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Role of metal proteinases in the fruit-body formation of *Hypsizygus marmoreus*

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Abstract We investigated the possible role of metal proteinase on the fruit-body formation of *Hypsizygus marmoreus*. The addition of a specific metal proteinase inhibitor, phosphoramidon, to the culture medium (10 μg/ml) completely inhibited fruit-body formation. Metal proteinase activity in both the medium and the mycelia of this fungus increased markedly during vegetative mycelial growth, and activity was maximal 25 days after inoculation. When phosphoramidon was added to the culture medium during vegetative mycelial growth, the metal proteinase activity in the mycelium decreased to 56% of the control (without inhibitor) level. Isoelectric focusing analysis showed that two kinds of metal proteinases with a pI of 7.7 and 8.4, respectively, were obtained from 29-day-old mycelia. Uptake of phosphoramidon into the mycelia was confirmed as the result of inhibition of thermolysin activity by the mycelial extracts. The degree of inhibitor uptake into mycelia was about 2.0% and was independent of the initial concentration of the inhibitor administered. The addition of peptone and amino acids to medium treated with phosphoramidon resulted in fruit-body dry weight yields that were about 50% that of the control.

Key words *Hypsizygus marmoreus* · Fruit-body · Phosphoramidon · Edible mushrooms · Metal proteinase

Introduction

Hypsizygus marmoreus (Peck) Bigelow (bunashimeji in Japanese) is usually cultured in sawdust rice bran medium using the bottle cultivation method. This mushroom requires a long spawn-running process (60–70 days) to allow mycelial maturation. Without adequate time for this process, a high yield of fruit-body production cannot be achieved.

To clarify the physiological traits of the mycelial maturation of *H. marmoreus*, Kinugawa and Tanesaka[1] investigated the changes in CO_2 liberation from culture during the cultivation process. Amano et al.[2] examined changes in the activity of several extracellular enzymes of this mushroom upon sawdust cultivation and detected both proteolytic and cellulolytic enzyme activity in the medium. In addition, using X-ray analysis they detected[3] decomposition in cellular tissue when sawdust was used as the substrate by the fungus. In a previous study we examined[4] changes in hydrolytic enzyme activities in the culture filtrate and vegetative mycelia during vegetative growth of *H. marmoreus* on potato dextrose liquid medium and found both metal and carboxyl proteinase activity according to the classification of Hartley,[5] the former being higher than the latter. In addition, Dohmae et al.[6] have shown that metal proteinase producd in mushroom fungi (e.g., *Pleurotus ostreatus*, *Grifora frondosa*, and *Armillariella mellea*) are zinc enzymes.

In our previous paper[7] we found that the development of fruit-bodies in *Flammulina velutipes* (Curt.: Fr.) Sing. was completely inhibited by addition of the metal proteinase inhibitor talopeptin (MK-I) to the medium. Chao and Gruen[8] have reported a similar effect for the metal proteinase inhibitor *o*-(1,10)-phenanthroline. These noteworthy effects of metal proteinase inhibitors suggest that metal proteinases play a major role in the fruit-body formation of mushroom fungi. In the present report the role of metal proteinases in the fruit-body formation of *H. marmoreus* was investigated using metal proteinase inhibitors.

Materials and methods

Hypsizygus marmoreus (Peck) Bigelow was isolated from a fruit-body obtained commercially from a grocery store in Osaka, Japan in 1989. The stock was subcultured in potato

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dextrose (PD) agar medium. Ethylenediaminetetraacetic acid (EDTA) and phosphoramidon (microbial metal proteinase inhibitor isolated from *Streptomyces tanashiensis*)[9] were purchased from Wako Pure Chemical Industustries (Osaka, Japan).

A 200-ml glass bottle containing 130g sawdust (*Fagus crenata* Blume) rice bran medium [sawdust/rice bran 5:1 (w/w), moisture content about 65%] was used for cultivation. Spawn running, which consists of two successive stages – linear mycelial growth (spawnrun I, for 25 days) and mycelial maturation (spawnrun II, for 35 days) – was performed under the culture conditions and processes described in a previous paper[10] The fresh weight of mature fruit-bodies per bottle in this cultivation system was about 30g. EDTA or phosphoramidon was added to the cultures 18 days after inoculation, and the effect of these inhibitors was examined 30 days after inoculation.

