Purification, characterization and cDNA cloning of an endo-exonuclease from the basidiomycete fungus *Armillaria mellea*

Vincent HEALY, Shawn DOONAN¹ and Tommie V. McCARTHY²

Department of Biochemistry, University College Cork, Lee Maltings, Prospect Row, Cork, Republic of Ireland

We have purified an endo-exonuclease from the fruiting body of the basidiomycete fungus *Armillaria mellea* by using an ethanol fractionation step, followed by two rounds of column chromatography. The enzyme had an apparent molecular mass of 17500 Da and was shown to exist as a monomer by gel-filtration analysis. The nuclease was active on both double-stranded and single-stranded DNA but not on RNA. It was optimally active at pH 8.5 and also exhibited a significant degree of thermostability. Three bivalent metal ions, Mg²⁺, Co²⁺ and Mn²⁺, acted as cofactors in the catalysis. It was also inhibited by high salt concentrations: activity was completely abolished at 150 mM NaCl. The nuclease possessed both endonuclease activity on supercoiled DNA and a 3'–5' (but not a 5'–3') exonuclease activity. It generated 5'-phosphomonoesters on its products that,

after a prolonged incubation, were hydrolysed to a mixture of free mononucleotides and small oligonucleotides ranging in size from two to eight bases. Elucidation of its N-terminal amino acid sequence permitted the cDNA cloning of the *A. mellea* nuclease via a PCR-based approach. Peptide mapping of the purified enzyme generated patterns consistent with the amino acid sequence coded for by the cloned cDNA. A BLAST search of the SwissProt database revealed that *A. mellea* nuclease shared significant amino acid similarity with two nucleases from *Bacillus subtilis*, suggesting that the three might constitute a distinct class of nucleolytic enzymes.

Key words: *Bacillus subtilis*, NucA, NucB, thermostable.

INTRODUCTION

The basidiomycete *Armillaria mellea* originally comprised a broad spectrum of pathogenic root-infecting fungi. In recent years the genus has been reclassified into over 15 individual species [1]; however, to the non-specialist these fungi are still collectively known as *A. mellea*. The interest in the *Armillaria* genus stems from the fact that it is arguably the single most important cause of root-rot in temperate forests and has been recorded as a parasite of a very wide range of dicotyledonous and coniferous trees [2].

The fruiting body or mushroom of basidiomycete species is a specialized structure developed by the vegetative mycelium in which meiosis of diploid nuclei is followed by the discharge of haploid spores. Nucleolytic enzymes have important roles in the short life span of a fruiting body. During meiosis and DNA recombinational repair, nucleases that generate single-stranded nicks or gaps, or double-stranded breaks, are required [3–7]. In addition, autolysis of the mushroom cap by various hydrolytic enzymes, including nucleases, has been documented to occur in many fungal species after spore release [8].

Nucleases that are believed to have a role in DNA recombination have previously been isolated from fungal species. The best studied is an endo-exonuclease originally purified from *Neurospora crassa*; however, closely related homologues have been isolated from, or detected in, *Aspergillus nidulans*, *Saccharomyces cerevisiae* and other eukaryotic species such as *Drosophila melanogaster*, monkey CV-1 cells, human leukaemic CEM cells and more recently *Pneumocystis carinii* [9,10]. These endo-exonucleases share similar enzymic properties and are immunologically related to each other. In addition, antiserum against the *N. crassa* enzyme cross-reacts with the *Escherichia coli* recombinational RecBCD nuclease [11]. Like RecBCD, the

eukaryotic endo-exonucleases are thought to act at double-stranded breaks in DNA and generate single-stranded tails with 5'-P termini that are believed to be substrates for strand-exchange enzymes during recombination [9]. Mutations in the RAD52 gene of *Saccharomyces* and the *uvs-3* gene of *Neurospora* result in a very low level of expression of the endo-exonucleases, which causes increased sensitivity to mutagenic agents owing to deficiencies in DNA recombinational repair [12,13]. In more recent work, the endo-exonuclease detected in leukaemic CEM cells has also been implicated in chromatin degradation during programmed cell death [14].

Three nucleases have been purified from the fruiting body of the fungus *Coprinus cinereus*. These are two endonucleases that generate single-stranded nicks in supercoiled DNA [15,16] and an endo-exonuclease specific for single-stranded DNA (ssDNA) [17]. Expression of these enzymes has been localized to the gill tissue during meiotic prophase, which strongly implicates them in the deliberate nicking of, or creation of, gaps in DNA during meiotic recombination.

Here we report the purification, characterization and cDNA cloning of a novel endo-exonuclease from the mushroom cap of *A. mellea*.

EXPERIMENTAL

Purification of *A. mellea* nuclease

Unless stated otherwise, all procedures were performed at 4 °C. Protein concentrations were determined with the Bradford assay [18].

