Full Paper

Purification and characterization of a novel extracellular neutral metalloprotease from *Cerrena albocinnamomea*

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We selected a fungus secreting a neutral protease from soil and identified it as the basidiomycete fungus *Cerrena albocinnamomea* according to its ITS-5.8S rDNA and 28S rDNA-D1/D2 sequences. A major extracellular protease isolated from *C. albocinnamomea* was purified approximately 44-fold through two purification steps. SDS-PAGE analyses of the purified protease revealed a single band, and its molecular mass of 39,756 Da was determined using MALDI-TOF-MS. The enzyme was optimally active at approximately pH 7.0 and 45°C. The K_m and V_{max} values for the hydrolysis of azocasein were 2.46 mg/mL and 989 units/min/mg protein, respectively. The enzyme was stable at pH 3.6–8.6 for 16 h and at temperatures $\leq 35^\circ\text{C}$ for 1 h. Enzymatic activity was completely inhibited by Cu^{2+} and Zn^{2+} and markedly by EDTA and phosphoramidon. The N-terminal amino acid sequence ASYRVLPIT is highly similar to those of the members of the metalloprotease family M36, such as keratinase and elastinase. However, the protease did not detectably hydrolyze keratin or elastin. In contrast, the protease hydrolyzed fibrinogen, although there were no significant sequence similarities to the N-terminal amino acid sequences of other fibrinolytic enzymes. These results suggest that the purified protease represents a new neutral metalloprotease with fibrinogenolytic activity.

Key Words: *Cerrena albocinnamomea*; fibrinogenolysis; metalloprotease; neutral protease

Introduction

Proteases mediate numerous biological activities and are essential for producing foods, detergents, pharmaceuticals, leather goods, and fine chemicals. For example, proteases represent 60% of industrial enzymes (Rao et al., 1998). Proteases for biotechnological and industrial use are acquired from numerous sources, such as bacteria, insects, and fungi. In particular, approximately two-thirds of commercial proteases are produced by bacterial and fungal species, including *Aspergillus* spp. and *Anticarsia gemmatilis* (Oliveira et al., 2005), and *Bacillus* spp. (Hadj-Ali et al., 2007). Proteases produced by such microorganisms all have properties essential for biotechnological applications; namely, they are produced extracellularly at higher yields compared with those produced by animals and plants (Gupta et al., 2002).

Neutral proteases account for the largest share of industrial enzymes because they are active under mild catalytic conditions that are less harmful to the environment (Kasana et al., 2011). These enzymes are mainly active within narrow ranges of pH, temperature, and ionic strength. Therefore, the search continues for new microbial neutral proteases for various applications. Here, we describe the purification and characterization of a novel neutral metalloprotease from *Cerrena albocinnamomea*.

Materials and Methods

Protease activity assay. The reaction mixture (final volume, 0.4 mL) containing 0.5% azocasein (Sigma-Aldrich, St. Louis, MO, USA) and 100 mM Tris-HCl buffer (pH 7.5) was incubated at 30°C for 5 min, and enzyme was added to initiate the reaction at 30°C for 1 h, which was then terminated by the addition of 800 μL 10% trichloro-

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Fig. 1. Phylogenetic tree based on ITS-5.8S rDNA and 28S rDNA-D1/D2 sequences of *Cerrena albocinnamomea*.

Scale bar = 0.02 nucleotide substitutions per base. Numbers at the nodes indicate the bootstrap percentage of 1,000 replicates. GeneBank accession numbers are presented in parentheses.

Table 1. Summary of the purification of extracellular protease.

Purification step	Total activity (units)	Specific activity (U/mg)	Protein (mg)	Yield (%)	Purification (-fold)
Crude extract	16,600	748	22.2	100	1
Butyl Toyopearl	2,050	14.8	139	12.3	6.26
CM52 cellulose	375	0.382	982	2.26	44.2

acetic acid. After centrifugation at 13,000 *g* for 15 min at 4°C, the absorbance of the filtered supernatant was measured at 400 nm. One unit of protease activity was defined as an increase in absorbance by 0.1 at 400 nm under the above conditions.

