A survey of proteases in edible mushrooms with synthetic peptides as substrates

doi: 10.1007/s10267-010-0089-9

Mycoscience (Revised2)

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Total pages: 22

Tables: 5

Figures: 2

Supplementary materials

Total page: 1

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Abstract The protease activities in six edible mushrooms were surveyed using synthetic fluorogenic substrates that have different specificities for each protease group. The activity was determined by measuring the fluorogenic intensity of the 7-amino-4-methylcoumarin (AMC) liberated by an enzyme. Various types of activities were found in all mushrooms, and their activities depended largely on the mushroom species, but also on pH and localization. *Flammulina velutipes* and *Pleurotus eryngii* had the widest and highest proteolytic activities among the six mushrooms examined. The proteasome-like protease activities were generally much higher than those of other proteases. High caspase activities, which occur during apoptosis in cells, were detected in two mushrooms, *F. velutipes* and *Hypsizigus marmoreus*. The pH optima of the proteolytic activities were largely divided into two groups, acidic pH 5 to 6 for caspases and neutral to alkaline (pH 6.5 to 11) for the others. In *F. velutipes,* higher proteolytic activity was observed in the basement of stem than in the cap and stem. Purification and characterization of protease was also carried out to identify a protease from *Grifola frondosa* using *t*-butyloxycarbonyl-Leu-Arg-Arg-4-methylcoumaryl-7-amide（Boc-LRR-MCA）as the substrate.

Keywords Basidiomycetes · Edible mushroom · Protease activity · Protease survey · Synthetic peptide

Introduction

Edible mushrooms, the fruiting bodies of the basidiomycetes, are quite popular in Asia and commercially cultivated worldwide. They are commonly used as food, food flavoring, and also in traditional Chinese medicines (Park et al. 2007). Eight million tons a year are consumed. Recently, mushrooms have attracted attention as 'functional foods' because of their various physiologically active compounds. It has been reported that the extracts exert hematological, antiviral, antitumorigenic, hypotensive, and hepatoprotective effects (Chang and Miles 1989; Chang 1996; Hobbs 1995). Mushrooms can help prevent life-style related diseases, such as diabetes, apoplectic ictus, cardiac disease, hyperlipemia, elevated blood pressure, and adiposis.

Proteases are a group of fundamentally hydrolytic enzymes that acylate and deacylate to peptide bonds. The enzymes are categorized into four groups, serine proteases, cysteine proteases, aspartic proteases, and metalloproteases, based on their active sites (van der Hoorn 2008). Proteases are characteristically distributed in cells and tissues and function in such major physiological processes as protein turnover, sporulation and conidial discharge, germination, enzyme modification, nutrition, and regulation of gene expression (Horikoshi 1996). Proteases also have important applications in industry including the detergent, food, pharmaceutical, leather, and silk industries, and recovery of silver from used X-ray films (Lee et al. 1991; Kembhavi et al. 1993; Gessesse and Gashe 1997; Anwar and Saleemuddin 1998; Rao et al. 1998). In particular, protease used in the detergent industries, account for 30% of the total world enzyme production (Manachini and Fortina 1998). Microorganisms are the most important sources of enzyme production. However, proteases from edible mushrooms such as *Agaricus bisporus*, *Armillariella mellea*, *Flammulina velutipes*,

Grifola frondosa, *Pleurotus ostreatus*, and *P. eryngii* also play an important role (Wang and Ng 2001; Nishiwaki et al. 2009). As procedures for commercial cultivation of common mushrooms are established, it is considered that mushrooms have a potential as bioresouces as well as nutritious foods.

Although that mushrooms are familiar to the public and commonly used as foods, they have many unknown components, and there are a few reports on the existence and function of their proteases. Metalloendopeptidases (Terashita et al. 1985a; Terashita et al. 1985b; Nonaka et al. 1995; Healy et al. 1999) and aminopeptidases (Abdus Sattar et al. 1989; Nishiwaki and Hayashi 2001) have been purified and characterized from the culture fluids or fruiting bodies of several mushrooms with authentic substrates such as casein and aminoacyl-*p*-nitroanilide. However, the other peptidases from edible mushrooms have received little attention. In this study, to find and characterize peptidase species, we surveyed the proteolytic activities of six species of common edible mushrooms using synthetic fluorogenic peptides as substrates which have different specificities for each protease group. Purification and characterization of a protease from *G. frondosa* are also described.

Materials and methods

Fungi

Six common mushrooms (Japanese name in parenthesis), *F. velutipes* (enokitake), *G. frondosa* (maitake), *Hypsizigus marmoreus* (bunashimeji), *Lentinula edodes* (shiitake), *Pholiota nameko* (nameko), and *P. eryngii* (eringi), were obtained from a local market. Mushrooms without basement of stem were used for experiments.