A. mellea fruiting bodies were collected from the wild at Fota Arboretum (Fota Island, Co. Cork, Ireland). The mushroom caps were removed and immediately stored in a freezer at

Abbreviations used: CNBr, cyanogen bromide; ds, double-stranded; Endo Lys-C, endoproteinase Lys-C; ss, single-stranded.

¹ Present address: Faculty of Science and Health, University of East London, Romford Road, London E15 4LZ, U.K.

² To whom correspondence should be addressed (e-mail t.mccarthy@ucc.ie).

–70 °C. Frozen mushroom caps (530 g) were thawed and were homogenized with an equal volume (500 ml) of water in a Kenwood blender for 2 min at maximum speed. The homogenate (1350 ml) was centrifuged at 600 *g* for 30 min at 4 °C. The crude extract (1000 ml) was placed in a stainless steel 10 litre bucket contained in a salt-ice bath. An equal volume of prechilled (–70 °C) ethanol was added dropwise with constant stirring, after which the solution was kept stirring for a further 1 h. Precipitated protein was removed by centrifugation at 600 *g* for 30 min at 0 °C. The clarified ethanol-soluble fraction (1880 ml) was returned to the steel bucket contained on salt/ice and its ethanol concentration was increased dropwise to 70 % (v/v) with constant mixing. Stirring was continued for 1 h, after which precipitated protein was recovered by centrifugation at 600 *g* for 30 min at –5 °C. After removal of the supernatant, the pellets were dried with a gentle stream of N₂ gas and the protein was then resuspended in 75 ml of 10 mM citrate/NaOH, pH 6.0.

The resuspended pellet 1 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 before elution with a 2 litre linear gradient of 10–200 mM citrate/NaOH, pH 6.0. Active fractions were pooled and were concentrated by freeze-drying. The freeze-dried protein was resuspended in 35 ml of 10 mM citrate/NaOH, pH 6.0 and was desalted 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 (70 ml).

The CM-23 eluate was applied in 10 ml 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; the enzyme was eluted with a 22 ml gradient of 0–0.25 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 (1.5 ml), was stored at –20 °C.

Preparation of radiolabelled substrates

Radiolabelled 500 bp PCR product was used as the double-stranded DNA (dsDNA) substrate. PCR amplification was performed with 100 ng of DNA as template, 0.2 mM dATP, dGTP and dTTP, 0.02 mM dCTP and 1 μ Ci of [α -³²P]dCTP, 3 pmol of both primers and 1 unit of *Taq* polymerase in a total volume of 10 μ l. After removal of primers and desalting by G-50 spun-column chromatography [19], a 100-fold excess of identical, but unlabelled, purified PCR product was added. The specific radioactivity (c.p.m./ μ g of DNA) of this substrate was measured by scintillation counting and A_{260} .

PCR products served as ssDNA substrate as described above, except that a biotinylated primer was used in the reactions. Amplified products were pooled and NaCl was added to a final concentration of 1 M before immobilization of the DNA on streptavidin-coated beads (Dynabeads M-280 Streptavidin). Beads [30 μ l, prewashed in 10 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 M NaCl] were added to the sample and incubated at room temperature for 5 min. The beads were pelleted with a Dynal MPC-E (magnetic particle concentrator for Microfuge tubes) and the supernatant was discarded. PCR fragments were denatured by the addition of 100 μ l of 0.15 M NaOH and incubation at room temperature for 5 min. After pelleting with the MPC-E, the supernatant was removed and neutralized by the addition of 100 μ l of 0.15 M HCl. ssDNA was then precipitated

with ethanol and resuspended in water; its specific radioactivity was determined as described previously.

Radiolabelled RNA was prepared by growing *E. coli* cells overnight in culture medium in the presence of [³H]uridine. [³H]RNA was isolated from the cells with an Ultraspec-3 RNA isolation system (Biotech). Specific radioactivity was determined as described above.

DNA substrates, specifically labelled at the 3' end, were prepared by adding [α -³²P]dCTP to a fill-in reaction of the 3' recessed ends of *Bam*HI-digested pUC 18 by using the Klenow fragment [19]. For 5' labelling, the linearized plasmid was filled in with the Klenow fragment in an unlabelled reaction. Unincorporated nucleotides were then removed by spun-column chromatography and the DNA was 5' end-labelled in an exchange reaction with [γ -³²P]ATP by using T4 polynucleotide kinase [19]. Single-stranded 5'- and 3'-labelled DNA were generated by being heated at 95 °C for 10 min followed by incubation on ice for 5 min.

