Characterization of a proteinase inhibitor isolated from the fungal pathogen *Coccidioides immitis*

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A proteinase inhibitor was isolated from the cytosol and cell wall of the fungal respiratory pathogen *Coccidioides immitis*. The inhibitor was purified to apparent homogeneity by acid precipitation, gel filtration and reverse-phase h.p.l.c. It has an M_r of about 5000 under reducing conditions, as revealed by SDS/polyacrylamide-gel electrophoresis, and is both heat-stable and tolerant to low pH. The inhibitor can efficiently block activity of a 36000- M_r serine proteinase previously isolated from the same cell-wall fraction of *C. immitis*, and the two molecules react in a 1:1 stoichiometry. The dissociation constant (K_i) of the enzyme-inhibitor complex is 2.3×10^{-8} M. We suggest that the low- M_r inhibitor may play a role in regulation of the activity of the cell-wall-associated 36000- M_r proteinase during sporulation of *C. immitis*.

INTRODUCTION

Proteinases produced by certain fungi pathogenic in humans have been recognized as potentially important virulence factors (Kwon-Chung *et al.*, 1985). It has been suggested that extracellular proteinases of such fungi may play roles in adherence and persistence of the pathogen on host mucosal surfaces (Borg & Rchel, 1988), invasion of host tissues (Rchel, 1983; Odds, 1985; Rchel *et al.*, 1986; Cole *et al.*, 1988) and digestion of immunoglobulins (Rchel, 1986; Yuan & Cole, 1987). We have previously isolated an alkaline serine proteinase from *Coccidioides immitis* (Yuan & Cole, 1987), which is a respiratory and systemic pathogen of humans capable of dissemination to almost all organs of the body (Stevens, 1980). The proteinase was isolated from both extracellular products and cell-wall extracts of the pathogen (Yuan & Cole, 1987; Yuan *et al.*, 1988). The enzyme was characterized by an M_r of 36000 under reducing conditions, broad substrate specificity, temperature optimum of 35–40 C and stability at pH 4.0–9.0. The *C. immitis* proteinase can digest human collagen, elastin, haemoglobin and both IgG and secretory IgA. We have also demonstrated (Yuan *et al.*, 1988) that this same proteinase may perform an important function in fungal morphogenesis during the parasitic cycle. The tiny endospores of *C. immitis* (2–4 m diam.), which can disseminate in body fluids of the host from foci of infection in the lungs, are released from the parental cells (spherules) by autolysis of an endogenous network of cross-walls, referred to as the segmentation apparatus (Cole & Sun, 1985). We have presented evidence that the serine proteinase becomes localized within the segmentation apparatus, is capable of digestion of the segmentation-apparatus wall components and can be isolated in active form from endosporulating spherules (Yuan *et al.*, 1988).

The serine proteinase was shown to be concentrated in the segmentation apparatus even during early stages of spherule segmentation. We proposed that activity of the wall-associated enzyme was somehow inhibited until the

endospores had differentiated and were about to be released from the maternal cell. In the present paper we describe a low- M_r peptide capable of inhibiting the activity of the 36000- M_r proteinase. The inhibitor was isolated from wall and cytosol fractions of the saprobic and parasitic phases of *C. immitis*.

MATERIALS AND METHODS

Materials

Sephadex G-50 (particle size 20–80 m) was obtained from Pharmacia. Trichloroacetic acid (6.1 M, approx. 100 %, w/v), Bz-Tyr-OEt, angiotensin I (hypertensin I, acetate salt, synthesized human form), α_1 -antitrypsin (human plasma), *N*-chlorosuccinimide (crystallized) and L-methionine were from Sigma Chemical Co. Trifluoroacetic acid was obtained from Advanced Chem-Tech. Acetonitrile (h.p.l.c. grade) was purchased from Fisher Scientific Co. The chemicals for gel electrophoresis and protein standards for calibration were obtained from Sigma Chemical Co. and Bio-Rad Laboratories respectively. Amino acid standards and HCl for amino acid analysis were from Pierce Chemical Co. All other chemicals were obtained from commercial sources. Wet cellulose dialysis tubing (M_r cut-off 1000) used throughout this study was obtained from Spectrum Medical Industries.