Chemicals

Chemicals and reagents were obtained from either Wako (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted. The following synthetic peptides and 7-amino-4-methylcoumarin (AMC) were obtained from the Peptide Institute (Osaka, Japan): acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide (MCA) (Ac-DEVD-MCA), acetyl-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA), *t*-butyloxycarbonyl-Leu-Arg-Arg-MCA (Boc-LRR-MCA), *t*-butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA), *N*-succinyl-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), and benzyloxycarbonyl-Leu-Leu-Gln-MCA (Z-LLE-MCA). Protease species that catalyze lysis of the substrates used in this study are summarized in Table 1. *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) was obtained from Sigma (Tokyo, Japan) and other buffers, 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-[Tris(hydroxymethyl)methyl]glycine (tricine), *N*-cyclohexyl-2-amino ethanesulfonic acid (CHES), and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) were from Dojindo (Kumamoto, Japan). Miracloth was purchased from Calbiochem (Darmstadt, Germany).

Extraction of enzyme

All steps were carried out at 4ºC unless otherwise noted. The proteases were extracted from each mushroom as follows. Mushrooms (about 5 g fresh weight) were frozen with liquid N_2 and ground in mortar with 20 mM tris (hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 8.0). The homogenate was filtered through two layers of Miracloth, and the resulting filtrate was centrifuged at 20, 000 x g for 30 min. The

supernatant was collected as a crude extract and used for enzyme assay.

Protease assay

Proteases in the extracts of six edible mushrooms were surveyed using six synthetic fluorogenic substrates, according to the previously reported method (Usui et al. 2007; Watanabe et al. 2009). The reaction mixture consisted of 100 mM MES-HEPES-Tricine (MHT) buffer in the pH range of 5 to 9 at intervals of around 1 pH unit for each substrate, 10 µl of enzyme solution, and 10 mM substrate dissolved in dimethyl sulfoxide (DMSO) in a total volume of 100 µl (the final concentration of DMSO was less than 5% to prevent inhibition). The reaction was started by adding 10 µl of enzyme solution and incubated for 30 min at 37ºC. After incubation, the reaction was stopped by adding 2 ml of 0.1 M borate-NaOH buffer (pH 9.0) containing 1% (w/v) sodium dodecyl sulfate (SDS), and the fluorescence was measured with a fluorescence spectrophotometer, model F-2500 (Hitachi, Tokyo, Japan). The hydrolysis was monitored by fluorescence emission at 460 nm with excitation at 360 nm. The activity was expressed as the amount (nmoles) of 7-amino-4-methylcoumarin (AMC) released from the fluorogenic peptide per g fresh weight (or mg protein) per h at 37ºC. AMC concentrations were estimated by comparing the fluorescence intensity to that of authentic AMC.

Effects of pH on protease activity

The effects of pH on protease activity of crude extracts were assayed in the pH range of 3 to 11. Peptide hydrolytic activity was assayed in a reaction mixture containing 50 mM succinate-borax (SB) buffer (pH range 3-5), 50 mM MHT buffer (pH range

5-9), and 50 mM CHES-CAPS (CC) buffer (pH range 9-11) using 0.1 mM fluorogenic substrate. The enzymatic reaction was carried out for 30 min at 37ºC.

Localization of protease activity

The localization of proteolytic activity was measured using the fruiting body of *F. velutipes.* The mushroom body was cut into three parts (cap, stem, and basement of stem), and the peptide hydrolytic activity of each part was measured as described above.

Effects of growth on protease activity

To elucidate the effects of growth on protease activity, the fruiting body of *F. velutipes* was examined. This mushroom increases in height as it grows. After separating the mushrooms into three groups (less than 4 cm, 4-9 cm, and more than 9 cm according to their height without basement of stem), peptide hydrolytic activity was measured as described in the text.

Purification of protease from *G. frondosa*

In this experiment, protease activity was measured in a similar manner as described in **Protease assay**, except for Boc-LRR-MCA as a substrate. All procedures were carried out at 4ºC unless otherwise noted. Mushroom fruiting bodies (360 g fresh weight) were homogenized in 400 ml of 20 mM Tris-HCl buffer (pH 7.0) with a blender. The homogenate was filtrated through four layers of Miracloth and centrifuged at 22,000 x g for 50 min. The resulting supernatant was used as the crude extract. Then $(NH_4)_2SO_4$ was added to the crude extract with stirring to give a 40%