Nuclease assay

Nuclease activity was determined by measuring the release of acid-soluble material from radiolabelled substrates. A standard nuclease assay contained 25 mM glycine/NaOH, pH 8.5, 1.25 mM MgCl₂, 0.1 μ g/ μ l BSA and 500 ng of radiolabelled substrate (ds/ssDNA or RNA), preincubated at 45 °C for 5 min. After the addition of the enzyme/column fraction, the reaction (final volume 50 μ l) was incubated at 45 °C for a further 5 min, at which point it was terminated by the addition of 50 μ l of ice-cold 0.8 M trichloroacetic acid and 2 μ l of 1 μ g/ μ l calf thymus DNA with immediate vortex-mixing. The sample was placed on ice for 15 min before centrifugation at 17000 *g* for a further 15 min. The quantity of acid-soluble material in the supernatant was measured by scintillation counting.

One unit of nuclease activity was defined as the amount required to render 1 μ g of substrate acid-soluble in 1 min at 45 °C. All assays were performed in their linear ranges and were conducted in triplicate for construction of the purification table and for the quantitative characterization experiments.

Electrophoresis

SDS/PAGE was performed by the method of Laemmli [20] with 4 % (w/v) polyacrylamide in the stacking gels and 15 % or 20 % (w/v) polyacrylamide in the resolving gels. Proteins were stained with 0.5 % Coomassie Brilliant Blue R-250.

An SDS/PAGE DNase detection gel was prepared as described by Rosenthal and Lacks [21], with some modifications. Calf thymus DNA was added to the resolving gel mix, to a final concentration of 10 μ g/ml, before pouring and setting. After electrophoresis of the enzyme, the gel was washed in water for 2 h to remove SDS and to allow renaturation of the nuclease. The gel was then incubated in 25 mM glycine/NaOH (pH 8.5)/1.25 mM MgCl₂/1 μ g/ml ethidium bromide for 8 h. A zone of hydrolysis due to nuclease activity was detected by exposure of the gel to UV.

Determination of native molecular mass

The native molecular mass of *A. mellea* nuclease was determined by applying 100 μ g of the purified enzyme to an FPLC Superose 12 gel-filtration column, equilibrated in 0.05 M sodium phosphate (pH 7.0)/0.15 M NaCl at a flow rate of 0.3 ml/min. All fractions were collected and the elution volume of the nuclease

was recorded. The column was calibrated with thyroglobulin (669 kDa), aldolase (160 kDa), transferrin (75 kDa), BSA (66 kDa), ovalbumin (45 kDa), chymotrypsin (25.7 kDa) and lysozyme (14 kDa).

Analysis of the type of phosphomonoester generated by the nuclease

Radiolabelled dsDNA (5 µg) was hydrolysed with 1 ng of *A. mellea* nuclease to render 50 % of the substrate acid-soluble. The precipitated DNA was washed with 70 % (v/v) ethanol and resuspended in 20 µl of water. The sample was then divided into four equal parts and the following were added to each: (1) standard assay buffer with no enzyme, (2) standard assay buffer and 1 ng of *A. mellea* nuclease, (3) standard assay buffer and 50 µg of snake-venom phosphodiesterase (Boehringer Mannheim) and (4) 50 units of λ exonuclease (Gibco BRL) and its reaction buffer. Reaction volumes were all 50 µl and incubations were performed at 37 °C for 1 h. The release of acid-soluble radioactivity was measured as described.

Determination of the size of the products of enzyme hydrolysis

A 100 µl reaction, consisting of the standard assay buffer, 500 ng of radiolabelled dsDNA and 2 µg of *A. mellea* nuclease, was incubated at 45 °C. At timed intervals, 10 µl aliquots were removed and immediately mixed with 2 µl of agarose-gel loading dye. These samples were analysed by electrophoresis on a 7 M urea/20 % (w/v) polyacrylamide gel. End-labelled (³²P) oligonucleotides, of sizes 15, 22, 24 and 27 bp, were used as size markers. After electrophoresis the hydrolysis products were detected by autoradiography.

Isolation of total RNA from *A. mellea* fruiting bodies and reverse transcriptase-mediated PCR

Total RNA was isolated from *A. mellea* mushroom caps by using the Ultraspec-3 RNA isolation system (Biotecx); 1 µg of this RNA was used as template in a first-strand cDNA synthesis reaction directed by an oligo(dT) primer with a nine-base clamp (T-primer, 5'-CGCGGATCCTTTTTTTTTTTTTTTT-3') in a 20 µl volume employing avian myeloblastosis virus reverse transcriptase (Promega) under conditions recommended by the manufacturer.

