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FULL PAPER

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

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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 the 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 5–6 for caspases and neutral to alkaline (pH 6.5–11) for the others. In *F. velutipes,* higher proteolytic activity was observed in the basement of the stem than in the cap and stem. Purification and characterization of protease were 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.

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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 lifestyle-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, proteases 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 potential as a bioresource as well as a nutritious food.

Although mushrooms are familiar to the public and commonly used as foods, they have many unknown components, and there are few reports on the existence and function of their proteases. Metalloendopeptidases (Terashita et al. 1985a, b; 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 the basement of the stem were used for the 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 4°C 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 a 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×*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

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