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Purification and Characterization of Metalloproteases from *Pleurotus sajor-caju*

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Abstract Fibrinolytic protease activity was detected in the fruit body of *Pleurotus sajor-caju* using a fibrin plate method. Two fibrinolytic activities (FPI and II) were found at the regions of 14.5 and 86.0 kDa by using gel-filtration column chromatography. FPII was identified as an alkaline protease, whereas FPI was a neutral protease. Both were inhibited by phenanthroline and EDTA, suggesting that they are metalloproteases. Inactivated enzyme activities were restored by adding Co^{2+} or Zn^{2+}. Iodoacetate inhibited FPI, but not FPII. Both enzymes cleaved B_β and γ chains of the human fibrinogen. FPII showed a preference to hydrophobic and bulky residues of nitroanilidine compounds as substrates, whereas FPI preferred positively charged residues.

Key words: Metalloprotease, Zn^{2+}, fibrinolytic, *Pleurotus sajor-caju*

Proteolytic cleavage is one of the important modifications of proteins. Modification occurs in many different ways and is catalyzed by different kinds of proteases. The limited localization and temporal gene expression of some proteases indicate important functions for the organisms. It is thought that proteases play an important role in germination [6, 10], cell growth [3], morphogenesis [14], and differentiation [1, 4]. Intracellular metalloproteases have been purified and characterized from *Lentinus edodes* fruiting bodies. When the ability of fruit body formation became weak, the metalloprotease activities in the mycelium reached a low level [14], therefore, it has been suggested that these enzymes play a key role in fruiting body formation in these organisms [13].

The fibrinolytic protease was found in the mycelium and fruit body of *Pleurotus sajor-caju*. The specific enzyme activities were 0.29 and 1.03 U/mg for the mycelial culture and fruit body, respectively. The enzyme was completely inactivated by 1,10-phenanthroline. When 1,10-phenanthroline was added, it significantly inhibited the mycelial growth and this indicated that the metalloprotease is indeed important for growth. In this paper, two metalloproteases from the fruit body of *P. sajor-caju* were purified to homogeneity and a preliminary study was carried out in order to provide information on the cellular significance of the enzyme function.

Fruit bodies of *P. sajor-caju* were obtained from a mushroom farm which was supported by the Korean Agricultural Improvement Department (Suwon). Two hundred grams of fruit bodies were suspended in 250 ml of 10 mM Tris-HCl, pH 8.0 (Buffer A) and disrupted in an ice-chilled bead beater (Biospec Products, U.S.A.) for 5 min with pulse [9]. The homogenate was centrifuged at 10,000 ×g for 20 min in a refrigerated centrifuge T-324 using an A-8.24 rotor (Kontron Instruments, Sweden), concentrated by ultrafiltration (Amicon) using the PM 10 membrane, and then heated at 50°C for 10 min (only in the case of FPI). The solution was applied to a phenyl Sepharose column (2×7 cm), equilibrated with 10 mM Tris-HCl, pH 8.0, containing 1.7 M ammonium sulfate (Buffer B). The active fraction was applied to a Sephadex G-150 column (2.8×105 cm), and then to Phenylsuperose (Pharmacia; H/R, 5/5), hydrophobic column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1.7 M ammonium sulfate. The protease fractions were eluted with decreasing concentration of ammonium sulfate, and then it was concentrated, and the buffer was exchanged by ultrafiltration. The fibrinolytic activity was measured using fibrin plates by a method modified by Jeon *et al.* [7] Twelve ml of 0.8% bovine fibrinogen was dissolved in 0.2 M borate buffer, pH 7.8, containing 50 mM NaCl and finally 10 U/ml of thrombin was added to form a fibrin clot layer. After loading each sample on a fibrin clot layer at 30°C for 15 h, the zones of clearance areas were measured and the relative activities were compared. One unit of activity was defined as the amount of enzyme which produced a clear zone of 1 mm^2 in a fibrin plate. In comparison, one unit of Subtilisin