Cultivation of fungi

Two strains of *C. immitis* (C634 and C735) recently isolated from patients with disseminated coccidioidomycosis were used in this study. The mycelial (saprobic phase) and spherule (parasitic phase) cultures were grown in defined media and under incubation conditions that have been described previously (Yuan & Cole, 1987).

Isolation of cell-wall and cytosol fractions

Mycelia grown in defined media for 7 days (30 C) were collected by vacuum filtration (Yuan & Cole, 1987),

Abbreviation used: Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester.

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and the retained material was resuspended in distilled water. The mycelial fraction was washed three times with distilled water by repeated centrifugation (10000 *g* for 10 min), and the pellet was then suspended in distilled water and homogenized in a Braun homogenizer as described previously (Cole *et al.*, 1983). The homogenate was passed through a glass-wool filter and the filtrate was centrifuged (13000 *g* for 20 min). The supernatant was frozen and stored at -20°C until further analysis.

Spherules grown in defined media for 96 h were obtained by centrifugation (10000 *g* for 10 min), washed with distilled water and homogenized as above. The total wall fraction, including the segmentation apparatus, was obtained from these endospore-forming spherules as described previously (Cole *et al.*, 1985). The endospore-forming-spherule wall fraction was freeze-dried and stored at -20°C .

Trichloroacetic acid precipitation

Initial concentration of cellular fractions suspected of containing the proteinase inhibitor was performed by using a modification of the method of Aronson (1986). Approx. 5.0 ml of the isolated cytosol fraction, or 5.0 mg of the endospore-forming-spherule wall fraction suspended in 5.0 ml of distilled water, was heated in a 95°C water bath for 5 min and then rapidly cooled on ice. After centrifugation (10000 *g* for 10 min), 100% (w/v) trichloroacetic acid was added to the supernatant of each fraction to give a final concentration of 5% trichloroacetic acid. Each suspension was kept on ice for 20 min, then centrifuged as described above, and trichloroacetic acid was then added to the supernatants to give a final concentration of 10%. The resulting precipitates were collected by centrifugation (10000 *g* for 10 min), suspended separately in 500 μl of 0.02 M-Tris/HCl buffer, pH 9.0, and dialysed against the same buffer (three changes) at 4°C for 16 h. The materials retained in the dialysis bags were freeze-dried.

Purification of proteinase inhibitor

The inhibitor was isolated and purified from the freeze-dried trichloroacetic acid precipitates of both the mycelial cytosol and the endospore-forming-spherule wall fractions. The former was subjected to gel filtration followed by reverse-phase h.p.l.c. The latter was subjected only to reverse-phase h.p.l.c.

(i) Gel filtration. The dialysed 10%-trichloroacetic acid precipitate of the mycelial cytosol was fractionated by gel filtration as described in Fig. 1 legend. Pooled fractions of the mycelial cytosol-derived material showing high proteinase-inhibitory activity, as described below, were selected for further fractionation by reverse-phase h.p.l.c. The pooled gel-filtration fractions were dialysed against distilled water (at 4°C for 48 h, three changes) and freeze-dried.

(ii) Reverse-phase h.p.l.c. A Hewlett-Packard model HP1090 chromatograph equipped with a diode array detector was used. The selected mycelial cytosol fractions from the trichloroacetic acid precipitate of the endospore-forming-spherule wall fraction were fractionated by reverse-phase h.p.l.c. as described in Fig. 2 legend.

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed with a 20.0% slab gel and the discontinuous buffer system described by Laemmli (1970). Freeze-dried preparations of the purified proteinase inhibitor obtained by reverse-phase h.p.l.c. of the mycelial cytosol and endospore-forming-spherule wall fractions were examined for homogeneity by SDS/polyacrylamide-gel electrophoresis by using the method previously described (Yuan & Cole, 1987).

