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Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*

Zhao Minglian
Mo Minghe
Zhang Keqin[1]

Key Laboratory of Industrial Microbiology & Fermentation Technology of Yunnan Province, Yunnan University, Kunming 650091, Yunnan, P.R. of China

Abstract: A neutral serine protease (designated Aoz1) was purified to homogeneity from a strain of *Arthrobotrys oligospora*, obtained from soil in Yunnan Province. The purified protein showed a molecular mass of approximately 38 000 Dalton, pI 4.9 and displayed optimal activity at 45 C and pH 6–8. The protein could hydrolyze gelatin, casein and the chromogenic substrate azocoll, and it could immobilize nematodes in vitro (*Panagrellus redivivus* L. [Goodey]). The level of activity in culture medium was found to increase with increasing gelatin concentration. Scanning electron micrographs demonstrated dramatic structural changes in nematode cuticle treated with the purified protease. A partial peptide sequence obtained by N-terminal sequence analysis was used to design degenerate primers for the isolation of a cDNA gene encoding the mature protease. Analysis of the cDNA and corresponding genomic sequence revealed 97% identity with *PII,* a gene previously described from *A. oligospora*, and we conclude that this gene is likely a PII ortholog.

Key words: *Arthrobotrys oligospora* Fres, full-length cDNA gene, neutral serine protease, 3′ RACE, SMART-RACE, virulence factor

INTRODUCTION

Parasitic nematodes cause great damage to crops and livestock. During the past 50 years, nematicides have been used extensively to control nematodes in both plants and animals, but their use has become increasingly restricted due to public health and environmental concerns. Nematophagous fungi are natural enemies of nematodes because they may attack living nematodes or their eggs and use them as a source of nutrients. Because of the role these fungi may play in the natural or applied biocontrol of nematodes, and because of the interesting infection biology and biochemistry associated with the capturing, cuticle penetration and colonization process, nematophagous fungi have attracted much attention (Dijksterhuis et al 1994).

Extracellular proteases have been implicated in the penetration and digestion of host tissues by many plant and animal parasitic fungi (Geremia et al 1993, Rhodes 1995, St. Leger 1995). During the infection of nematodes, nematophagous fungi must penetrate the nematode cuticle, which is a rigid and flexible exoskeleton composed mainly of proteins, including collagens (Cox et al 1981, Maizels et al 1993). Although the proteinaceous nematode cuticle is an effective barrier against most pathogens, nematophagous fungi can breach it using these enzymes.

Several extracellular proteases isolated from nematophagous fungi belong to serine proteases, and it has been demonstrated that they have high homology to members of the subtilase family (Segers et al 1994, Tunlid et al 1994, Bonants et al 1995). The importance of serine proteases during the infection of nematodes has been indicated by treating nematophagous fungi with various protease inhibitors (Tunlid and Jansson 1991) by localizing the protease during the infection of eggs (Lopez-Llorca and Robertson 1992), by examining the effects of purified proteases on the mobility of nematodes or on the development and hatching of eggs (Tunlid et al 1994, Bonants et al 1995) and by showing that the production of protease can be stimulated in the presence of nematode cuticle or egg shells (Bonants et al 1995, Åhman et al 1996).

To date, relatively small amounts (1–2 μg/L) of native cuticle-degrading protease have been obtained from *A. oligospora* culture media (Tunlid et al 1994). Toward the goal of acquiring higher quantities for study, Åhman et al (2000) successfully expressed the serine protease gene *PII* from *A. oligospora* in *Aspergillus niger*. Here we report the characterization of an apparent *PII* homolog, which we have designated *Aoz1* in accord with gene nomenclature in other fungi, and its protein (Aoz1) from an isolate of *A. oligospora* from Yunnan Province. We demonstrate that expression of this protease is enhanced by addition of gelatin to the culture medium. In addition, we

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[1] Corresponding author. E-mail: kqzhang1@yahoo.com.cn

demonstrate the biochemical and ultrastructural consequences of exposure of nematodes and nematode cuticle to the enzyme.