PCR was performed with the primer AMN1, a 27-mer degenerate primer designed from the first nine N-terminal residues of the nuclease (5'-GCIGGICCIGAYTTYGAYTYTGAYTAY-3') and the T-primer described above. PCR was performed in a 25 µl volume containing 10 mM KCl, 20 mM Tris/HCl (pH 8.8 at 25 °C), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 % (v/v) Triton X-100, dNTPs (each at 200 mM), 250 ng of AMN1 primer, 50 ng of T-primer, 2 µl of first-strand cDNA template and 0.5 unit of Vent DNA polymerase (New England Biolabs), with the use of 35 thermocycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min.

Cloning and sequencing of PCR products

Amplified products were 'A-tailed' by incubation with 1 unit of *Taq* DNA polymerase at 72 °C for 30 min. After electrophoresis on a 1.5 % (w/v) NuSieve GTG agarose (FMC) gel in 0.04 M Tris-acetate/0.001 M EDTA buffer [19], the four PCR products were rapidly excised and transferred to microtubes. Each product was then individually T/A cloned by adding 6.5 µl of the melted gel slices to ligation reactions containing the pTag vector supplied in 'The LigATor' PCR fragment cloning kit (Ingenius).

DNA sequencing was performed by radioactive cycle sequencing with the Thermo Sequenase cycle sequencing kit (Amersham Life Sciences).

Cleavage of *A. mellea* nuclease with endoproteinase Lys-C (Endo Lys-C) and CNBr

Purified nuclease (5 µg) was digested with 0.1 µg of Endo Lys-C (Boehringer Mannheim) in 50 µl of 25 mM Tris/HCl (pH 8.5)/1 mM EDTA for 18 h at 37 °C, followed by precipitation with acetone. For cleavage with CNBr (Sigma), 5 µg of purified nuclease was resuspended in 50 µl of 70 % (v/v) formic acid containing 50 µg of CNBr. The tube was flushed with N₂ gas, sealed with Parafilm, wrapped in tinfoil and left at room temperature for 48 h, at which point the mixture was concentrated by freeze-drying. Both digestions were then resolved by SDS/PAGE [20 % (w/v) gel].

RESULTS AND DISCUSSION

Purification of *A. mellea* nuclease

The mushrooms collected for this study were identified as *A. mellea* by examination of morphological characteristics [22]; all batches were found to express the nuclease of interest. The results of a typical purification are given in Table 1 and an SDS/PAGE analysis of each stage is shown in Figure 1(A). During chromatography on the CM-23 column, most contaminating protein did not bind and was eluted in the flow-through. The enzyme itself was eluted over a broad range of the citrate gradient, peaking at approx. 65 mM. Final purification of the nuclease was accomplished with the FPLC MonoS column: all nuclease activity was eluted as a single sharp protein peak at 130 mM NaCl.

Almost 4 mg of nuclease was purified 357-fold from 530 g of mushrooms, with a yield of 11 %. However, as a general nuclease assay was employed during the course of the purification, other DNases in the *A. mellea* fruiting body would have contributed to the total activity value of the crude extract, so the purification factor and yield were in reality higher than the values in Table 1. The nuclease was stable at -20 °C for at least 18 months but highly diluted solutions of the enzyme (500 pg/µl), prepared for characterization experiments, required supplementation with BSA (0.1 µg/µl) to prevent a loss of activity.

SDS/PAGE analysis of the eluate from the MonoS column revealed a single band (Figure 1A). To ensure that this protein and not some trace contaminant was responsible for the nuclease activity, 2 µg of this sample was analysed on a DNase detection gel as described in the Experimental section. The presence of a zone of hydrolysis at a position corresponding to the molecular mass of the purified protein (Figure 1B) can be taken as strong evidence that this protein was a nuclease enzyme.

Structural characterization of *A. mellea* nuclease

Analysis by SDS/PAGE (Figure 1A) revealed that the enzyme had an apparent molecular mass of approx. 17.5 kDa. Size-exclusion chromatography of the purified nuclease yielded a single peak corresponding to a molecular mass of 18 kDa, indicating that the enzyme exists as a monomer (results not shown).

The sequence of the 26 N-terminal residues of the nuclease was determined as AGPDFDLDYRTYPQSSSENIXYSWFXN by gas-phase sequencing of the purified enzyme (200 µg).

Table 1 Purification of *A. mellea* nuclease

Nuclease activity was measured with the use of ^{32}P -radiolabelled dsDNA as described in the Experimental section. Abbreviation: n.d., not determined.

Step	Volume (ml)	Protein (mg)	Nuclease activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Homogenate	1350	n.d.	n.d.	—	—	—
Crude extract	1000	13 000	1800 000	138	100	1
Ethanol-soluble fraction	1880	1 030	470 000	456	26	3.3
Pellet 1	74	14.2	390 000	27 500	22	200
CM23 eluate	70	5.1	230 000	45 100	13	327
MonoS eluate	1.5	3.9	195 000	50 000	11	357

Figure 1 SDS/PAGE and DNase detection gel analysis of *A. mellea* nuclease

(A) Samples from each purification step were analysed by SDS/PAGE [15% (w/v) gel]. Lane 1, 80 μg of crude extract; lane 2, 50 μg of ethanol-soluble fraction; lane 3, 20 μg of pellet 1; lane 4, 5 μg of CM 23 eluate; lane 5, 5 μg of MonoS eluate; lane 6, molecular mass markers (molecular masses are indicated at the right). (B) The MonoS eluate (2 μg) was analysed on a 15% (w/v) DNase detection gel as described in the Experimental section.