Isolation and cultivation of microorganisms. The fungal species studied here was isolated from soil from a field located on the campus of Hirosaki University. Candidate fungi were isolated on potato dextrose agar supplemented with 20 g/L glucose. To prepare a crude protease extract, each isolate was inoculated from an agar plate into the same liquid medium and cultured at 27°C for 1 month without shaking. The supernatants were collected, and protease activity was measured at pH 5.0 (0.1 M acetate buffer) as well as at pH 7.0 and 9.0 (0.1 M Tris-HCl buffer), according to the assay conditions described above.

Species identification. To identify the fungal isolates, TechnoSuruga Laboratory Co., Ltd. conducted nucleotide sequencing of ITS-5.8S rDNA and 28S rDNA-D1/D2 and generated a phylogenetic tree. PCR was performed using PrimeSTAR HS DNA polymerase (Takara Bio, Japan). Previously reported primers sets were used to amplify the ITS5 and ITS4 sequences of ITS-5.8S rDNA (White et al., 1990) and the NL1 and NL4 sequences of 28S rDNA-D1/D2 (O'Donnell, 1993). The nucleotide sequences of ITS-5.8S rDNA and 28S rDNA-D1/D2 were determined

using the BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). Sequence comparisons were performed using BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST>), and phylogenetic analyses were conducted using MEGA software version 5.2 (Tamura et al., 2011). Distances and clusters were calculated using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis (1,000 replicates) was performed to evaluate the tree topology generated by neighbor-joining data (Felsenstein, 1985).

Enzyme purification. All purification steps were performed at 4°C. The cells were grown in 2 L of medium under the conditions described above. The supernatant was harvested by filtering the culture through two layers of gauze to remove fungi, and a 0.1 volume of 0.2 M acetate buffer (pH 5.0) was added to the filtrate. The cell-free supernatant was adjusted to 20% saturated (NH₄)₂SO₄, centrifuged at 10,000 × *g* for 20 min at 4°C, and loaded onto a Butyl Toyopearl column (25 × 150 mm; TOSOH, Japan) equilibrated with 20% saturated (NH₄)₂SO₄ buffer A (20 mM acetate buffer, pH 5.0). After washing the column with the same buffer, proteins were eluted using a linear gradient of 20–0% saturated (NH₄)₂SO₄ in buffer A. The active fractions were pooled and dialyzed against buffer A and then fractionated using a CM52 Cellulose

Fig. 2. SDS-PAGE analysis (A), and MALDI-TOF/MS spectrum (B), of the purified protease.

Standard proteins (Lane M): Myosin (229 kDa), β -galactosidase (136 kDa), phosphorylase- β (94.6 kDa), bovine serum albumin (71.3 kDa), ovalbumin (45.1 kDa), carbonic anhydrase (32.2 kDa), and soybean trypsin inhibitor (26.8 kDa). Lanes 1 and 2 contain crude extract and purified protease, respectively.

column (15 $\times$ 150 mm; Whatman, GE Healthcare Bio-Science, Piscataway, NJ, USA) equilibrated with buffer A. Proteins were eluted using a linear gradient of 0–0.3 M NaCl in buffer A. The active fractions were pooled and concentrated using a Vivaspin centrifugal filtration device, 10 kDa cutoff (Vivascience, Hanover, Germany), and then stored at -20°C in 50% glycerol.

Gel electrophoresis and mass spectrometry. SDS-PAGE was performed using a slab gel prepared with 3% and 10% acrylamide stacking and separating gels, respectively (Laemmli, 1970). Proteins were detected using Coomassie Brilliant Blue or silver stain. The purified protein solution was desalted using a PD-10 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), and molecular mass was determined using the linear and positive-ion modes of an Autoflex III TOF/TOF (Bruker, Bremen, Germany) MALDI-TOF/MS and a matrix comprising 2,5-dihydroxybenzoic acid.

Enzymatic assays. When the optimum pH was determined, enzyme assays were performed in McIlvaine buffer (pH 4.0–7.0) or 100 mM Tris-HCl buffer (pH 7.0–9.0) at 30°C for 30 min. For pH stability, the enzyme was stored in Brinton-Robinson buffer (diluted 1:2, pH 2.0–12.0) at 4°C for 16 h, and the protease activity was measured in 100 mM Tris-HCl buffer, pH 7.5. To determine the optimum temperature, the purified enzyme preparations were reacted at temperatures ranging from 25°C to 60°C for 15 min under the standard conditions described above. To determine the thermal stability, purified protein preparations were heated at temperatures ranging from 25°C to 50°C for 1 h and then immediately cooled on ice. The catalytic constants of the purified protease using the substrate azocasein were determined using Lineweaver-Burk plots.

