A 33 kDa serine proteinase from *Scedosporium apiospermum*

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An extracellular proteinase produced by the filamentous fungus *Scedosporium apiospermum* has been purified and characterized. Initially, *in vitro* conditions for enzyme synthesis were investigated. The highest yield of enzyme production was obtained when the fungus was cultivated in modified Czapek–Dox liquid medium supplemented with 0.1 % bacteriological peptone and 1 % (w/v) glucose as the nitrogen and carbon sources respectively. Purification to homogeneity of the proteinase was accomplished by (NH₄)₂SO₄ precipitation, followed by gel filtration through Sephadex G-75 and finally affinity chromatography through immobilized phenylalanine. Analysis of the purified enzyme by SDS/PAGE revealed a single polypeptide chain with an apparent molecular mass of 33 kDa. Further investigation of its physical and biochemical properties disclosed numerous similarities with

those of the previously described serine proteinase of *Aspergillus fumigatus*. The enzyme was not glycosylated and its pI was 9.3. Proteinase activity was optimum between 37 and 50 °C and at pH 9.0, but remained high within a large range of pH values between 7 and 11. The inhibition profile and N-terminal amino acid sequencing confirmed that this enzyme belongs to the subtilisin family of serine proteinases. In agreement with this, the specific synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide proved to be an excellent substrate for the proteinase, with an estimated *K_m* of 0.35 mM. Like the alkaline proteinase of *A. fumigatus*, this enzyme was able to degrade human fibrinogen, and thus may act as a mediator of the severe chronic broncho-pulmonary inflammation from which cystic fibrosis patients suffer.

INTRODUCTION

Over the past few years it has become increasingly clear that proteinases produced by the pathogenic fungi play an important role in host tissue invasion. In a previous study we purified and characterized a serine proteinase secreted by *Aspergillus fumigatus* [1]. This opportunistic fungus determines various infections involving principally the lungs [2]. In particular, *A. fumigatus* is well known for its ability to colonize the respiratory tract of cystic fibrosis (CF) patients [3], and proteinases produced by this micro-organism have been suggested to contribute to the deterioration of respiratory function [4].

Recently more attention has been paid to another filamentous fungus, *Scedosporium apiospermum*, the anamorph of *Pseudallescheria boydii*, since its recognition as an emerging pathogen [5–9]. This fungus elicits various respiratory infections similar to those induced by *A. fumigatus* [6,9–12], and numerous cases of pseudallescheriasis have been reported in children with CF [9,13]. These observations led us to examine the putative virulence factors of this fungus. In the present study we demonstrate the secretion of an extracellular serine proteinase by a clinical isolate of *S. apiospermum*. We have purified this enzyme and specified its biological properties in order to compare it with the *A. fumigatus* alkaline proteinase.

EXPERIMENTAL

Micro-organism and culture conditions

Scedosporium apiospermum 4595.90, originally isolated from human sputum in our hospital laboratory, was used throughout.

The isolate was propagated on yeast extract/peptone/dextrose (YPD) agar plates at 37 °C, and inocula were prepared from 7-day-old cultures by flooding with approx. 10 ml of sterile distilled water and scraping off the agar plates.

Enzyme production

Enzyme production in 100 ml liquid submerged cultures was studied in three types of media, always supplemented with 0.1 % chloramphenicol and 0.1 % cycloheximide, and containing the following (per litre). (1) Malt medium: bacto-malt extract (Difco Laboratories), 20 g; (2) YPD medium: bacteriological peptone (Grosseron Laboratoires), 10 g; yeast extract (Biokar), 5 g; glucose, 20 g; (3) modified Czapek–Dox medium: glucose, 10 g; K₂HPO₄, 1.0 g; MgSO₄, 0.5 g; KCl, 0.5 g; FeSO₄·7H₂O, 0.01 g. This last basal medium was supplemented with BSA at 0.1 or 0.5 % (w/v) final concentration, bacteriological peptone at 0.1 or 1 % (w/v) final concentration or inorganic nitrogen compounds such as NaNO₃ and NaNO₂ at 0.1 % final concentration. The media were inoculated with fungal suspensions prepared as described above. After incubation at 37 °C for 6 days without shaking, the cultures were filtered through pre-sterilized filters which were then lyophilized for determination of the mycelial dry weight, and serine proteinase activities were assayed in the culture filtrates.

For the kinetic study of enzyme production, triplicate cultures were carried out in 20 ml sterile tubes, each containing 5 ml of culture medium, which were incubated at 37 °C for different periods ranging from 24 h to 10 days.

Abbreviations used: Ac, acetyl; Bz, benzoyl; Cbz, benzoyloxycarbonyl; CF, cystic fibrosis; *p*NA, *p*-nitroanilide; SBTI, soybean trypsin inhibitor; Suc, succinyl; TBS, Tris-buffered saline; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK'); YPD, yeast extract/peptone/dextrose.

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Enzyme purification

Crude enzyme preparation

For enzyme production, *S. apiospermum* was grown in modified Czapek–Dox liquid medium supplemented with 0.1 % bacteriological peptone as the nitrogen source. Flasks (2 litres) containing 1 litre of culture medium were inoculated with the fungal suspension, and incubated for 6 days at 37 °C. Cultures were then checked for bacterial contamination, and filtered successively through filter paper no. 3 (Whatman) and 0.2 µm-pore-size filters (Millipore).

Ammonium sulphate precipitation

Solid (NH₄)₂SO₄ was added to the supernatant to 80 % saturation. After 1 h of stirring at 4 °C, the suspension was centrifuged at 4 °C for 30 min at 20 000 g. The pellet was then resuspended in distilled water at one-tenth of the original volume and dialysed against 50 mM Tris/HCl buffer, pH 7.5, (Tris buffer) containing 100 mM NaCl (Tris-buffered saline; TBS). After removal of the insoluble material by centrifugation at 4 °C for 20 min at 45 000 g, the supernatant was concentrated with poly(ethylene glycol) 35000.

