Short Communication

Isolation and characterization of extra- and intra-cellular metal proteinases produced in the spawn-running process of *Hypsizygus marmoreus*

Takao Terashita, Takaaki Inoue, Yoko Nakaie, Kentaro Yoshikawa and Jiko Shishiyama

Laboratory of Food Microbiology, Faculty of Agriculture, Kinki University, 3327-204, Nakamachi, Nara 631, Japan

Isolation and characterization of extra-(PE-1) and intra-cellular (PE-2) metal proteinases produced during the spawn-running process of *Hypsizygus marmoreus* were carried out. These enzymes were the most active toward Hammarsten casein at pH 7.0 (PE-1) and pH 6.5–7.5 (PE-2). The molecular weight and pI value of PE-1 were 29,500, 8.8, and those of PE-2 were 21,500, 8.4. K_m values against the synthetic peptide substrate Z-Gly-L-Leu·NH_2 were 0.9×10^{-3} M (PE-1) and 1.2×10^{-3} M (PE-2). PE-1 was strongly inhibited by phosphoramidon, whereas PE-2 was weakly inhibited. These enzymes are considered to play an important role in providing nitrogenous substrates during fruit-body formation.

Key Words——fruit-body formation; *Hypsizygus marmoreus*; metal proteinase; mushroom; proteinase.

The sawdust culture of *Hypsizygus marmoreus* (Peck) Bigelow requires a long spawn-running process (60–70 d) to allow the mycelial maturation. Without an adequate period of mycelial maturation in the cultivation process, a high yield of fruit-body production cannot be achieved.

To clarify the physiological significance of mycelial maturation of *H. marmoreus*, Amano et al. (1992) examined the changes in activities of several extracellular enzymes in sawdust cultivation. They reported that the activity of proteolytic enzymes was detected in sawdust medium at neutral pH range. We have also examined some hydrolytic enzyme activities in the culture filtrate and the vegetative mycelia during the vegetative growth of *H. marmoreus* on potato dextrose liquid medium (Terashita et al., 1995b). Activities of neutral and acid proteinases were detected, the former being higher than the latter. Protein in the culture media and the mycelia may be broken down by the action of proteinases and serve as the main source of nitrogen substrate (Kitamoto et al., 1980).

In a previous paper (Terashita et al., 1985), we found that the addition of a metal proteinase inhibitor, Talopeptin (MK-I), to the medium completely inhibited the development of fruit-bodies in *Flammulina velutipes* (Fr.) Karst. Chao and Gruen (1987) have also reported that the fruit-body growth of *F. velutipes* was completely inhibited by the addition of a metal proteinase inhibitor, 1, 10-phenanthroline. These facts suggest that the metal (neutral) proteinase may play an important role in turnover of nitrogenous compounds during fruit-body production.

In the present study, isolation and characterization

of extra- and intra-cellular metal proteinases of *H. marmoreus* in bottle cultivation with sawdust-rice bran medium are described as the first step in clarifying the possible role of proteinases in mycelial maturation in the spawn-running process.

The stock culture of *H. marmoreus* was isolated from commercial fruit-bodies obtained at a store in Osaka, Japan in 1989 and was subcultured on potato dextrose agar (PDA) medium.

A substrate consisting of 130 g of sawdust rice bran (sawdust : rice bran=5 : 1 (w/w); moisture content, about 65%) contained in a 200-ml glass bottle was autoclaved at 119°C for 60 min and used for the cultivation of this mushroom. The spawn-running process, which consists of two successive stages, linear mycelial growth (for 25 d) and mycelial maturation (for 35 d), was carried out at 24°C for 60 d under a fluorescent lamp of 50 lx and a relative humidity of 45 to 50%. To induce fruiting, the cultures were treated by *kinkaki* (removal of both old spawn and the uppermost layer of mycelium-medium complex) and grown at 10°C under light illumination (100–200 lx) and a relative humidity of 85 to 95%. The fruit-bodies were initiated after ca. 75 d and matured after ca. 90 d of cultivation.

