Purification and characterization of an extracellular serine protease from the nematode-trapping fungus Arthrobotrys oligospora

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When grown in liquid cultures allowing the formation of nematode traps, the fungus Arthrobotrys oligospora produced two extracellular proteases hydrolysing the chromogenic substrate Azocoll. The protease activity was separated into two fractions (FI and FII) using anion-exchange chromatography. In bioassays, protease(s) present in FII immobilized the free-living nematode Panagrellus redivivus indicating that the enzyme(s) might be involved in the infection of nematodes. A protease designated PI1 was purified from FII to apparent homogeneity by hydrophobic interaction and size-exclusion chromatography, resulting in an approximately 15-fold increase in specific activity. The purified enzyme was glycosylated, had a molecular mass of approximately 35 kDa (gel filtration) and an isoelectric point of pH 4.6. PI1 immobilized P. redivivus in bioassays and hydrolysed proteins of the purified cuticle. The enzyme hydrolysed several protein substrates including casein, bovine serum albumin and gelatin, but not native collagen. Examination of substrate specificity with synthetic peptides showed that PI1 readily hydrolysed tripeptides with aromatic or basic amino acids including N-benzoyl-L-phenylalanyl-L-valyl-L-arginine-4-nitroanilide (Bz-Phe-Val-Arg-NA) and succinyl-glucyl-glucyl-L-phenylalanine-4-nitroanilide (Suc-Gly-Gly-Phe-NA). Mono-peptides were hydrolysed at considerably slower rates. PI1 had an optimum activity between pH 7 and 9 and was susceptible to autodegradation. PI1 was inhibited by several serine protease inhibitors including phenylmethylsulfonyl fluoride (PMSF), chymostatin and antipain. The protease was N-terminally blocked, but the sequence of one internal peptide showed a high homology with a region containing the active site histidine residue of the subtilisin family of serine proteases.

Keywords: Arthrobotrys oligospora, extracellular serine protease, nematophagous fungus, subtilisin, cuticle-degrading enzyme

INTRODUCTION

Nematophagous fungi infect their hosts through a sequence of events: attachment to the host surface, penetration, followed by invasion and digestion of the host cells (Jansson & Nordbring-Hertz, 1988). The molecular mechanisms of this sequence are not well known, but, on the basis of studies of entomopathogens (St Leger, 1993), it is likely that hydrolytic enzymes are involved in several steps and processes during the infection: releasing nutrients for pathogen growth, facilitating penetration by solubilizing the cuticle, inducing cytotoxic effects on the nematode, digesting the host tissue, and inhibiting secondary invasion of micro-organisms.

Among the hydrolytic enzymes proteases are of special interest since the nematode cuticle is composed of...
proteins, including collagens (Cox et al., 1981). Extracellular proteases have also been detected and partly characterized from a few nematode-trapping fungi (Schenk et al., 1980; Aswani & Jaffar, 1992), as well as from endoparasites of cyst nematodes (Dackman et al., 1989; Lopez-Llorca, 1990). However, the importance and function of these proteases in the infection of nematodes are not known.

We recently demonstrated that the nematode-trapping fungus Arthrobotrys oligospora produces extracellular serine proteases when grown in liquid medium allowing the formation of nematode traps. Furthermore, incubating the trap-bearing mycelium with inhibitors against serine proteases significantly decreased the immobilization of captured nematodes, indicating an important function of such proteases during infection (Tunlid & Jansson, 1991). In this study, an extracellular serine protease present in the culture filtrates of A. oligospora has been purified and characterized. The purified protease hydrolysed cuticle proteins and immobilized free-living nematodes, indicating that it may be an important virulence factor for the infection of nematodes by A. oligospora.

**METHODS**

Cultures of A. oligospora and Panagrellus redivivus. A. oligospora Fres. (ATCC 24927) was maintained on cornmeal agar (Difco) supplemented with 2 g KH₂PO₄ 1⁻¹. Liquid cultures of A. oligospora were obtained by inoculating 4500 ml medium with a water suspension of conidia prepared from a 2- to 3-week-old culture grown on the cornmeal agar. Trap-containing mycelium [T++] was grown in medium containing 0.01% soya peptone (neutralized; Oxoid) supplemented with 0.05 g phenylalanine and 0.05 g valine 1⁻¹ (Friman et al., 1985). The cultures were grown for 7 days at room temperature with constant aeration, achieved by vigorous air bubbling from the bottom of the vessel (Friman et al., 1985). To compare proteases produced by hyphae without traps [T--], the fungus was grown in the same medium supplemented with a phosphate buffer to a final concentration of 12 mM (pH 7.0).

The nematode P. redivivus L. (Goodey) was grown axenically in a soya peptone–liver extract medium (Nordbring-Hertz, 1977).

**Purification of extracellular serine proteases.** Typically, mycelium from four vessels (4 × 4500 ml) was harvested by filtering through a nylon mesh, and washed with 4 × 50 ml 10 mM Tris/HCl (pH 7.5).