Gel-filtration chromatography

The concentrate was loaded on to a column (100 cm × 2.5 cm) of Sephadex G-75 (Sigma) previously equilibrated with TBS. Samples of the effluent (3 ml) were collected at a flow rate of 15 ml/h. Fractions with high proteinase activity were pooled, concentrated with poly(ethylene glycol) 35000 and dialysed against TBS.

The molecular mass of the native proteinase was estimated by gel filtration along with proteins of known molecular mass: BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and pancreatic ribonuclease (13.7 kDa).

Affinity chromatography

Finally, the dialysate was applied on an [*N*^ε-benzoyloxycarbonyl (Cbz)]-D-phenylalanine-agarose (Pierce) column (10 cm × 1.14 cm) equilibrated with TBS. The proteinase was eluted with 0.1 M acetic acid, pH 3, at a flow rate of 12 ml/h. To avoid enzyme denaturation, fractions (1 ml) were collected in tubes containing 300 µl of 0.5 M Tris/HCl buffer, pH 10, to give neutral pH. Fractions with enzyme activity were pooled, dialysed against Tris buffer and divided into aliquots which were stored at –20 °C.

The last two steps of the purification were monitored for protein by measuring the absorbance at 280 nm, and for enzyme activity by measuring the rates of hydrolysis of chromogenic substrates.

Protein estimation

Protein concentrations were determined as described by Bradford [14], using BSA as standard.

Enzyme activity assay

Substrates were dissolved in DMSO to give stock solutions of 5 mM. Unless otherwise stated, the assay was performed on polystyrene microtitre plates and the reaction mixture contained, per well, 180 µl of a suitably diluted proteinase solution in Tris buffer and 20 µl of chromogenic substrate. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of

100 µl of 2 % (v/v) acetic acid, and the amount of *p*-nitroaniline released was measured at 405 nm using a Titertek Multiskan spectrophotometer (Labsystem).

Enzyme activities were expressed in arbitrary units, defined as the amount of enzyme that liberated 1 nmol of *p*-nitroaniline per min under the assay conditions. All assays were performed in triplicate, except for the fractions collected during chromatographic steps. Three different chromogenic substrates were used on these fractions to detect serine proteinase activities: *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *N*-Ac-Ile-Glu-Ala-Arg-*p*NA and *N*-Suc-Ala-Ala-Pro-Leu-*p*NA (Sigma) (where Suc is succinyl, *p*NA is *p*-nitroanilide and Ac is acetyl), titrating the chymotrypsin/subtilisin, trypsin and elastase activities respectively.

Kinetic constants of the purified enzyme were measured with several synthetic peptide substrates (Sigma). To 180 µl (per well) of a suitably diluted proteinase solution in 0.1 M glycine/NaOH, pH 9, buffer was added 20 µl of a 10-fold range of substrate concentrations in DMSO to achieve a final concentration of 0.125–8.0 mM. Initial rates were determined at 37 °C by monitoring *p*NA appearance at 405 nm at 5, 10, 15 and 20 min incubation times. The *K_m* and *V_{max}* for each substrate were obtained by analysing the experimental data using Morrisson (Enzfitter) non-linear regression [15]. All kinetic parameters are the means of four determinations.

Enzyme characterization

Effect of pH and temperature on activity and stability

These effects were determined using the standard proteinase assay with *N*-Suc-Ala-Ala-Pro-Phe-*p*NA as chromogenic substrate. Determination of the optimum pH was performed at 37 °C with the following buffer systems: 0.2 M sodium acetate (pH 3.5–5), 0.2 M Tris/maleate/NaOH (pH 6–8) and 0.1 M glycine/NaOH (pH 9–11).

pH stability was investigated in these buffer solutions at various pH values. Enzyme solution (100 µl) was incubated with 1.9 ml of the buffer solutions at room temperature. After neutralization of the mixtures, the residual activities were measured.

For determination of the optimum temperature, the reaction was carried out at temperatures ranging from 37 to 100 °C in 0.1 M glycine/NaOH, pH 9. The effect of temperature on enzyme stability was investigated by measuring the residual activities after a 20 min incubation of the proteinase solution at the same temperatures, and subsequent cooling.

Inhibition studies

Proteinase inhibitors were tested for activity against purified enzyme with the optimized protocol (i.e. pH 9.0 and 37 °C). Aliquots of the purified proteinase solution (160 µl at 14.5 µg/ml in Tris buffer) were preincubated for 10 min at 37 °C with 20 µl of 10-fold concentrated stock solutions of each reagent. Then, 20 µl samples of chromogenic substrates *N*-Suc-Ala-Ala-Pro-Phe-*p*NA or *N*-Ac-Ile-Glu-Ala-Arg-*p*NA (5 mM) were added, and proteinase activity was assayed as described above. PMSF, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (Tos-Lys-CH₂Cl; 'TLCK') and 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (Tos-Phe-CH₂Cl; 'TPCK') were prepared as stock solutions in methanol, and chymostatin was prepared in DMSO. Residual activity was determined as a percentage of the activity in control samples without reagent. Appropriate solvent controls were run in parallel when required.

The effects of several metal ions at 1 and 10 mM final concentrations on proteinase activity were investigated using *N*-

Suc-Ala-Ala-Pro-Phe-pNA as substrate, as described above for commercially obtained proteinase inhibitors.