Table 2. Effects of metal ions, various inhibitors and reducing agents on the purified protease activity.

Reagents	Concentration	Relative activity (%)
None		100.0 $\pm$ 6.9
Mg ²⁺	5 mM	89.4 $\pm$ 2.3
Ca ²⁺	5 mM	94.0 $\pm$ 4.8
Mn ²⁺	5 mM	41.1 $\pm$ 1.8
Fe ²⁺	5 mM	23.0 $\pm$ 1.8
Co ²⁺	5 mM	83.3 $\pm$ 5.3
Ni ²⁺	5 mM	12.6 $\pm$ 1.0
Cu ²⁺	5 mM	0
Zn ²⁺	5 mM	0
PMSF	5 mM	80.2 $\pm$ 3.3
Benzamidine	5 mM	70.5 $\pm$ 1.0
Aprotinin	5 $\mu\text{g/ml}$	65.5 $\pm$ 2.3
Leupeptin	50 $\mu\text{g/ml}$	83.7 $\pm$ 3.4
Antipain	3 $\mu\text{g/ml}$	100.8 $\pm$ 3.1
Indoacetoamide	5 mM	88.3 $\pm$ 2.8
Pepstatin A	3 $\mu\text{g/ml}$	99.6 $\pm$ 1.1
Phosphoramidon	1 mM	37.4 $\pm$ 1.5
EDTA	10 mM	43.9 $\pm$ 1.2
2-mercaptoethanol	10 mM	22.5 $\pm$ 2.8
DTT	1 mM	38.9 $\pm$ 2.5
Tween 20	15 % (V/V)	90.8 $\pm$ 0.8
Triton X-100	15 % (V/V)	78.7 $\pm$ 2.0
CTAB	25 mM	2.7 $\pm$ 0.9

All values represent the mean of three independent experiments at each substrate concentration.

Effect of metal ions, inhibitors, and detergents on enzyme activity. We tested the effects of 5 mM MgSO₄, CaCl₂, MnCl₂, FeSO₄, CoSO₄, NiCl₂, CuSO₄, and ZnSO₄

Fig. 3. Enzymatic properties of the purified protease.

(A) and (B) show the effects of pH and temperature on the enzymatic activity, respectively. Values are shown as percentages of the optimal activity. The solid and open circles in (A) indicate activity in McIlvaine buffer (pH 4.0–7.0) and Tris-HCl buffer (pH 7.0–9.0), respectively. (C) The residual activity was determined after 16 h incubation at different pH values at 4°C. The maximum activity at pH 7.8 was defined as 100%. (D) The residual activity was determined after 60 min incubation at different temperatures. The maximum activity at 25°C corresponds to 100%. (E) Lineweaver-Burk plots of reactions using the substrate azocasein. Double-reciprocal plots were generated from the initial velocity of the purified protease as a function of substrate concentration.

on protease activity. To classify protease activity, we tested the following protease inhibitors: phenylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, antipain, iodoacetamide, pepstatin A, phosphoramidon, and EDTA. We tested the effects of the reducing agents β -mercaptoethanol (BME) and dithiothreitol (DTT), as well as the detergents Tween-20, Triton X-100, and cetyltrimethylammonium bromide (CTAB), on catalytic activity. The activity of the enzyme without any additive was taken as 100%.

Fibrinogenolytic assay. To determine the fibrinogenolytic pattern, the reaction mixture (final volume, 500 μ l) containing 2.5 mg/mL fibrinogen (Sigma-Aldrich) and 100

mM Tris-HCl buffer (pH 7.5) was incubated with 8 μ g purified enzyme at 30°C. The fibrinogenolytic activity was analyzed using 5 μ l of reaction mixture for 10% SDS-PAGE.