Figure 2 Activity of *A. mellea* nuclease on nucleic acid substrates

Radiolabelled dsDNA ($\square$), ssDNA ($\bullet$) and RNA ($\circ$) were prepared as described in the Experimental section. Standard assays (200 μl) containing 2 μg of substrate and 1 ng of enzyme were prepared. At timed intervals 10 μl aliquots were removed and precipitated with trichloroacetic acid. The activity of acid-soluble material is expressed as a percentage of that of an unprecipitated aliquot of the respective assay.

Activity of *A. mellea* nuclease on various substrates

The nuclease degraded both dsDNA and ssDNA into acid-soluble products but exhibited no reactivity towards RNA (Figure 2). Prolonged incubation with RNA did result in a small degree of RNA hydrolysis, but this was most probably due to trace contamination of either the enzyme sample or the reaction buffer with RNase. It is also apparent from Figure 2 that the nuclease was more active towards dsDNA than ssDNA.

Effect of pH, bivalent metal ions, salt concentration and temperature on nuclease activity and stability

The activity of the nuclease on dsDNA was optimal at approx. pH 8.5 (Figure 3A). The enzyme was active, however, over a broad pH range, with substantial digestion occurring between pH 6.5 and 9.5. Preincubation in very acidic and very basic buffers before assaying under optimal conditions revealed that the nuclease was stable in the pH range 2–11 (results not shown).

The enzyme was active in 25 mM glycine, pH 8.5, alone (presumably owing to trace metal ion contaminants in the buffer). However, the addition of 0.1 mM EDTA completely abolished this activity, indicating an absolute requirement for bivalent metal cations by *A. mellea* nuclease. The addition of Mg^{2+} , Mn^{2+} or Co^{2+} restored and enhanced this lost activity, whereas other metal ions such as Ni^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , Cd^{2+} and Sr^{2+} inhibited the enzyme when added at various concentrations. Figure 3(B) shows the effect of various concentrations of the three stimulatory metal ions on nuclease activity: maximum activity was attained with 2 mM Mg^{2+} . Co^{2+} and Mn^{2+} (the optimal concentration for both ions was 1.0 mM) yielded activities that were 80% and 60% respectively of that achieved with Mg^{2+} .

The enzyme's activity was not enhanced by the addition of either NaCl or KCl. Indeed even low concentrations of both salts had an inhibitory effect, with activity being abolished at only 150 mM NaCl and 120 mM KCl (results not shown). The preference for an environment of low ionic strength is typical of many nucleolytic enzymes including DNase I and of some type II restriction endonucleases [23]. The significance of this property for *A. mellea* nuclease is unclear but the enzyme would still exhibit activity at physiological conditions, albeit at a much lower level.

Nuclease thermostability was assessed by preincubation at temperatures of 60 °C and higher before measuring activity under optimal conditions at 45 °C. Incubation for 5 min and longer at 75 °C caused the rapid denaturation of the enzyme; however, on being returned to the assay temperature approx. 25% of the activity was restored. Similar results were obtained for incubations performed at higher temperatures and for longer

Figure 3 Effect of pH (A) and bivalent metal ion concentration (B) on nuclease activity

Standard assays containing 500 ng of ³²P-labelled dsDNA and 1 ng of enzyme were performed with the following modifications. (A) The pH was varied by the addition of the following buffers: ○, 25 mM sodium acetate/acetic acid; ■, 25 mM Mes/HCl; ●, 25 mM Tris/HCl; □, 25 mM glycine/NaOH. (B) The following bivalent metal ions were added at the concentrations shown: ■, Mg²⁺; ●, Co²⁺; □, Mn²⁺.

periods. For example, boiling the enzyme for 30 min before assay resulted in a level of activity 25% of that of the control. These results indicated that the enzyme was rapidly denatured above 75 °C but that it also had the ability to renature partly when returned to more favourable conditions.

The results described above were obtained with dsDNA as substrate. Similar values were obtained when the effect of varying these parameters was tested on ssDNA (results not shown).

