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NOTE

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Changes in carbohydrase activities during vegetative growth and development of fruit-bodies of *Hypsizygus marmoreus* grown in sawdust-based culture

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Abstract Carbohydrase production of *Hypsizygus marmoreus* cultured on sawdust rice bran medium by bottle cultivation was investigated to elucidate the carbohydrase utilized as the growth substrate for the fruit-body formation of this fungus. Among the extracellular enzymes assayed, xylanase showed the highest activity. This activity greatly increased during the end of vegetative mycelial growth and fruit-body formation. Among the cellulases. CM-cellulase showed higher activity than avicelase. The activities of β-1,3-glucanase, amylase, and chitinase were low. Among the intracellular enzymes, both xylanase and amylase showed higher activity levels than the other carbohydrases. In contrast, β-1,3-glucanase, avicelase, and chitinase activities in mycelia were considerably lower. These results suggest that xylanase, CM-cellulase, and amylase play an important role in mycelial maturation and fruit-body growth of *H. marmoreus*.

Key words *Hypsizygus marmoreus* · Carbohydrase · Mushroom fungi · Enzyme production · Fruit-body formation

Introduction

The results of our studies (unpublished data) of the effects of specific proteinase inhibitors on the fruit-body formation of *Hypsizygus marmoreus* (Peck) Biglow (Bunashimeji in Japanese) revealed that the metal proteinase may play an important role in the turnover of nitrogenous compounds (e.g., protein and amino acids) during fruit-body formation. In addition to these nitrogen substrates, growth of the fruit-bodies of mushroom fungus required a supply of carbohydrates hydrolyzed by enzymes from the culture medium and mycelia.[1] Many carbohydrate hydrolyzing enzymes are produced during the growth and development processes of mushroom fungi.[2–6]

In a previous paper,[7] we examined the production of hydrolytic enzymes in culture filtrate and vegetative mycelia during the vegetative growth of *H. marmoreus* on potato dextrose liquid medium. The results showed that β-1,3-glucanase in the culture filtrate and CM-cellulase and avicelase in the culture filtrate and mycelia have relatively high activity. Amano et al.[8] examined changes in the activity of several extracellular enzymes produced during commercial cultivation of this mushroom. They showed that cellulase and xylanase activity increase during the growth of fruit-body primordia. The details of intracellular enzyme activity are not clear.

In the present study, extracellular and intracellular enzyme activity involved in substrate degradation for fruit-body growth produced in sawdust medium by *H. marmoreus* was assayed throughout the cultivation stages.

Materials and methods

Microorganism

Hypsizygus marmoreus (Peck) Bigelow was isolated from fruit-body obtained commercially in Osaka, Japan in 1989. The stock was subcultured in potato dextrose (PD) agar medium.

Culture conditions

A 200-ml glass bottle containing 130g sawdust (*Fagus crenata* Blume) rice bran medium [sawdust: rice bran 5:1 (v/v); moisture content: about 65%] was used for the cultivation. The culture process is shown in Fig. 1. The spawnrunning process, which consists of two successive stages – linear mycelial growth (spawnrun I, 25 days) and mycelial maturation (spawnrun II, 35 days) – was allowed to proceed under the culture conditions and processes de-

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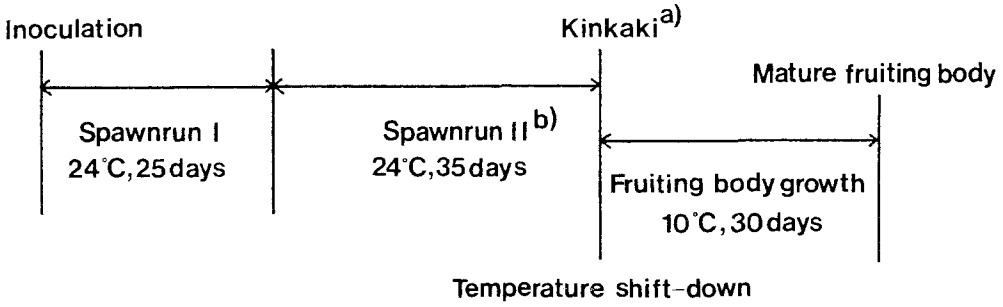

Fig. 1. Culture process of *Hypsizygus marmoreus* in sawdust rice bran medium. [a]Removal of both spawn and the uppermost layer of the medium. [b]Mycelial maturation during the spawn-running process

scribed in our previous paper.[9] The yield of fresh fruit-bodies per bottle in this system was about 30 g.