N-terminal amino acid sequence analysis. The purified enzyme was subjected to 10% SDS-PAGE and transferred onto PVDF membranes using an electroblotting system (ATTO, Japan). Edman degradation to determine the N-terminal sequence was performed on the membranes using an automatic protein sequencer (PPSG-31A, Shimadzu, Japan). The amino acid sequence database was searched using the BLAST program.

Table 3. Alignment of the N-terminal amino acid sequences of the purified enzyme with matched peptides using the NCBI or other fibrinolytic enzymes from mushroom.

Protein	Origin	N-terminal amino acid sequences	Identity (%)
Purified protein	<i>Cerrena albocinnamomea</i>	¹ ASYRVLPIT	100
Metallo protease M36	<i>Heterobasidion irregulare</i> TC32-1	²²⁶ ASYRVLPIT	100
Hypothetical protein	<i>Dichomitus squalens</i> LYAD-421 SS1	²²⁷ ASYRVLPIT	100
Hypothetical protein	<i>Punctularia strigosozonate</i> HHB-11173 SS5	²²⁹ ASYRVLPIT	100
17 kDa fibrinolytic enzyme	<i>Schizophyllum commune</i>	HYNIXNSW	0
21 kDa fibrinolytic enzyme	<i>Lyophyllum shimeji</i>	ITFQSASP	0
32 kDa fibrinolytic enzyme	<i>Pleurotus ostreatus</i>	ALRKGGA	11

Results and Discussion

Screening and identification of strains producing neutral proteases

The five newly isolated fungi were incubated with azocasein at pH 5.0, 7.0, and 9.0. To avoid selecting alkaline proteases, we identified a unique strain that exhibited a comparatively higher activity at pH 7.0 than at pH 9.0 (data not shown). The ITS-5.8S rDNA and 28S rDNA-D1/D2 sequences of the isolate were very similar to those of *C. albocinnamomea* and *C. aurantiopora*. The 28S rDNA-D1/D2 sequence was completely consistent with that of *Cerrena albocinnamomea* and *Cerrena aurantiopora*, and the ITS-5.8S rDNA sequence was 99.7% identical to that of *C. albocinnamomea* (Fig. 1).

C. aurantiopora is also called *C. albocinnamomea* because their 28S rDNA-D1/D2 sequences are completely consistent (Yuan, 2014). Therefore, we conclude that the fungal species isolated here was *C. albocinnamomea*. In the same genus, *C. unicolor* is a well known fungus producing laccase (Rogalski and Janusz, 2010), as well as new antiviral, immunomodulatory, and anticancer compounds (Mizerska-Dudka et al., 2015). However, to our knowledge, there are no reports on the purification and characterization of proteases produced by *Cerrena* spp. Therefore, the present study is the first to report the production of a protease by a *Cerrena* sp.

Purification of an extracellular protease

The neutral protease produced by *C. albocinnamomea* was purified to near homogeneity using the methods described above (Table 1). The final enzyme preparation (specific activity, 982 U/mg) contained 0.382 mg protein (2.6% yield), equivalent to a 44.2-fold purification (Table 1). In SDS-PAGE analysis, the purified enzyme migrated as a single 40 kDa band (Fig. 2A), and MALDI-TOF/MS detected a 39,756 Da single, symmetrical peak (Fig. 2B).

N-terminal amino acid sequence

The N-terminal amino acid sequence ASYRVLPIT of the purified protein is identical to residues 226–234 of metalloprotease M36 from *Heterobasidion irregulare* TC32-1 and to those of several hypothetical proteins annotated as fungalyisin metalloprotease M36 (Table 3). These data suggest that the enzyme purified from *C. albocinnamomea* represents a cleavage product of a member of the fungalyisin metalloprotease family M36 that includes endopeptidases of pathogenic fungi. Although M36

Fig. 4. Fibrinolytic pattern analysis of the purified protease.

The purified protease (8 μ g) was incubated with fibrinogen (2.5 mg/mL) at 30°C in 100 mM Tris-HCl (pH 7.0).

endopeptidases cleave extracellular matrix proteins, such as elastin and keratin, they were not detectably hydrolyzed by the purified protease.