The effect of inhibitors on the fruit-body formation of *H. marmoreus* was also investigated using plastic bottle cultivation [850ml; sawdust/rice bran 5:1 (v/v), moisture content about 65%]. In this case, inhibitors (phosphoramidon 10μg/ml, EDTA 100μg/ml] were added to the culture bottles 20 days after inoculation. The results were determined 110 days after inoculation.

Preparations of crude enzyme solutions

To obtain extracellular enzymes, 0.1M Költhoff buffer solution (pH 6.0) was added to the culture medium (0–90 days after inoculation) and mixed for 3h at 4°C. The culture filtrate was collected by filtration with filter paper. To obtain intracellular enzymes from mycelia, the debris of the culture substrate after extracting the extracellular enzymes was used. To the mycelia–sawdust mixture, 0.1M Költhoff buffer (pH 6.0) was added and homogenized with a mixer (MX 740G) for 15min at 0°C. The homogenate was then centrifuged at 15000*g* for 15min and the supernatant used for the enzyme assay.

Assays for proteinase activity

Carboxyl proteinase and metal proteinase activity were assayed at pH 3.0 and pH 7.0, respectively. These enzyme reactions were carried out at 37°C for 30min with Hammarsten casein (Wako) dissolved in 0.1M McIlvaine buffer (pH 2.8) and Bacto hemoglobin (Difco Laboratories) dissolved in 0.1M Költhoff buffer (pH 7.2) as the respective substrates. One unit of enzyme activity was defined as the quantity of enzyme that liberates 1μg tyrosine per milliliter of reaction mixture per minute.

The harvested fruit-bodies were dried in an oven at 80°C to constant weight. After cooling in a desiccator, the average weights of 16 replicates were obtained.

After extracting the extracellular enzyme, 10g of mycelia–sawdust mixture was homogenized with 30ml of 0.1M Költhoff buffer pH 6.0. After centrifugation (15000*g*, 15min), the supernatant was used for isoelectric focusing analysis, which was performed on an LKB column (110ml) containing carrier ampholytes (LKB) with a pH range of 7.0–9.0. Electrophoresis was performed at a constant voltage of 700V for 48h, and the column temperature was maintained at 0°C using a cooling system with chilled methanol.

Hypsizygus marmoreus was cultured in a 100-ml Erlenmeyer flask containing medium consisting of 20ml of PD (200g potato extract and 20g glucose per liter) liquid medium. The initial pH of the medium was adjusted to 5.6 before sterilization at 119°C for 8min. The metal proteinase inhibitor phosphoramidon was added to the liquid medium after culturing for 15 days at 24°C and the effects examined 30 days after inoculation. The uptake of inhibitor into the mycelia was determined as the relative inhibition rate of thermolysin[11,12] activity by the mycelial extract (30-day old cultures) in 0.1M Költhoff buffer pH 6.0. After heating (100°C, 3min), the mycelial extract was mixed with thermolysin and the mixture then preincubated at 37°C for 10min. Using Hammarsten casein as the substrate, the inhibition rate was measured at 37°C for 30min.

Peptone (polypeptone), glutamic acid, and arginine (200mg/bottle, dissolved in 5ml of distilled water) (Wako) were added to the culture medium at the time of the temperature shift-down (60 days after inoculation).

Results and discussion

Effect of metal proteinase inhibitors on fruit-body formation

Metal proteinases may play an important role in the fruiting, of mushroom fungi, as demonstrated by the inhibition of fruit-body growth of *Flammulina velutipes* upon addition of the metal proteinase inhibitor talopeptin (MK-I, 10μg/ml).[7] In the present study, we investigated the effect of other metal proteinase inhibitors, such as EDTA and phosphoramidon, on the fruit-body formation of *H. marmoreus.* Figure 1 shows that addition of phosphoramidon to the culture medium (10μg/ml) completely inhibited fruit-body formation, but that addition of EDTA caused only partial inhibition. These results suggest that metal proteinases play an important role in the turnover of proteins and amino acids during fruit-body formation of this mushroom fungi.