(w/v) saturation. The stirring was continued for 30 min, and the solution was left to stand for 1 h. The solution was centrifuged at 22,000 x g for 15 min, and the pellet was discarded. $(NH_4)_2SO_4$ was again added to the supernatant to 60% (w/v) saturation and stirring continued for 30 min. The pellet was collected by centrifugation at 22,000 x g for 15 min and dissolved in a small volume of 20 mM Tris-HCl buffer (pH 7.0). The enzyme solution was dialyzed against the same buffer, and then it was applied to a column (2.5 x 12 cm) of DE52 (Whatman, Kent, England) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was washed with three column volumes of the same buffer and eluted with 400 ml of 20 mM Tris-HCl buffer containing a linear gradient of NaCl from 0 to 0.5 M at pH 7.0. The fractions with high protease activity (0.25 M NaCl) were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). The dialyzed sample was applied to a column (1.6 x 6 cm) of DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was washed with three column volumes of the same buffer and eluted with 100 ml of 20 mM Tris-HCl buffer containing a linear gradient of NaCl from 0 to 0.5 M at pH 7.0. The fractions with high activity (0.17 M NaCl) were collected and concentrated with a Centriplus YM-10 (Millipore, Bedford, MA). The concentrated sample was loaded onto a column (1.6 x 60 cm) of Superdex 200 HiLoad 16/60 (GE Healthcare, Buckinghamshire, England) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl with an ÄKTAprime (GE Healthcare). Proteins were eluted with the same buffer, and fractions with high activity were concentrated as described above. The concentrated sample was loaded onto a column (0.5 x 5 cm) of MonoQ (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) and eluted with 30 ml of 20 mM Tris-HCl buffer (pH 7.0) containing a linear

gradient of NaCl from 0 to 0.4 M at pH 7.0. The proteins fractions with high activity (0.3 M NaCl) were used for characterization (see Table).

Molecular weight determination

Molecular weight was determined by gel filtration with a Superdex 200 HiLoad 16/60 using an ÄKTAprime system and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The column was equilibrated and eluted with 20 mM Tris-HCl (pH 7.0) containing 150 mM NaCl at a flow rate of 0.2 ml per min. Fractions (0.3 ml) were collected. The column was calibrated with the following molecular weight (M_r) markers (Sigma): blue dextran (M_r 2,000 k), thyroglobulin (M_r 669 k), β-amylase (M_r 200 k), γ-globulin (M_r 160 k), bovine serum albumin (BSA) (M_r 67 k), and cytochrome *c* (M_r 12.4 k).

Electrophoresis

SDS-PAGE was performed using 12% (w/v) polyacrylamide gel under reducing conditions. Heat treatment was performed at 95°C for 5 min. Fixing and staining were done in aqueous methanol (25%, v/v) containing acetic acid (7.5%, v/v) and 0.25% (w/v) Coomassie brilliant blue R-250, respectively. Molecular markers used were SDS-PAGE molecular weight standards, broad range (Bio-Rad, Tokyo, Japan).

Protein assay

Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, IL, USA) with BSA as a standard.

Results

Proteolytic activity in mushrooms

Proteolytic activities of common edible mushrooms were surveyed using substrates that have different specificities for protease. As shown in Table 2, mushrooms have various types of activities. *P. eryngii* and *G. frondosa* had relatively high activity for all substrates, except for Ac-DEVD-MCA and Ac-YVAD-MCA. In contrast, *Ph. nameko* and *L. edodes* showed low activities for most substrates. *F. velutipes* and *H. marmoreus* had high activities for Ac-DEVD-MCA and Ac-YVAD-MCA, which are substrates for caspases that function in apoptosis of the cells. Despite differences in mushroom species, quite similar acidic pH optima in the rage of 5 to 6 were obtained for caspases using Ac-DEVD-MCA and Ac-YVAD-MCA as the substrate, while a broader spread, neutral to alkaline pH optima in the range of 6.5 to 11, was obtained for the other substrates (Table 2).

Localization of proteolytic activity

F. velutipes has morphologically distinct parts that were used for localization of the protease activity. This mushroom mostly showed the highest activity in the basement of stem, and low and comparable activities in the stem and cap (Table 3).

Effects of height of fruiting body on protease activity

F. velutipes obtained from a local market has different height of fruiting body. The effects of height as an indicator of growth on the protease activity were examined (Supplementary Fig. S1). The enzyme from a higher sample had slightly higher proteolytic activity with all substrates tested than the lower samples. However, the

differences were within 1.5-fold, indicating that the activities are not affected severely by height.

Purification and characterization of protease

An attempt was made to identify a protease by purification and characterization of *G. frondosa* using Boc-LRR-MCA as the substrate. This enzyme was simply selected from its higher enzyme activity and easy purification in preliminary experiments. The protease was purified almost to homogeneity from the crude extracts of the fruiting body by successive chromatographies using DE52, DEAE-Toyopearl 650M, Superdex 200 HiLoad 16/60 gel filtration, and MonoQ columns (Table 4 and Figure 1). The purified enzyme showed K_m of 16.5 μM for Boc-LRR-MCA with a broad pH optimum at 8 to 11 (Figure 2A) and optimum temperature of 50ºC. The temperature at which the activity of the enzyme was decreased by half was 57ºC (Fuigure 2B). The substrate specificity of the enzyme was determined using 6 synthetic peptide substrates (Table 1). This enzyme digested mainly Boc-LRR-MCA (100%) and Suc-LLVY-MCA (18.6%), which is a substrate of proteasomes, whereas activities of other substrates were less than 2%. The enzyme was not affected by the serine protease inhibitor phenyl methanesulphonylfluoride (PMSF) (1 mM) or the cysteine protease inhibitor E-64 (0.1 mM), but it was severely inhibited by EDTA (97% inhibition at 4 mM) (Table 5). However, instead of activation, a rather slight inhibition was observed by the addition of common divalent and monovalent metal ions (4 mM) such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{+}, except for Ca^{2+} (13% increase). The molecular weight of the enzyme was estimated to be 33.3 k by SDS-PAGE (Figure 1) and 128 k by gel filtration, indicating that this enzyme is a tetramer. The N-terminal amino acid sequence of the