Preparation of crude enzyme solution

To obtain extracellular enzymes, 0.1 M Költhoff buffer solution (pH 6.0) was added to the culture medium and mixed for 3 h at 4°C. The culture filtrate was collected by filtration with filter paper. To obtain mycelial intracellular enzyme solutions, the debris of the culture substrate after extracting the extracellular enzymes was used. To the mycelia–sawdust mixture, 0.1 M Költhoff buffer (pH 6.0) was added and homogenized with a mixer (MX 740G, Matsushita Electric Industustries) for 15 min at 0°C. The homogenate was then centrifuged at 15000 g for 15 min, and the supernatant solution was used for the enzyme assay.

Carbohydrase assay

CM-Cellulase and avicelase were assayed by determining the respective amounts of reducing sugar liberated from carboxymethyl cellulose (CMC-Na; Wako Pure Chemical Industries) in 0.1 M sodium acetate buffer (pH 5.0) and avicel-SF (Asahi Kasei Co.) in the same buffer, respectively. Chitinase activity was determined as reported previously.[10] The reaction mixture, consisting of 0.5 ml enzyme solution and 0.5 ml colloidal chitin in 0.2 M sodium acetate buffer (pH 5.0), was incubated at 37°C for 60 min followed by boiling at 100°C for 2 min to stop the reaction. The substrates for β-1,3-glucanase, amylase, and xylanase were laminarin (Nacalai tesque Co.) in 0.1 M McIlvaine buffer (pH 5.0), soluble starch (Wako Pure Chemical Industries) in the same buffer, and xylan (Sigma) in distilled water (pH 6.2), respectively. These reactions were carried out at pH 5.0, and the activity was estimated by determining the amount of reducing sugar liberated by the Somogyi-Nelson method[10] using D-glucose as the standard. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μg of glucose per minute at 37°C. β-*N*-Acetylglucosaminidase (β-GlcNAcase) activity[11] was assayed by incubating the reaction mixtures, which consisted of 0.2 ml enzyme solution, 0.2 ml 4 mM p-nitrophenyl-β-*d*-*N*-acetylglucosamine (Wako) and 0.1 M McIlvaine buffer (pH 4.5), at 37°C for 10 min, followed by addition of 2.0 ml of 0.2 M Na_2CO_3 to stop the reaction. The amount of p-nitrophenol released was measured by reading the absorbance at 420 nm. One unit of β-GlcNAcase was defined as the amount of enzyme liberating 1 μg of p-nitrophenol per minute at 37°C.

Table 1. Changes in extracellular carbohydrase activities during vegetative growth and development of fruit-bodies of *Hypsizygus marmoreus* in sawdust-based culture

Enzyme	Specific activity (1 μg/min/mg protein), by culture period (10–90 days)					
	10	25	40	55	70	90
β-1,3-Glucanase	2.13	3.55	2.96	2.57	8.75	8.85
Xylanase	29.5	60.7	31.2	20.3	20.5	72.4
CM-cellulase	9.22	21.20	11.40	12.50	13.30	35.50
Avicelase	4.34	11.60	10.70	12.60	14.00	14.20
Amylase	7.22	13.30	9.64	5.23	3.18	2.11
Chitinase	1.54	4.77	1.55	0.74	1.42	3.68
β-GlcNAcase[a]	10.6	27.5	12.8	13.1	21.4	21.4

[a]β-*N*-Acetyl-D-glucosaminidase.