The proteinase activities were assayed by the modified casein-Folin method (Terashita and Kono, 1987) at pH 6.5 for 30 min with Hammarsten casein as the substrate. Their substrate specificities were investigated using Hammarsten casein, milk casein, bovine serum albumin, ovalbumin (egg albumin), hemoglobin and human serum γ-globulin at a 1.33% (w/v) concentration. One unit of enzyme (PU) corresponds to 1 μg of tyrosine released per ml of reaction mixture per min. The

Michaelis constants were calculated from Lineweaver-Burk plots using carbobenzoxy-L-glycyl-L-leucineamide (Z-Gly-L-Leu·NH$_2$) as the substrate. A reaction mixture containing the substrate at concentrations ranging from 0.001 M to 0.1 M and 0.75 mg of enzyme protein/ml in a defined solution of pH 7.0 was incubated at 37°C, and amino acids in the hydrolyzate were measured by the ninhydrin method (Moore and Stein, 1954).

Various enzyme inhibitors (S-PI (*Streptomyces*-pepsin inhibitor, Pepstatin Ac), MAPI (microbial alkaline and thiol proteinase inhibitor), and Talopeptin (MK-I, microbial metal proteinase inhibitor)) were prepared as reported previously (Terashita et al., 1995a). Phosphoramidon (microbial metal proteinase inhibitor from *Streptomyces tanashiensis* Hata et al.), EDTA and 1, 10-phenanthroline were purchased from Wako Pure Chemical Industry.

In preliminary experiments to determine the time course of production of proteinases in the culture of *H. marmoreus*, a high activity of metal proteinase was detected in the culture filtrate (70 PU/mg protein) and the vegetative mycelia (35 PU/mg protein) at the linear mycelial growth stage (25 d after inoculation) in the spawn-running process. Acid proteinase activities (extracellular enzyme: 20 PU/mg protein; intracellular enzyme: 12 PU/mg protein) were also detected, but at lower levels compared to metal proteinase.

For purification of the extracellular metal proteinase (PE-1), 0.1 M Költhoff buffer solution, pH 6.0, was added to the culture substrate at the linear mycelial growth stage in the spawn-running process (21-d-old cultures). After mixing for 3 h at 4°C, the broth was collected by filtration through filter paper on an aspirator. Ammonium sulfate was added to the filtrate (6 L) to 70% saturation. The precipitate was dissolved in 0.1 M Költhoff buffer, pH 6.5, and dialyzed against the same buffer for 2 d. The dialyzed solution (1.1 L) was put on a Phenyl Sepharose CL-4B column (2.6 × 24.5 cm) equilibrated with 0.02 M Költhoff buffer, pH 6.5, containing ammonium sulfate at 20% saturation. The enzyme was eluted with linear gradient of both ammonium sulfate from 20% saturation to zero and ethylene glycol from 50% to zero in 0.02 M Költhoff buffer, pH 6.5. The active fractions (ethylene glycol, 28%; ammonium sulfate, 7.9%) were concentrated by ultrafiltration (Amicon PM-10) and by use of a collodion bag at 4°C. The enzyme was charged on an isoelectric focusing column containing carrier ampholyte with a pH range of 3.5 to 9.5.

For purification of the intracellular metal proteinase (PE-2) from mycelia, the debris of the culture substrate after extracting the PE-1 enzyme was used. The mycelium-sawdust complex was added to 0.1 M Költhoff buffer, pH 6.5, and homogenized with a cell homogenizer for 15 min at 0°C, then the homogenate was centrifuged at 20,000×g for 15 min. Ammonium sulfate was added to the supernatant (5.8 L) to 80% saturation. The dialyzate (420 ml) was mixed with DEAE-Cellulose that had been equilibrated with 0.1 M Költhoff buffer, pH 6.5. The non-retained fraction (530 ml) was put on a Phenyl Sepharose CL-4B column, and the active fractions (150 ml) were eluted (ethylene glycol, 17.5%; ammoni-

um sulfate, 13.5%). The subsequent procedures were the same as those used for the extracellular metal proteinase.