(i) Binding to Q Sepharose. The pH of the culture filtrate was adjusted to 7.5 by adding Tris/HCl to a final concentration of 10 mM. Approximately 20 ml of a suspension (80% in 0.01 M Tris/HCl pH 7.5) of Q Sepharose (Pharmacia LKB Biotechnology) was added per 4500 ml of culture filtrate and placed on a magnetic stirrer at 4 °C for 15 min. After sedimenting for at least 3 h, the supernatant was discarded, and the resin was transferred to a funnel with a Whatman glass microfibre filter (GF/F). The Q Sepharose was washed with 3.4 M (NH₄)₂SO₄ in a proportion of 3:2 (v/v) sample:buffer. The sample was applied to a Phenyl Sepharose HR 5/5 column (Pharmacia) connected to the Pharmacia HPLC system. Buffers used were: A, 50 mM sodium phosphate buffer (pH 7.0) and 1.7 M (NH₄)₂SO₄; B, 50 mM sodium phosphate buffer (pH 7.0). The gradient was 0% B for 5 min, 0 to 100% B for 37 min, 100% B for 5 min, with a flow of 0.4 ml min⁻¹. Elution of proteins was followed at 280 nm. Fractions of 0.4 ml were collected and assayed for protease activity (Azocoll).

(ii) Anion-exchange chromatography. The crude protease extract was applied to a Mono Q HR 5/5 column (Pharmacia) connected to an HPLC system (Pharmacia LKB; pump 2248, detector Variable Wavelength Monitor 2141, fraction collector HellFrac). Buffers used were: A, 10 mM Tris/HCl (pH 7.5); B, 10 mM Tris/HCl (pH 7.5) and 0.5 M NaCl. The gradient was 0.5% B for 5 min, 0.5% to 100% B in 35 min, and 100% B for 5 min with a flow of 1.0 ml min⁻¹. Elution of proteins was followed at 280 nm. Fractions of 1.0 ml were collected and assayed for protease activity (Azocoll).

(iii) Hydrophobic interaction chromatography. Fractions containing protease activity from the Mono Q column were pooled and mixed with 34 M (NH₄)₂SO₄ in a proportion of 3:2 (v/v) sample:buffer. The sample was applied to a Phenyl Superose HR 5/5 column (Pharmacia) connected to the Pharmacia HPLC system. Buffers used were: A, 50 mM sodium phosphate buffer (pH 7.0) and 1.7 M (NH₄)₂SO₄; B, 50 mM sodium phosphate buffer (pH 7.0). The gradient was 0% B for 5 min, 0 to 100% B for 37 min, 100% B for 5 min, with a flow of 0.4 ml min⁻¹. Elution of proteins was followed at 280 nm. Fractions of 0.4 ml were collected and assayed for protease activity (Azocoll or chromogenic peptides).

(iv) Size-exclusion chromatography. Fractions containing protease activity from the Phenyl Superose column were pooled, concentrated by ultrafiltration (Filtron, cut-off 10 kDa, final volume of sample ≤ 0.35 ml), and applied to a Superdex 75 HR 10/30 size exclusion column (Pharmacia) connected to the Pharmacia HPLC system. The buffer used was 0.100 M NH₄HCO₃ (pH 7.7) with a flow of 0.4 ml min⁻¹. Elution was followed at 280 nm. Fractions of 0.4 ml were collected and assayed for protease activity (Azocoll or chromogenic peptides). The enzyme purified from the Superdex column was designated PII. Before biochemical characterization, the ammonium carbonate buffer was evaporated (Speedvac) and PII was dissolved in the appropriate buffer.

**Extraction of cell-bound proteases.** The filtered mycelium of A. oligospora was washed with 3 × 50 ml PBS (20 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl) and cell-bound proteins were extracted using a homogenization sonication procedure previously described (Tunlid et al., 1991). The extract was concentrated by ultrafiltration (YM-3 membrane), desalted (PD-10 column) and separated chromatographically on the Mono Q column as described above.

**Protease assays.** Protease activity was measured in the extracts and HPLC fractions using the chromogenic protein substrate Azocoll (Sigma) (Chavira et al., 1984; Tunlid & Jansson, 1991). Incubations were performed at 37 °C. The activities were expressed as A₅₇₀, or as proteolytic units (PU) defined as the increase of A₅₂₀ ml⁻¹ min⁻¹.