Endonuclease activity associated with the enzyme

The effect of incubating supercoiled DNA with *A. mellea* nuclease is shown in Figure 4. As can be seen, the supercoiled form of the plasmid pUC 18 was rapidly converted into linear DNA before it was completely degraded, presumably by hydrolysis by a combination of endonuclease and exonuclease activity. The fact that no nicked form of the plasmid was created suggests that, under the conditions used in the reaction, the enzyme converted supercoiled DNA directly into the linear form by creating a double-stranded break. The nuclease was also able to degrade single-stranded circular M13 DNA (results not shown) indicating that it also has an endonucleolytic mechanism of action on ssDNA.

Figure 4 Analysis of the endonuclease activity of *A. mellea* nuclease

A 100 μ l standard assay, containing 5 μ g of pUC 18 plasmid DNA and 0.5 ng of enzyme, was incubated at 45 °C. At timed intervals 10 μ l aliquots were removed, immediately mixed with 2 μ l of agarose-gel loading dye and analysed by 0.8% agarose-gel electrophoresis. Lanes 1–9 contained samples from 0, 0.5, 1, 2, 3, 4, 5, 15 and 40 min of incubation respectively. Lane C, all three forms of plasmid DNA used as markers.

Exonuclease activity associated with the enzyme

5'-labelled dsDNA and ssDNA and 3'-labelled dsDNA and ssDNA were treated with the nuclease (Figure 5). The immediate and rapid release of acid-soluble radioactivity from both 3'-labelled substrates strongly suggests that this enzyme has a 3' exonuclease activity. However, the release of radioactivity from 5'-labelled substrates occurred very gradually and was most probably due to degradation from the 3' end. In addition, exonuclease activity was greater towards dsDNA than towards ssDNA.

To confirm that the same protein molecule was responsible for both the endonuclease and the 3' exonuclease activities, the purified enzyme was applied to an FPLC Superose 12 gel-filtration column; the two activities were co-eluted (results not

Figure 5 Analysis of directionality of exonuclease activity of *A. mellea* nuclease

Standard assays (100 μ l), containing 500 ng of 5'-³²P-labelled dsDNA (●), 5'-³²P-labelled ssDNA (■), 3'-³²P-labelled dsDNA (□) and 3'-³²P-labelled ssDNA (○) and 1 ng of enzyme were prepared. At timed intervals 10 μ l aliquots were removed and precipitated with trichloroacetic acid. The activity of acid-soluble material is expressed as a percentage of that of an unprecipitated aliquot of the respective assay.

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1  GCGGGGCGGACTTCGATTGGATTATAGACCTACCTCAATCATCCGAGAACATATGC
   A G P D F D L D Y R T Y P Q S S E N I C
61  TATCTTGGTTCTGTAAACAATGGGCTCACTCTGTAGCTCTGACCGACCCATGCTGCT
   Y S W F C N N G P H S V A P D R T H A A
121 GCCCATCGCGCTAGCACCAGCTGCGGTAAACCAACCGGTGCAGCATCCGCGTG
   A H R A S N S C G N V N P N R C S I R V
181 GGCCATGTCAGTGGGTACCACTGCGATGAATGGCCATGGGCAAACTCCAACGCTGGGGGT
   G H V S G Y Q C D E W P W A N S N A G G
241 GCCAATGCCGCTACGAGGTGCATTCCCACTGCGGATAATACTGGATCTGGTTCACAATGG
   A N A A T R C I P T A D N T G S G S Q W
300 GGAATTTTCATTAATAACAGAGGATCCCAAGCTGTTGGATACGTTTTCAGGATAACGTT
   G N F I N N R G S Q A V G Y V L Q D N V
361 GTTTTCGCCACCATCGAGATTAGCAATATCCCACTACCGCGGAATTCGCAAGGAGTT
   V F A T I E I S N I P T T A E F C K G V
421 CTTGGAACGGCTATCACGCAACGATGTGCAGACAGGTGCGAATGGTCAGCGTATTTG
   L G T A I T A T M C R Q V A N G Q P Y L
481 CAAAGGATTGGTTGAACAATCGCTTCTGCAATGATGTTGGACTACCACAATTTGATCTTC
   Q R I G *
541 CGATAGTTTCAGACATTGCGCAACCATTTTCTTCATACCTTCCAATAGTCTGATCGTA
601 TTTCTTGTAACCTTGGATTGCGATTGCACGCTTAACCTCCAAAAA
661 AAAAAA

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Figure 6 DNA sequence and deduced amino acid sequence of the cDNA fragment encoding the active *A. mellea* nuclease

The stop codon is indicated with an asterisk.

shown). In addition, both activities were co-eluted as a very sharp peak from the MonoS column on the FPLC, which was then judged by SDS/PAGE to consist of a single polypeptide. These results indicate that the 17.5 kDa protein was responsible for both activities and this enzyme could therefore be described more properly as an endo-exonuclease.