Assay of *C. immitis* proteinase inhibitor activity

Fractions derived from gel filtration (Fig. 1) and reverse-phase h.p.l.c. (Fig. 2) were tested for inhibitor activity in the presence of the previously isolated *C. immitis* proteinase, which was purified by the method described previously (Yuan & Cole, 1987). Bz-Tyr-OEt was used as the substrate for the assay of activity of the *C. immitis* proteinase (Yuan *et al.*, 1988). The inhibitor activity of chromatographic fractions was measured in a spectrophotometric assay based on comparison of the digestion of Bz-Tyr-OEt by the purified proteinase preincubated with the sample containing the suspected inhibitor with digestion of Bz-Tyr-OEt in the presence of proteinase alone. Digestion of the substrate was measured as change in absorbance of the reaction mixture at 256 nm during a 15 min period of incubation at 24°C .

Initial analysis of inhibitor activity in the gel-filtration fractions of the mycelial cytosol-derived material was tested by addition of 80 μl portions of each fraction to a solution containing 5 μg of purified proteinase solubilized in 20 μl of enzyme buffer (0.1 M-Tris/HCl buffer, pH 8.0, containing 10 mM- CaCl_2). The mixture was preincubated at 37°C for 15 min. After addition of substrate (Bz-Tyr-OEt) solution [1.3 ml of 0.1 M-Tris/HCl buffer, pH 8.0, and 0.15 ml of 1.0 mM-Bz-Tyr-OEt in 30% (v/v) methanol] and incubation at 24°C for 15 min, activity of the proteinase in the reaction mixture was measured spectrophotometrically as described above. In subsequent analysis of the freeze-dried reverse-phase h.p.l.c. fractions (Fig. 2), different amounts of sample (1–10 μg) were added to a solution containing 30 μg of purified proteinase in 50 μl of enzyme buffer. The h.p.l.c. fractions plus proteinase were preincubated, then added to substrate solution, and the inhibitor activity of each mixture was determined spectrophotometrically as described above. Purified proteinase preincubated with enzyme buffer alone served as a positive control in each assay. One unit of inhibitor was defined as the amount of substance able to arrest the activity of 1.0 μg of purified proteinase under these assay conditions.

Assay of *C. immitis* proteinase activity

The ability of the inhibitor to block cleavage of a synthetic peptide (angiotensin I) of known amino acid sequence by the purified *C. immitis* proteinase was also examined in a separate assay by using a modification of the procedures described by Elliott & Cohen (1986). The synthetic peptide (100 μg) was first dissolved in 100 μl of 0.1% (v/v) trifluoroacetic acid and fractionated by reverse-phase h.p.l.c. (Fig. 3). Only the major peak of angiotensin I was collected (monitored at 214 nm; Fig. 3a). Confirmation that this fraction was angiotensin I was based on analysis of its amino acid composition, which was conducted as described below. The purified

fraction was freeze-dried, resuspended in diethyl ether and dried under vacuum to remove residual trifluoroacetic acid. The substrate was then used in the following experiments.

Portions (10 μ l) of purified proteinase (from stock solution containing 100 μ g/ml of enzyme buffer) were each added to 90 μ l of the same buffer alone or of buffer plus different amounts of chromatographically purified inhibitor (0.01–1.0 μ g) obtained by reverse-phase h.p.l.c. separation of either the cytosol or endospore wall fractions. These solutions were preincubated for 15 min at 37 °C, followed by addition of 10 μ g of chromatographically purified angiotensin I. The reaction mixture was then incubated for 5–30 min at 37 °C. As a negative control, 10 μ l of distilled water was substituted for the proteinase solution. At the end of the incubation period, reactions were arrested by addition of 100 μ l of 0.2% (v/v) trifluoroacetic acid. The samples were then centrifuged (10000 *g* for 3 min). The supernatants (200 μ l) were separately fractionated by reverse-phase h.p.l.c. as described in Fig. 3 legend. The fractions were collected and directly subjected to hydrolysis for amino acid analysis.