The metal proteinases were purified 112-fold (extracellular enzyme; PE-1) and 812-fold (intracellular enzyme; PE-2) over the original materials with about 8.3% and 6.3% recoveries, respectively. Each enzyme preparation gave a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Table 1 shows some properties of the purified metal proteinases. Their estimated molecular weights by SDS-PAGE were 29,500 for PE-1, which is almost the same as the extracellular metal proteinase of *F. velutipes* (30,000) (Terashita et al., 1995a), and 21,500 for PE-2. These enzymes had similar enzymatic properties of optimum pH and optimum temperature and thermal stability. Km values were 0.9 × 10^{-3} M for PE-1 and 1.2 × 10^{-3} M for PE-2 against the synthetic peptide substrate Z-Gly-L-Leu·NH$_2$. Both enzymes showed the highest activity against Hammarsten casein among the substrates tested. These results are in contrast to carboxyl proteinase (Terashita and Kono, 1987), which showed the highest activity against hemoglobin but very low activity against Hammarsten casein. On the other hand, the metal proteinases differed from each other in their responses to various inhibitors. In particular, the extracellular proteinase (PE-1) was strongly inhibited by phosphoramidon, a specific inhibitor of the metal proteinase Thermolysin (Terashita et al., 1995a), whereas the intracellular metal proteinase (PE-2) was weakly inhibited.
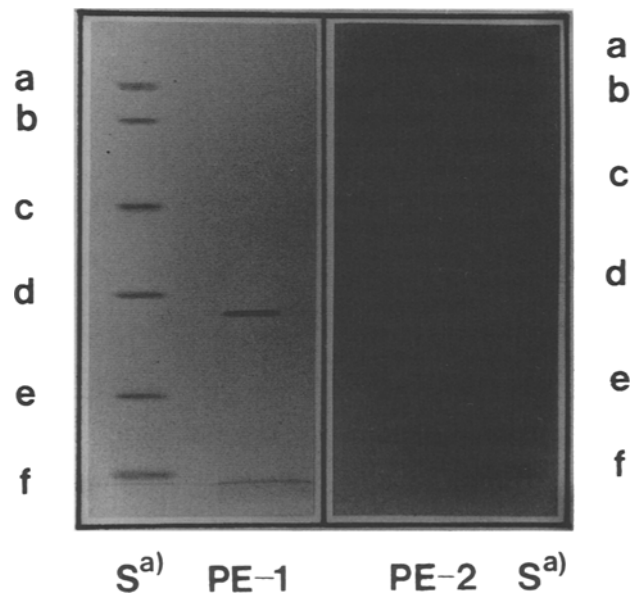
We confirmed that the fruit-body formation in *H.*

Fig. 1. SDS-polyacrylamide gel electrophoresis patterns of extracellular (PE-1) and intracellular (PE-2) metal proteinases from *Hypsizygus marmoreus*.
a) Standard proteins. a: phosphorylase b (M.W. 97,400), b: serum albumin (66,300), c: aldolase (42,400), d: carbonic anhydrase (30,000), e: trypsin inhibitor (20,000), f: lysozyme (14,400).

Table 1. Some properties of purified metal proteinases from culture filtrate (PE-1) and vegetative mycelia (PE-2) produced in the spawn running process (21 d after inoculation) of *Hypsizygus marmoreus*.