Electrophoretic analysis

SDS/PAGE

Protein purity and the molecular mass of the purified enzyme were evaluated by SDS/PAGE using the discontinuous buffer system of Laemmli [16]. Samples (10 µg of protein per lane) were analysed under reducing or non-reducing conditions on 1.5-mm-thick slab gels [12.5 % (w/v) polyacrylamide resolving gel; 3 % (w/v) polyacrylamide stacking gel]. Gels were stained with silver reagent [17] and the electrophoretic migration of the purified proteinase was compared with that of low-molecular-mass protein markers (Pharmacia).

Zymogram

The proteolytic activity of the purified proteinase (20 µg per lane) was also assessed in SDS/12.5 %-polyacrylamide gels containing human fibrinogen at 0.02 % final concentration, as described by Heussen and Dowdle [18]. After electrophoresis, the gel was rinsed for 3 × 15 min with distilled water and then incubated in TBS for 16 h at room temperature. After Coomassie Blue staining and subsequent destaining, proteolytic activity was observed as non-stained bands on a blue background of non-digested fibrinogen.

pI determination

Isoelectric focusing using LKB-IEF gels (pI 3.5–9.5) was performed with an LKB Multiphor apparatus according to the manufacturer's recommendations. An appropriate LKB calibration kit was used and silver staining was done. Proteinase activity was detected by incubation of the gel for 30 min at room temperature in Tris buffer containing 0.5 mM of the fluorogenic substrate *N*-Suc-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (Sigma).

Glycoprotein detection

The purified proteinase was subjected to SDS/PAGE, then electroblotted to Immobilon membranes (Millipore) and finally analysed with digoxigenin glycan/protein double-labelling kit (Boehringer). A positive glycosylation control was run with the proteinase.

N-terminal sequence determination

N-terminal amino acid sequencing was accomplished by *in situ* Edman degradation [19] in Eurogentec Laboratory with an Applied Biosystems Procise sequencer.

RESULTS

Enzyme production

The growth of the fungus and its ability to produce proteinases was monitored in various media (Table 1). Nitrogen-restricted Czapek medium supplemented with 0.1 % bacteriological peptone induced maximum serine proteinase activity. Conversely, enzyme production was low in YPD medium and in modified Czapek medium supplemented with BSA. Serine proteinase activities were also decreased when the fungus was cultivated in media lacking a nitrogen source (Malt medium and nitrogen-restricted Czapek medium). Determination of the mycelial dry

weights did not show significant differences between these media (0.232 ± 0.092 g). Finally, increasing the concentration of peptone to 1 % resulted in maximal growth, but did not enhance enzyme synthesis. Moreover, in culture medium containing 0.1 % peptone, an intense activity was detected with *N*-Suc-Ala-Ala-Pro-Phe-pNA, whereas trypsin and elastase activities were weak or very weak, and similar serine proteinase profiles were observed in all the media tested, with varying intensities. The enzyme activities were dramatically affected by the addition of inorganic nitrogen compounds such as NaNO₃ or NaNO₂ to the 0.1 % peptone-containing medium. Moreover, when NaNO₃ or NaNO₂ was used as the sole nitrogen source the level of enzyme activity was so altered that it could be considered negligible. In contrast, the growth of the fungus did not vary significantly, irrespective of the culture media, suggesting a tight regulation of proteinase secretion induced by external protein substrate.

The kinetics of enzyme synthesis were studied in nitrogen-restricted Czapek–Dox liquid medium supplemented with 0.1 % peptone. Enzyme synthesis reached a maximum at day 6 (results not shown). Thus, for further study, the incubation time for fungal growth was established at 6 days.

Purification of the fungal proteinase

Figure 1 shows the elution profiles obtained on Sephadex G-75 chromatography (Figure 1a) and immobilized phenylalanine affinity chromatography (Figure 1b). The peaks of the three serine proteinase activities superimposed exactly, both in the size-exclusion and in the affinity-purification steps. In each case a single protein peak with proteinase activity was observed. Analysis by SDS/PAGE gave effectively a single band (Figure 2, lane 2). This typical purification protocol produced a highly purified product, but at a low yield (5 % of the initial enzyme activity) (Table 2). The fact that only 14.9-fold purification was sufficient to achieve homogeneity suggests that this proteinase is one of the dominant species of secreted proteins in *S. apiospermum* culture filtrate. In addition, no protein peak was detected on the elution profile when the final step of the purification procedure was substituted by soybean trypsin inhibitor (SBTI)–agarose affinity chromatography (results not shown).

Molecular properties

Molecular mass and isoelectric point

The molecular mass of the proteinase was estimated by gel filtration to be 33 kDa (Figure 1a). SDS/PAGE analysis revealed that the proteinase was one of the major components of the culture filtrate (Figure 2, lane 1) and that the purification procedure resulted in a homogeneous protein with a molecular mass of 33 kDa (Figure 2, lane 2), confirming the value obtained by gel filtration. When 2-mercaptoethanol was added to the sample buffer, no change could be observed in the molecular mass, suggesting a monomeric structure for the proteinase (Figure 2, lane 3). In addition, this protein was not detected in the culture filtrate of the fungus grown with NaNO₃ as the sole nitrogen source (standard Czapek medium) (results not shown).

On isoelectric focusing, the enzyme migrated as a sharp band with a pI of 9.3, closely related to its activity detected with the fluorogenic substrate *N*-Suc-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin.

Fibrinogenolytic activity

In addition to the SDS/PAGE analysis, a zymogram of the purified proteinase was carried out (Figure 2, lanes 4 and 5).