MATERIALS AND METHODS

Microorganisms and culture conditions.—In initial experiments we examined protease production by one isolate from each of four species: *Arthrobotrys oligospora*, *A. dactylioides*, *A. brochopaga* and *Monacrosporium gephyrophagum*. All isolates were obtained from field soil in Yunnan Province and were maintained on cornmeal-extract agar (CMA) slants with transfer every 3–4 mo. Single-spore isolates of four species were chosen to be examined for protease production and were activated on CMA plates for 3–14 d at 28 C before inoculation into liquid medium (see below).

The nematode *Panagrellus redivivus* L. (Goodey) was grown axenically in semiliquid oat medium at 28 C for 5 d and then stored at 4 C until use. Nematodes were washed extensively with autoclaved distilled water, penicillin solution (400 000 IU) and streptomycin solution (320 000 IU) before being used in bioassays.

Screening of strains for protease production.—A modified liquid medium (LMZ, 2 g gelatin, 8 g peptone, 1 g yeast extract, 0.5 g $[NH_4]_2SO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$ in 1 L phosphate buffer [pH 6.5, $Na_2HPO_4 \cdot 12H_2O$/$NaH_2PO_4 \cdot 2H_2O$] [Schench et al 1980], supplemented with an autoclaved suspension of *Panagrellus redivivus*) was used for protease production. Fifty μL of LMZ was poured into a 500 mL flask, and two drops of oil were added to avoid foaming during culture periods. Several agar pieces with fungal mycelia from four single-spore isolates were inoculated and grown aerobically in an incubator at 25–28 C for 6 d by shaking at 150–200 rpm. Cultures were examined daily for bacterial contamination by inoculation of medium onto LB plates (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar in 1 L distilled water, pH 7.0). At harvesting, agar pieces were removed by centrifugation at 4 C, 7800 rpm (Beckman Avanti J-20 Centrifuge) and general filtration. Supernatants were stored at 4 C before concentration of enzymes by ammonium sulfate precipitation.

Protease activity assays.—A semiquantitative casein-plate method was employed to screen supernatant and purification fractions for protease activity. Casein plates were prepared by combining molten agar in phosphate-citrate buffer (35 mM citric acid, 65 mM trisodium phosphate, 4.0% agar, pH 7.0, dissolved by microwaving and cooled to 55 C) with an equal volume of 1.0% casein dissolved in the same buffer at 55 C, followed by pouring 15 mL into standard 10 cm petri dishes. After cooling, wells were created in plates with a punch. Each well received 30 μL of an enzyme sample, and plates were incubated overnight at 45 C before being examined for enzyme halos.

A quantitiative method employing chromogenic azocoll as substrate was used to assess the effects of temperature and culture age on protease activity as well as to monitor activity in purification fractions. Twenty μL of protease sample was added to 200 μL of azocoll in 25 mM Tris-HCl buffer (pH 7.4) in a 1.5 mL microcentrifuge tube, and the tube was incubated in a Thermomixer (Eppendorf) for 30 min at 400 rpm. Absorbance of 100 μL of sample was read at 520 nm with a nucleic acid/protein analyzer Du-7000 (Beckman Coulter). The effect of pH on enzyme activity was examined using the azocoll method but substituting a mixed buffer system (pH 2–11), which included 100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM $CaCl_2$, 150 mM KCl and 0.01% Triton X-100. Each solution was adjusted to the desired pH with HCl or NaOH.

Enrichment and purification of protease.—Cultures were grown 6 d in gelatin-supplemented medium as described above. Three liters of culture filtrate were fractionated using four concentrations of ammonium sulfate (0–20%, 20–45%, 45–65% and 65–85% saturation) in an ice bath. The precipitates were collected by centrifugation, dissolved in 25 mM Tris/HCl buffer (pH 7.4), and dialyzed against the same Tris buffer through several changes until the conductivity of the dialyzed solution was below 2.0 ms/cm. Protease activity in the four fractions was assayed using the casein-plate method (pH 7.0). The dialyzed sample from the 45–65% precipitate contained the highest activity and was selected for further purification. A sample (20 mL) filtered through a 0.45 μm filter was applied to a Q Sepharose Fast Flow column (20 mL; Bio-Rad BioLogic Liquid Chromatography System) equilibrated with the buffer used for dialysis. Bound proteins were eluted with a linear gradient of increasing NaCl (0–1.0 M), and 6 mL fractions were collected. All fractions with absorbance at 280 nm, including flow-through peaks, were assayed for protease activity using the casein-plate method.