Proteolytic activity **versus** protein substrates and purified nema-tode cuticle (see below) were assayed by mixing 10 µl PII (typically 0.01 µg µl⁻¹) with 100 µl 0.1 M Tris/HCl (pH 7.5) containing 2.5 mg substrate ml⁻¹. After appropriate periods of incubation at 30 °C, the reaction was terminated by adding 200 µl 5% (w/v) trichloroacetic acid and the tubes were allowed to stand for more than 1 h. Undigested protein was removed by centrifugation and the released peptides were assayed by measuring the absorbance at 280 nm. One unit is defined as the increase in A₅₂₀ ml⁻¹ min⁻¹ (1 cm path length). The following substrates were used: casein (BDH), bovine serum albumin fraction V (Boehringer Mannheim), collagen (Sigma, type I insoluble) and gelatin (Sigma). Fragments of cuticle were prepared from the nematode P. redivivus by sonication and treatment with 1% (w/v) SDS according to Cox et al. (1981). Before being used in the assays, the fragments were thoroughly washed with 0.01 M Tris/HCl (pH 7.5) and lyophilized.
Hydrolysis of chromogenic peptide substrates was measured using peptides conjugated to 4-nitroaniline. The following peptides were used: N-benzoyl-l-arginine-4-nitroanilide. HCl (Bz-Arg-Na), N-benzoyl-tyrosine-4-nitroanilide. HCl (Bz-Lys-Na), N-benzoyl-l-tyrosine-4-nitroanilide (Bz-Tyr-Na), N-benzoyl-l-phenylalanine-4-nitroanilide (Bz-Val-Na), N-benzoyl-phenylalanine-4-nitroanilide (Bz-Phe-Na), succinyl-l-tyrosyl-l-leucyl-l-arginine-4-nitroanilide (Ac-Bz-Phe-Val-Arg-NA). N-benzoyl-phenylalanyl-leucyl-arginine-4-nitroanilide. HCl (Bz-Phe-Leu-Arg-Na), succinyl-1-tyrosyl-l-tyrosyl-l-phenylalanine-4-nitroanilide (Suc-Ala-Ala-Phe-NA), succinyl-l-tyrosyl-l-tyrosyl-l-phenylalanine-4-nitroanilide (Suc-Ala-Ala-Ala-NA), succinyl-glycyl-glycyl-l-phenylalanine-4-nitroanilide (Suc-Gly-Gly-Phe-NA). All were obtained from Serva Feinbiochemica, except Bz-Phe-Leu-Arg-NA, which was from NovaBiochem. Stock solutions (10 mM) of the substrates were prepared in DMSO, and diluted with H2O to 10 mM just before use. Ten microlitres of sample (typically 0.001 mg μl⁻¹) was added to 70 μl 100 mM Tris/HCl buffer (pH 7.5), and the reaction was started by adding 10 μl substrate (final concentration 0.025 mM). After a suitable incubation time (5-15 min) at 25 °C, the reaction was stopped by adding 10 μl 50% (v/v) acetic acid, and the absorbance was measured at 405 nm. Specific activities are given in nmol 4-nitroaniline liberated min⁻¹ (mg protein)⁻¹, using a molar absorption coefficient for 4-nitroaniline of 10 500 M cm⁻¹ (Sarah et al., 1989).

The kinetic constants (Km, Vmax) were determined from the initial rate of hydrolysis of the substrate Bz-Phe-Val-Arg-NA at five different concentrations by using Lineweaver-Burk plots.

### Protease inhibitors and pH.

The effects of inhibitors on the protease activity were examined by incubating 0-10 μg PII with various inhibitors for 5 min at room temperature, before adding the Tris buffer and the substrate Bz-Phe-Val-Arg-NA. The proteolytic activity was measured as described above. The following inhibitors were used: phenylmethylsulfonyl fluoride (PMSF), tosyl lysyl chloromethyl ketone (TLCK), tosyl phenylalanoyl chloromethyl ketone (TPCK), N-(β-l-carboxyiso- pentyl)-carbamoyl-α-(2-iminohexahydro-4(3H)-pyrimidinyl)-l-Gly-Phe-al (chymostatin), (β-l-carboxy-2-phenylthyl)-carbamoyl-Arg-Val-Arg-al (antipain), 3,4-dichloroisocoumarin (DCI), β-hydroxymercuribenzoate (pCMB), l-trans-e-epoxy-succinyl-leucyl-γleucylamide (4-guanidino)-butane (E-64), peptatin A, 1,10-phenanthroline, EDTA, and dithiothreitol (DTT). All inhibitors were obtained from Sigma, and they were prepared in stock solutions and applied in concentrations within their effective range, as given by Beynon & Salvesen (1989).

The effects of pH on the proteolytic activity were investigated by mixing enzyme extracts or purified PII with the Britton Robinson universal buffer system at pH values between 3 and 12, followed by protease assay.

### Stability of PII.

PII (10 μl, 0.002 μg μl⁻¹) and incubated at 4, 20, 37 or 55 °C. The protease activity was determined in the samples after 0, 1, 2, or 24 h using the peptide Bz-Phe-Val-Arg-NA.

### PAGE, detection of glycoproteins and molecular mass.

Discontinuous SDS-PAGE was performed according to Laemmli (1970) using a Mini-Protein II electrophoresis unit (Bio-Rad) and 12% running gels. Samples were diluted (1:1, by vol.) with 50 mM Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 11.6% (v/v) glycerol and 0.001% (w/v) bromophenol blue in the presence or absence of 5% (w/v) DTT. The samples were heated at 100 °C for 3 min. The molecular mass was calculated using a Bio-Rad prestained SDS-PAGE standard kit including phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.3 kDa). Gels were stained with Coomassie Brilliant Blue.