Measurement of protein content

The protein content was determined by the Lowry method.[12]

Results and discussion

In the present study, carbohydrase productions by *H. marmoreus* cultured in sawdust rice bran medium by bottle cultivation was assayed to elucidate the carbohydrates utilized as the growth substrate for fruit-bodies. The changes in extracellular enzyme activity are shown in Table 1. Xylanase showed the highest activity among the enzymes assayed. This activity in culture medium greatly increased (60.7 U/mg protein) during the end of vegetative mycelial growth (spawnrun I) and decreased (20.5 U/mg protein) toward the end of mycelial maturation (spawnrun II). The activity increased (72.4 U/mg protein) again during the growth of fruit-bodies. The production pattern of β-1,3-glucanase, CM-cellulase, avicelase, chitinase, and β-

Table 2. Changes in intracellular carbohydrase activities during vegetative growth and development of fruit-bodies of *Hypsizygus marmoreus* in sawdust-based culture

Enzyme	Specific activity (1 μg/min/mg protein), by culture period (10–90 days)					
	10	25	40	55	70	90
β-1,3-Glucanase	3.52	6.73	5.48	5.22	11.20	10.80
Xylanase	12.2	31.7	28.9	19.5	32.6	25.7
CM-cellulase	6.48	12.10	9.72	9.74	26.70	29.40
Avicelase	0.42	2.58	3.45	2.78	5.63	7.64
Amylase	18.4	45.9	38.8	37.0	25.5	18.9
Chitinase	1.87	2.66	1.10	1.05	1.68	4.44
β-GlcNAcase	4.55	9.82	15.70	22.40	29.30	37.20

GlcNAcase were similar to that of xylanase. Among the cellulases, CM-cellulase showed higher activity than avicelase. The activities of β-1,3-glucanase, amylase, and chitinase were low.

Wood and Goodenough[13] studied the changes in extracellular enzyme activity during the vegetative growth and fruiting of *Agaricus bisporus* (J. Lange) Imbach. They found that the laccase and cellulase activity changed markedly upon fruit-body development. Ohga[6] showed that cellulase and xylanase activities of *Lentinus edodes* (Berk) Sing. increased rapidly during the early exponential growth phase and were maintained at this high level until the fruiting stage. Matsumoto[5] also studied the changes in enzyme activity during fruiting of *L. edodes* in sawdust culture and showed that the neutral proteinase and glycogen phosphorylase activity in the vegetative mycelia was high during the initial stage of fruit-body development.

The changes in intracellular enzyme activity are shown in Table 2. Intracellular xylanase and amylase had higher levels of activity than other carbohydrases, and their maximum levels (xylanase 31.7 U/mg protein; amylase 45.9 U/mg protein) were attained at the end of the mycelial growth period (25 days after inoculation). In contrast, β-1,3-glucanase, avicelase, and chitinase activities in mycelia were considerably lower.

In a previous study[7] we examined the nutritional environment for mycelial growth and hydrolytic enzyme activity during the vegetative growth of *H. marmoreus* on PD liquid medium and found that the activity of β-1,3-glucanase in culture filtrate and intracellular chitinase was greatly increased by prolonging the incubation time. These results obtained from the fungus grown on PD liquid medium are markedly different from those obtained with the sawdust rice bran culture.

Amano et al.[8] reported that the cellulase and xylanase activities in sawdust rice bran cultures of *H. marmoreus* greatly increased after the primordia of the fruit-body had been formed. The results of the present study suggest that xylanase, CM-cellulase, and amylase play an important role in mycelial maturation and fruit-body growth of this mushroom. In addition, xylose and glucose, produced from xylan, starch, and cellulose by the action of carbohydrases, may act as good substrates for the growth of *H. marmoreus*. In fact, we have already reported[7] that these low-molecular-weight carbohydrates are especially good carbon sources for the vegetative mycelial growth of this mushroom.

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