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The Lysine-Specific Proteinase from *Armillaria mellea* Is a Member of a Novel Class of Metalloendopeptidases Located in Basidiomycetes

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The fruiting body of the basidiomycete fungus *Armillaria mellea* produces a lysine-specific proteinase which exhibits both potent fibrinolytic activity and a remarkable resistance to denaturing agents. An improved purification protocol has been developed for this enzyme and the sequence of the 26 N-terminal amino acid residues of the pure protein has been determined by gas-phase sequencing. Searches of the SwissProt database showed that the N-terminal sequence of *A. mellea* proteinase is highly similar to those of lysine-specific metalloendopeptidases from the basidiomycetes *Grifola frondosa* and *Pleurotus ostreatus.* These results support the view that the *A. mellea* proteinase is a member of a novel class of lysine-specific metalloendopeptidases which may be exclusive to basidiomycete fungi. © 1999 Academic Press

The basidiomycete fungus *Armillaria mellea* produces a metalloendoproteinase which cleaves protein substrates specifically at the amino-terminal side of lysine residues (1, 2). This enzyme is of interest for two particular reasons; first, its cleavage specificity has been used as a tool in amino acid sequence analysis of proteins (3, 4) and secondly, it has potent fibrinolytic activity and has been investigated as a potential therapeutic agent in the treatment of thrombosis (1, 5). In addition to the above properties, the *A. mellea* proteinase also displays unusual stability to anionic detergents, retaining substantial catalytic activity at 50°C in 5% (w/v) SDS (6).

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The enzyme is optimally expressed in the fruiting body of the fungus but, for reasons unknown, enzyme levels are very variable with a large proportion of *A. mellea* fruiting bodies collected from the wild not containing the proteinase (1). In order to further study the enzyme, we developed a new purification protocol for the *A. mellea* proteinase which significantly shortened the procedure and gave improved yields compared with earlier methods (1, 2). We also sequenced the first 26 N-terminal residues of the purified enzyme as a basis for oligonucleotide probe synthesis with a view to cDNA cloning of the proteinase.

During the course of this work reports have appeared describing purification and sequencing of lysine-specific metalloendopeptidases from the basidiomycetes *Grifola frondosa* and *Pleurotus ostreatus* (7–9). These enzymes show strong similarity in chemical, physical and catalytic properties to those of the proteinase from *A. mellea*. The sequence data presented here confirms that these three proteins are members of a same family of metalloendoproteinases which may be exclusive to basidiomycetes.

MATERIALS AND METHODS

Purification of A. mellea proteinase. Unless otherwise stated, all procedures were carried out at 4°C. Protein concentrations were determined using the Bradford assay (10).

A. mellea fruiting bodies were collected from the wild in Cork county, Ireland and the mushroom caps were removed and immediately stored in a −70°C freezer. Frozen mushroom caps (280 g) were thawed and were homogenised with an equal volume (250 ml) of water in a Kenwood blender for 2 min at maximum speed. The homogenate (780 ml) was centrifuged at 600 ***g*** for 30 min at 4°C. The crude extract (500 ml) was placed in a stainless steel 10 litre bucket contained in a salt-ice bath. An equal volume of pre-chilled (−70°C) ethanol was added, drop-wise, with constant stirring after which the solution was kept stirring for a further 1 hr. Precipitated protein was removed by centrifugation at 600 ***g*** for 30 min at 0°C. The clarified ethanol-soluble fraction (950 ml) was returned to the steel bucket contained on salt-ice and its ethanol concentration was increased, drop-wise, to 70% with constant mixing. Stirring was continued for 1 hr after which precipitated protein was recovered by centrifugation at 600 ***g*** for 30 min at −5°C. Following removal of the supernatant,

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TABLE 1

Purification of the Lysine-Specific Proteinase from *A. mellea*

Step	Volume (ml)	Protein (mg)	Proteinase activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Homogenate	780	n.d.	n.d.	—	—	—
Crude extract	500	5176	9835	1.9	(100)	(1)
Pellet	70	12	5155	429	52	225
CM23 eluate	19.5	5.1	2436	477	25	251
MonoS eluate	0.5	1.2	1144	953	12	501

Note. n.d., not determined. Proteinase activity was measured using the azocaesin assay as described under Materials and Methods.

the pellets were dried and the protein was then re-suspended in 70 ml of 10 mM citrate-NaOH, pH 6.0. Insoluble material was removed by centrifugation at 10000 ***g*** for 10 min at 4°C.