Properties of the purified protease

The purified protease was most active at approximately pH 7.0 and 45°C (Figs. 3A and B), indicating that it is a neutral protease. Its activity was maintained at pH 3.5–8.5 (Fig. 3C), and >80% of activity was retained at temperatures $\leq 35^\circ\text{C}$ for 1 h at temperatures ranging from 25°C to 50°C (Fig. 3D). The values of $V_{\max}$ and K_m for azocasein hydrolysis were 989 units/min/mg and 2.46 mg/mL, respectively, (Fig. 3E).

Neutral proteases may be active at an alkaline pH. For example, the metallo-neutral protease produced by *Bacillus amyloliquefaciens* and the neutral serine protease produced by *Penicillium italicum* retain approximately 60% of their activities at pH 10.0 compared with their maximum activities at pH 7.0 (Abidi et al., 2014; Wang et al., 2013). However, the purified protease was active in a narrow pH range, and little activity was detected at pH 9.0.

Effect of metal ions, inhibitors, and detergents on the purified protease activity

The purified protease activity was completely inhibited by Cu^{2+} and Zn^{2+} (0%), markedly reduced by Ni^{2+} , Fe^{2+} , and Mn^{2+} (12.6%, 23.0%, and 41.1%, respectively), and moderately reduced by Co^{2+} , Mg^{2+} , and Ca^{2+} (83.3%, 89.4%, and 94.0%, respectively) (Table 2).

Proteases can be classified according to their sensitivity to various inhibitors (Rao et al., 1998). Interestingly, the purified protease activity was not strongly inhibited by specific protease inhibitors (Table 2). The thiol protease inhibitors antipain and 2-iodoacetamide, as well as the aspartic protease inhibitor pepstatin-A, had almost no influence on enzyme activity. The serine protease inhibitors phenylmethylsulfonyl fluoride, benzamidin, aprotinin, and leupeptin, moderately inhibited proteolytic activity. In contrast, the divalent-cation chelator EDTA and the thermolysin-like protease inhibitor phosphoramidon inhibited activity by 43.9% and 37.4%, respectively, indicating that the enzyme is a member of the metalloprotease family. These data are consistent with those of the members of the metalloprotease family M36.

The purified protease was inhibited by BME and DTT, indicating that both catalytic function and protein structure are maintained by disulfide bonds. Proteolytic activity was slightly reduced by some surfactants like Triton X-100 and Tween 20 (at 15% concentration). In contrast, 25 mM CTAB inhibited activity by 97% (Table 2).

Analysis of fibrinogenolysis

The N-terminal amino acid sequence suggested that the purified protease is a member of the metalloprotease family M36, which includes endopeptidases that cleave extracellular matrix proteins, such as elastin and keratin. However, the enzyme did not hydrolyze azoelastin or azokeratin (data not shown), suggesting that the substrate specificity of the purified protease is unique compared with that of other members of the M36 family.

Certain metalloproteases produced by the edible mushroom (*Lyophyllum shimeji*) and *B. subtilis* hydrolyze fibrin and fibrinogen (Moon et al., 2014; Velusamy et al., 2015). In SDS-PAGE analysis, the purified protease hydrolyzed the $\text{A}\alpha$ -, $\text{B}\beta$ -, and γ -chains of fibrinogen in order of decreasing rates (Fig. 4). Fibrinogen is the precursor of fibrin, which is the primary protein component of blood clots. Therefore, this protease may be useful for preventing thrombotic disease. However, the N-terminal amino acid sequence of the purified protease was 0–11% identical to those of other fibrinolytic enzymes produced by mushrooms (Table 3). These data indicate that the purified enzyme is a novel fibrinogenolytic metalloprotease.

Conclusions

We report here the discovery of a major extracellular protease produced by the soil fungus *C. albocinnamomea*. We classified the purified protease as a member of the metalloprotease family M36 according to its N-terminal amino acid sequence and the effects of enzyme inhibitors on its activity. However, it did not detectably hydrolyze the M36 substrates elastin or keratin. Although it

hydrolyzed fibrinogen, its N-terminal amino acid sequence was not similar to those of fibrinolytic proteases. These results, together with its catalytic properties, suggest that this protease represents a novel neutral metalloprotease. Further studies focusing on cloning the gene encoding this protease are required to facilitate its further characterization.

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