Table 1 Influence of culture medium on exoproteinase synthesis by *S. apiospermum*

After 6 days at 37 °C, the mycelium of *S. apiospermum* 4595.90 was separated from the culture medium by filtration on pre-tered filters and enzyme activity was estimated by measuring the rate of hydrolysis of the following synthetic substrates: *N*-Suc-Ala-Ala-Pro-Phe-*p*NA for chymotrypsin/subtilisin activity, *N*-Ac-Ile-Glu-Ala-Arg-*p*NA for trypsin activity and *N*-Suc-Ala-Ala-Pro-Leu-*p*NA for elastase activity. Assays were carried out as described in the Experimental section. Specific activities are expressed as total enzyme activity of the culture filtrate per g of mycelial dry weight.

Culture medium	Mycelial dry weight (g)	Serine proteinase specific activity (units/g)		
		Chymotrypsin/subtilisin	Trypsin	Elastase
Malt	0.249	32	16	20
YPD	0.200	75	45	25
Modified Czapek with:				
No nitrogen source	0.170	53	12	18
+ NaNO ₃ (0.1%)	0.231	160	80	30
+ NaNO ₂ (0.1%)	0.286	70	20	10
Peptone (0.1%)	0.175	1080	240	97
+ NaNO ₃ (0.1%)	0.154	200	123	49
+ NaNO ₂ (0.1%)	0.443	149	38	25
Peptone (1%)	0.335	39	18	21
BSA (0.1%)	0.139	86	43	43
BSA (0.5%)	0.167	78	30	42

Figure 1 Purification of *S. apiospermum* proteinase

(a) Gel filtration through Sephadex G-75 and (b) affinity chromatography on immobilized phenylalanine. Both purification steps were performed as described in the Experimental section. ●, enzyme activity; ○, absorbance at 280 nm. Molecular masses of calibration standards were: a, BSA (67 kDa); b, ovalbumin (45 kDa); c, chymotrypsinogen (25 kDa); d, pancreatic ribonuclease (13.7 kDa).

Major degradation of human fibrinogen was observed in the upper part of the gel (R_F 0.05) associated with a scarcely discoloured zone appearing at an R_F of 0.45, which is identical to the mobility of the purified proteinase on SDS/PAGE (Figure 2, lane 2), demonstrating that the enzyme shows fibrinogenolytic activity.

Figure 2 Electrophoretic analyses of *S. apiospermum* proteinase

(A) SDS/PAGE (12.5% gel) pattern of culture filtrate (lane 1) and purified proteinase (10 µg per lane) without (lane 2) or with (lane 3) reducing agent, after silver staining. (B) Fibrinogen substrate-gel electrophoresis of the unboiled (lane 4) and boiled (lane 5) purified proteinase (20 µg per lane) after Coomassie Blue staining. The positions of molecular mass standards (in kDa) are indicated on the left: phosphorylase b (94 kDa), BSA (68 kDa), ovalbumin (44 kDa), carbonic anhydrase (30 kDa) and SBTI (20 kDa).

Glycan detection

An investigation of the carbohydrate residues on the molecule revealed that the enzyme was not glycosylated (results not shown).

N-terminal amino acid sequencing

As shown in Figure 3, N-terminal sequencing of the purified proteinase disclosed numerous similarities with other fungal subtilisins, particularly those produced by the *Aspergilli*.

Enzymic properties

The linearity of the enzyme activity assay with time was checked by measuring the rate of hydrolysis after 5, 10, 15, 20, 25, 30, 40, 50 and 60 min of incubation. The reaction was linear until 30 min

Table 2 Summary of purification of *S. apiospermum* proteinase

For experimental details, see the Experimental section. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 nmol of *p*-nitroaniline per min under the assay conditions.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg)	Purification factor (fold)
Culture filtrate	6000	45.40	605000	100	13000	1.0
(NH ₄) ₂ SO ₄ precipitation	60	2.05	230000	38	112000	8.6
Sephadex G-75	58	1.0	121000	20	121000	9.3
Immobilized phenylalanine	11	0.16	31000	5	194000	14.9

1	A	Y	T	G	Q	T	G	A	P	W	G	L	A
2	A	L	T	T	Q	K	G	A	P	W	G	L	G
3	G	L	T	T	Q	K	S	A	P	W	G	L	G
4	A	L	T	S	Q	S	G	A	P	W	G	L	G
5	D	L	T	T	Q	S	D	A	P	W	G	L	G
6	A	L	T	T	Q	S	G	A	P	W	G	L	G
7			A	A	Q	T	N	A	P	W	G	L	A
8	E	F	D	T	Q	N	S	A	P	W	G	L	A

Figure 3 Sequence similarity of *S. apiospermum* serine proteinase with other fungal subtilisins

1, *S. apiospermum* alkaline proteinase; 2, *A. fumigatus* alkaline proteinase [20]; 3, *Aspergillus oryzae* alkaline proteinase [21]; 4, *Aspergillus nidulans* alkaline proteinase [22]; 5, *Aspergillus flavus* alkaline proteinase [23]; 6, *Trichoderma harzianum* alkaline proteinase [24]; 7, *Tritirachium album* Limber proteinase K [25]; 8, *Saccharomyces cerevisiae* proteinase B [26]. Boxes enclose highly conserved amino acid residues.

Table 3 Effects of various substances on the activity of *S. apiospermum* proteinase

For experimental details, see the Experimental section. The following substances did not inhibit the enzyme activity significantly (residual activity above 80%) at the concentrations tested and indicated in parentheses: NH₄Cl, NaNO₂, NaNO₃ (50 mM); iodoacetamide (10 mM); *N*-ethylmaleimide (2 mM); dithiothreitol, pepstatin, *o*-phenanthroline (1 mM); leupeptin (100 μ M); E-64, bestatin (10 μ M); DMSO, methanol (10%); 2-mercaptoethanol (1%). Metal ions which gave a residual activity above 50% at 1 and 10 mM final concentrations were: Ca²⁺, Co²⁺, Mg²⁺, Mn²⁺ and Ni²⁺. ND, not determined. Substrate abbreviations: -Phe-*p*NA and -Arg-*p*NA correspond respectively to *N*-Suc-Ala-Ala-Pro-Phe-*p*NA and *N*-Ac-Ile-Glu-Ala-Arg-*p*NA.