Amino acid analysis

The procedures for acid hydrolysis and amino acid analysis were the same as described previously (Yuan & Cole, 1987).

Stoichiometry of inhibition

A fixed amount of purified proteinase was preincubated with different amounts of inhibitor, and the remaining activity of the proteinase was determined by addition of a fixed amount of substrate as described in Fig. 4 legend.

Oxidation of methionine residues

Methionine residues were oxidized to methionine sulphoxide by the method of Johnson & Travis (1979).

The *C. immitis* proteinase inhibitor (5.0 μ g in 25 μ l of distilled water) was incubated with 5 μ l of *N*-chlorosuccinimide (30 mg/ml in 0.1 M-Tris/HCl buffer, pH 8.0) at 25 °C for 30 min. The reaction was stopped by addition of 20 μ l of an aqueous methionine solution (2 mg/ml). The same concentration of inhibitor was incubated in enzyme buffer under identical conditions. In a separate experiment 5.0 μ g of human α_1 -antitrypsin, which was used as a positive control, was oxidized by the same method. The latter has already been shown to be an inhibitor of the *C. immitis* proteinase (Yuan & Cole, 1987). The assay for inhibitor activity after methionine oxidation, or incubation with enzyme buffer alone, was conducted as described above.

Kinetics of inhibitor activity

Double-reciprocal-plot analysis of enzyme rate data was conducted by using essentially the same methods as previously reported (Yuan *et al.*, 1988) to determine whether the nature of the proteinase inhibition was competitive or non-competitive. The dissociation constant (K_i) of the enzyme-inhibitor complex was determined as described in Fig. 5 legend.

RESULTS

Purification and characterization of proteinase inhibitor

The crude inhibitor was obtained from both the cytosol fraction of the saprobic phase and the endospore wall of the parasitic phase of *C. immitis* by using a trichloroacetic acid precipitation method that has proved successful for the isolation of bacterial proteinase inhibitors (Aronson, 1986). Although gel filtration was not well suited for further fractionation of the dialysed trichloroacetic acid precipitate of the cytosol material (Fig. 1), it did provide a method for separation of higher- M_r components and concentration of the low- M_r inhibitor-containing fractions. Subsequent reverse-phase

Fig. 1. Elution profile of Sephadex G-50 gel-filtration chromatography (●) of trichloroacetic acid precipitate of mycelial cytosol, and profile of inhibitor activity (○) of gel-filtration-chromatography fractions in the presence of *C. immitis* proteinase

The column (25 ml bed volume) was first equilibrated with 0.1 M-Tris/HCl buffer, pH 7.0, at room temperature. Samples (50 μ g) of the dialysed and freeze-dried trichloroacetic acid precipitates of the cytosol were eluted with the same buffer and monitored by absorbance at 280 nm. Portions (80 μ l) of each fraction (400 μ l of eluate) were tested for *C. immitis* proteinase-inhibitor activity by the assay described in the Materials and methods section.

Fig. 2. Purification of inhibitor by reverse-phase h.p.l.c. and examination of homogeneity by SDS/polyacrylamide-gel electrophoresis

Reverse-phase h.p.l.c. profiles of pooled gel-filtration fractions (nos. 28–43 in Fig. 1) of mycelial cytosol and of resolubilized 10% trichloroacetic acid precipitate of isolated endospore-spherule wall (ESW) are shown in (a) and (b) respectively. Samples (approx. 50 µg each) dissolved in 500 µl of 0.1% (v/v) trifluoroacetic acid prepared in filtered distilled water were fractionated on a Beckman Ultrasphere-ODS C₁₈ column (10 mm × 25 cm). Approx. 150–200 µl portions of samples were injected separately and eluted with a linear solvent gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 30 min. The flow rate was 1 ml/min and fractions were monitored simultaneously at 214 nm and 280 nm. Each fraction (1–7) was dialysed and freeze-dried, and tested for inhibitor activity as described in the Materials and methods section. The major fraction (3) of the cytosol and endospore-spherule wall was examined separately by SDS/polyacrylamide-gel electrophoresis (c) as described in the Materials and methods section. These same fractions were pooled and subjected to co-chromatography (d).