Glycoproteins were detected on SDS-PAGE blots using a digoxigenin (DIG) glycan detection kit (Boehringer Mannheim) according to the manufacturer's instructions. Approximately 2 μg PII was applied on the gels. Transfer and creatinase served as positive and negative controls for glycoproteins, respectively.

The apparent molecular mass of PII was determined by size-exclusion chromatography (using the Superdex 75 HR 10/30 column and conditions described above), and comparing the elution volume of the peak containing the protease activity with those of bovine serum albumin (67 kDa), ovalbumin (45 kDa), α-chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa) (Pharmacia low molecular weight standard kit).

### Isoelectric focusing and chromatofocusing.

Isoelectric focusing (Pharmacia Immobiline DryPlate, pH 4-7) was performed in a Multiphore II system (Pharmacia) according to the manufacturer's instructions with the temperature set at 10 °C. The isoelectric point (pl) was estimated using a broad pl calibration kit (pH 3-10, Pharmacia). The gel was stained with Coomassie Brilliant Blue.

Chromatofocusing was performed with a Mono P HR 5/20 column (Pharmacia) connected to the Pharmacia HPLC system (see above). The column was equilibrated with 0.025 M Bis-Tris/HCl (pH 5.8) and the sample was eluted with Polybuffer 74 (Pharmacia) pH 3.5. The chromatography was performed at 21 °C with a flow rate of 1-0 ml min⁻¹. Elution of proteins was followed at 280 nm. Fractions of 1-0 ml were collected and assayed for protease activity (using the peptide Bz-Phe-Val-Arg-NA).

### Protein content.

The protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

### Protein cleavage.

Purified PII (approx. 10 μg) was digested with Acrobacter lyticus protease (Wako Pure Chemical Industries) in 2 M guanidine hydrochloride essentially as described by Riviere et al. (1991). The peptides were separated by HPLC on a Brownlee C8 Aqueapore column (2.1 x 30 mm). Pumps were System Gold (Beckman) and the detector a photo diode array 990 (Waters). Buffers used for the reversed-phase chromatography were: A, 0.1% (v/v) trifluoroacetic acid (TFA) in H2O; and B, 0.1% TFA (v/v), 10% H2O (v/v), 90% acetonitrile (v/v). The gradient was 2 to 62% B in 60 min and 62 to 90% B in 3 min with a flow rate of 100 μl min⁻¹. Elution was followed at 214 nm and fractions were collected manually.

### Peptide sequencing.

Peptides were sequenced with an on-line phenylthiohydantoin (PTH) amino acid analyser (model 120, Applied Biosystems) according to the manufacturer's protocol.

### Bioassay.

P. redivivus nematodes were washed thoroughly with a 10 mM sodium phosphate buffer (pH 7.2) before being used in the assays. Portions of the nematode suspension containing 20-30 nematodes (100 μl) were transferred to microtitre wells and 5-10 μl protease extracts were added. After incubating (static) the wells at 20 °C for 20-22 h, the numbers of mobile and immobile (i.e. with arrested movements) nematodes were counted in a light microscope. The experiments were performed with five parallels and repeated at least twice. Controls were incubated without protease extracts. In one experiment, the extract PII (from Mono Q) was boiled for 10 min before being added to the nematodes. PMSF-treated PII was obtained by
The statistical significance of the differences in frequency of mobile and immobile nematodes in treated versus control samples was tested by $\chi^2$ analysis.

**RESULTS**

**Production of serine proteases**

When grown in dilute liquid medium allowing the formation of trap cells [T(+)], *A. oligospora* secreted very low levels of proteins (< 0.1 mg ml$^{-1}$). Proteolytic activity hydrolysing the chromogenic substrate Azocoll was, however, detected in culture filtrates concentrated by ultrafiltration. Experiments showed that it was possible to bind at least 95% of this activity to the anion exchange resin Q-Sepharose at pH 7.5.

Chromatography on Mono Q of the extracts eluted from the Q-Sepharose separated the proteolytic activity into two fractions, FI and FII (Fig. 1a). The proteolytic activities in both FI and FII were briefly characterized. The activities in both fractions were completely inhibited by the serine protease inhibitor PMSF, but not by the metalloprotease inhibitor phenanthroline (FI, 107% of the activity in controls; FII, 101%), nor the aspartate protease inhibitor pepstatin (FI, 118%; FII, 114%). The proteolytic activities of FI and FII had their maxima at basic pH values (approx. 7.5–10.0). Further purification of FI and FII by size exclusion chromatography (Superdex column) showed that the protease activity of FI eluted in one peak with an apparent molecular mass of 60 kDa and FII in one peak of 35 kDa. Of the 4-nitroanilide peptide substrates tested (see Methods), both FI and FII hydrolysed the tripeptide Bz-Phe-Val-Arg-NA best.