Reagent	Final concn.	Residual activity (%)	
		-Phe- <i>p</i> NA	-Arg- <i>p</i> NA
PMSF	1 mM	4	0
Tos-Phe-CH ₂ Cl	1 mM	53	61
Tos-Lys-CH ₂ Cl	1 mM	69	67
Chymostatin	100 μ M	3	0
Elastatinal	10 μ M	89	87
SBTI	50 μ M	22	23
EDTA	10 mM	75	100
EGTA	10 mM	81	98
SDS	1%	5	1
Nonidet P-40	1%	66	83
Triton X-100	1%	74	70
Ethanol	10%	50	53
Cu ²⁺	10 mM	19	ND
	1 mM	46	ND
Hg ²⁺	10 mM	44	ND
	1 mM	70	ND
Zn ²⁺	10 mM	37	ND
	1 mM	57	ND

of incubation under all experimental conditions, except at pH > 11 and at *T* > 42 °C.

Inhibition studies

The effects of different reagents were tested on the activity of *S. apiospermum* purified proteinase (Table 3). Inhibition profiles for the two chromogenic substrates *N*-Suc-Ala-Ala-Pro-Phe-*p*NA and *N*-Ac-Ile-Glu-Ala-Arg-*p*NA were very similar. The enzyme was strongly inactivated by PMSF and chymostatin. Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl and SBTI, which are trypsin inhibitors, had a significant effect on proteinase activity. A slight effect was observed for elastatinal and metal chelators such as EDTA and EGTA. Neither alkylating agents nor reducing agents modified the enzyme activity. Likewise, the proteinase was not inhibited by pepstatin, *o*-phenanthroline, leupeptin, *L*-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) or bestatin.

Non-ionic detergents such as Triton X-100 and Nonidet P-40 had a slight effect on enzyme activity. In contrast, SDS induced a drastic decrease in activity. Organic solvents such as DMSO and methanol did not inhibit the enzyme, whereas ethanol partially inactivated it. Finally, the proteinase activity was not affected by the presence of NH₄Cl, NaNO₂ or NaNO₃ at a final concentration of 50 mM.

Proteinase activity against *N*-Suc-Ala-Ala-Pro-Phe-*p*NA was sensitive to bivalent cations (Table 3). Ca²⁺ and Mn²⁺ gave no effect on proteinase activity. Concentrations of 1 and 10 mM Co²⁺, Mg²⁺ and Ni²⁺ caused partial inhibition (residual activities above 50%), whereas enzyme activity was decreased drastically to 19%, 37% and 44% with 10 mM Cu²⁺, Zn²⁺ and Hg²⁺ respectively.

Optimum pH and effect of extreme pH

Enzyme assays were performed from pH 3.5 to pH 11. The proteinase was found to have an alkaline optimum pH of 9 (Figure 4). Enzyme activity decreased rapidly in the acidic part of the curve, the activity at pH 5 being about 20% of the maximum detected. Exposure of the proteinase to extreme pH below 5 or above 11 resulted in the abolition of enzyme activity. However, the inactivation by acid pH (3.5, 4 or 5) was reversible at incubation times up to 60 min.

Heat stability and optimum temperature

The temperature profile of the purified proteinase was conducted from 37 to 100 °C. To check the temperature stability, the proteinase was subjected to these temperatures for 20 min. The

Figure 4 Effect of pH on proteinase activity

A standard proteinase assay with *N*-Suc-Ala-Ala-Pro-Phe-*p*NA was performed at 37 °C with the following buffer systems: 0.2 M sodium acetate (pH 3.5–5), 0.2 M Tris/maleate/NaOH (pH 6–8) and 0.1 M glycine/NaOH (pH 9–11). The maximal activity was set as 100% relative enzyme activity.

Figure 5 Effect of temperature on proteinase activity

Optimum temperature (●) was determined by carrying out a proteinase assay with *N*-Suc-Ala-Ala-Pro-Phe-*p*NA at temperatures ranging from 37 to 100 °C in 0.1 M glycine/NaOH, pH 9. Thermal stability (○) was investigated by measuring at 37 °C the residual activities after a 20 min incubation of the proteinase solution at the same temperatures and subsequent cooling. Maximal activities observed were set as 100% relative enzyme activity.

proteinase was thermolabile, a significant decrease in activity due to protein denaturation being observed at temperatures higher than 42 °C (Figure 5). Therefore the enzyme activity assay was not linear under these conditions, and initial rates were determined to establish the optimum temperature curve.

The optimum temperature was found to be between 37 and 50 °C. Only 49% of the activity remained at 60 °C, and no activity was detected at 70 °C (Figure 5). As 37 °C was not far from the optimum temperature, and in our desire to use a physiological temperature, 37 °C was chosen as the temperature for the optimized enzyme assay.