Changes in proteinase activity during mycelial growth and development of fruit-bodies

Based on the results shown in Fig. 1, we examined the changes in carboxyl and metal proteinase activities that occur during the mycelial growth and development of the fruit-bodies of *H. marmoreus* cultured in sawdust–rice bran medium by bottle cultivation. The results are shown in Fig. 2. Metal proteinase activity in the medium and mycelia increased markedly during vegetative mycelial growth (spawnrun I), and activity was maximal 25 days after inoculation. However, a decrease in metal proteinase activity

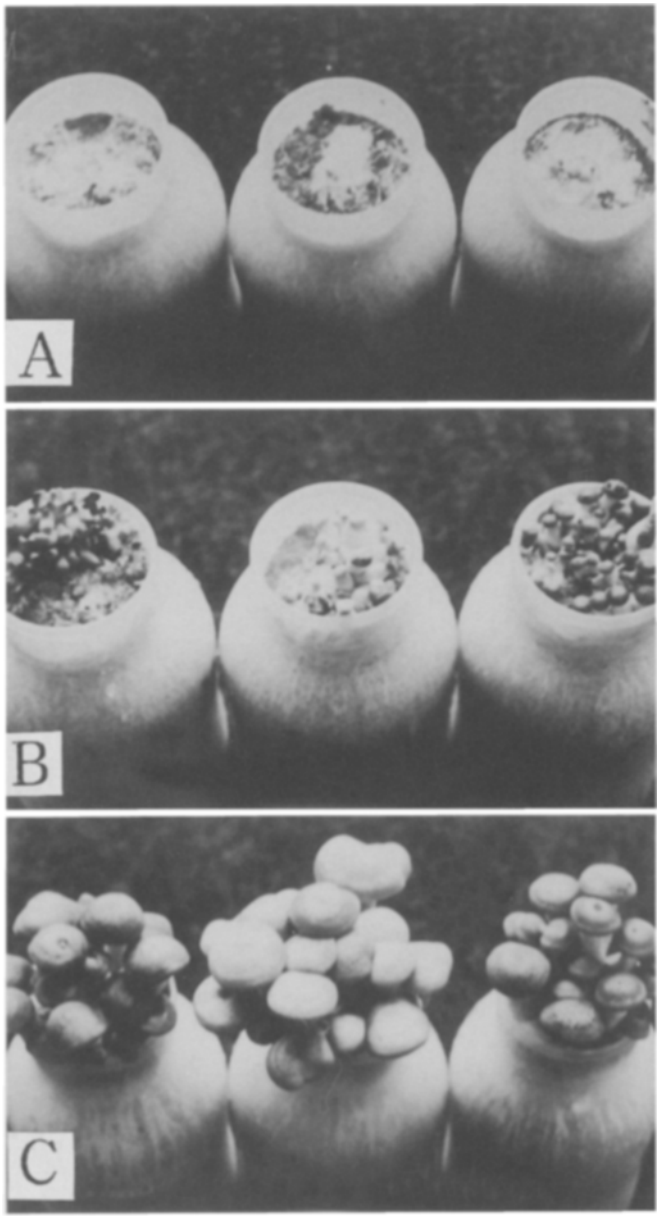

Fig. 1. Effect of metal proteinase inhibitors on fruit-body formation of *Hypsizygus marmoreus* using plastic bottle cultivation. **A** Phosphoramidon [8.5 mg, dissolved in water (17 ml)/bottle]. **B** EDTA [85 mg, dissolved in water (17 ml)/bottle]. **C** Control (no inhibitor added). Inhibitor was added to the culture bottle after culturing for 15 days. Effects were analyzed 110 days after inoculation