h.p.l.c. permitted isolation of a single major component (fraction 3 in Fig. 2a) that demonstrated inhibitor activity when preincubated with the *C. immitis* proteinase

followed by addition of substrate (Bz-Tyr-OEt). A summary of results of the inhibitor assay at steps in purification of the inhibitor from the cytosol fraction is presented in Table 1. The resolubilized trichloroacetic acid-precipitated fraction of the endospore-spherule wall, which was applied directly to the reverse-phase h.p.l.c. column (Fig. 2b), contained a major fraction with the same retention time as fraction 3 derived from the mycelial cytosol (Fig. 2a), and also demonstrated inhibitor activity.

These two inhibitory fractions were subsequently examined for homogeneity by SDS/polyacrylamide-gel electrophoresis. In each case a single polypeptide band with an estimated M_r of 5000 was revealed in the Coomassie Blue-stained gels (Fig. 2c). The same two fractions were combined (1:1 by dry wt.) and subjected to co-chromatography (Fig. 2d) on the C₁₈ column under the same conditions as used for the original separations. A single peak was observed when monitored at either 214 nm or 280 nm, with the same retention time as the major fractions obtained from the cytosol and endospore-spherule wall samples. The single fraction collected during co-chromatography also demonstrated inhibitor activity.

Results of amino acid analysis of the purified inhibitor are presented in Table 2. Acidic and polar amino acids were the major components detected.

Inhibition of cleavage of angiotensin I

The chromatographically purified peptide (angiotensin I; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) was eluted from the C₁₈ column as a single peak with a retention time of 21.3 min. Fractionation of the cleavage products of the peptide after incubation with the *C. immitis* proteinase yielded four fractions (A–D), each of which was subjected to amino acid analysis (Table 3). Fraction A was composed of only two amino acid residues (His-Leu). The sum of the amino acid composition of two additional cleavage products (B and C) was equal to that of fraction D, suggesting the sequence of Asp-Arg-Val-Tyr for fraction B and Ile-His-Pro-Phe for fraction C (Table 2). No further cleavage of the peptide occurred after longer incubation with the proteinase. On the basis of these data, the primary and secondary cleavage sites of angiotensin I were predicted (Fig. 3b).

The amount of inhibitor purified by reverse-phase h.p.l.c. that totally blocked cleavage of angiotensin I (10 µg) in the presence of a fixed concentration of the *C. immitis* proteinase (1.0 µg/100 µl of enzyme buffer) was approx. 0.15 µg (Fig. 3d).

Stoichiometry of inhibition

A graph comparing the amount of inhibitor added to a mixture of *C. immitis* proteinase plus substrate and residual proteolytic activity is presented in Fig. 4. Extrapolation to zero proteinase activity remaining corresponds to 1.1 nmol of inhibitor.

Methionine oxidation

The inhibitor activity of human α_1 -antitrypsin against the *C. immitis* proteinase (100% inhibition) was completely lost after oxidation of methionine residues. No loss of activity of the chromatographically purified inhibitor was noted in the presence of the *C. immitis*

Table 1. Purification of *C. immitis* proteinase inhibitor from mycelial cytosol

For experimental details of the purification procedure see the Materials and methods section. Equal portions (80 μ l) of samples obtained at each step in the purification process were tested for inhibitor activity. A unit of inhibitor activity is described in the assay of *C. immitis* proteinase inhibitor activity. Abbreviation: TCA, trichloroacetic acid.