Affinity for various substrates

To ascertain the substrate specificity of the proteinase, its ability to hydrolyse a number of synthetic substrates was assessed. Kinetic parameters were determined using a non-linear regression computer kinetics program and are reported in Table 4. These studies showed a high affinity of the proteinase for hydrolysing a tetrapeptide containing an aromatic residue at the P-1 site. A K_m of 0.35 mM was calculated for *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, a specific substrate for chymotrypsins and subtilisins. The highest k_{cat}/K_m value was obtained for this substrate. The trypsin substrate *N*-Ac-Ile-Glu-Ala-Arg-*p*NA was hydrolysed by the enzyme with a similar K_m value of 0.31 mM, but the k_{cat}/K_m for this substrate was about 4-fold lower than the value obtained for *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. The K_m of the proteinase with the substrate *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, which can be cleaved by both elastase and chymotrypsin, was estimated to be 2.59 mM. Another substrate more specific for elastase, *N*-Suc-Tyr-Leu-Val-*p*NA, was not hydrolysed by the enzyme. Likewise, the proteinase did not react with *N*-Suc-L-Phe-*p*NA, *N*-Ac-DL-Phe-*p*NA or *N*-Bz-L-Tyr-*p*NA (where Bz is benzoyl), which demonstrates that a minimum length of synthetic peptide chain was required for cleavage. Substrates for subtilisin, such as *N*-Cbz-Gly-Gly-Leu-*p*NA and *N*-Cbz-Ala-Ala-Leu-*p*NA, were also tested; no hydrolysis was observed.

DISCUSSION

In this study we have demonstrated and isolated a major proteolytic enzyme secreted by *S. apiospermum* in its environment. To our knowledge, a proteinase has never previously been purified from this fungus.

Prior to enzyme purification, *S. apiospermum* was cultivated in different media to determine the optimum medium for enzyme production. The maximum rate of secretion was observed in the presence of 0.1% peptone, with 1% glucose as carbon source. In contrast, some authors have reported that glucose and/or peptone repress proteinase production in fungi such as *Aspergillus oryzae* [27], *Candida lipolytica* [28], *Trichophyton rubrum* [29] and *Microsporium canis* [30,31]. Also, a drastic decrease in enzyme accumulation was observed by us in the presence of a higher

Table 4 Kinetic constants for the hydrolysis of chromogenic substrates by *S. apiospermum* proteinase

Assay conditions are described in the Experimental section. K_m and V_{max} values are the means $\pm$ S.D. of four determinations. Hydrolysis was not detected for the following substrates: *N*-Suc-Tyr-Leu-Val-*p*NA, *N*-Suc-L-Phe-*p*NA, *N*-Ac-DL-Phe-*p*NA, *N*-Bz-L-Tyr-*p*NA, *N*-Cbz-Gly-Gly-Leu-*p*NA and *N*-Cbz-Ala-Ala-Leu-*p*NA. Substrate abbreviations: -Phe-*p*NA, -Arg-*p*NA and -Leu-*p*NA correspond respectively to *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *N*-Ac-Ile-Glu-Ala-Arg-*p*NA and *N*-Suc-Ala-Ala-Pro-Leu-*p*NA.

Substrate	K_m (mM)	V_{max} (units)	k_{cat} (s^{-1})	$10^3 \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
-Phe- <i>p</i> NA	0.35 ± 0.05	1032 ± 35	189	545
-Arg- <i>p</i> NA	0.31 ± 0.02	234 ± 4	43	139
-Leu- <i>p</i> NA	2.59 ± 0.06	613 ± 10	112	43

Table 5 Characteristics of proteinases isolated from micro-organisms responsible for respiratory infections in CF patients

Micro-organism	Class	Molecular mass (kDa)	pI	Optimum pH	Ref.
<i>Staphylococcus aureus</i>	Metalloproteinase	29	—	7.5–8.0	42
	Metalloproteinase	38	—	7.0	43
<i>Haemophilus influenzae</i>	Metalloproteinase	100	—	Neutral	44
<i>Pseudomonas aeruginosa</i>	Metalloproteinase	48	4.1	8–9	45
	Metalloproteinase	33	5.9	7–8	46
<i>Pseudomonas cepacia</i>	Metalloproteinase	34	6.0	—	47
<i>Candida albicans</i>	Aspartyl proteinase	45	4.4	2.5–3.9	48
	Aspartyl proteinase	42	4.0	4.0	49
	Aspartyl proteinase	46	4.2	3.5–4.0	50
<i>Aspergillus fumigatus</i>	Serine proteinase	33	8.75	9.0	1
	Metalloproteinase	40	5.5	6.5–9.0	51
<i>Aspergillus terreus</i>	Serine proteinase	37	—	—	52
	Metalloproteinase	21	6.0–7.0	—	52

concentration of peptone (1 %, w/v). We believe that the free amino acids released by hydrolysis of this high quantity of peptone may cause enzyme repression. This idea is supported by the findings that individual amino acids curtail proteinase production by various fungi such as *T. rubrum* [29], *Neurospora crassa* [32] and *Rhizopus oligosporus* [33]. Likewise, proteinase secretion by *S. apiospermum* seemed to be repressed by the products of starch hydrolysis (Malt media) and by inorganic nitrogen. This phenomenon appears to be common in fungi, especially in filamentous fungi such as *Aspergillus* [34–36] and *Rhizopus* [37] species.

In fact, the results on the whole suggested that easily metabolized nitrogen substrates such as free amino acids and inorganic nitrogen compounds repressed proteinase secretion [29,38] and that an exogenous protein as the sole nitrogen source in the culture medium was required to induce enzyme synthesis by *S. apiospermum*. However, all proteins were not as efficient at triggering enzyme synthesis: 0.1 % peptone was the best, while lower yields of enzyme occurred with BSA.