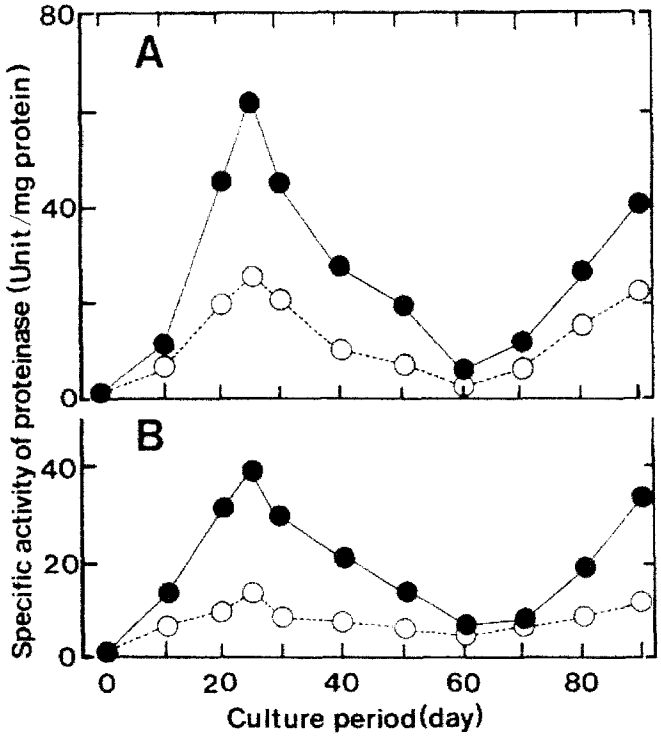

Fig. 2. Changes in proteinase activity during mycelial growth and development of the fruit-body of *H. marmoreus* cultured in sawdust rice bran medium by bottle cultivation. **A** extracellular enzymes. **B** Intracellular enzymes. *Filled circles*, metal proteinase; *open circles*, carboxyl proteinase. Carboxyl proteinase activity was assayed at pH 3.0 and metal proteinase activity at pH 7.0. The enzyme reaction was carried out at 37°C for 30 min with Hammarsten casein in 0.1 M McIlvaine buffer (pH 2.8) and Bacto hemoglobin in 0.1 M Költhoff buffer (pH 7.2) as the substrates, respectively

was observed upon the initiation of mycelial maturation (spawnrun II). The activity increased again upon initiation of the formation of primordia of the fruit-bodies. Carboxyl proteinase activity showed similar tendencies, although the level of activity was considerably lower than that of the metal proteinases.

Kitamoto et al.[13] investigated changes in the amounts of cellular nitrogenous compounds during development of the mycelia and fruit-bodies of *Favolus arcularius* and found that the mycelial proteins served as the major nitrogen substrate for the formation of fruit-bodies. Thus, proteins are broken down by the action of intracellular proteinases.[14]

Amano et al.[2] have reported that extracellular proteinases are produced by *H. marmoreus* in sawdust medium. These proteinases, which have an optimum pH in the neutral range, play an important role in fruit-body formation. In a previous paper[4] we reported that the activity of neutral proteinases in the medium and vegetative mycelia of *H. marmoreus* increases during vegetative growth of this mushroom in PD liquid medium. In the present study, high levels of metal proteinase activity in the culture medium and vegetative mycelia were found during vegetative mycelial growth and fruit-body formation.

Furthermore, the changes in extracellular proteinase activity correlated well with the rate of CO_2 liberation from the culture of this fungus, as reported by Kinugawa and Tanesaka.[1] They suggested that the decrease in the rate of CO_2 liberation from the culture during cultivation, most likely due to the consumption of nutrients in the medium, may be one of the physiological characteristics of this fungus.

Isoelectric focusing analysis of metal proteinases

Isoelectric focusing analysis was used to examine in detail the metal proteinase production in the vegetative mycelia during mycelial maturation in the spawn-running process of