purified enzyme up to 20 residues was determined to be RTQDNAPWGLNRISQGPPLA. A database search using BLAST revealed that the sequence obtained in our study completely matched that of the serine protease predicted for the cloned cDNA encoding of 379 amino acids from the same mushroom, *G. frondosa* (Islam 2008), suggesting that this enzyme is the same enzyme reported previously.

Discussion

In the present study, we examined various proteolytic activities of mushrooms. The proteolysis depended on the mushroom speices used, pH, substrate, and localization on the fruiting body. In particular, *F. velutipes* and *P. eryngii* had higher enzymatic activity for most of the substrates than the other mushrooms used in this study. The extracted enzymes recognized the C-terminal residue of amino acids in the fluorogenic substrate and cleaved it to liberate AMC. The C-terminal residues of the substrate showing high activity on *F. velutipes* and *P. eryngii* were Asp and Glu and Arg, Lys, and Tyr, respectively. Although the pH optima of the proteolytic activities were largely divided into two groups, acidic pH 5 to 6 for caspase and neutral to alkaline (pH 6.5 to 11) for the others, the pH optima for the caspases were quite similar among mushrooms. These results may imply that these enzymes are activated in similar organs or events and play a role in all mushroom species. Additionally, high activity was found in a base called the basement of stem in *F. velutipes* and this is probably related to the main place where absorption and distribution of nutrients occur in this mushroom. The effects of growth on the activity were not conspicuous, however.

We purified and characterized the protease from *G. frondosa* using Boc-LRR-MCA as a substrate. The N terminal amino acid sequence exactly matched up to 20 residues that the sequence predicted for the serine protease that was previously cloned from cDNA of this mushroom and expressed (Islam 2008). Although limited information is available concerning the properties of this recombinant enzyme, similar broad pH and temperature optima in the recombinant protein were reported (Islam 2008) (see Figure 2). The results of characterization of purified protease show that the enzyme clearly belongs to family S8 pepetidase (MEROPS) (Rawlings et al. 2010). Members of this family have a catalytic Ser residue in the active center and most of the members are active at neutral-mildly alkaline pH and are thermostable. Moreover, our enzyme belongs to members of subfamily S8B from its specificity with a preference to cleave after dibasic amino acids. Some of the enzymes of this subfamily are resistant to PMSF. In contrast, many members of the family bind calcium for stability and inhibition can be seen with EDTA and EGTA, as in the case of this study (Table 5).

At present, studies of mushrooms are increasing but mostly in the fields of pharmacology, angiology, and clinical medicine. There are few reports on dietary components, especially proteases. In addition, because mushrooms have a simple structural system compared to that of animals and plants, they are useful to understand the mechanisms of protease actions *in vivo* and localization of proteases in cells. Interestingly, activities of caspases, which are a family of cysteine proteases, were found in several mushrooms. Caspases are essential for apoptosis, one of the main types of programmed cell death in development and most other stages of adult life, and are conserved widely from *Caenorhabditis elegans* to mammals (van der Hoorn 2008).

Recently, evidence from inhibitor studies and biochemical approaches as well as homology with genes that control apoptosis in animals are encoded in plant genomes that a caspase-like protease may also be involved in cell death in higher plants was reported (Sanmartin et al. 2005; Lam and Pozo 2000). There has been, however, little information on caspases in mushrooms until now. Our finding indicates that caspases are involved in induction of apoptosis in mushrooms as in other biological systems. We are now cloning and characterizing the caspase from *F. velutipes* and *H. marmoreus.*

Acknowledgements

This study was financially supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The research was partially carried out using facilities at the Institute for Genetic Research and Biotechnology.