Analysis of the phosphomonoester type and the sizes of the products generated by *A. mellea* nuclease

DNA partly degraded by *A. mellea* nuclease was rendered totally acid-soluble by subsequent treatment with snake-venom phosphodiesterase, which is a 3' exonuclease acting only on free 3'-OH groups [24]. Similarly, λ exonuclease, which degrades DNA strands from the 5' end only if a 5'-P group is present [25], also completely hydrolysed the products of *A. mellea* nuclease digestion (results not shown). This supports the conclusion that the enzyme cuts DNA molecules leaving 5'-P and 3'-OH termini on its products.

Analysis of the breakdown products on a denaturing 20% (w/v) polyacrylamide gel revealed that the enzyme degraded dsDNA to a mixture of free nucleotides (consistent with its exonuclease activity) and small oligonucleotides of two to eight bases in length (results not shown).

Amplification of *A. mellea* nuclease cDNA by PCR

First-strand cDNA, prepared from total RNA isolated from *A. mellea* fruiting bodies, served as template in a PCR as described in the Experimental section. Agarose gel (0.8%) analysis of this PCR revealed that four distinct products of approximate sizes 1000, 650, 500 and 350 bp were amplified (results not shown). All four fragments were individually T/A cloned into a pTag vector and then sequenced. A translation of the DNA sequence of the 5' region of the 650 bp product was identical with the established N-terminal sequence of the enzyme, indicating that it was amplified from *A. mellea* nuclease cDNA. The entire PCR clone was then sequenced on both strands.

Figure 7 Peptide mapping of *A. mellea* nuclease

The enzyme (5 μ g) was digested with Endo Lys-C and CNBr as described in the Experimental section. Cleavage reactions were analysed by SDS/PAGE [20% (w/v) gel]. Lane I, molecular mass markers (molecular masses are indicated at the left); lane II, 5 μ g of undigested enzyme; lane III, 5 μ g of enzyme cleaved with Endo Lys-C; lane IV, 5 μ g of enzyme cleaved with CNBr.

Amino acid sequence of *A. mellea* nuclease

A translation of the coding strand of the PCR clone reveals an open reading frame for a 164-residue protein followed by a 3' untranslated region of 174 bases (Figure 6). The calculated molecular mass of the translated polypeptide was 17576 Da, which is identical with the apparent molecular mass of the purified enzyme on SDS/PAGE (Figure 1A) and its theoretical pI was 6.44, which is consistent with the nuclease's behaviour during column chromatography on CM-23 and MonoS.

To provide further confirmation that the translated protein sequence was identical with that of the *A. mellea* nuclease, peptide mapping of the purified enzyme was performed with the protease Endo Lys-C and the chemical cleavage reagent CNBr, which cut at the C-terminus of Lys and Met residues respectively. From the translated sequence, Endo Lys-C should generate fragments of 14.8 and 2.7 kDa, and CNBr should generate fragments of 15.9 and 1.6 kDa. Although the smaller fragments were not detected under the electrophoretic conditions used, the peptide maps of the purified enzyme were consistent with this prediction (Figure 7).

Relationship of *A. mellea* nuclease to NucA and NucB

A BLAST [26] search of the SwissProt protein database revealed that the *A. mellea* nuclease shares significant amino acid similarity with two closely related *Bacillus subtilis* nucleases, NucA and NucB. An alignment of the nuclease with both bacterial nuclease sequences is given in Figure 8. The fungal nuclease shares 31% identity (55% similarity) with NucB and 29% identity (48% similarity) with NucA. However, a 38-residue segment is 42% identical (74% similar) to a 38-residue segment of both bacterial nucleases (region highlighted in Figure 8).

This degree of sequence similarity suggests that *A. mellea* nuclease, NucA and NucB might be members of the same class of nucleolytic enzymes. In addition, the 38-residue region with the highest degree of similarity might constitute the active site of these enzymes. A run of four residues (Asp-Trp-Glu-Pro) in this section is conserved and contains two acidic residues, one or both of which might act as a general acid catalyst or might co-ordinate the bivalent ion (Mg^{2+}) required for catalysis. The

NucB	-----MKKWMAGLFLAAAVLLCLMVP-----QQIQGASS	29
NucA	MTTDTIKITLLLVITIAAAVGLIKGDFPSADQKTSQTKE	40
AMN	-----AGPDFLDYR-----TYPQSSE	17
	*. . . *	
NucB	YDKVLYFPLSRYPETGSHIRDAIEGHPDICTIDRDGADK	69
NucA	YDETHAFPSDRYPETAKHIKDAINEGHSEVCTIDRDGAE	80
AMN	NICYSWFCNNGPHSVAPDRTHAAHRASNSCGVNPNCRS	57
	* *	
NucB	RREESLKGIPTKGYDRDEWPMACEGGAGADVRYVTPS	109
NucA	RREQSLKDVPSKNGYDRDEWPMAMCKEGEGASVEYISPA	120
AMN	IR-----VGHVSGYOCDEWPMANSNAGGANAATRCITPA	91
	* : : : : *	
NucB	DNRGAGSWVGNDMSYPDGTRLFIVQ-----	136
NucA	DNRGAGSWVGHRLTDYDGTKVLFTIQ-----	147
AMN	DNTSGSGOWGNFINNRGS-QAVGVVLQDNVVFATIEISNI	130
	** : : : *	
NucB	-----	136
NucA	-----	147
AMN	PTTAEFCCKVGLGTATATMCRQVANGPYLQRIQ	164