All fractions with a protease activity halo on casein plates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 12% resolving and 5% stacking gel (Bio-Rad MiniProtean II gel device), following standard methods (Sambrook et al 1989). Molecular mass standards were phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14.4 kDa). Gels were stained with Gelcode® Blue Stain Reagent (Coomassie G-250, Pierce). Fractions with the same electrophoresis band pattern were pooled, concentrated by ultrafiltration (Amicon, YM-3 membrane, cut-off 3 kDa), and exchanged in 25 mM Tris/HCl buffer containing 0.15 M NaCl. The final concentrated sample (2 ml) was loaded onto a Sephacryl S-100 High Resolution gel filtration column (180 mL) equilibrated with 25 mM Tris/HCl buffer containing 0.15 M NaCl (pH 7.4). The column was eluted with the equilibration buffer and 3 mL fractions collected. Fractions with protease activity and a single band by gel assay were pooled, desalted by ultrafiltration and lyophilized.

Protein assay.—Protein was measured with a BCA Protein Assay Reagent Kit using Bicinchoninic Acid, according to the manufacturer (Pierce), with bovine serum albumin (BSA) as the standard.

Isoelectric focusing for pI assay.—Isoelectric focusing was performed at 10 C using Immobiline DryPlate in a Multiphor II Electrophoresis Unit (Pharmacia) according to the manufacturer's instructions. The isoelectric point was estimated with a broad pI calibration kit (pI 3.5–9.3). The IEF gel was stained with Coomassie Brilliant Blue.

Inhibition of the purified protease.—Twenty μL of protease sample and 5 μL PMSF (10 μM), EDTA (0.5 M, pH 8.0) and SSI (10 μM) were added to microcentrifuge tubes and incubated 5 min at room temperature. Protease activity was assayed using the azocoll method described above.

Scanning electron microscopy of nematode cuticle.—Nematode suspensions (1 mL) were treated for 24 h at 25 C with crude proteases, purified serine protease, and purified bacterial collagenase (Worthington), followed by examination with a scanning electron microscope. An untreated nematode sample served as a control.

N-terminal amino acid analysis.—The N-terminal region of the purified protease was analyzed by Edman degradation using a PE/ABI 491 protein sequenator. After electrophoresis, the purified protease was electroblotted onto a PVDF transfer membrane (0.2 μm, Bio-Rad, Sequi-Blot™) and stained with Gelcode® Blue Stain Reagent for protein microsequencing. Sequencing conditions were: sample amount, 30.0 picomoles; standard amount, 10.0 picomoles; sampling rate, 4.0 Hz; detector scale, 1.000 AUFS. PTH amino acid peaks were identified in the HPLC elution profile with an Applied Biosystems Procise sequencer.

cDNA and genomic cloning.—Agar pieces from colony edges of *A. oligospora* isolate 807 were inoculated into LMZ and incubated with shaking at 150 rpm for 4 d at 28 C. The mycelia were harvested, and samples immediately were flash-frozen in liquid nitrogen and stored at −70 C. Fungal total RNA was isolated using an RNeasy Plant Mini Kit™ (Qiagen) according to the manufacturer's protocol.

The RACE (Rapid Amplification of cDNA Ends) method (3′ RACE System for Rapid Amplification of cDNA Ends [Invitrogen]; SMART-RACE cDNA Amplification Kit [Clontech]) was employed to obtain the full-length *Aoz1* cDNA. Three degenerate primers deduced from a portion of the Aoz1 N-terminal amino acid sequence (E-Q-T-D-S-T-W) were designed for the 5′ end of the mature protease gene (primer 0118-52: 5′ > GA(AG) CA(AG) AC(AGCT) GA(C T) AG(CT) AC(AGCT) TGG < 3′, primer 0118-53: 5′ > G A(AG) CA(AG) AC(AGCT) GA(CT) TC(AGCT) AC(AC) T GG < 3′, and primer 0118-54: 5′ > GA(AG) CA(AG) AC (AGCT) GA(CT) TC(AGCT) AC(TG) TGG < 3′). These primers were used in combination with reverse transcriptase (RT) and an oligo-dT adapter primer to amplify the cDNA from fungal total RNA. PCR conditions using Taq DNA polymerase were: predenaturation at 94 C for 3 min before polymerase addition, followed by 30 cycles consisting of denaturation at 94 C for 30 s, primer annealing at 55 C for 30 s and primer extension at 72 C for 1 min. After cycling, the reaction mixture was kept at 72 C for 10 min and cooled to 4 C.