FI and FII were also the major fractions containing proteolytic activity in extracts purified from fungal cultures containing no traps [T(−)]. There was no significant differences in the ratio of the proteolytic activity of FI and FII between extracts recovered from T(+) and T(−) cultures; the ratio of the maximum $A_{280}$ of FI to maximum $A_{280}$ of FII in T(+) was 0.334 (mean) (SD, 0.168; $n = 3$) and in T(−) 0.202 (mean) (SD, 0.288; $n = 3$).

Chromatography on Mono Q of cell-bound proteases extracted from *A. oligospora* showed the presence of protease activity in a fraction corresponding to FI of the extracellular proteases, but no activity was detected corresponding to FII. Chromatography of FI from the cell extract on the Superdex column showed that the protease activity eluted as a peak with an apparent molecular mass of approximately 60 kDa.

**Bioassays**

Crude extracts from both T(+) and T(−) cultures contained materials that immobilized the nematode *P. redivivus* in microtitre assays (Table 1). After chromatography on Mono Q, the fraction FII immobilized the nematodes, but FI failed to do so. No significant effect however, was observed after boiling or treating FII with PMSF, indicating that the effect was due to the presence of...
Table 1. Immobilization of the nematode Panagrellus redivivus by protease extracts from A. oligospora

Extracts were incubated with nematodes in microtitre wells. After 20–22 h, the numbers of mobile and immobile nematodes were counted in a light microscope.

<table>
<thead>
<tr>
<th>Extract*</th>
<th>Protein (µg ml⁻¹)</th>
<th>PU† (x 10⁻⁹)</th>
<th>No. of nematodes</th>
<th>Statistics‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mobile</td>
<td>Immobile</td>
</tr>
<tr>
<td>Crude T(+)</td>
<td>1.36</td>
<td>4.4</td>
<td>254</td>
<td>87 (25.5)§</td>
</tr>
<tr>
<td>Crude T(−)</td>
<td>1.03</td>
<td>2.6</td>
<td>204</td>
<td>70 (25.5)</td>
</tr>
<tr>
<td>FI (Mono Q)</td>
<td>1.00</td>
<td>0.2</td>
<td>173</td>
<td>51 (22.8)</td>
</tr>
<tr>
<td>FII (Mono Q)</td>
<td>1.2</td>
<td>10.4</td>
<td>131</td>
<td>175 (57.2)</td>
</tr>
<tr>
<td>FII, boiled</td>
<td>1.2</td>
<td>0.4</td>
<td>275</td>
<td>68 (19.8)</td>
</tr>
<tr>
<td>FII, PMSF</td>
<td>1.2</td>
<td>0.0</td>
<td>273</td>
<td>72 (20.9)</td>
</tr>
<tr>
<td>PII</td>
<td>1.0</td>
<td>17.5</td>
<td>70</td>
<td>232 (76.8)</td>
</tr>
</tbody>
</table>

*Crude T(+) and T(−) extracts designate extracellular proteases concentrated from the medium using Q Sepharose. Mono Q FI and FII are two pooled fractions containing protease activity purified by anion exchange chromatography; PII is the purified extracellular serine protease (cf. Fig. 1).
† PU, proteolytic units. For definition see Methods.
‡ χ² tests (d.f. = 1) comparing the frequencies of mobilized and immobilized nematodes in treated versus control samples. NS, Not significant.
§ Percentage (of the total numbers) of nematodes that were immobilized. Values in controls (without enzyme) varied between 17 and 22%.

Table 2. Purification of the major extracellular serine protease (PII) from A. oligospora

The extracts were recovered from trap-containing cultures [T(+)]. Crude extract was obtained by binding of proteases in culture filtrate (4 x 4500 ml) to Q Sepharose, followed by elution in salt buffer and concentration by ultrafiltration. Crude extract was chromatographed on Mono Q, followed by hydrophobic interaction and size-exclusion chromatography as shown in Fig. 1(a–c). The purification scheme and calculations were repeated three times with similar results.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (PU)*</th>
<th>Specific activity (PU mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2.18</td>
<td>0.27</td>
<td>0.12</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mono Q</td>
<td>0.33</td>
<td>0.22</td>
<td>0.67</td>
<td>5.47</td>
<td>82.4</td>
</tr>
<tr>
<td>Phenyl Superose</td>
<td>0.060</td>
<td>0.039</td>
<td>1.56</td>
<td>12.74</td>
<td>35.1</td>
</tr>
<tr>
<td>Superdex</td>
<td>0.013</td>
<td>0.031</td>
<td>1.88</td>
<td>15.32</td>
<td>9.29</td>
</tr>
</tbody>
</table>

* PU, proteolytic units. Protease activity was assayed using the substrate Azocoll (see Methods).

Serine proteases in FII. On the basis of these data, we decided to further purify the protease(s) present in FII.

Purification of PII

A protease designated PII was purified from fraction FII by hydrophobic-interaction and size-exclusion chromatography (Fig. 1b, c; Table 2). The purification scheme and calculations were repeated three times, giving a mean specific activity of 3.14 PU mg⁻¹ (sd, 1.68), a 15.5-fold increase in specific activity (sd, 2.0), and a yield of 15.5% (sd, 16.7). Further purification of PII using chromatofocusing (Mono P) or hydroxylapatite chromatography (Bio-Rad HTP cartridge) did not increase the specific activity of the protease.