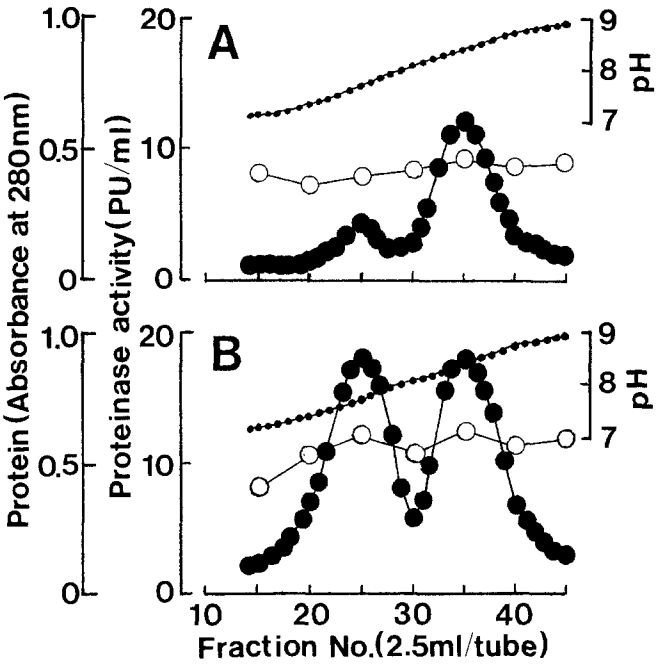

Fig. 3. Isoelectric focusing analysis of metal proteinases produced in the vegetative mycelia during the mycelial maturation of *H. marmoreus* by bottle cultivation. **A** Phosphoramidon (1.3 mg/bottle). **B** Control (no inhibitor added). *Filled circles*, proteinase activity at pH 7.0; *open circles* absorbance at 280 nm; *small filled circles*, pH of the fractions. Inhibitor was added 18 days after inoculation (spawnrun I), and the proteinase activity was assayed 29 days after inoculation (spawnrun II). Isoelectric focusing analysis was carried out on an LKB column (110 ml) containing carrier ampholytes at pH 7.0–9.0. Electrophoresis was performed at a constant voltage of 700 V for 48 h at 0°C with chilled methanol

the fungus. The fractionation patterns in Fig. 3 indicate that two kinds of neutral (metal) proteinases, with pI values of 7.7 and 8.4, respectively, were present in the 29-day-old vegetative mycelia (Fig. 3B). When this mushroom fungus was cultured with phosphoramidon (1.3 mg/bottle), production of the neutral (metal) proteinase with pI 7.7 was almost completely inhibited (Fig. 3A). These results suggest that these two proteinases (pI 7.7 and 8.4) have markedly different interactions with phosphoramidon.

Effect of metal proteinase inhibitors on proteinase activities

The fruiting of *H. marmoreus* was completely inhibited by addition of the metal proteinase inhibitor phosphoramidon to the medium. The effect of EDTA and phosphoramidon, on carboxyl and metal proteinase activity in the medium and the mycelia of this fungus cultured in sawdust medium was also investigated.

The results are shown in Table 1. Addition of the inhibitors resulted in an increase (about 1.32–1.37 times) in the extracellular carboxyl proteinase activity compared to that of the control (no inhibitor added). The metal proteinase activity decreased to about 0.34–0.44 times that of the control. On the other hand, the intracellular (in vegetative mycelium) metal proteinase activity during mycelial maturation (spawnrun II) in the spawn-running process decreased significantly when EDTA or phosphoramidon was added to the culture medium. The enzyme activity of the samples treated with EDTA (13 mg/bottle) or phosphoramidon (1.3 mg/bottle) was about 60% or 56% that of the control, respectively. Thus, the metal proteinases in the culture medium and vegetative mycelia were inhibited by phosphoramidon.

Uptake of phosphoramidon by the mycelia

Table 2 shows the results of uptake of the specific metal proteinase inhibitor phosphoramidon by fungal mycelia. Inhibitor content in the mycelia was found to be 2.39–3.28 μg/flask (20 ml). The uptake of the inhibitor into the mycelia was determined to be about 2.0%, regardless of the concentration of the inhibitor in the culture medium [100 and 200 μg per flask (20 ml culture)]. The results in Fig. 1 indicate that the formation of fruit-bodies is completely inhibited by addition of phoshoramidon (10 μg/ml) to the culture medium. Similarly, a previous study of *Lentinus edodes*[15] showed that proteinase activity changes significantly despite very little uptake of the inhibitor by the vegetative mycelia (<2%) when the specific carboxyl proteinase inhibitor *Streptomyces*-pepsin inhibitor (S-PI)[16] is added to the culture medium (5–10 μg/ml).

Effect of adding peptone and amino acids on the yield of fruit-bodies with and without addition of phosphoramidon

The effect of adding peptone, glutamic acid, and arginine to the sawdust medium on the yield of fruit-bodies cultivated in the presence and absence of phosphoramidon was also examined. The results are shown in Table 3. The fruit-body yield based on dry weight decreased to 6.8% (0.22 ± 0.03 g/bottle) by adding phosphoramidon [10 μg/medium (g)] compared to that of the control (3.24 ± 0.36 g/bottle). However, when peptone, glutamic acid, and arginine were added (200 mg/bottle) to sawdust medium along with phosphoramidon 60 days after inoculation, the fruit-body yields were 64.3% (2.77 ± 0.83 g/bottle), 49.2% (1.83 ± 0.28 g/bottle), and 55.9% (2.23 ± 0.30 g/bottle), respectively, compared to that of the control (without phosphoramidon but with peptone and amino acids). These results suggest that the added amino acids were translocated into the vegetative mycelium and were utilized for the growth of the fruit-body in the fungus.