Enzyme properties		Metal proteinase	
		Extracellular	Intracellular
Optimum pH (Hammarsten casein)		7.0	6.5–7.5
Optimum temperature (°C)		50	45–55
pH stability (37°C, 30 min)		6.2–7.6	6.5–8.0
Thermal stability (pH 6.5, 30 min)		40°C	40°C
Inhibition rate (%)[a] :	EDTA (10 mM)	92	42
	Phosphoramidon (1 mM)	87	20
	Talopeptin (1 mM)	88	85
	1, 10-Phenanthroline (10 mM)	97	100
	MAPI (1 mM)	3	2
	S-PI (1 mM)	0	0
Substrate specificity (%) :	Hammarsten casein	100	100
	Milk casein	—	85
	Ovalbumin (egg albumin)	3	6
	Hemoglobin	19	22
	Human serum γ-globulin	—	3
	Bovine serum albumin	13	6
Molecular weight (SDS-PAGE)		29,500	21,500
Isoelectric point		8.8	8.4
Km (M)(Z-Gly-L-Leu·NH$_2$)		0.9×10^{-3}	1.2×10^{-3}

Hammarsten casein as substrate was dissolved in 0.1 M Költhoff buffers of various pHs. The reaction for optimum pH was carried out at various pHs and 37°C for 30 min. Optimum temperature was determined by measuring activites at various temperatures (pH 7.0) for 30 min. The pH stability was determined by incubating the proteinases in 0.1 M Költhoff buffers of various pHs at 37°C for 30 min and assaying the remaining activity. The thermal stability was determined by incubating the proteinases in 0.1 M Költhoff buffer, pH 6.5, at each temperature for 30 min and measuring the remaining activity.

a) Inhibition rate (%) was estimated from the residual enzyme activity after incubation with various inhibitors in 0.1 M Költhoff buffer, pH 6.5, for 10 min at 37°C.

marmoreus was strongly inhibited by the addition of the metal proteinase inhibitor phosphoramidon (1.3 mg/bottle) to the medium on the 18th day after inoculation (Terashita et al., unpublished). These results indicate the major role of metal proteinase during fruit-body formation.

However, more detailed information is needed about the time course of metal proteinase production during the fruit-body growth and the effect on the fruit-body production of feeding amino acids into the culture medium containing phosphoramidon. Further studies are in progress.

Literature cited

Amano, Y., Nishizawa, K., Tokoo, R., Matsuzawa, T. and Kanda, T. 1992. Extracellular enzymes produced by *Lyophyllum ulmarium* (*Hypsizygus marmoreus*) in commercial cultivation. Mokuzai Gakkaishi **38**: 411–416. (In Japanese.)

Chao, E. E. and Gruen, H. E. 1987. Intracellular activity of mycelial proteinases during fruit-body development in *Flammulina velutipes*. Can. J. Bot. **65**: 518–525.

Kitamoto, Y., Matsumoto, T., Hosoi, N., Terashita, T., Kono, M.

and Ichikawa, Y. 1980. Nitrogen metabolism in *Favolus arcularius*: Changes in cellular nitrogen compounds during development of the mycelium and fruit-bodies. Trans. Mycol. Soc. Japan **21**: 237–244. (In Japanese.)

Moore, S. and Stein, W. H. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. **211**: 907–913.

Terashita, T. and Kono, M. 1987. Purification and some properties of carboxyl proteinases from *Tricholoma matsutake*. Trans. Mycol. Soc. Japan **28**: 245–256. (In Japanese.)

Terashita, T., Oda, K., Kono, M. and Murao, S. 1985. Proteinase systems in *Flammulina velutipes* and *Pleurotus ostreatus*. Trans. Mycol. Soc. Japan **26**: 397–409. (In Japanese.)

Terashita, T., Yoshikawa, K. and Shishiyama, J. 1995a. Purification and some properties of metal proteinase from the pilei: Essential for the growth of pileus and basidiospore formation in the fruit-body of *Flammulina velutipes*. Food Sci. Technol., Int. **1**: 18–21.

Terashita, T., Ro, S., Yoshikawa, K. and Shishiyama, J. 1995b. Nutritional environment for mycelial growth and hydrolytic enzyme activities during the vegetative growth of *Hypsizygus marmoreus*. Mushroom Sci. Biotechnol. **2**: 15–20. (In Japanese.)