Supporting Information

Supplementary Figure S1. Effects of height of fruiting body on proteolytic activity

References

Abdus Satter, A M K, Yoshimoto, T, Tsuru, D (1989) *Lyophyllum cinerascens* aminopeptidase: Purification andenzymatic properties. Arch Biochem Biophys 274: 241-250

Aki M, Shimbara N, Takashina M, Akiyama K, Kagawa S, Tamura T, Tanahashi N, Yoshimura T, Tanaka K, Ichihara A (1994) Interferon-gamma induces different subunit organizations and functional diversity of proteasomes. J Biochem 115: 257-269

Anwar A, Saleemuddin M (1998) Alkaline proteases: a review. Bioresource Technol 64: 175-183

Chang S T, Miles P G (1989) Edible Mushrooms and Their Cultivation, CRC Press: Boca Raton, p 345

Chang S T (1996) Mushroom research and development, equality and mutual benefit. International Conf. Mushroom Biology and Mushroom Products, 2nd ed., Royse D J Ed., The Pennsylvania State University Press, Philadelphia, pp 1-10

Gessesse A, Gashe B A (1997) Production of alkaline protease by an alkaliphilic bacteria isolated from an alkaline soda lake. Biotechnol Lett 19: 479-481

Healy, V, O'Connell, J, McCarty, T V, Doonan, S (1999) The lysine-specific proteinase from *Armillaria mellea* is a member of a novel class of metalloendopeptidases located in Basidiomycetes. Biochim Biophys Res Commun 262: 60-63

Hobbs C (1995) Medicinal mushrooms: An exploration of tradition, healing and culture. Botanica Press, Santa Cruz, p 252

Horikoshi K (1996) Alkalophiles-from an industrial point of view. FEMS Microbiol Rev 18: 259-270

Ishiura S, Sano M, Kamakura K, Sugita H (1985) Isolation of two forms of the high-molecular-mass serine protease, ingensin, from porcine skeletal muscle. FEBS Lett 189: 119-123

Islam M M (2008) Molecular cloning, expression and characterization of a serine proteinase from Japanese edible mushroom, *Grifola frondosa*: solving the structure-function anomaly of a reported aminopeptidase. Elect J Biotechnol 11: 1-12

Kato H, Adachi N, Ohno Y, Iwanaga S, Takada K, Sakakibara S (1980) New fluorogenic peptide substrates for plasmin. J Biochem 88: 183-190

Kembhavi A A, Kulkarni A, Pant A (1993) Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis*. Appl Biochem Biotechnol 38: 83-92

Lam E, Pozo O D (2000) Caspase-like protease involved in the control of plant cell death. Plant Mol Biol 44: 417-428

Lee W J, Cho Y J, Son G M, Choi C (1991) Characteristic and action pattern of alkaline protease production from *Bacillus* sp. Korean Biochem J 24: 537-542

Manachini P L, Fortina M G (1998) Production in seawater of thermostable alkaline protease by a halotolerant strain of *Bacillus licheniformis*. Biotechnol Lett 20: 565-568

Mizuno K, Nakamura T, Takada K, Sakakibara S, Matsuo H (1987) A membrane-bound, calcium-dependent protease in yeast alpha-cell cleaving on the carboxyl side of paired basic residues. Biochem Biophys Res Commun 144: 807-814

Nicholson D W, Ali A, Thornberry N A, Vaillancourt J P, Ding C K, Gallant M, Gareau Y, Griffin P R, Labelle M, Lazebnik Y A et al. (1995) Identification and

inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376: 37-43

Nishiwaki T, Hayashi K (2001) Purification and characterization of an aminopeptidase from the edible basidiomycete *Grifola frondosa*. Biosci Biotechnol Biochem 65: 424-427

Nishiwaki T, Asano S, Ohyama T (2009) Properties and substrate specificities of proteolytic enzymes from the edible basidiomycete *Grifola frondosa*. J Biosci Bioeng 107: 605-609

Nonaka, T, Ishikawa, H, Tsumuraya, Y, Hashimoto, Y, Dohmae, N, Takio, K (1995) Characterization of a thermostable lysine-specific metalloendopeptidase from the fruiting bodies of a basidiomycete, *Grifola frondosa*. J Biochem 118: 1014-1020

Ozaki M, Fujinami K, Tanaka K, Amemiya Y, Sato T, Ogura N, Nakagawa H (1992) Purification and initial characterization of the proteasome from the higher plant *Spinacia oleracea.* J Biol Chem 267: 2168-21684

Park S E, Li M H, Kim J S, Sapkota K, Kim J E, Choi B S, Yoon Y H, Lee J C, Lee H H, Kim C S, Kim S J (2007) Purification and characterization of fibrinolytic protease from a culture supernatant of *Flammulina velutipes* mycelia. Biosci Biotechnol Biochem 71: 2214-2222

Rao M B, Tanksale A M, Ghatge M S, Deshpande V V (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597-635

Rawlings, N D, Barrett, A J, Bateman, A (2010) MEROPS: the peptidase database. Nucleic Acids Res 38: D227-D233

Sanmartin M, Jaroszewski L, Raikhel N V, Rojo E (2005) Caspases. Regulating death since the origin of life. Plant Physiol 137: 841-847

Sasaki T, Kikuchi T, Yumoto N, Yoshimura N, Murachi T (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. J Biol Chem 259: 12489-12494