Figure 8 Alignment of the amino acid sequence of *A. mellea* nuclease with sequences of NucA and NucB from *B. subtilis*

Protein sequences of *A. mellea* nuclease (AMN), NucA and NucB (accession numbers NUCA_BACSU and NUCB_BACSU from the SwissProt Sequence Database) were aligned by using CLUSTAL W 1.7 [31]. Amino acid identities are designated by asterisks, strong similarities by colons and weaker similarities by full points. The 38-residue segment in the alignment with the highest degree of similarity is shown in a box.

discovery of further members of this class would cast more light on this particular structural aspect. Many other nucleases have been grouped into discrete classes with members from different species. The group of endo-exonucleases isolated from *N. crassa*, *S. cerevisiae* and a human cell line, among others, is a prime example [9]. Even more diverse is a class containing non-specific nucleases from species ranging from mammals to *Streptococcus pneumoniae*, of which the *Serratia* nuclease is the paradigm [27].

NucA and NucB have been recently studied and cloned [28]. NucA is a 16.5 kDa membrane-associated and manganese-stimulated DNase that is believed to be involved in competence development in *B. subtilis*. This nuclease shows specific activity towards dsDNA, producing predominantly single-stranded nicks in the presence of Mg^{2+} , which indicates an endonucleolytic mode of cleavage. In the presence of Mn^{2+} , nuclease activity is stimulated, resulting in extensive DNA degradation. However, it is not known whether this is due solely to an increase in endonuclease activity or whether it involves any exonuclease activity. Interestingly, NucA forms a 75 kDa equimolar complex with an 18 kDa inhibitor, termed the nin protein.

NucB, in contrast, is a 12 kDa Mn^{2+} -stimulated nuclease, which is secreted into the medium during sporulation. It has, however, very similar enzymic properties to NucA in that it possesses specific activity towards dsDNA at an optimum pH of 7.5. It has been shown to possess endonuclease activity but was not investigated for any exonuclease activity. The nin protein has also been shown to inhibit NucB's activity *in vitro*.

Despite their sequence similarity, *A. mellea* nuclease's enzymic activity is significantly different (i.e. pH optima, metal ion co-factor requirements and exonuclease activity) from those of NucA and NucB [28]. In addition, in preliminary work we have shown that the nin protein does not inhibit *A. mellea* nuclease activity *in vitro*.

Possible biological function(s) of *A. mellea* nuclease

The *A. mellea* nuclease's enzymic properties do not exactly match any known nuclease yet studied. The endo-exonucleases from other fungi such as *Neurospora*, *Aspergillus* and *Saccharomyces*, implicated in DNA recombination [9], have ssDNA-specific endonuclease activities that nick supercoiled DNA as

well as 5'-3'-processing exonuclease activities on dsDNA. In addition, all these endo-exonucleases possess RNase activity and are not inhibited by high concentrations of salt. Finally, the genes for both the nuclear (NUD1) and the mitochondrial (NUC1) endo-exonucleases from *S. cerevisiae* have been sequenced [29,30] and their protein products bear no significant similarity in amino acid sequence to that of the *A. mellea* nuclease. It is therefore unlikely that the *A. mellea* nuclease is another member of this class. The endo-exonuclease from the fruiting body of another basidiomycete, *Coprinus cinereus*, does possess a 3'-5' exonuclease activity but there are again some key differences in enzymic activities. The *Coprinus* enzyme has a ssDNA-specific endonuclease activity that generates 3'-phosphomonoesters on its products [17]. However, the associated 3'-5' exonuclease activity cannot act on these products without prior treatment *in vitro* with phosphatase enzyme.

The biological function of *A. mellea* nuclease is unclear at this point but it does possess the correct properties for involvement in DNA recombination. It is capable of generating double-stranded breaks in supercoiled DNA, and the 3'-5' exonuclease activity, which is more active towards dsDNA, could then be involved in substrate preparation before recombination events. However, these enzymic properties are also consistent with a role in DNA degradation during autolysis of the mushroom cap. Further detailed analysis will be necessary to elucidate the biological function(s) of this novel enzyme.

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