Step	Volume (μ l)	Absorbance (A_{280})	Inhibitor activity units (A_{256})	Purification (fold)	Yield (%)
Cytosol	5000	39000	123.0	1.0	100.0
5%-TCA supernatant	5000	296	81.2	87.0	66.0
10%-TCA pellet	300	187	77.0	130.5	62.6
Sephadex G-50 fractions (nos. 28-43)	2000	21	30.3	457.5	24.6
Reverse-phase h.p.l.c. fraction 3	1500	2.8	23.1	2615.8	18.8

Table 2. Amino acid composition of the proteinase inhibitor

The samples of proteinase inhibitor were obtained from reverse-phase h.p.l.c. fraction 3 of either endospore-forming wall or pooled mycelial cytosol fraction (Fig. 2). The results given are average values from 24 h hydrolysates.

Amino acid	Composition (no. of residues/molecule)
Asx	2
Thr	2
Ser	8
Glx	6
Pro	1
Gly	8
Ala	3
Val	1
Met	1
Ile	1
Leu	1
Lys	4
Tyr	1
His	2
Phe	1

Table 3. Amino acid analysis of cleavage products of angiotensin I isolated by reverse-phase h.p.l.c.

Cleavage products of the purified substrate (S; angiotensin I) in presence of the *C. immitis* proteinase were collected as fractions (A-D) by reverse-phase h.p.l.c. as described in Fig. 3 legend. The values shown were normalized to the apparent number of residues in the synthetic peptide (shown in parentheses).

Amino acid	Fraction...	Composition (no. of residues/molecule)				
		Control S	Digest			
			A	B	C	D
Asp		1.2 (1)		0.8 (1)		0.9 (1)
Arg		1.1 (1)		0.7 (1)		0.8 (1)
Val		1.1 (1)		0.9 (1)		0.9 (1)
Tyr		0.9 (1)		0.9 (1)		1.1 (1)
Ile		0.8 (1)			1.2 (1)	1.0 (1)
His		1.9 (2)	0.9 (1)		1.0 (1)	1.1 (1)
Pro		1.1 (1)			1.0 (1)	0.7 (1)
Phe		1.1 (1)			1.1 (1)	0.9 (1)
Leu		1.1 (1)	1.1 (1)			

proteinase after preincubation of the inhibitor with *N*-chlorosuccinimide.

Kinetics of inhibitor activity

The same fixed concentration of proteinase, preincubated with enzyme buffer alone or different concentrations of inhibitor, yielded different slopes for plots of $1/v$ versus $1/[S]$ for six or seven different $[S]$ values (Fig. 5). A common intercept occurs for the extrapolated lines through the ordinate. The calculated value for the dissociation constant (K_i) of the enzyme-inhibitor complex is 0.23 nM.

DISCUSSION

The same proteinase inhibitor was isolated and purified from both the cytosol of the saprobic phase and the cell wall of the parasitic phase of the respiratory pathogen *Coccidioides immitis*. The inhibitor was purified to

homogeneity by reverse-phase h.p.l.c., as revealed by SDS/polyacrylamide-gel electrophoresis. The active fraction was shown in a reducing gel to be a single polypeptide band with an M_r of 5000. The inhibitor is both acid- and heat-stable, characteristics that have been reported for low- M_r proteinase inhibitors of other fungi (Beck *et al.*, 1980; North, 1982). The predominance of acidic and polar amino acid components of the purified inhibitor may contribute to its solubility in aqueous buffer.

Examination of the cleavage of purified angiotensin I provided insights into the activity of both the *C. immitis* proteinase and inhibitor. The primary and secondary cleavage sites of angiotensin I were the Phe-His bond and Tyr-Ile bond respectively. Our determination that 0.15 μ g of inhibitor was required to block digestion of a fixed amount of angiotensin I by 1.0 μ g of proteinase is consistent with the stoichiometry of inhibition. On the

Fig. 3. Separation of cleavage products of a synthetic peptide substrate (angiotensin I) by reverse-phase h.p.l.c.