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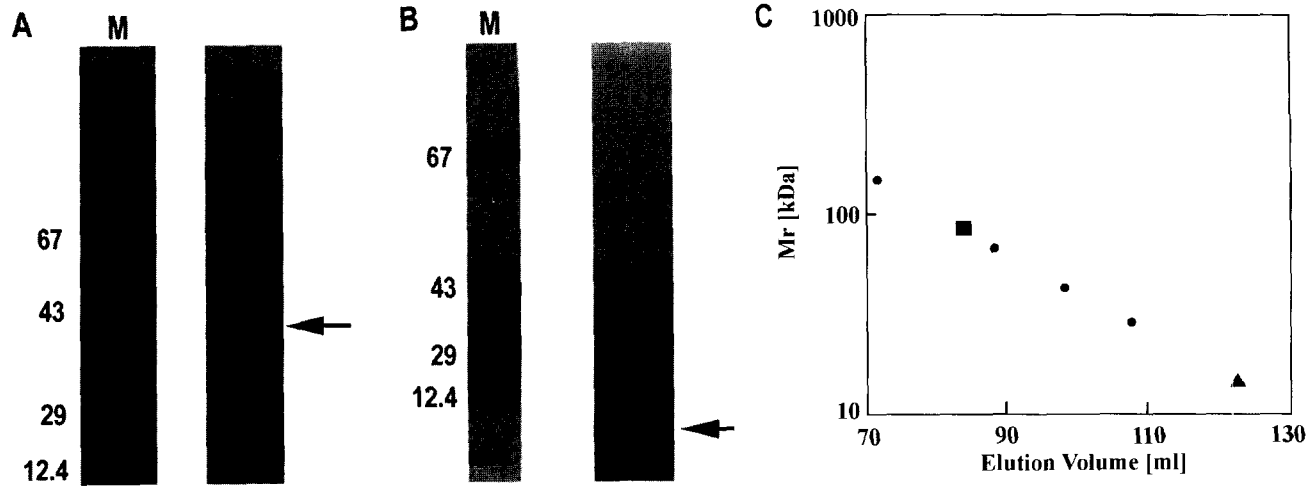

Fig. 1. Estimation of M.W. of protease by SDS-PAGE (A, B) and Sephadex G-150 column chromatographies (C).
The active fraction after phenylsuperose column chromatography was applied. The M.W standards for SDS-PAGE consisted of bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). The M.W. of FPI (A) and FPII (B) were calculated to be 42 and 7.3 kDa, respectively. The M.W. standards for gel filtration consisted of alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). The M.W. of FPI (■) and FPII (▲) were calculated to be 86 and 14.5 kDa, respectively.

Carlsberg (Sigma) produced a clear zone of 43,250 mm^2 under the same condition.

Two fibrinolytic protease activities (FPI and FPII) were found in the fruit body of *P. sajor-caju,* and they were purified by using phenyl Sepharose and gel filtration chromatographies. FPI preparation was heated at 50°C to inactivate the activity of FPII. FPII bound less tightly to phenyl Sepharose, therefore, it was eluted first with decreasing concentration of salt, although FPI appeared after elution without ammonium sulfate. FPI and FPII were purified 20.4 and 6.6-folds with yields of 17 and 4 %, respectively. The purity of isolated enzymes was confirmed by SDS-PAGE. The molecular masses of purified FPI and FPII were estimated by SDS-PAGE to be 7.3 (Fig. 1A) and 42.0 kDa (Fig. 1B), respectively. Apparent molecular masses of native enzymes were estimated to be 14.5 (FPI) and 86 kDa (FPII) by Sephadex G-150 gel filtration (Fig. 1C), indicating that both were dimers. The optimum pHs were in the range of 8.0 to 8.5 for FPI and 7.0 to 7.5 for FPII (Fig. 2A). Thermal stability of the purified protease was determined at different temperatures for 15 min, and it was found that FPI was more stable and resistant to heat than FPII. The remaining activities of FPI and FPII after 15 min at 50°C were 83 and 0%, respectively (Fig. 2B). Lyophilization did not affect the activity of FPI, but it reduced that of FPII by 63%.

When the purified FPII was treated with human fibrinogen, the B_β and γ chain disappeared during the incubation (Fig. 3A). It was of interest to mention that FPI also showed a similar pattern. Substrate specificity with synthetic substrates was measured by a modified method of Kang *et al.* [8]. Both enzymes reacted with azoalbumin, but not with azocasein. However, purified enzymes showed different activities with 4-nitroaniline compounds as shown in Table 1. FPI reacted best with Lys pNA among the compounds tested, and it was more efficient with positively-charged residues compared to the hydrophobic ones. The K_m and V_{max} values of Lys pNA were determined to be 3.4×10^{-5} M and 2.4×10^{-1} M $sec^{-1}g^{-1}$, respectively. The enzyme cleaved *N-p*-Tosyl-Gly-Pro-Arg pNA with a much lower rate than the corresponding aminopeptidase substrate and no cleavage was observed with *N-p*-Tosyl-Gly-Pro-Lys pNA. This suggested that the enzyme reacted much better as an aminopeptidase than an endopeptidase. However, FPII hydrolyzed Phe pNA the most actively among the synthetic substrates and had a preference for a bulky hydrophobic residue. The K_m and V_{max} values of Phe pNA were determined to be 8.0×10^{-5} M and 3.4×10^{-1} M $sec^{-1}g^{-1}$, respectively.