The resulting amplified cDNA fragment (approximately 1.2 kb) was separated by electrophoresis in a 1.0% agarose gel in 0.5× Tris-borate-EDTA and visualized with EtBr and UV irradiation using standard procedures (Sambrook et al 1989). A slice of agarose containing the DNA band was excised with a clean razor blade and purified with a Waters Gel Extraction Kit according to the instructions of the manufacturer (TaKaRa). The purified cDNA fragment was cloned into the pGEM-T® vector, following the instructions of the manufacturer (Promega Corporation, Madison, Wisconsin).

A cDNA fragment representing the 5′ end of the *Aoz1* transcript was obtained using a SMART™ RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech). The procedure employed a 25-nucleotide primer (5′ > GCG AAT ACC GGT ATC GAT GAC GTA G < 3′) based on a portion of the 3′ sequence of the cDNA clone.

A portion of the gene was amplified from genomic DNA using two specific primers based on the assembled cDNA sequence (Forward: 5′ > GCT GAA CAG ACT GAC TCC < 3′, Reverse: 5′ > CTA GCT AGC AAC AAT CGT GAC < 3′). Extraction of total fungal DNA employed a DNeasy® Plant Mini Kit (Qiagen). The PCR conditions were: predenaturation at 94 C for 3 min, followed by 30 cycles consisting of denaturation at 94 C for 1 min, primer annealing at 50 C for 1 min and primer extension at 72 C for 1 min. After cycling, the reaction mixture was kept at 72 C for 10 min and cooled to 4 C.

DNA sequencing.—Plasmid DNA and PCR products were sequenced using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (version 2.0, Perkin Elmer) and an ABI PRISM™ 377 DNA Sequencer. The sequencing primers were T7 (forward) and SP6 (reverse) universal primers and an internal specific primer (5′ > GCT GTT AAG GTT CTT AGC GCT < 3′).

RESULTS

Strain selection.—Of the four nematode-trapping fungal strains examined, only that identified as *A. oligospora* (isolate 807) exhibited substantial protease activity with the casein plate assay. This strain was used for the analyses reported here. The strain produces three-dimensional networks (traps) when grown on CMA plates and LMZ media. All conidia have only one septation (FIG. 1).

Protease activity in response to gelatin and fermentation time.—Protease activity in culture filtrates increased with the concentration of gelatin as an inducing substrate when added to LMZ medium (data not shown). Protease activity also increased over time during the first 6 d after inoculation (FIG. 2).

Purification of cuticle-degrading protease.—By incorporating gelatin in the culture medium and growing mycelium 6 d before harvest, we were able to maximize protease production for purification (TABLE I). Protease activity was monitored during the course of pu-