PII was found to be almost homogeneous on SDS-PAGE (Fig. 2). Electrophoresis of the purified protease, in the presence or absence of DTT, showed a major band at an estimated molecular mass of approximately 40 kDa.

The amount of PII recovered from 4 x 4500 ml of culture medium ([T(+) or T(−)]) varied and was relatively small, typically between 3 and 30 µg. Growth in medium with a higher concentration of soya peptone, in medium dialysed to remove low molecular mass peptides and proteins (cut-off 3 or 12 kDa), or adding nematodes (P. redivivus) to the mycelium did not considerably improve the amount of recovered PII (data not shown).

Molecular mass, isoelectric point and glycosylation

Gel filtration of the purified PII showed that it corresponded to a protein with an apparent molecular mass of approximately 35 kDa. The isoelectric point of PII was 4.6 on the Immobiline gel with the temperature set at
**Fig. 2.** SDS-PAGE of samples from the purification of PII. Lanes: A, crude extract; B, Fraction FII from the Mono Q column (cf. Fig. 1a); C, Fraction FII from the Phenyl Superose column (cf. Fig. 1b); D, PII from the Superdex column (cf. Fig. 1c). Molecular mass (kDa) of marker proteins is indicated at the left of the figure.

10 °C. Using chromatofocusing at 21 °C, PII eluted as a sharp peak at pH 4.4 in which the $A_{280}$ and proteolytic activity coincided.

PII was stained on blots using the DIG kit, indicating that the protease was a glycoprotein.

**Hydrolysis of protein substrates and nematode cuticle**

PII showed high hydrolytic activity against denatured casein and moderate hydrolysis of BSA, gelatin, denatured collagen and the preparation of cuticle from the nematode *P. redzii* (Table 3). The activity against native collagen was very low.

**Hydrolysis of peptide substrates**

The substrate specificity of PII was investigated in more detail with a number of synthetic peptide substrates, including those with one or three amino acids. The substrates were blocked at the N-terminus and bore the chromogenic group at the C-terminus. The most completely cleaved substrate was the tripeptide Bz-Phe-Val-Arg-NA (Table 4). Substituting Val for Leu at position $P_2$ in this peptide (for notation see Schechter & Berger, 1967) significantly decreased the rate of hydrolysis. PII was also active against the tripeptide Suc-Ala-Ala-Phe-NA. Substitutions at position $P_1$ (-Ala-Ala-Ala-) or at $P_3$ and $P_3$ (-Gly-Gly-Phe-) in this peptide significantly decreased the rate of hydrolysis. The one-peptide substrates tested were not cleaved or were active at considerably lower rates.

**Table 3.** Hydrolysis of various protein substrates by the serine protease PII, purified from *A. oligospora*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (denatured)*</td>
<td>100</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>5-99</td>
</tr>
<tr>
<td>Gelatin</td>
<td>10-5</td>
</tr>
<tr>
<td>Collagen</td>
<td>0-42</td>
</tr>
<tr>
<td>Collagen (denatured)*</td>
<td>16-2</td>
</tr>
<tr>
<td>Nematode cuticle†</td>
<td>9-84</td>
</tr>
</tbody>
</table>

* Denatured by heating at 100 °C for 15 min.
† Fragments of cuticle prepared from the nematode *P. redzii*.

**Table 4.** Substrate specificity of the serine protease PII

Purified enzyme (0.1 μg) was incubated with the substrates (1.0 mM) at 25 °C for 20 min. The maximum proteolytic activity corresponding to 100% was 1370 (sn, 85) nmol NA ml⁻¹ min⁻¹ (mg protein)⁻¹, for three replicates.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-Arg-NA</td>
<td>1-4</td>
</tr>
<tr>
<td>Bz-Lys-NA</td>
<td>1-5</td>
</tr>
<tr>
<td>Bz-Tyr-NA</td>
<td>0-0</td>
</tr>
<tr>
<td>Suc-Phe-NA</td>
<td>1-6</td>
</tr>
<tr>
<td>Bz-Phe-Val-Arg-NA</td>
<td>100-0</td>
</tr>
<tr>
<td>Bz-Phe-Leu-Arg-NA</td>
<td>28-9</td>
</tr>
<tr>
<td>Suc-Ala-Ala-Phe-NA</td>
<td>45-6</td>
</tr>
<tr>
<td>Suc-Ala-Ala-Ala-NA</td>
<td>2-8</td>
</tr>
<tr>
<td>Suc-Gly-Gly-Phe-NA</td>
<td>2-4</td>
</tr>
</tbody>
</table>

The mean values ($n = 3$) of $K_m$, $k_{cat}$, and catalytic efficiency ($k_{cat}/K_m$) of PII for the hydrolysis of the peptide Bz-Phe-Val-Arg-NA were 0.0962 mM (sd, 0.0145), 7.62 s⁻¹ (sd, 1.45), and 78900 M⁻¹ s⁻¹ (sd, 3200), respectively.

