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Short Communication

Purification and some properties of an isoform of metal proteinases from *Hypsizygus marmoreus* grown on sawdust culture

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Purification and some properties of an isoform of metal proteinase from *Hypsizygus marmoreus* are described. This enzyme was purified 711-fold with 5.44% recovery. The molecular weight and pI value were 41,500 and pH 7.7, respectively. The highest activity was observed against milk casein as the substrate. This enzyme was strongly inhibited by metal proteinase inhibitors such as phosphoramidon, EDTA, and *o*-phenanthroline.

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

The sawdust culture of *Hypsizygus marmoreus* (Peck) Bigelow requires a long spawn-running process to allow the mycelial maturation.

Amano et al. (1992) reported that the potential activity of neutral proteinase was detected in sawdust medium during vegetative mycelial growth of this fungus. We also studied the possible role of proteinase on the fruit-body formation of this fungus by using its specific inhibitors. We found (Terashita et al., 1998) that the addition of a metal proteinase inhibitor, phosphoramidon (Suda et al., 1973), to the medium completely inhibited the fruit-body growth. These facts suggest the metal (neutral) proteinase may play an important role in turnover of nitrogenous compounds during fruit-body production. Both the extra- (PE-1 from culture) and intracellular (PE-2 from mycelia) metal proteinase during vegetative mycelial growth (21-d cultures) were purified and characterized as previously reported (Terashita et al., 1997). PE-1 (pI 8.8) from culture medium was strongly inhibited (87%) by phosphoramidon. However, PE-2 (pI 8.4) purified from vegetative mycelium showed very weak inhibition (20%) by phosphoramidon. We cannot fully explain the remarkable decrease in the yield of fruit-bodies and the striking inhibition of the metal proteinase activity in vegetative mycelia when phosphoramidon was added to the culture medium (Terashita et al., 1998). This prompted us to seek an isoform of metal proteinase in vegetative mycelium of this fungus.

During the study of metal proteinase production and the isozyme analysis during development of fruit-body, a metal proteinase with an isoelectric point of 7.7 was found in addition of the metal proteinase having pI 8.4 (Terashita et al., 1997) in vegetative mycelia during the mycelial maturation (29-d cultures).

In the present study, purification procedures and some properties of a metal proteinase (pI 7.7) produced in vegetative mycelia of *H. marmoreus* are described.

The stock culture of *H. marmoreus* was isolated from commercial fruit-bodies previously reported (Terashita et al., 1997). A medium consisting of 130 g of mixed sawdust and rice bran (sawdust : rice bran = 5 : 1 (w/w); moisture content, about 65%) contained in a 200-ml glass bottle was used for its cultivation under the conditions described in the previous paper (Terashita et al., 1997).

After cultivation for various periods (21, 29, or 81 d), 10-g portions of cultured medium were treated to extract extra-cellular enzyme, then homogenized with 30 ml of 0.1 M Költhoff buffer, pH 6.0, and centrifuged (20,000 × *g*, 15 min). The supernatant solution was subjected to isoelectric focusing analysis on an LKB column (110 ml) containing carrier ampholytes (LKB) with a pH range of 7.0 to 9.0. Electrophoresis was performed at a constant voltage of 700 V for 48 h, and the column temperature was maintained at 0°C with chilled methanol.

Metal proteinase activity was assayed at pH 7.0 by the casein-Folin method with slight modification (Terashita et al., 1997). One unit of enzyme (PU) corresponds to 1 μg of tyrosine released per min.

For the purification of the intracellular metal proteinase of pI 7.7 from mycelia, the debris of the cultured sawdust-rice bran medium after extracting the extracellular enzyme was used. Cultured medium (2.5 kg) at the mycelial maturation stage in the spawn-running process (29-d cultures) was added to 0.1 M Költhoff buffer, pH 6.5, and homogenized with a cell homogenizer (Nissei homogenizer AM-1 (18,000 rpm), Nihon Seiki) for 7 min at 0°C and a mixer (MX-740G, Matsushita Electric Industrial) for 8 min at 0°C, then the homogenate was

centrifuged at 20,000×*g* for 15 min. After collecting the extract (6.3 l) by filtration, ammonium sulfate was added to make 80% saturation. The dialyzed solution (450 ml) was mixed with DEAE-cellulose equilibrated with 0.1 M Költhoff buffer, pH 6.5. The non-retained fraction (550 ml) was collected and loaded on a Phenyl Sepharose CL-4B column. The active fractions (260 ml) obtained from the column were concentrated by ultrafiltration. The concentrated fraction (18.9 ml) was charged for 48 h on an electrofocusing column (LKB column, pH 7.0–9.0). Electrophoresis was performed at a constant voltage of 700 V at 0°C.