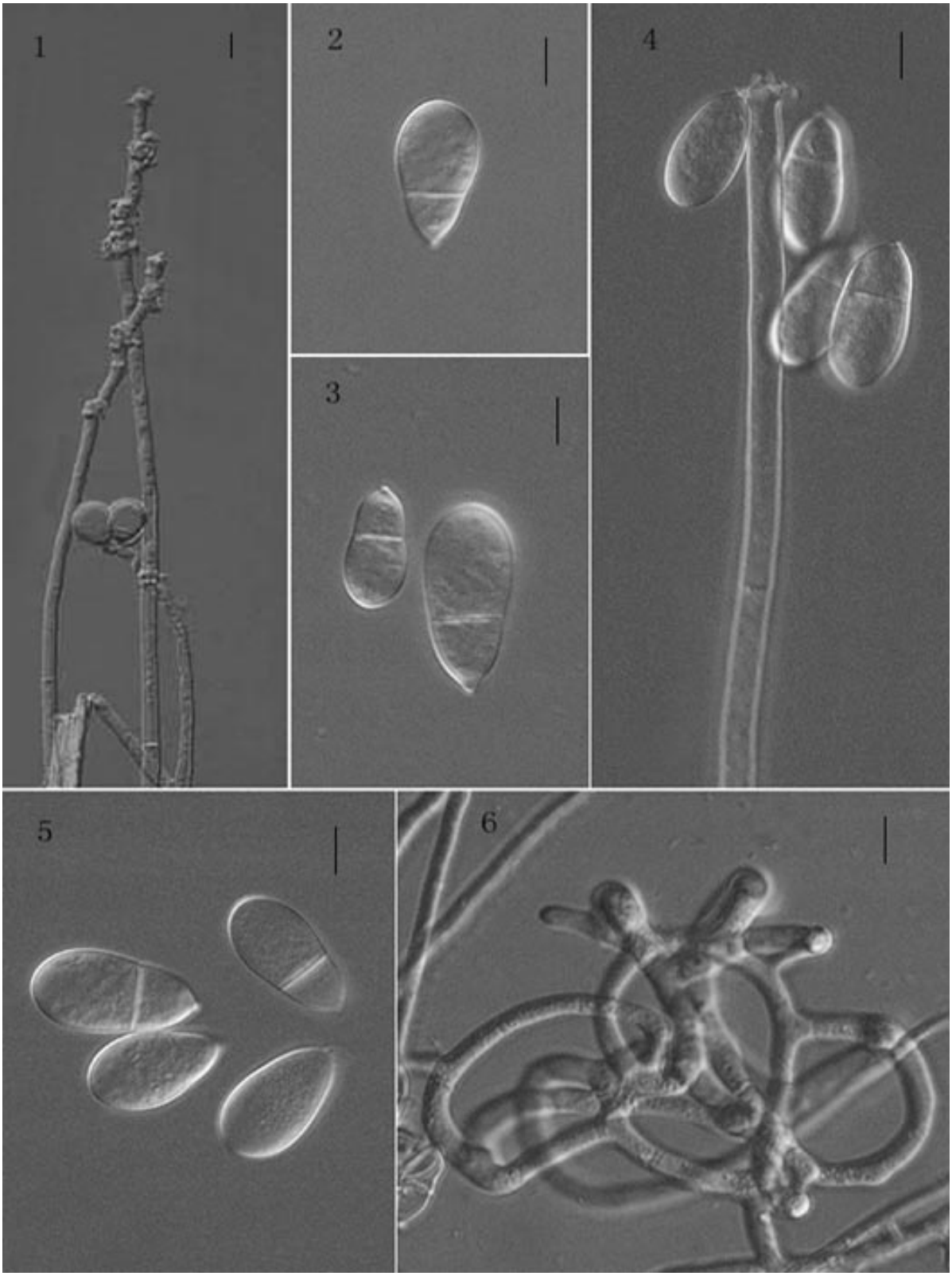

FIG. 1. Characteristics of *A. oligospora*. Isolate 807 produces three-dimensional traps and conidia with one septation each.

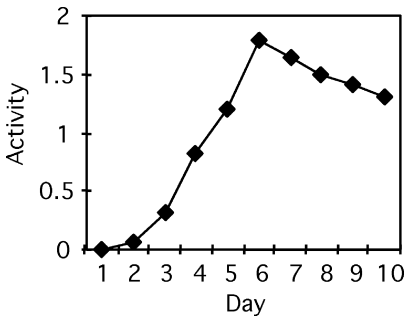

FIG. 2. Relationship between protease production and fermentation time. The maximum protease activity was at Day 6 of the culture period. Each point represents the average of triplicate assays.

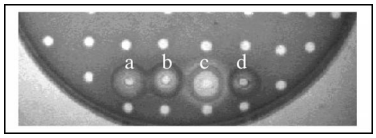

FIG. 3. Casein plate method for monitoring protease activity. Results are shown for four $(NH_4)_2SO_4$ fractionation samples from the purification procedure: (a) 0–20%, (b) 20–45%, (c) 45–65%, and (d) 65–85% saturation. The 45–65% fraction was chosen for subsequent purification steps.

rification by a combination of casein-plate assays (FIG. 3), SDS-PAGE and quantitative assays using azocoll.

Molecular mass determination.—The protease sample from gel filtration chromatography showed a single protein band. The mobility of the target protein band using SDS-PAGE corresponded to a molecular mass of 38 000 Dalton (FIG. 4).

N-terminal region.—The purified Aoz1 protein was electroblotted to a PVDF membrane for protein sequencing. PTH amino acid peaks in the HPLC elution profile revealed an N-terminal amino acid sequence of NH_2-A-E-Q-T-D-S-T-W-G-L-D-R-I-S-H-E-D-Y-S-A. This sequence has been deposited in the SWISS-PROT protein sequence database (accession number P83290).