The re-suspended pellet was applied to a column (7 cm × 16 cm) of CM-23 cellulose (Whatman Scientific Ltd) equilibrated in 10 mM citrate-NaOH, pH 6.0. The column was washed with 1 litre of this buffer prior to elution with a 2 litre linear gradient of 10 mM to 200 mM citrate-NaOH, pH 6.0. Active fractions were pooled and were dried by lyophilisation. The lyophilised protein was re-suspended in 25 ml of 10 mM citrate-NaOH, pH 6.0 and was equilibrated by passage through a column (4 cm × 20 cm) of Sephadex G-25 (Pharmacia Biotech) equilibrated in the same buffer. This solution was designated the CM-23 eluate.

The CM-23 eluate was applied in 2 equal aliquots to a MonoS HR 5/5 (FPLC system) column equilibrated in 10 mM citrate-NaOH, pH 6.0. The column was washed with 3 ml of this buffer and the enzyme was eluted with a 22 ml gradient of 0 to 0.25 M NaCl in 10 mM Citrate-NaOH buffer at a flow rate of 0.5 ml/min. The active protein peaks from each run were pooled and dialysed against 10 mM citrate pH 6.0, 1 mM dithiothreitol. This sample, designated the MonoS eluate, was stored at −20°C.

General proteinase assay. Proteinase activity was determined by measuring the release of acid-soluble material from azocasein (Sigma). Enzyme sample/column fraction (50 μl) was added to 300 μl of 1% (w/v) azocaesin (prepared in 50 mM Tris-HCl, pH 7.0). Following incubation at 37°C for 20 min, 600 μl of ice-cold 10% (w/v) trichloroacetic acid was added with immediate vortexing. The sample was placed on ice for 10 min before centrifugation at 17000 ***g*** for 15 min. The quantity of acid-soluble material in the supernatant was measured by absorbance at 366 nm. One unit of proteinase activity was defined as the amount required to produce enough acid-soluble material from azocaesin to yield an absorbance of 0.1 at 366 nm following a incubation at 37°C for 20 min.

Fibrinolysis assay. Enzyme sample (5 μl) was added to 500 μl of 3 mg/ml human fibrinogen (Sigma) (prepared in 0.1 M sodium phosphate, pH 7.4, 0.15 M NaCl). After 1 min at room temperature, 10 μl of 500 units/ml thrombin (Sigma) was added. As a control 5 μl of water was added instead of enzyme. A solid clot formed in the control within 20 sec while no clot appeared in the presence of *A. mellea* proteinase.

N-terminal sequence analysis. Purified proteinase (150 μg) was concentrated onto a polyvinyldene difluoride (PVDF) membrane using a Prospin column (Applied Biosystems) as described in the manufacturer's instructions. Gas-phase automated sequencing was performed on an Applied Biosystems 477A Protein Sequencer.

RESULTS AND DISCUSSION

Purification of A. mellea proteinase. Approximately 30% of the batches of *A. mellea* collected from the wild expressed the proteinase of interest. The results of a typical purification are given in Table 1. Ethanol fractionation proved to be of particular value in purifying this enzyme, resulting in a 225-fold increase in purity. During chromatography on the CM-23 column, the majority of contaminating protein did not bind and was eluted in the flow-through. Proteinase activity, as measured by the azocaesin assay, was eluted over a broad range of the citrate gradient, peaking at approximately 80 mM (not shown). The elution profile from the MonoS (FPLC) column is shown in Fig. 1. Proteinase activity as measured by both the azocaesin and fibrinolysis assays was confined exclusively to protein peak 1.

The purification protocol for *A. mellea* proteinase presented here greatly simplifies the original protocol designed by Broadbent *et al.* (1, 2). Batch adsorption onto CM cellulose was replaced with a single column-chromatography step using the same resin. Final purification was then achieved by using the superior resolution achievable with a MonoS column on the FPLC system rather than the two further rounds of chromatography (CM-52 ion-exchange and Sephadex G-75 gel filtration) employed in the original protocol. As can been seen from Table 1, 1.2 mg of enzyme was purified 501-fold from 280g of *A. mellea* fruiting bodies with a yield of 12%. However, as a general proteinase assay was employed during the course of the purification, other proteinases in the *A. mellea* fruiting body would

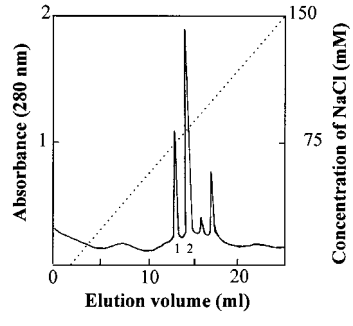

FIG. 1. Elution profile of proteins applied to the MonoS (FPLC) column. Elution of protein peaks was monitored by absorbance at 280 nm. The dashed line represents the NaCl gradient applied to the column. Both proteinase and fibrinolytic activities were confined exclusively to peak 1.

have contributed to the total activity value of the crude extract. Therefore, the purification factor and yield were in reality higher than the values in Table 1. However, as stated above, levels of this enzyme are very variable in the *A. mellea* fruiting body and, depending on the mushroom batches collected from the wild, greater or smaller quantities of proteinase will be purified from the same amount of starting material.