Our previous study showed[14] that free amino acids in the culture medium are translocated into the vegetative mycelia to be utilized for fruit-body formation. These amino acids, which act as the growth substrate, are believed to be supplied from the proteins in the culture medium and the vegetative mycelia by the action of proteinases.[13,14] Yoshida et al. reported[17] that the predominant free amino acids in vegetative mycelia of *H. marmoreus* are glutamic acid and

Table 1. Effects of metal proteinase inhibitors on proteinase activity of *H. marmoreus* cultured in sawdust–rice bran medium by bottle cultivation

Inhibitor added	Proteinase activity (units/mg protein)			
	Extracellular		Intracellular	
	Carboxyl	Metal	Carboxyl	Metal
Phosphoramidon	28.2 (132%)	15.1 (34%)	8.85 (104%)	18.4 (56%)
EDTA	29.4 (137%)	19.5 (44%)	9.10 (107%)	19.5 (59%)
Control (none)	21.4 (100%)	44.7 (100%)	8.50 (100%)	32.8 (100%)

EDTA: 13 mg/bottle [100 μg/g (sawdust medium)]. Phosphoramidon: 1.3 mg/bottle [10 μg/g (sawdust medium)].
Inhibitors were added (5.0 ml/bottle) 18 days after inoculation (spawnrun I), and the enzyme activity was assayed 30 days after inoculation (spawnrun II)

Table 2. Uptake of metal proteinase inhibitor phosphoramidon into the mycelia of *H. marmoreus* in potato dextrose liquid medium

Concentration of phosphoramidon (μg/flask)	Trial no.	Inhibitor content in mycelia (μg/flask)		Percent inhibitor uptake by mycelia
		Amount	Average	
100	1	2.53	2.39	2.39
	2	2.37		
	3	2.26		
200	1	3.18	3.28	1.64
	2	3.14		
	3	3.52		

Phosphoramidon was added to the liquid medium (20 ml) after culturing for 15 days at 24°C, and the effects were examined 30 days after inoculation. The uptake of phosphoramidon into the mycelia was determined as the relative inhibition rate of thermolysin activity by the mycelial extract in 0.1 M Költhoff buffer pH 6.0. After heat treatment (100°C, 3 min), the mycelial extract was mixed with thermolysin, and the mixture was then preincubated at 37°C for 10 min. Using Hammarsten casein as the substrate, the inhibition rate was measured at 37°C for 30 min. The data show the residual percent of inhibitor in the mycelium

Table 3. Effect of adding peptone, glutamic acid, and arginine to culture medium on the yield of fruit-bodies with and without addition of phosphoramidon

Addition of peptone and amino acids (200 mg/bottle)	Yield of fruit-bodies [dry weight (g)/bottle][a]	
	Control[b]	Phosphoramidon[c]
Glutamic acid	3.72 ± 0.59 (100%)	1.83 ± 0.28 (49%)
Arginine	3.99 ± 0.51 (100%)	2.23 ± 0.30 (56%)
Peptone	4.31 ± 0.67 (100%)	2.77 ± 0.83 (64%)
Control (none)	3.24 ± 0.36 (100%)	0.22 ± 0.03 (7%)

Phosphoramidon was added to the culture (5.0 ml/culture bottle) after culturing for 18 days. Peptone and amino acids were added to the culture medium after culturing for 60 days. The average weight of 16 replicates was obtained.
[a] Fruit-body yield based on dry weight (mean ± SE) was measured 90 days after inoculation.
[b] No phosphoramidon added.
[c] Addition of phosphoramidon [10 μg/(g) sawdust medium]

arginine. Previously, we showed that the major free amino acids in peptone (polypeptone; Wako) were lysine (14%) and leucine (9.2%).[18]

In the present study, the use of metal proteinase inhibitors clearly demonstrated that metal proteinases play an important role in the degradation of proteins for fruit-body formation. However, a detailed investigation is required to clarify the physiology of mycelial maturation for formation of the fruit-body of this fungus.

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