Cultivating *S. apiospermum* in the defined liquid medium gave a high level of proteolytic activity after an incubation of 6 days. The proteinase was purified by a simple two-step method involving size-exclusion chromatography on Sephadex G-75 and affinity chromatography on phenylalanine-agarose. SDS/PAGE analysis of the enzyme disclosed, after silver staining, a unique band of 33 kDa, confirming the molecular mass obtained by gel filtration. This protocol resulted in a highly purified end-product at a modest but workable yield. The biochemical characterization of this proteinase proved to be quite interesting. Determination of inhibition profiles, optimum pH and kinetic constants revealed the enzyme to be a chymotrypsin/subtilisin-like serine proteinase. Surprisingly, the purified enzyme was not completely free of trypsin activity. This corresponded to a lack of specificity of the enzyme. Indeed, during the purification procedure the peaks of chymotrypsin/subtilisin and trypsin activities superimposed exactly, and no protein was eluted when an SBTI-agarose affinity column was used. Furthermore, inhibition profiles of the purified enzyme performed with *N*-Suc-Ala-Ala-Pro-Phe-pNA and *N*-Ac-Ile-Glu-Ala-Arg-pNA were similar. The same phenomenon was observed with the *A. fumigatus* alkaline proteinase that we isolated previously [1]. In addition, these two enzymes presented many similarities with regard to their molecular mass (33 kDa) and their alkaline pI (approx. 9). They are both composed of a single polypeptide chain and are not glycosylated. The *S. apiospermum* proteinase presented an inhibition profile and a substrate specificity similar to those obtained for the *A. fumigatus*

proteinase. Like the extracellular proteinase of *A. fumigatus*, this new fungal proteinase is sensitive to PMSF and chymostatin. Both enzymes show pronounced activity against the synthetic substrate *N*-Suc-Ala-Ala-Pro-Phe-pNA, with K_m values of 0.62 mM and 0.35 mM for the *A. fumigatus* and *S. apiospermum* enzymes respectively. Among numerous chromogenic substrates, the highest k_{cat}/K_m values were obtained for this substrate. Physico-chemical characteristics, such as optimum pH and optimum temperature, were identical (9.0 and 42 °C respectively) for the two fungal proteinases. N-terminal amino acid sequencing of the purified *S. apiospermum* enzyme revealed 69 % identity with the sequence of *A. fumigatus* alkaline proteinase [20], and confirmed that it belongs to the subtilisin family of serine proteinases. Finally, substrate-gel electrophoresis revealed that the *S. apiospermum* enzyme shows fibrinogenolytic activity. Interestingly, we previously showed similar fibrinogenolytic activity in *A. fumigatus* culture filtrate and demonstrated the inhibitory effect of heavy-metal ions such as Zn^{2+} and Cu^{2+} on this activity [1,39].

Infections caused by *S. apiospermum* largely resemble those due to *A. fumigatus* [6,9,10], and there is presumptive evidence for a role for these fungal proteolytic enzymes in the pathogenesis of infection. In our laboratory, a recent study performed on a large population of CF patients demonstrated a significant occurrence of *A. fumigatus* and *S. apiospermum* compared with other filamentous fungi. The respiratory tracts of these patients are chronically infected by microbial agents which contribute to pulmonary damage by degrading host proteins such as fibrinogen [39] and basement membrane laminin [40], or indirectly by hypersensitivity mechanisms [12,41]. Together, these clinical considerations and the biochemical properties demonstrate that these fungal proteinases are closely related. Interestingly, among the micro-organisms infecting the airways of CF patients, serine proteinases seemed to be secreted exclusively by the filamentous fungi, whereas metalloproteinases are produced by bacteria such as *Staphylococcus aureus* [42,43], *Haemophilus influenzae* [44], *Pseudomonas aeruginosa* [45,46] and *P. cepacia* [47], and aspartyl proteinases by yeasts such as *Candida albicans* [48–50] (Table 5).

In summary, we have isolated and characterized a serine proteinase of the subtilisin family that is secreted by *S. apiospermum*. Further investigations are necessary to determine the biological significance of this extracellular proteinase *in vivo*, and particularly in CF patients. Indeed, proteinase inhibitors might be employed in an effort to lessen the damage caused by the poorly controlled inflammatory response and/or to reduce the overall level of inflammation.