Sawada H, Yokosawa H, Hoshi M, Ishii S (1983) Ascidian sperm chymotrypsin-like enzyme; participation in fertilization. Experientia 39: 377-378

Terashita, T, Oda, K, Kono, M, Murano, S (1985a) Purification and some properties of metal proteinases from *Lentinus edodes*. Agric Biol Chem 49: 2293-2300

Terashita, T, Oda, K, Kono, M, Murano, S (1985b) Proteinases systems in *Flammulina velutipes* and *Pleurotus ostreatus.* Trans Mycol Soc Jpn 26: 397-409

Thornberry N A, Bull H G, Calaycay J R, Chapman K T, Howard A D, Kostura M J, Miller D K, Molineaux S M, Weidner J. R, Aunins J et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356: 768-774

Thornberry N A, Rano T A, Peterson E P, Rasper D M, Timkey T, Garcia-Calvo M, Houtzager V M, Nordstrom P A, Roy S, Vaillancourt J P, Chapman K T, Nicholson DW (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272: 17907-17911

Tsukahara T, Ishiura S, Sugita H (1988) An ATP-dependent protease and ingensin, the multicatalytic proteinase, in K562 cells. Eur J Biochem 177: 261-266

Usui M, Tanaka S, Miyasaka H, Suzuki Y, Shioi Y (2007) Characterization of cysteine

protease induced by oxidative stress in cells of *Chlamydomonas* sp. strain W80. Physiol Plant 131: 519-526

van der Hoorn R A L (2008) Plant proteases: from phenotypes to molecular mechanisms. Annu Rev Plant Biol 59: 191-223

Wang H X, Ng T B (2001) Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom *Pleurotus eryngii*. Biochem Biophys Res Commun 289: 750-755

Watanabe Y, Matsushima S, Yamaguchi A, Shioi Y (2009) Characterization and cloning of cysteine protease that is induced in green leaves of barley. Plant Sci 176: 264-271

Figure legends

Figure 1. SDS-PAGE of purified serine protease from *G. frondosa*. Purified protease was electrophoresed in a 14% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Purified protease is shown in arrow. M, Molecular markers. The molecular mass of each marker protein is given on the right in kDa. E, enzyme; M, molecular markers.

Figure 2. (A) pH optimum of serine protease activity from *G. frondosa*. The enzyme assay was performed with 50 mM MHT buffer (pH 5-9) and 50 mM CC (pH 9-12) buffer at the indicated pH value from 5 to 12, as described in the text. Experiment was done three times and its average is shown with SE. (B) Thermal staibility of serine protease from *G. frondosa*. The thermal stability of the enzyme was examined at temperatures from 10 to 60 ℃. Residual activity of the enzyme was assayed in the standard conditions after incubation at the indicated temperature for 10 min in the absence of substrate.

Table 1. Substrates used in this study

Substrate	Protease	Reference
Ac-DEVD-MCA	Caspase-3/-7/-8	Nicholson et al. 1995; Thornberry et al. 1997
Ac-YVAD-MCA	Caspase-1	Thornberry et al. 1992
Boc-LRR-MCA	Carboxyl side of paired basic residue cleaving enzyme and proteasome	Mizuno et al. 1987; Aki et al. 1994
Boc-VLK-MCA	Plasmin and Calpain	Kato et al. 1980; Sasaki et al. 1984
Suc-LLVY-MCA	Chymotrypsin, Ingensin/Proteasome and Calpain	Sawada et al. 1983; Ishiura et al. 1985; Tsukahara et al. 1988; Sasaki et al. 1984
Z-LLE-MCA	Proteasome, V8 protease-like	Ozaki et al. 1992

Table 2. Proteolytic activities and their pH optima in six edible mushrooms assayed with synthetic peptides

Species	Substrate											
	Ac-DEVD-MCA		Ac-YVAD-MCA		Boc-LRR-MCA		Boc-VLK-MCA		Suc-LLVY-MCA		Z-LLE-MCA	
	Activity	pH optimum	Activity	pH optimum	Activity	pH optimum	Activity	pH optimum	Activity	pH optimum	Activity	pH optimum
Flammulina velutipes	42.88	6.0	141.12	6.0	126.53	7.0	16.98	10.0	89.96	7.0	65.15	8.0
Grifola frondosa	5.06	5.5	26.79	6.0	304.25	11.0	24.30	6.5	598.83	7.0	42.67	6.5
Hypsizigus marmoreus	30.99	5.0	80.46	5.5	97.27	9.0	29.32	7.5	68.37	7.0	21.05	6.5
Lentinula edodes	2.38	5.5	1.39	5.5	34.20	8.0	2.24	9.0	168.03	7.5	25.53	7.5
Pholiota nameko	2.02	6.0	1.38	5.0	215.25	8.0	4.69	7.5	29.05	9.0	8.14	7.0
Pleurotus eryngii	3.51	5.5	7.05	5.0	500.66	7.0	62.20	7.5	1089.78	8.5	27.67	7.5

The results are averages of more than three experiments, and SE is less than ±17%.

