant origin; fungi generally produce two types of exoamylase, glucoamylase and α-glucosidase. α-Glucosidases catalyze the liberation of α-D-glucose from the non-reducing terminus of substrates, such as maltooligosaccharides, α-glucosides and α-glucans. Glucoamylases, which belong to glycosidase hydrolase family 15, catalyze hydrolysis of α-1, 4 and α-1, 6-glycosidic linkages to release β-D-glucose units from the non-reducing ends of starch and related polysaccharides.

The basidiomycetous fungus *P. microspora* is an economically important edible mushroom in Japan. The glucoamylase gene *PnGlu1* was cloned and characterized in a previous study. Following the completion of sequencing of the genome of *P. microspora*, we identified a different glucoamylase gene, named *PnGlu2*. We also identified three α-amylase genes (*PnAmy1*, *PnAmy2* and *PnAmy3*) and an α-glucosidase (*PnGcs*).

To analyze the effect of carbon source on gene expression, *P. microspora* strain NGW19-6/12-163 was grown on potato dextrose agar at 25°C for 1 week, and then 5 circular agar blocks (5 mm diameter) of mycelia were transferred into 10 ml minimal medium (1.5 g/l $(NH_4)_2HPO_4$, 1 g/l KH_2PO_4, 20 g/l glucose, 25 mg/ml thiamine hydrochloride, pH 5.5) in an Erlenmeyer flask and grown at 25°C for 5 days. The mycelia were collected by filtration and washed three times with minimal medium containing no carbon source. The washed mycelia were suspended in 10 ml minimal medium containing 20 g/l glucose, 20 g/l cellobiose, 20 g/l sucrose, 20 g/l maltose, 20 g/l soluble starch, 20 g/l corn starch, 20 g/l potato starch, 20 g/l wheat starch, 20 g/l amylose, 20 g/l amylopectin, or no carbon source. After 24 h of incubation, mycelia were harvested by filtration for RNA extraction.

Fruiting bodies of *P. microspora* were cultivated on a sawdust substrate. The sawdust substrate was prepared as follows: beech sawdust was mixed with rice bran at a gravimetric ratio of 5: 1 and adjusted to 65% moisture using tap water, and the medium was put into a 100 mL Erlenmeyer flask and autoclaved at 121°C for 60 min. After cooling the medium, it was inoculated with circular agar blocks of NGW19-6/12-163 mycelia (5 mm diameter) and incubated at 25°C. When the mycelia had colonized the substrate (about 40 days after inoculation), the surface layer of the mycelia was scratched by a spatula and then 50 ml sterilized distilled water was poured into the flask. The water was removed after the flasks had been incubated at 15°C overnight, and then cultivation was continued at 15°C until the fruiting body developed. We defined the day after removing the water as day 0. The RNA from mycelia in sawdust substrate was isolated at 0 d, 10 d, 20 d, 30 d, 40 d, 60 d, 80 d and 100 d. We also isolated the RNA from primordia (about 120 d after removing water) and three stages of fruiting bodies (total length included cap and stipe is 1 cm, 2 cm and 3 cm, respectively) in sawdust substrate.

Glucoamylases are present in many fungi and show a conservative pattern in evolution. This conserved evolution in glucoamylases reflects their important roles in fungi, and suggests that they may be essential. Based on intron position and amino acid sequence identity, PnGlu1 and PnGlu2 are likely duplicated genes that originated from a common ancestor. The clustering of PnGlu1 and PnGlu2 on the genome sequence, separated by less than 2 kb (data not shown), strongly supports such an event. In minimal media, the highest level of *PnGlu1* expression was observed when maltose was used as the sole carbon source, while the transcript level of *PnGlu2* was much higher in the amylose culture than in other culture media. *PnGlu1* and *PnGlu2* expression in minimal media containing different carbon sources are far lower than that in sawdust medium. In sawdust medium, transcription level of *PnGlu1* is high in vegetative dikaryotic mycelia and that of *PnGlu2* is high in primordia and at the fruiting body stage. We thus suggested that the two glucoamylase gene PnGlu1 and PnGlu2 may differentially regulate in each developmental stages such as mycelial growth and fruiting body formation, respectively.

The α-amylase PnAmy1, PnAmy2 and PnAmy3 are members of glycoside hydrolase family 13, belonging to subfamily 32, 5 and 1, respectively. Phylogenetic analyses showed that PnAmy1, PnAmy2, and PnAmy3 might have originated from three sources, and that enzymes might have different functions. Transcription profiling of α-amylase genes may provide useful evidence for illustrating the physiological role of these three different types of α-amylase in *P. microspora*.

PnAmy1, which is closely related to bacterial α-amylases, belongs to GH13_32. The expression of PnAmy1 during fruiting body development was higher than in mycelia in sawdust medium. Abundant expression of PnAmy1 in the fruiting body stage suggests that it is important for mature fruiting body formation. PnAmy2 is an intracellular enzyme belonging to GH13_5. The enzymes from this subfamily in other fungi are involved in the synthesis or modification of α-glucan in the fungal cell wall. Starch and maltose did not induce *PnAmy2* expression in minimal medium, and the highest expression appeared at the primordia stage in sawdust medium, which implies that PnAmy2 may participate in α-glucan synthesis for the cell wall of primordia from *P. microspora* instead of in starch decomposition. PnAmy3 is a member of GH13_1. We hypothesized that PnAmy3 may be a key α-amylase decomposing starch in *P. microspora*. The highest *PnAmy3* expression appeared in minimal medium containing amylose, and expression in mycelia at 20, 30, 40, and 100d was higher than at the primordia or fruiting body stages in sawdust medium, which supports this conjecture.

The α-glucosidase gene, *PnGcs*, was identified as a GH31 family protein based on amino acid sequence similarity. Expression of the α-glucosidase gene in minimal medium containing maltose was the highest of the tested carbon sources in this study, which also included amylopectin, amylose, wheat starch, potato starch, corn starch, soluble starch, sucrose, cellobiose and glucose, as well as in the absence of a carbon source. The lowest *PnGcs* expression appeared when cellobiose was added to the minimal medium. *PnGcs* was equally expressed when cultured both in the presence and absence of glucose, and expression in the culture medium containing maltose was approximately two times higher in the presence or absence of glucose. Thus, *PnGcs* appears to be constitutively transcribed by *P. microspora*, but maltose promotes its expression.

The α-glucosidase activity at the primordia and fruiting body formation stages dramatically increased over the mycelial growth stage. The highest enzyme activity was observed in the primordia throughout the whole *P. microspora* development cycle. To determine the reasons for the increased α-glucosidase activity on sawdust medium, quantitative reverse transcription-PCR was used to study gene expression. In the mycelial stage, the number of transcripts of *PnGcs* decreased rapidly, dropping to the lowest levels at 40 days, and then increased from 60 days until the primordia had developed. α-Glucosidase gene expression at the primordia and fruiting body formation stages also dramatically increased relative to the mycelia growth stage. The highest *PnGcs* expression during the entire *P. microspora* development cycle appeared in 1 cm fruiting bodies, and then decreased.The enzyme activity and expression levels of PnGcs at the fruiting body developmental stage were higher than in mycelium when grown on sawdust. Abundant expression of PnGcs in the fruiting body stage suggests that it is important for mature fruiting body development.