Metalloendopeptidases from edible mushrooms: Unique properties and structural analyses

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Acyl-lysine bond (-X-Lys-) specific zinc metalloendopeptidases, GFMEP and POMEP, have been purified from *Grifola frondosa* and *Pleurotus ostreatus*, which are popular mushrooms called "MAITAKE" and "HIRATAKE", respectively, in Japan. Prominent features of GFMEP are its high optimum pH, heat stability and affinity for β-glucans and chitin. Sequence homology around the putative zinc binding sites suggests that they belong to a new subfamily of metalloendopeptidases. Several fluorogenic peptides have been synthesized to examine the detailed specificity of the enzymes.

Intracellular metalloendopeptidases and acid proteases with broad specificity toward hydrophobic amino acid residues have been purified and characterized from *Lentinus edodes* and *Flammulina velutipes* fruiting bodies, called as "SHIITAKE" and "ENOKITAKE", respectively, in Japan, and the enzymes have been suggested to play a key role in fruiting body formation.[1)]

Zinc-metalloendopeptidases, GFMEP and POMEP, have been purified and characterized from the fruiting bodies of *Grifola frondosa*[2)] and *Pleurotus ostreatus*[3)] (Fig. 1) known as "MAITAKE" and "HIRATAKE", respectively. Both of them consist of single polypeptide chains with apparent molecular masses of 20 kDa and contain 1 atom zinc/molecule which is indispensable for their proteolytic activity. Strict specificity of the enzymes toward acyl-lysine (X-Lys) bonds was established by the digestion of horse heart cytochrome *c*. While POMEP has its pH optimum at 5.6, GFMEP is most active at pH 9.5 and exhibits more than 50% of the maximal activity within the pH range of 6–10.5. These enzymes can be inactivated by chelating agents such as EDTA and 1,10-phenanthroline and the activity of apo-enzymes can be restored by the addition of Mn^{2+}, Zn^{2+}, Ca^{2+}, or Co^{2+}. The most effective divalent metal ion for apo-GFMEP is Mn^{2+}, with which the enzyme regains the activity 2.2 times higher than the original activity at 50 μM. This enzyme is stable even at 80°C, because about 60% of the original activity remains after incubation for 3 hrs, as shown in Fig. 2. GFMEP also retains full activity in the presence of 4 M urea. Stability against denaturing agents and high temperature is similar to POMEP.

Fig. 1. *Pleurotus ostreatus*, an edible mushroom called "HIRATAKE".

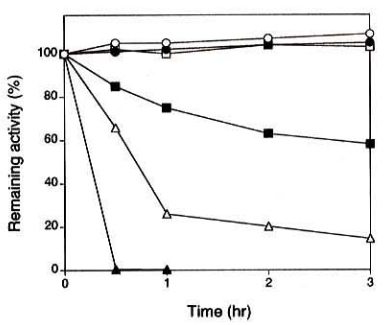

Fig. 2. Effects of temperature on GFMEP. ○, 50°C; ●, 60°C; □, 70°C; ■, 80°C; △, 90°C; ▲, 100°C.

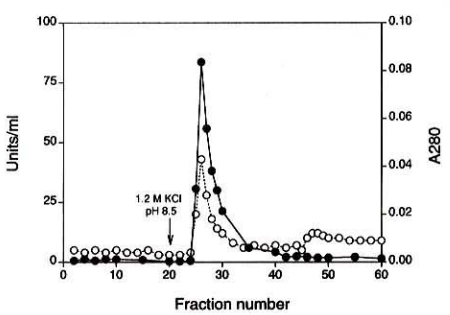

Fig. 3. Binding of GFMEP to cellulose. GFMEP was applied to a cellulose powder column equilibrated with 20 mM Na-acetate buffer, pH 5.0. The enzyme was eluted from the column with 0.1 M Tris-HCl buffer, pH 8.5, containing 1.2 M KCl. ●, Proteinase activity; ○, Absorbance at 280 nm.

Furthermore, GFMEP binds tightly to cellulose, curdlan (β-1,3-glucan) and chitin. No appreciable activity could be detected in the breakthrough fraction (Fig. 3), when GFMEP

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was loaded onto a cellulose column at pH 5.0. Attempts to recover GFMEP from the cellulose column with 1.2 M KCl, 20 mM EDTA containing 4 M urea or 0.5 M cellobiose were unsuccessful. GFMEP can be eluted almost quantitatively only with a pH 8.5 buffer containing 1.2 M KCl. GFMEP is fully active in the presence of cellulose. The Zn^{2+}-depleted or Mn^{2+}-substituted GFMEP can be equally retained on cellulose. However, GFMEP passed through a Sephadex G-25 column at pH 5.0, indicating that the enzyme lacks affinity for dextran, α-1,6-linked glucose polymer. Its strong interaction with both β-1,3-glucan and chitin, major polysaccharides constituting the fungous cell wall, may provide an indication for the cellular localization and function of this enzyme.