The fibrinolysis assay was employed in this work as a specific assay for the *A. mellea* proteinase and peak 1 from the MonoS column (Fig. 1) was found to exhibit potent fibrinolytic activity indicating that it contained the proteinase of interest. Protein peak 2 (Fig. 1) contained a pure endo-exonuclease which has also been recently characterised (11).

Purity and molecular weight of the proteinase. SDS-PAGE analysis of 10 μg of the MonoS eluate revealed a single protein band (Fig. 2). Further evidence of purity was provided by the fact that a single amino acid residue was detected at each step of N-terminal amino sequence analysis.

The molecular weight of *A. mellea* proteinase has been variously reported to be in the range 13.5–21.5 kDa (1, 2, 6) although there is common consensus that the enzyme is a monomer. Inclusion of molecular weight markers in the gel shown in Fig. 2 gave an estimate of 19–20 kDa for the apparent molecular weight of the enzyme. This is very similar to the values of 20 kDa and 19 kDa reported for the metalloendopeptidases from *G. frondosa* (7) and *P. ostreatus* (8), respectively.

N-terminal sequence analysis of A. mellea proteinase. Gas-phase sequencing of the MonoS eluate yielded the first 26 N-terminal residues of the *A. mellea* proteinase. The sequence obtained was as follows: **ISYNGWTSSRQTTLVSAAAAWQTYAQ.** Consistently, the N-terminal amino acid had previously been reported as isoleucine (1). This sequence data should greatly assist in future attempts to clone this interesting enzyme.

Homology with other known proteins. A BLAST (12) search of the SwissProt database with the sequence shown above produced one "hit" namely the metalloendopeptidase from *G frondosa* (accession number P81054). Residues 2–24 of the *A. mellea* enzyme showed 52% identity with residues 1–23 of the proteinase from *G. frondosa*. A Fasta3 (13) search of the same database detected, in addition, the metalloendopeptidase from *P ostreatus* (accession number P81055) which showed 46% identity in a 24 residue overlap with the *A. mellea* enzyme. The complete amino acid sequences of the *G. frondosa* and *P. ostreatus* proteinases, established by protein sequencing methods, have recently been published (9).

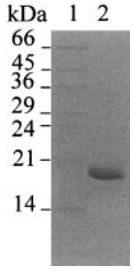

FIG. 2. Analysis of the MonoS eluate by 15% (w/v) SDS-PAGE. Lane 1, molecular weight markers; lane 2, 10 μg aliquot of peak 1 from the MonoS (FPLC) column.

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gf  -TYNGCSSSEQSALAAAASAAQSYVA
po  ATFVGCSATRQTQLNAAASQAQTYAA
am  ISYNGWTSSRQTTLVSAAAAWQTYAQ
     :: * :::.*: * :**:  *:*.
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FIG. 3. An alignment of the amino-terminal sequence of *A. mellea* proteinase (am) with those of the enzymes from *P. ostreatus* (po) and *G. frondosa* (gf). The comparison was carried out using CLUSTAL W (14). Amino acid identities are designated by asterisks, strong similarities by colons and weaker similarities by full points. The amino acid sequence data reported in this paper will appear in the PIR amino acid sequence database under Accession No. A58955.

An alignment of the N-terminal amino acid sequences of the three proteins obtained using CLUSTAL W (14) is shown in Fig. 3. The sequences share 7 identical residues in a 25-residue overlap clearly demonstrating that they belong to a single family of lysine-specific metalloendopeptidases. Moreover, the results lend support to the postulate that this family is exclusive to the basidiomycota phylum (9). Cloning of the cDNA for the *A. mellea* proteinase, as well as allowing a more detailed comparison with the other members of the family, will also answer the interesting question as to whether the enzyme is synthesised in a precursor form. In addition, it will also reveal what amino acid sequence motifs might contribute to the extreme stability of the proteinase.

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