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REFERENCES

- Larcher, G., Bouchara, J. P., Annaix, V., Symoens, F., Chabasse, D. and Tronchin, G. (1992) *FEBS Lett.* **308**, 65–69
- Bodey, G. P. and Vartivarian, S. (1989) *Eur. J. Clin. Microbiol. Infect. Dis.* **8**, 413–437
- Bhargava, V., Tomashevski, J. F., Stern, R. C. and Abramowsky, C. R. (1989) *Hum. Pathol.* **20**, 977–986
- Robinson, B. W. S., Venaille, T. J., Mendis, A. H. W. and McAleer, R. (1990) *J. Allergy Clin. Immunol.* **86**, 726–731
- Travis, L. B., Roberts, G. D. and Wilson, W. R. (1985) *Mayo Clin. Proc.* **60**, 531–537
- Rippon, J. W. (1988) in *Medical Mycology: Pseudallescheriasis* (Rippon, J. W., ed.), pp. 651–680. W. B. Saunders, Philadelphia
- Schwartz, D. A. (1989) *Arch. Pathol. Lab. Med.* **113**, 476–480
- Patterson, T. F., Andriole, V. I., Zervos, M. J., Therasse, D. and Kauffman, C. A. (1990) *Mycoses* **33**, 297–303
- Dupont, B., Improvisi, L. and Ronin, O. (1991) *J. Mycol. Méd.* **1**, 33–42
- Walker, D. H., Adamec, T. and Krigman, M. (1978) *Arch. Pathol. Lab. Med.* **102**, 158–160
- Rosen, P., Adelson, H. T. and Burleigh, E. (1969) *Am. J. Clin. Pathol.* **52**, 182–186
- Lake, F. R., Tribe, A. E., McAleer, R., Fraudist, J. and Thompson, P. J. (1990) *Thorax* **45**, 489–492
- Chabasse, D., Bouchara, J. P., Chazalotte, J. P., Carrère, J., Giniès, J. L., De Gentile, L. and Cimon, B. (1991) *J. Mycol. Méd.* **1**, 152–155
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Leatherbarrow, R. J. (1987) *ENZIFIT: A Non-linear Regression Data Analysis Program for the IBM PC*, Elsevier Science Publishers, Amsterdam
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Merrill, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981) *Science* **211**, 1437–1438
- Heussen, C. and Dowdle, E. B. (1980) *Anal. Biochem.* **102**, 196–202
- Kratzin, H. D., Wiltfang, J., Karas, M., Neuhoff, V. and Hilschmann, N. (1989) *Anal. Biochem.* **183**, 1–8
- Jaton-Ogay, K., Suter, M., Cramer, R., Falchetto, R., Fatih, A. and Monod, M. (1992) *FEMS Microbiol. Lett.* **92**, 163–168
- Tatsumi, H., Ohsawa, M., Tsuji, R. F., Murakami, S., Nakano, E., Motai, H., Masaki, A., Ishida, Y., Murakami, K., Kawabe, H. and Arimura, H. (1988) *Agric. Biol. Chem.* **52**, 1887–1888
- Katz, M. E., Rice, R. N. and Cheetham, B. F. (1994) *Gene* **150**, 287–292
- Ramesh, M. V., Sirakova, T. and Kolattukudy, P. E. (1994) *Infect. Immun.* **62**, 79–85
- Geremia, R. A., Goldman, G. H., Jacobs, D., Ardiles, W., Vila, S. B., Van Montagu, M. and Herrera-Estrella, A. (1993) *Mol. Microbiol.* **8**, 603–613
- Jany, K. D., Lederer, G. and Mayer, B. (1986) *FEBS Lett.* **199**, 139–144
- Moehle, C. M., Tizard, R., Lemmon, S. K., Smart, J. and Jones, E. W. (1987) *Mol. Cell. Biol.* **7**, 4390–4399
- Klapper, B. F., Jameson, D. N. and Mayer, R. M. (1973) *Biochim. Biophys. Acta* **304**, 513–519
- Myers, S. P. and Ahearn, D. G. (1977) *Mycologia* **69**, 646–651
- Meevootisom, V. and Niederpruem, D. J. (1979) *Sabouraudia* **17**, 91–106
- Takiuchi, I., Higuchi, D., Sei, Y. and Koga, M. (1982) *Sabouraudia* **20**, 281–288
- Lee, K. H., Park, K. K., Park, S. H. and Lee, J. B. (1987) *Yonsei Med. J.* **28**, 131–138
- Cohen, B. L. and Drucker, H. (1977) *Arch. Biochem. Biophys.* **182**, 601–613
- Farley, P. C. and Ikasari, L. (1992) *J. Gen. Microbiol.* **138**, 2539–2544
- Cohen, B. L. (1972) *J. Gen. Microbiol.* **71**, 293–299
- Cohen, B. L. (1973) *J. Gen. Microbiol.* **77**, 521–528
- Bouchara, J. P., Larcher, G., Joubaud, F., Penn, P., Tronchin, G. and Chabasse, D. (1993) *FEMS Immunol. Med. Microbiol.* **7**, 81–92
- Schindler, J., Lehmann, R., Pleiffer, H. and Schmid, R. (1983) in *Enzyme Technology: Extracellular Acid Protease of *Rhizopus rhizopodiformis** (Lafferey, R. M., ed.), pp. 69–77, Springer Verlag, Berlin
- Apodaca, G. and McKerrrow, J. H. (1989) *Infect. Immun.* **57**, 3081–3090
- Bouchara, J. P., Larcher, G., Annaix, V., Bucher, P., Tronchin, G. and Chabasse, D. (1991) *J. Mycol. Méd.* **1**, 105–111
- Tronchin, G., Bouchara, J. P., Larcher, G., Lissitzky, J. C. and Chabasse, D. (1993) *Biol. Cell* **77**, 201–208
- Miller, M. A., Greenberger, P. A., Amerian, R., Toogood, J. H., Noskin, G. A., Roberts, M. and Patterson, R. (1993) *Am. Rev. Respir. Dis.* **148**, 810–812
- Hasche, K. D., Schaeg, W., Blobel, H. and Brückler, J. (1977) *Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. A* **238**, 300–309
- Drapeau, G. R. (1978) *J. Bacteriol.* **136**, 607–613
- Kornfeld, S. J. and Plaut, A. G. (1981) *Rev. Infect. Dis.* **3**, 521–534
- Moriwaka, K. (1963) *Biochim. Biophys. Acta* **73**, 113–124
- Moriwaka, K., Tsuzuki, H., Oka, T., Inoue, H. and Ebata, M. (1965) *J. Biol. Chem.* **240**, 3295–3304
- McKevitt, A. I., Bajaksouzian, S., Klinger, J. D. and Woods, D. E. (1989) *Infect. Immun.* **57**, 771–778
- Rüchel, R. (1981) *Biochim. Biophys. Acta* **659**, 99–113
- Negi, M., Tsuboi, R., Matsui, T. and Ogawa, H. (1984) *J. Invest. Dermatol.* **83**, 32–36
- Kaminishi, H., Hagihara, Y., Hayashi, S. and Cho, T. (1986) *Infect. Immun.* **53**, 312–316
- Monod, M., Paris, S., Sanglard, D., Jaton-Ogay, K., Bille, J. and Latgé, J. P. (1993) *Infect. Immun.* **61**, 4099–4104
- Stefanova, M. E. (1993) *J. Chromatogr.* **639**, 346–351