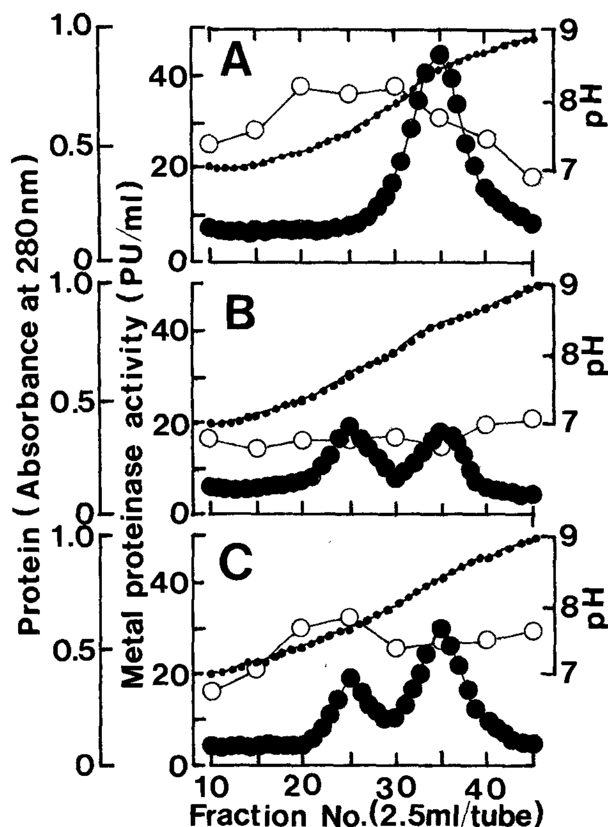

Fig. 1. Isoelectric focusing patterns of metal proteinases produced in vegetative mycelia during the growth of *H. marmoreus* cultured in sawdust-rice bran medium.
A: 21-d cultures (vegetative mycelial growth stage)
B: 29-d cultures (mycelial maturation stage)
C: 81-d cultures (fruit-body formation stage)
●: proteinase activity, ○: absorbance at 280 nm, •: pH of the fractions.
Isoelectric focusing column chromatography was carried out on a LKB column (110 ml) containing carrier ampholytes at pH 7.0 to 9.0. Electrophoresis was performed at a constant voltage of 700 V for 48 h at 0°C.

From the fractionation patterns by isoelectric focusing analysis (Fig. 1), a metal proteinase with pI 8.4 was detected in vegetative mycelia 21 d after inoculation (Fig. 1A, Terashita et al., 1997). In addition, a new metal proteinase with an isoelectric point of 7.7 was also found in mycelia during mycelial maturation (Fig. 1B, 29-d cultures) and during development of fruit-bodies (Fig. 1C, 81-d cultures).

We next tried to purify the proteinase (pI 7.7) to clarify its physicochemical and enzymatic properties. The process of purification is summarized in Table 1. The purified metal proteinase represented 711-fold purification over the original material with about 5.44% recovery. The homogeneity of the purified enzyme was demonstrated by SDS-PAGE (Fig. 2).

Some properties of the purified metal proteinase of pI 7.7 produced in mycelia during the mycelial maturation are shown in Table 2. The proteinase was most active at pH 7.0–7.5 toward Hammarsten casein and was sta-

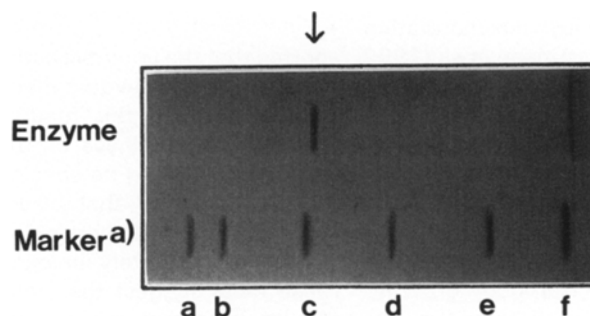

Fig. 2. SDS-PAGE analysis of purified metal proteinase of pI 7.7 from vegetative mycelia induced during the mycelial maturation of *H. marmoreus*.
a) reference proteins: a, phosphorylase b (MW 97,400); b, bovine serum albumin (66,300); c, aldolase (42,400); d, carbonic anhydrase (30,000); e, trypsin inhibitor (20,000); f, lysozyme (14,400).
Electrophoresis was done for 2 h on a 12.5% SDS-polyacrylamide gel. Enzyme protein (5 μg) was stained with Coomassie brilliant blue.

Table 1. Purification of intracellular metal proteinase (pI 7.7) from vegetative mycelia induced during the mycelial maturation of *H. marmoreus* cultured in sawdust rice bran medium.

Procedure	Total activity (Pro. Unit)	Specific activity (PU/mg protein)	Yield (%)
Crude extract	12,200	1.32	100
80% Sat. ammonium sulfate	10,100	1.63	82.8
DEAE-cellulose SH (Batch-wise, pH 6.5)	7,070	1.44	58.0
Phenyl sepharose CL-4B column	2,860	466	23.4
Isoelectric focusing column (pH 7.0–9.0)	664	938	5.44

ble only in the narrow pH range of 6.0–7.0, even under mild treatment (37°C, 30 min). Its molecular weight (MW) was estimated to be 41,500 by SDS-PAGE, the highest among the metal proteinases of this fungus (Terashita et al., 1997). The metal proteinase of pI 7.7 was not a dimer of that of pI 8.4. It showed highest activity against milk casein among the substrates tested. Additionally, it exhibited greater activity towards hemoglobin as substrate compared to the pI 8.4 enzyme.