The activity was expressed as nmoles of AMC released from the fluorogenic peptide per g fresh weight per h at 37ºC.

Table 3. Localization of proteolytic activity in the fruiting body of *F. velutipes*

Substrate	Protease activity (nmol/mg protein/h)		
	Cap	Stem	Basement of stem
Ac-DEVD-MCA	0.23	0.19	0.77
Ac-YVAD-MCA	3.33	1.95	10.8
Boc-LRR-MCA	1.74	2.03	6.86
Boc-VLK-MCA	0.52	0.19	4.06
Suc-LLVY-MCA	2.50	2.09	11.5
Z-LLE-MCA	0.46	0.91	0.70

The results are averages of three experiments and SE is less than 8.6%.

Table 4. Summary of purification of serine protease from *G. frondosa*

Purification step	Total protein (mg)	Total activity (nmol/h)	Specific activity (nmol/h/mg protein)	Purification (-fold)	Yield (%)
Crude extract	8773	298271	34.00	1.00	100
$(NH_4)_2SO_4$	1732	119573	69.03	2.03	40.1
DE52	87.01	22966	263.9	7.76	7.7
DEAE Toyopearl 650-M	4.75	8684	1826	53.7	2.9
Superdex 200 HiLoad	0.69	4239	6178	182	1.4
MonoQ	0.07	559.7	8425	248	0.19

Crude extract was obtained from 360 g (fresh weight) of fruiting body of *G. frondosa*. The enzyme activity was assayed as described in the text. The activity corresponds to the amount of hydrolyzed Boc-LRR-MCA.

Table 5. Effects of inhibitors on the serine protease from *G. frondosa*

Inhibitor	Concentration (mM)	Relative activity (%)
control		100±1.3
PMSF	1	111.6±4.6
Leupeptin	1	20.7±1.5
EDTA	5	2.6±0.1
EGTA	5	0.7±0.3
E-64	0.1	109.2±2.9
NEM	10	102.5±0.3
Pepstatin	10	113.5±5.2

The results are averages of three experiments and SE is shown.

Figure. 1　（Nakamura et al.）

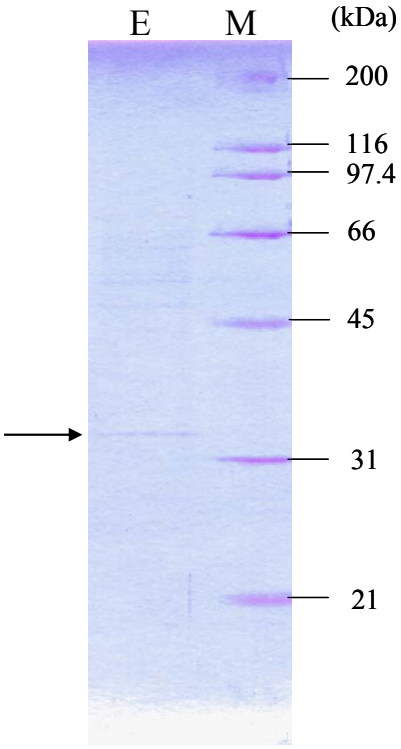
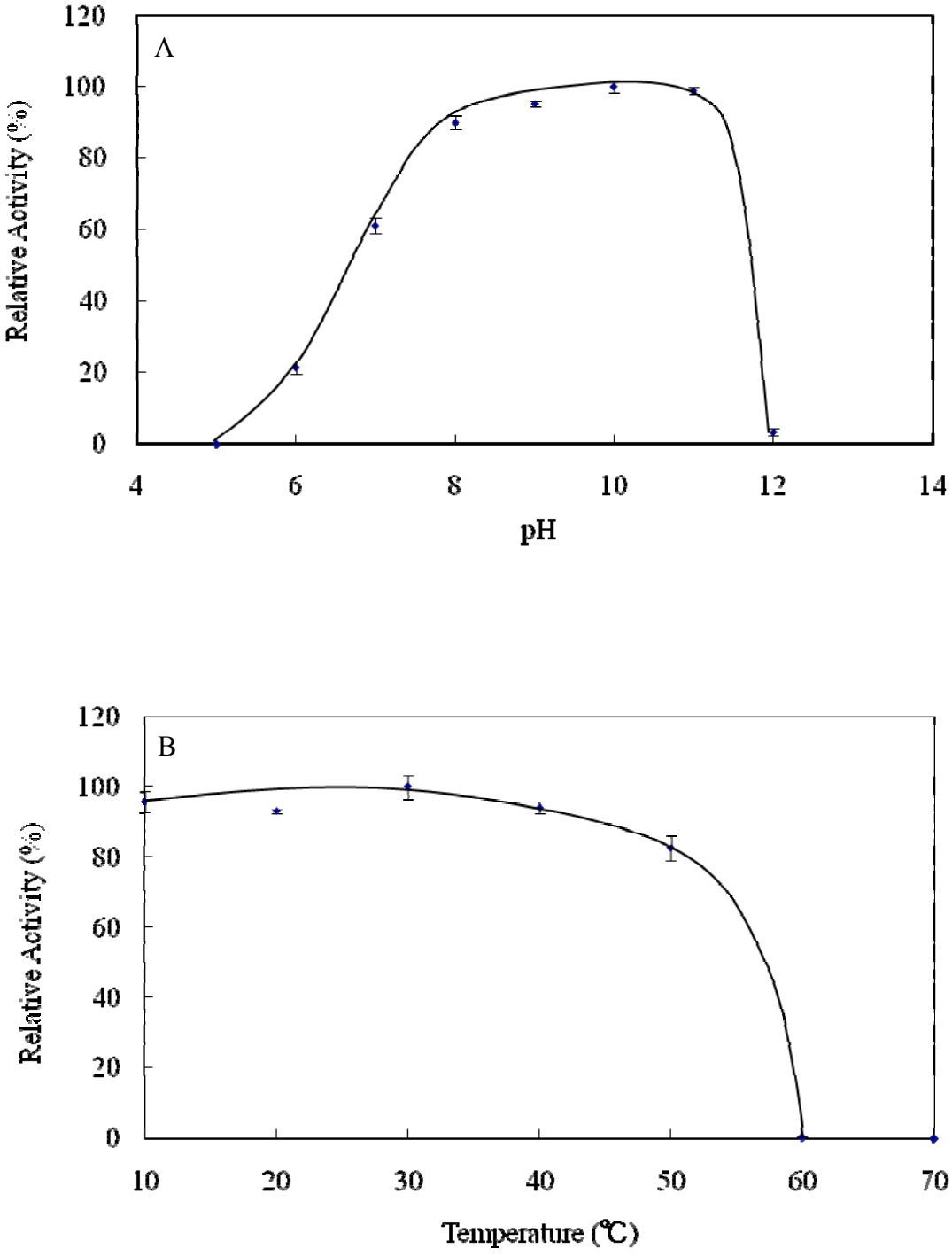