**Inhibition and pH effects**

PII was completely inhibited by the serine protease inhibitor PMSF (Table 5). The amino acid aldehydes chymostatin and antipain with a Phe and Arg residue, respectively, were also inhibitory. Other serine protease inhibitors, including the isocoumarin DCI, TLCK and TPCK were less inhibitory or completely ineffective. The thiol reagent pCMB inhibited the activity of the enzyme. The cysteine protease inhibitor E-64, the aspartic protease inhibitor pepstatin as well as DTT did not significantly affect the activity of PII. Minor effects on the proteolytic...
activity of PI1 were observed after treating the enzyme with the metal chelator EDTA at 5 mM concentration. The proteolytic activity of PI1 had a broad pH optimum at alkaline pH values (approx. 7–9) when using the Britton–Robinson universal buffer system.

**Stability**

The proteolytic activity of PI1 was sensitive to storage and the enzyme was subjected to rapid autolysis even at 4 °C (Fig. 3).

**Sequencing**

The N-terminal of PI1 was blocked. Cleavage with the α, β-epoxyproline-specific protease from *S. cerevisiae* yielded a peptide with a sequence that is partly bound to the mycelium of the fungus. Furthermore, the protease(s) present in FI had a higher molecular mass than those in FII, and analyses of cell extracts indicated that protease of FI, but not FII, was partly bound to the mycelium of the fungus. In preliminary experiments extracellular proteases produced from *A. oligospora* mycelium with traps [T(+)] were compared with those from vegetative mycelium [T(−)]. Analysis of proteases in culture filtrates using anion-exchange chromatography (Mono Q), peptide substrates (unpublished data), or results from previous work using protease inhibitors and substrate gel electrophoresis (Tunlid & Jansson, 1991) have so far not revealed any differences in the production of extracellular serine proteases between T(+) and T(−) cultures. Both T(+) and T(−) cultures contained two fractions of extracellular serine protease activity (FI and FII). The protease activity in these fractions was similar as examined by synthetic peptides, but the protease(s) present in FI had a higher molecular mass than those in FII, and analyses of cell extracts indicated that protease of FI, but not FII, was partly bound to the mycelium of the fungus. Furthermore, in bioassays the protease present in FII immobilized the nematode *P. redivivus*, but that in FI did not.

The sensitivity of the enzyme PI1, purified from FII, to the inhibitors PMSF, antipain and chymostatin indicated that it was a serine protease. The relatively low molecular mass (approx. 35 kDa) and isoelectric point (pH 4.6) of PI1 was consistent with those of other serine proteases purified from entomopathogens (Bi-dochka & Khachatourians, 1987; St Leger et al., 1987a), as well as from the nematophagous fungus *Verticillium albo-astrum*.

### Table 5. Effects of various inhibitors on the protease activity of PI1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Enzyme activity as % of control (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>1.0</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.010</td>
<td>86.2 (17.5)</td>
</tr>
<tr>
<td>TPCK</td>
<td>0.010</td>
<td>86.4 (10.7)</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>0.010</td>
<td>63.3 (3.9)</td>
</tr>
<tr>
<td>Antipain</td>
<td>0.010</td>
<td>48.4 (6.6)</td>
</tr>
<tr>
<td>DCI</td>
<td>0.050</td>
<td>86.8 (4.4)</td>
</tr>
<tr>
<td>pCMB</td>
<td>0.10</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>E-64</td>
<td>0.010</td>
<td>92.8 (10.1)</td>
</tr>
<tr>
<td>Phenanthroline</td>
<td>1.0</td>
<td>98.9 (7.2)</td>
</tr>
<tr>
<td>EDTA</td>
<td>5.0</td>
<td>84.4 (7.5)</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>0.010</td>
<td>92.2 (18.5)</td>
</tr>
<tr>
<td>DTT</td>
<td>5.0</td>
<td>98.9 (7.2)</td>
</tr>
</tbody>
</table>

**Fig. 3.** Stability of PI1 at various temperatures. Approximately 0.2 μg PI1 was incubated in 0.10 M Tris/HCl buffer (pH 7.5) at 4 °C (■), 20 °C (□), 37 °C (▲) or 55 °C (○). The protease activity was assayed using the substrate Bz-Phe-Val-Arg-NA. The maximum proteolytic activity in controls at the start of the incubation (corresponding to 100%) was between 324 and 480 nmol NA ml⁻¹ min⁻¹ (mg protein)⁻¹. Bars indicate ±1 SD (n = 3).