Several kinds of metal proteinases in mushroom fungi have been reported (Hashimoto, 1983; Chao and Gruen, 1987; Terashita, 1992) and characterized (Hashimoto, 1983; Terashita, 1992). Dohmae et al. (1995) reported the production of two kinds of intracellular metal proteinases (Pro B and Pro C) in *Pleurotus ostreatus* (Jacq.: Fr.) Kummer fruit-bodies. Pro B is a Zinc-enzyme of MW 18,000 and Pro C is a metal endopeptidase of MW 42,500. They mentioned that Pro C is very similar to the metal endopeptidases purified and characterized from mycelia or fruit-bodies of *Lentinula edodes* (Berk.) Pegler, *Flammulina velutipes* (Curt.: Fr.) Singer, and *Ganoderma lucidum* (Leyss.: Fr.) Karst. in terms of its properties (Terashita et al., 1985). The molecular weight of metal proteinases obtained from vegetative mycelia of *H. marmoreus* in this report was closely similar to those of Pro B and Pro C from *P. ostreatus* fruit-bodies.

The intracellular metal proteinase of pI 7.7 in *H. marmoreus* was not inhibited by MAPI (microbial alkaline, serine and thiol proteinase inhibitor, Shimizu et al., 1984) and S-PI (*Streptomyces*-pepsin (carboxyl proteinase) inhibitor, Murao and Satoi, 1971). In contrast, *o*-phenanthroline, ethylenediamine-tetraacetic acid (EDTA) and phosphoramidon exhibited strong inhibition. These results suggest that the metal proteinase of pI 7.7 is different from the metal proteinases of pI 8.4 or 8.8.

Mukai (1976) mentioned that the intracellular proteinases have important functions of limited proteolysis for the activation, inactivation, and modification of enzymes in addition to breakdown of enzymes and other proteins into peptide compounds and amino acids. Burton et al. (1993) suggested that the proteinase in *Agaricus bisporus* (J. Lange) Imbach var. *albidus* (J. Lange) Singer is important for the further growth of the harvested fruit-body. We showed (Terashita et al., 1993) that the carboxyl proteinase in fruit-bodies of *L. edodes* is essential for the maturation of basidiospores of this mushroom. In subsequent studies on the differntiation of the fruit-body of mushrooms also using *F. velutipes*, we found that a metal proteinase in the fruit-bodies is important for fruit-body growth and also exhibits a significant role in basidiospore formation (Terashita et al., 1995).

In our previous paper (Terashita et al., 1998), we showed that the activity of intracellular metal proteinase produced in mycelia during the spawn-running process of mycelial maturation (30-d cultures) decreased significantly on addition of phosphoramidon to the culture medium. As a result, the fruit-body yield (based on dry weight) of *H. marmoreus* was severely reduced (6.8% (0.22± 0.03 g/bottle) on addition of inhibitor compared to the control (3.24±0.36 g/bottle)). However, when glutamic acid or arginine was added (200 mg/bottle) to sawdust medium along with phosphoramidon 60 d after inoculation, the fruit-body yield was restored to 49.2% (1.83±0.28 g/bottle) and 55.9% (2.23±0.30 g/bottle), respectively, of the control (no phosphoramidon added). These results suggest that the added amino acids were translocated into the vegetative mycelium and utilized for the growth of the fruit-body.

The remarkable inhibition by phosphoramidon suggests that the metal proteinase of pI 7.7 produced in vegetative mycelia is important for mycelial maturation and fruiting body formation of this mushroom fungus.

Table 2. Some properties of purified metal proteinase from vegetative mycelia of pI 7.7 induced during mycelial maturation (29-d cultures) of *H. marmoreus* cultured in sawdust-rice bran medium.

Property		Value
Optimum pH (casein)		7.0–7.5
Optimum temperature (°C)		45
pH stability (37°C,30 min)		6.0–7.0
Thermal stability (pH 6.5, 30 min, °C)		40
Molecular weight (SDS-PAGE)		41,500
Inhibition (%)[a]	EDTA (10 mM)	97
	phosphoramidon (1 mM)	96
	o-phenanthroline (10 mM)	100
	MAPI (1 mM)	0
	S-PI (1 mM)	0
Substrate specificity(%)[b]	Hammarsten casein	82
	milk casein	100
	ovalbumin (egg albumin)	5
	bovine serum albumin	5
	hemoglobin	45
	human serum γ-globulin	1

a) The enzyme was treated with various inhibitors in 0.1 M Költhoff buffer, pH 6.5, for 10 min at 37°C.
b) Substrates (1.33%) were dissolved in 0.1 M Költhoff buffer, pH 7.0. The reaction was carried out at 37°C for 30 min. The values are expressed relative to milk casein.

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