Protease inhibition.—The enzymatic activity of the purified protease was not inhibited measurably by EDTA, but it was inhibited by PMSF (phenylmethylsulfonyl fluoride, Sigma) and SSI (an inhibitor of the subtilase family of serine proteases, Novozyme), suggesting that the enzyme contains a serine residue in its active site.

Effects of temperature and pH on enzyme activity.—The pH optimum for hydrolysis of azocoll by the purified protease was found to be pH 6–8. The optimal temperature for hydrolysis was 45 C (FIGS. 5, 6).

Substrate specificity.—The specificity of the purified protease was tested using various substrates (suspended in 25 mM Tris/HCl buffer, pH 7.4) at 45 C. The enzyme digested casein, AZCL-casein (a substrate for nonspecific protease, which releases blue dye when degraded), gelatin, azocoll, and nematode cuticle.

Degradation of nematode cuticle.—Scanning electron microscopy of nematode cuticle demonstrated dramatic structural changes resulting from treatment with purified Aoz1 (FIG. 7).

Immobilization of nematodes in bioassays.—Suspensions containing 30–50 washed nematodes in 150 μL sterile water were transferred to microtiter wells and incubated 24 h with 15 μL purified Aoz1. Nematodes exposed to protease were immobilized by the treatment, and microscopic examination revealed cuticle degradation. Control nematodes treated in the same manner, but without protease, were not immobilized and showed no evidence of cuticle degradation (FIG. 8).

Full-length cDNA sequence.—The full-length cDNA sequence for the *Aoz1* gene has been deposited in GenBank under accession number AF516146. The putative mature peptide is 303 amino acids in length. The calculated molecular mass of 38 390 Da is in accordance with the estimate of approximately 38 000 Da based on SDS-PAGE.

Sequencing of a genomic DNA fragment encoding

TABLE I. Purification of neutral serine protease from *A. oligospora*

Purification step	Protein/(mg)	Total activity (AU)[a]	Specific activity (AU/mg)	Purification (fold)	Recovery (%)
Crude filtrate	1.02	0.035	0.34	1.00	100
$(NH_4)_2SO_4$ fractionation[b]	0.18	0.80	—	—	—
Q Sepharose FF	0.07	0.0069	0.98	2.88	19.7
Sephacryl S-100	0.012	0.0134	1.12	3.29	8.3

[a] Protease activity was assayed using the chromogenic substrate azocoll (Sigma). One activity unit (AU) is defined as the increase in A_{520} mL^{-1} min^{-1} (1 cm path length).

[b] The 45–65% saturation precipitate.

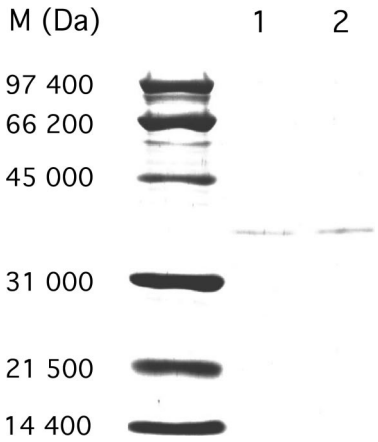

FIG. 4. SDS-PAGE pattern of purified serine protease preparation. Marker proteins for which molecular-mass values (Da) are indicated are shown on the left. Lanes 1 and 2 each received 40 μL of the purified protease sample.

the mature Aoz1 protein showed that *Aoz1* and the previously described *PII* gene (Åhman et al 1996) are 97% identical over the region sequenced and they share a 61-bp intron (see below).

DISCUSSION

Our results provide support for the important role of one or more proteases in the pathogenicity of *A. oligospora* toward nematodes. The protease characterized here, Aoz1, could immobilize nematodes and degrade cuticle. Based on BLASTP analyses, the deduced primary structure of Aoz1 revealed extensive similarity with proteases of the subtilase family of serine endopeptidases, including the conservation of serine, histidine and aspartate components of the active site in subtilisins. The strong similarity between Aoz1 and PII suggest that the two genes are orthologous, with slight differences in molecular mass, pI and sequence being the result of interisolate polymorphism.