**Fig. 4.** Comparison of the amino acid sequence of a peptide obtained by cleaving PI1 with *Achromobacter lyticus* lyticus protease with those of the subtilisin-family enzyme CA, subtilisin Carlsberg (from Nedkov et al., 1985); PB, Protease B of *Saccharomyces cerevisiae* (Moehle et al., 1987); PK, Proteinase K of *Trichirachium album* (Jany et al., 1986); Pr1, protease from *Metarhizium anisopliae* (St Leger et al., 1992). Identical amino acids are boxed. The asterisk denotes the active-site His residue.
Pseudomonas subtilisporium (Lopez-Llorca, 1990). Several peptides have been sequenced from PII (unpublished data). The reported amino acid sequence from a peptide recovered after A. byticas cleavage contained a sequence showing high homology with the region of the active site histidine residue of the subtilisin family of serine proteases (cf. Fig. 4). The total inactivation of PII by PMSF but not by the trypsin and chymotrypsin inhibitors TLCK and TPCK further supports the view that PII is a protease of the subtilisin family (Kominami et al., 1981a; Ogrydziak & Scharf, 1982; Ebeling et al., 1974; Ottesen & Svendsen, 1970).

The total loss of activity of PII caused by pCMB indicates that the protease requires a reactive sulfhydryl group. PII was not, however, inhibited by the cysteine protease inhibitor E-64 nor the reducing agent DTT, which further supports the view that PII is a protease of the subtilisin family. The effects of pCMB may reflect the close proximity of a cysteine residue to the active site of the enzyme. In such cases it has been observed that binding of pCMB can interfere with the binding of the substrate to the active site (Bai & Hayashi, 1979).

Studies of the various peptides showed that PII hydrolysed both trypsin (Bz-Phc-Val-Arg-NA) and chymotrypsin (Suc-Ala-Ala-Phe-NA) substrates. Mixed trypsin/chymotrypsin activities have previously been described for at least one other fungal serine protease of the subtilisin type, protease B from bakers’ yeast (Kominami et al., 1981b). St Leger et al. (1987b) demonstrated the presence of acid serine proteases in several entomopathogens with preference for the substrate Bz-Phc-Val-Arg-NA but cleavage of chymotrypsin substrates was not reported. The differences in the action of PII on triptides and monopeptides, and the effects of substitutions at position P₃ and P₄, indicate that the active site of PII extends over several amino acids and that the reactivity of a particular bond (P₃-P₄) is not only dependent on P₃ and P₄ but also on the residues at neighbouring subsites (cf. Schechter & Berger, 1967). The comparative non-specificity of PII accounts for it being a general protease with activity against a range of protein substrates.

Previous experiments treating A. oligospora with various protease inhibitors have indicated a role of fungal serine protease for the immobilization of nematodes captured by A. oligospora (Tunlid & Jansson, 1991). The most effective inhibitors used in these experiments (PMSF, antipain and chymostatin) were also the most potent inhibitors of PII. The immobilization of free-living nematodes by PII further indicates that the purified serine protease can be involved in the infection of nematodes by A. oligospora. On the basis of microscopic investigations it has been proposed that the immobilization of nematodes by A. oligospora occurs during the penetration of the cuticle (Nordbring-Hertz et al., 1986). This is a possible mechanism for the immobilization effect of PII on the nematode P. redivius, since the purified enzyme hydrolysed proteins in fragments of cuticles prepared from this nematode. Notably, PII did not degrade native collagen, which is supposed to be the major class of protein in the cuticle of nematodes (Cox et al., 1981). However, it has been shown that the cuticle of Panagrellus redivius contains proteins that are susceptible to proteases such as papain (Martin et al., 1986), which is a rather nonspecific cysteine protease with a preference for Arg or Lys at the subsite P₃ (Cleveland et al., 1977). In another study it has been demonstrated by scanning electron microscopy that adding papain to plant parasitic nematodes can produce structural changes in the cuticle (Miller & Sands, 1977). At this point, however, the possibility cannot be excluded that the enzyme has a more specific cytotoxic effect and that it can be transported inside the nematode during pumping of the feeding apparatus.

The relatively broad substrate specificity of PII and the production of this enzyme by vegetative mycelium indicates that PII has two independent functions, one during saprophytic growth and one during the infection of nematodes. Such a dual role has been suggested for the well-characterized serine protease Prl purified from Metarhizium anisopliae (St Leger, 1993). Notably, results from the inhibitor experiments on the fungal mycelium indicate that the active serine protease is present before contact with the nematode (Tunlid et al., 1991). Other proteases including collagenases might be synthesized after contact with the cuticle. Induction of protease activity in A. oligospora by peptone extract was demonstrated by Aswani & Jaffar (1992), but the enzyme was not purified and characterized. Collagenase(s) can be induced in the nematophagous fungus Arthrobotrys oligospora by growing the fungus in liquid medium containing collagen as a substrate (Schenk et al., 1980). This enzyme was not characterized in detail, but its sensitivity towards inhibitors showed that it was not identical with the purified PII.

There is an increasing demand to use nematophagous fungi or their products as biological control agents for plant and animal parasitic nematodes (Kerry, 1990). There are some data indicating that hydrolytic enzymes including proteases and collagenases can be added to soil to control plant parasitic nematodes (Miller & Sands, 1977; Galper et al., 1990). Isolation and characterization of hydrolytic enzymes from nematophagous fungi involved in the infection process can be used to develop more effective preparations or strains of organisms to be used for biological control of parasitic nematodes.

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REFERENCES


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