Figure. 2 (Nakamura et al.)



Supporting Information

Fig. S1. (Nakamura et al.)

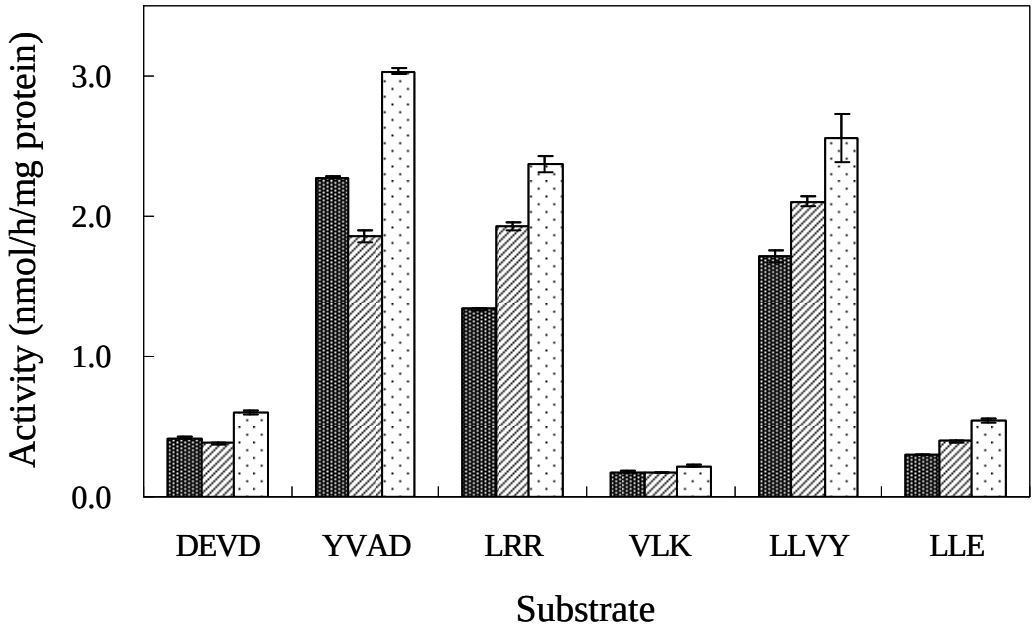


Fig. S1. Effects of length on proteolytic activity. Enzymatic activity was assayed using the indicated synthetic substrates in a medium containing 50 mM MHT buffer (pH 7.0) as described in the text. The results are averages of three experiments, and SE is shown. Bars: gray, <4 cm; stripe, 4-9 cm; dot, >9 cm