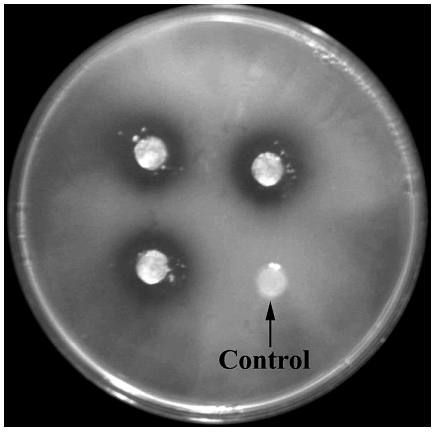

FIG. 5. Effects of pH on the activity of purified Aoz1. Each point represents the average of triplicate assays.

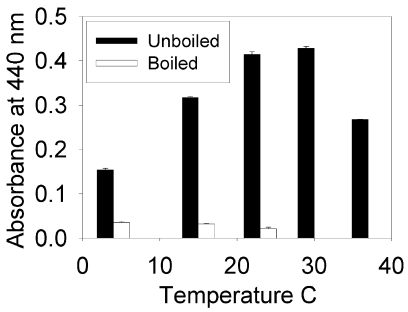

FIG. 6. Effects of temperature on the activity of purified protease. Each point represents the average of triplicate assays.

The apparent homology between our gene and that characterized previously aside, *A. oligospora* may contain multiple related proteases. PCR products derived from our degenerate primer pool revealed three DNA bands on agarose gels (900 bp, 1.2 kb, 1.5 kb; results not shown), only one of which was characterized in this study. In addition, Åhman et al

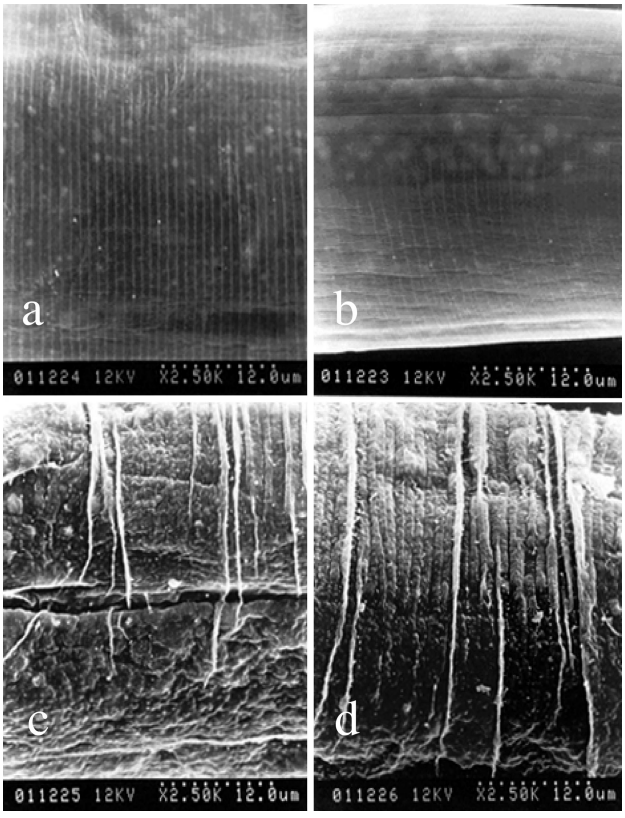

FIG. 7. SEM of nematode cuticle. (a) Control–native nematode cuticle (no protease treatment), (b) nematode cuticle treated by culture filtrate (crude proteases), (c) nematode cuticle treated with purified neutral serine protease Aoz1 and (d) nematode cuticle treated with pure collagenase (Worthington).

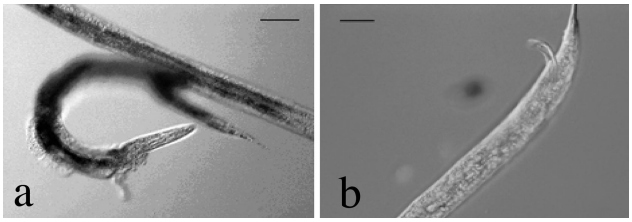

FIG. 8. (a) *P. redivivus* nematode immobilized and partially degraded by purified serine protease. (b) Background control of free-living *P. redivivus* nematode.