To determine primary structures of these unique enzymes, reduced and alkylated proteins were digested by several proteases or cleaved with various chemical methods. Amino acid composition, sequence and/or mass spectrometric analyses of the isolated fragments were performed. As a result, GF- and POMEP are found to be single-chain polypeptides of 167 and 168 amino acid residues, respectively, with two conserved intramolecular disulfide bonds. In addition, an unusual post-translational modification of GFMEP, partial attachment of a single mannose residue to threonine-42, was identified by mass spectrometry in combination with sugar analysis. Sequence identity between the two enzymes was 61.3%. These sequences somewhat resemble those of two fungous metalloendopeptidases, *Aspergillus oryzae* neutral proteinase II (NPII)[4] and penicillolysin[5] from *Penicillium citrinum*, which cleave specifically the peptide bonds involving the α-amino group of hydrophobic amino acid residues. The sequences of these four enzymes were found to contain the consensus zinc binding motif, HExxH, shared in most metalloproteinases. Metalloendopeptidases are classified into two major subfamilies, Gluzincins and Metzincins, based on homologous sequences around the other ligands to zinc.[6] As shown in Fig. 4, GF-, POMEP and two fungous enzymes have no consensus sequences of the two subfamilies except for HExxH. These suggest that these four fungous metalloendopeptidases must be categorized into a new subfamily.

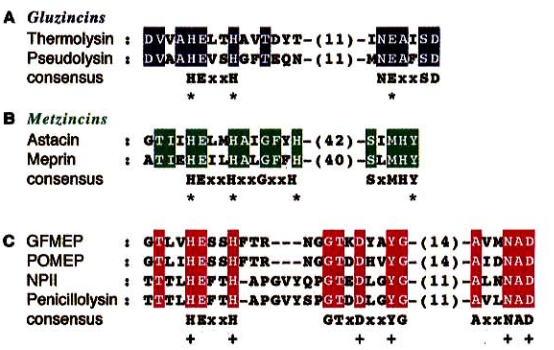

Fig. 4. Comparison of the sequences around the putative zinc binding sites. Panels A and B show consensus sequences of representative metalloproteinase subfamilies, Gluzincins and Metzincins, respectively. Sequences around HExxH of four fungous metalloendopeptidases are aligned in panel C. Asterisks indicate zinc ligands determined by X-ray crystallography. The putative zinc binding ligands are indicated by +.

To elucidate the three-dimensional structure of GFMEP by X-ray crystallography, crystallization has been tried by the hanging drop vapor diffusion method (Fig. 5). The crystal structure analysis is in progress.

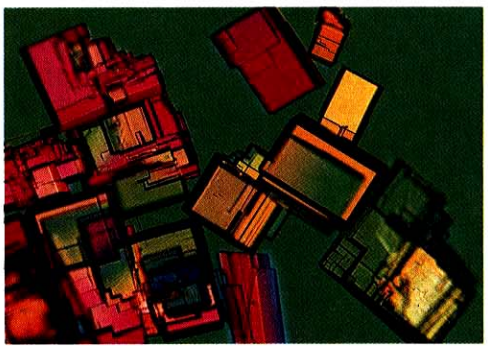

Fig. 5. Crystals of GFMEP.

To examine the detailed specificity of GF- and POMEP, we synthesized several intramolecularly quenched fluorescent peptides. Each of them has the fluorescent residue, tryptophan (Trp), at the carboxyl terminus and the quenching group, dinitrophenol (DNP), at the amino terminus. Quenching of the Trp fluorescence in the intact substrate is relieved on hydrolysis of the -X-Lys- bond, giving rise to continuous increase in fluorescence. The substrate, DNP-Ser-Thr-Ala-Thr-Lys-Leu-Ser-Trp was an efficient substrate for both enzymes, with kcat/Km values of 488.7×10^4 and 89.2×10^4 $M^{-1}s^{-1}$ for GF- and POMEP, respectively. Peptides containing aspartic acid (Asp) adjacent to Lys residue were found to be poor substrates for both enzymes. Neither the shortest peptide, DNP-Thr-Lys-Trp, nor peptides with substitution of arginine, ornithine (Lys analog) or D-Lys (stereo isomer) for Lys were hydrolyzed by both enzymes. These results provide proofs for the strict specificity of GF- and POMEP toward the peptide bonds, -X-Lys-. GF- and POMEP may become indispensable tools for structural studies on proteins owing to its strict specificity, high heat stability, resistance to denaturing agents, and ease of inhibition.

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