The fibrinolytic activity of the purified protease in the absence or presence of various reagents is summarized in Table 2. As it can be seen in Table 2, both had some similarities. The enzymes were strongly inhibited by 1,10-phenanthroline and EDTA, suggesting that both belonged to metalloproteases. Fibrinolytic proteases were not sensitive to serine protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), leupeptin, and tosyl phenylalanyl chloromethyl ketone (TPCK), or of the cysteine protease including *N*-ethylmaleimide (NEM) and trans epoxysuccinyl leucylamide 4-guanidinobutane (E-64). They were also insensitive to pepstatin which reacted with some aspartic proteases. However, it should be mentioned here that there were some differences between them. Phosphoamidon, a metalloprotease inhibitor, inhibited FPII more strongly than FPI, and FPI was sensitive to iodoacetate, but FPII was resistant to it.

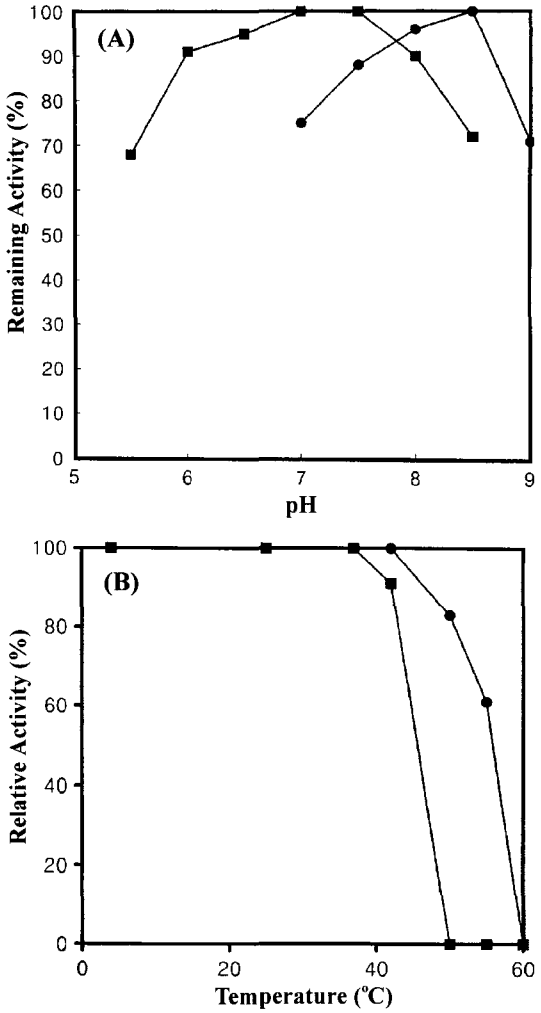

Fig. 2. Activities of FPI (●) and FPII (■) at different pHs (A) and temperatures (B).
For different pHs, each aliquot of enzyme was incubated at 50 mM of Tris-HCl (pH 7.0-9.0) and KPO_4 (pH 5.5-8.5) for FPI and FPII, respectively, for 30 min. The highest enzyme activity was assumed to have retained 100% activity. For temperature, each was incubated for 15 min at an indicated temperature and was assayed for the remaining activity. The 4°C sample was assumed to have retained 100% activity.

To elucidate the requirement for metal ions, the enzymes were preincubated with 1 mM of 1,10-phenanthroline for 30 min to completely reduce the initial activity. Various metal ions were added to test whether any of these ions could reverse the enzyme inactivation. As shown in Fig. 2, the activity of fibrinolytic protease was recovered by adding 1 mM of Zn^{2+} or Co^{2+}. The pattern of restoration of FPI activity was similar to that of FPII. Choi *et al.* [2] and Grimwood *et al.* [5] have reported that Co^{2+} activated the Zn^{2+}-containing enzyme. The metal content of the enzyme was determined by using an ICP spectrometer (Thermo Jarrell Ash Corporation, Model; Atomscan 25) to determine whether fibrinolytic protease contained Co^{2+} or Zn^{2+}. The purified FPI and FPII contained 3.31 and 0.67 μg of Zn^{2+}