(1996) reported that Southern-blot analysis of genomic DNA of *A. oligospora* performed under moderate stringency resulted in several minor bands in addition to that corresponding to *PII*. Moreover, studies indicate that parasitic fungi may have abilities to compensate for loss of proteolytic activity by the expression of other hydrolytic enzymes. From an evolutionary perspective this might be a useful strategy since several hosts, including plants, insects, and nematodes, are known to be capable of producing serine protease inhibitors (Peanasky et al 1984).

Finally, our characterization of this enzyme and its gene provides a good foundation for future investigation of structure-function relationships of cuticle-degrading proteases, improvement of the pathogenicity of nematophagous fungi and possibly the engineering of crop resistance.

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LITERATURE CITED

Åhman J, Ek B, Rask L, Tunlid A. 1996. Sequence analysis and regulation of a gene encoding a cuticle-degrading serine protease from the nematophagous fungus *Arthrobotrys oligospora*. Microbiology 142:1605–1616.

Åhman Johan. 2000. Extracellular serine proteases as virulence factors in nematophagous fungi: molecular characterization and functional analysis of the PII protease in *Arthrobotrys oligospora*. [Doctoral dissertation, Lund University] 100 p.

Bonants PJM, Fitters PFL, Thijs H, Den-Belder E, Waalwijk C, Henfling JWDM. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141:775–784.

Cox GN, Kusch M, Edgar RS. 1981. Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. J Cell Biol 90:7–17.

Cregg JM, Madden KR, Barringer KJ, Thill G, Stillman CA. 1989. Functional characterization of the two alcohol oxidase genes from the yeast, *Pichia pastoris*. Mol Cell Bio 9:1316–1323.

Dijksterhuis J, Veenhuis M, Harder W, Nordbring-Hertz B. 1994. Nematophagous fungi: physiological aspects and structure-function relationships. In: Rose AH, Jewpest DW, eds. Advances in microbial physiology. Vol. 36. Academic Press Limited. p 111–143.

Geremia RA, Goldman GA, Jacobs D, Vila SB, Ardiles W, Van Montagu MM, Herrera-Estrella A. 1993. Molecular characterization of the protease-encoding gene prb1 related to mycoparasitism by *Trichoderma harzianum*. Mol Microbiol 8:603–613.

Jansson HB, Tunlid A, Nordbring-Hertz B. 1997. Biological control: nematodes. In: Anke T, ed. Fungal biotechnology. Weinheim: Chapman and Hall. p 38–50.

Lopez-Llorca LV, Robertson WM. 1992. Immunocytochemical localization of a 32-kDa protease from the nematophagous fungus *Verticillium suchlasporium* in infected nematode eggs. Exp Mycol 16:261–267.

Mankau R. 1980. Biocontrol: fungi as nematode control agents. Journal of Nematology 12:244–252.

Maizels RM, Blaxter ML, Selkirk ME. 1993. Forms and functions of nematode surfaces. Exp Parasitol 77:380–384.

Peanasky RJ, Bentz Y, Paulson B, Graham DL, Babin DR. 1984. The isoinhibitors of chymotrypsin/elastase from *Ascaris lcmbricoides*: isolation by affinity chromatography and association with the enzymes. Arch Biochem Biophys 232:127–134.

Rhodes JC. 1995. Aspergillus proteinases and their interaction with host tissues. Can J Bot 73:S1126–S1131.

Sambrook J, Fritsch EF, Maniatis TM. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Schenck S, Chase T Jr, Rosenzweig WD, Pramer D. 1980. Collagenase production by nematode-trapping fungi. Appl Environ Microbiol 40:567–570.

Segers R, Butt TM, Kerry BR, Peberdy JF. 1994. The nematophagous fungus *Verticillium chlamydosporium* Goddard produces a chymoelastase-like protease which hydrolyses host nematode proteins in situ. Microbiology 140:2715–2723.

St. Leger RJ. 1995. The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73:S1119–S1125.

Tunlid A, Jansson S. 1991. Proteases and their involvement in the infection and immobilization of nematodes by the nematophagous fungus *Arthrobotrys oligospora*. Appl Environ Microbiol 57:2868–2872.

———, Rosén S, Ek B, Rask L. 1994. Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. Microbiology 140:1687–1695.