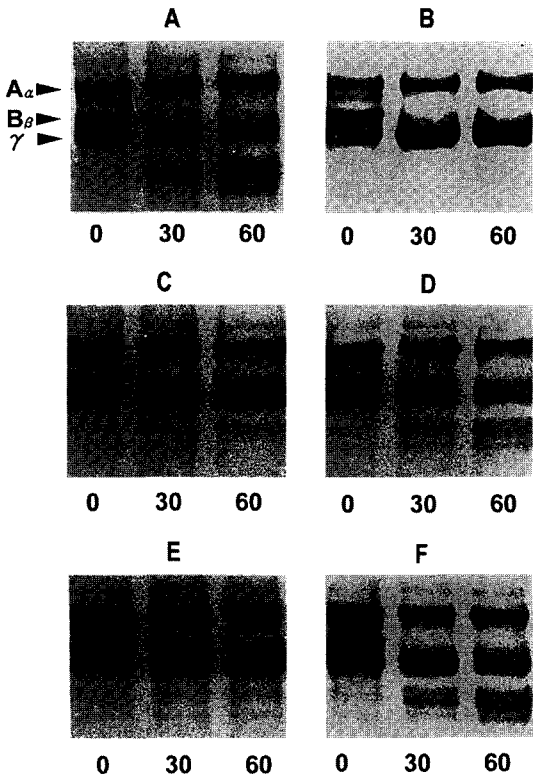

Fig. 3. Protease activity of FPII with human fibrinogen as a substrate with or without various inhibitors and metal ions.
For SDS-PAGE analysis, human fibrinogen was incubated for indicated time intervals with protease in 50 mM Tris-HCl, pH 8.0, at 37°C and electrophoresis was carried out on a 12% gel by using the Laemmli's method [9]. Degradation of B_β and γ chains of human fibrinogen was observed as the incubation time (0, 30, and 60 min) continued (A). Inhibition of degradation occurred with the same reaction mixture plus 1 mM 1,10-phenanthroline (B). Inhibition was reversed by the treatment of 0.1 mM of Zn^{2+} (C), 1 mM of Zn^{2+} (D), or 0.1 mM of Co^{2+} (E), 1 mM of Co^{2+} (F).

Table 1. Substrate specificity of two fibrinolytic proteases.

Substrate (0.1 mM)	FPI	FPII
	Enzyme activity (%)	
Azocasein	-	-
Azoalbumin	+	+
Lys pNA	100	0.1
Arg pNA	13	0
Phe pNA	7	100
Leu pNA	9	46
Pro pNA	ND[a]	11
Ala pNA	ND	2
Gly pNA	ND	0.2
Glu pNA	ND	0.2
N-p-Tosyl-GPR pNA	2	0
N-p-Tosyl-GPK pNA	0	0
N-Suc-AAPL pNA	4	0.1
N-Suc-AAPF pNA	2	0.1

+, positive hydrolysis ; -, negative hydrolysis.
ND[a], not determined.

Table 2. Effect of group-specific agents and various metal ions on enzyme activity.

Compound	Concentration (mM)	FPI	FPII
		Remaining activity (%)	
Control		100	100
PMSF	10	75	80
TPCK	5	75	80
Leupeptin	0.2	96	95
Pepstatin	0.2	89	100
Bestatin	0.2	71	95
E-64	0.2	93	70
Phosphoamidon	10	71	0
Phenathroline	10	0	0
EDTA	10	0	0
EGTA	10	80	0
NEM	1	96	115
PCMB	1	86	100
Iodoacetic acid	10	0	110

per mg of protein, respectively, but no other metals, including Co^{2+}, could be detected. Since the molecular masses of native proteins were 14,500 and 86,000 for FPI and FPII, respectively, the amount of zinc ions may be equivalent to 0.74 and 0.89 mol of Zn^{2+} per mol of the proteases. Both enzymes seemed to be Zn^{2+} metalloproteases.

Earlier, we discovered an another Zn^{2+} metalloprotease with a M.W. of 12 kDa from *Pleurotus ostreatus* [2]. The proteolytic activity was similar to those of the FPI and FPII described herein. However, there were differences in the hydrolyzing activity of synthetic substrates and the sensitivity towards iodoacetate. Due to a lack of these molecular properties of the enzymes, we are not certain at present whether they share the same gene sequence and/or undergo different post-translational modifications.

In this report, we presented the isolation and the characterization of two fibrinolytic metalloproteases from the fruit body of *P. sajor-caju*. Specific activities of metalloprotease increased during fruit body formation. Since the inhibition of metalloprotease by 1,10 phenanthroline was a main element in stopping the growth, a substantial level of protease could play an important role in providing necessary amino acids which could be utilized in complicated signaling pathways of differentiation. Both proteases hydrolyzed the B_β and γ chains of human fibrinogen, but they were not reactive with bovine serum albumin and human hemoglobin. The enzymes involved in intracellular protein degradation required the properties such as an optimum pH of neutral to alkaline range, a relative specificity, and a proper regulatory level under physiological conditions. Although their physiological role has not yet been clearly elucidated, the fibrinolytic metalloproteases from *P. sajor-caju* appear to satisfy these requirments.

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