Samples were fractionated on a Waters μ Bondapak C_{18} column (7.8 cm $\times$ 30 cm) with the same solvent system and flow rate as described in Fig. 2 legend. Isolation and identification of the purified synthetic peptide substrate (S, in *a*) and cleavage products (A–D) after incubation with the *C. immitis* proteinase for 5 min and 30 min (in *b* and *c* respectively) were conducted as described in the Materials and methods section. Inactivation of proteolysis of angiotensin I in the presence of approx. 0.15 μ g of the chromatographically purified inhibitor is shown in (*d*).

Fig. 4. Stoichiometry of inhibition

The purified proteinase (1.0 nmol, based on M_r 36000) in 10 μ l of enzyme buffer was preincubated with different amounts of chromatographically purified inhibitor (0.25–2.0 nmol) at 37 $^{\circ}$ C for 60 min. The long incubation

basis of the apparent M_r of 5000 for the inhibitor and 36000 for the proteinase (both determined under reducing conditions) and 1:1 stoichiometry, the predicted amount of inhibitor necessary to inactivate 1.0 μ g of proteinase is 0.14 μ g.

Methionine oxidation of the *C. immitis* inhibitor was performed to determine whether loss of inhibitor activity occurred after oxidation with *N*-chlorosuccinimide, which would indicate a critical requirement of the methionine residue (Tzeng *et al.*, 1982). No such requirement apparently exists for this inhibitor.

time was necessary to ensure that the reaction had gone to completion. The remaining activity of the *C. immitis* proteinase was determined by addition of 150 μ l of 1.0 mM-Bz-Tyr-OEt followed by incubation and spectrophotometric examination as described for the inhibitor assay in the Materials and methods section. The line represents a linear regression fitted to the data below 1.0 nmol. The control reaction mixture consisted of proteinase in enzyme buffer alone plus Bz-Tyr-OEt.

Fig. 5. Kinetics of inhibition of the *C. immitis* proteinase by the purified inhibitor

The purified proteinase (1.0 nmol) was preincubated in enzyme buffer alone (●) (37 °C for 20 min) or in the presence of different amounts of inhibitor [4.0 nmol (○); 6.0 nmol (△); 8.0 nmol (▲)]. The preincubated mixtures were then added separately to the substrate (S) solution (24 °C for 15 min), which consisted of 1.3 ml of enzyme buffer in 150 μ l of Bz-Tyr-OEt dissolved in 30 % (v/v) methanol. The final range of concentration of substrate ([S]) was 0.003–1.00 mM. The velocity of the enzymic reaction (v), based on rate of change in absorbance (A_{258}) of the supernatant of the reaction mixture, was determined for each concentration of Bz-Tyr-OEt used. Corresponding plots of v versus [S] are shown in the inset. The time is measured in seconds (s). The dissociation constant (K_i) of the enzyme inhibitor complex was calculated on the basis of a Dixon (1953) plot of $1/v$ versus $[I]$.

Results of kinetic studies of the inhibition of the *C. immitis* proteinase indicate that enzyme inactivation occurs by competitive inhibition. The calculated value of K_i (2.3×10^{-8} M) indicates a relatively high affinity of inhibitor for the *C. immitis* proteinase (Seemüller *et al.*, 1986). The purified inhibitor has been used to immunize rabbits for generation of monospecific antibody. The latter has been employed in immunoelectron microscopy for localization of the inhibitor in parasitic cells of *C. immitis* as previously described (Yuan *et al.*, 1988). Preliminary results have shown that the inhibitor is localized in the cell wall together with the serine proteinase, as well as in the cytoplasm (L. Yuan & G. T. Cole, unpublished work). Our ability to isolate the inhibitor directly from the endospore-spherule wall suggests the presence of abundant wall-associated inhibitor. A possible physiological function of the low- M_r inhibitor, as suggested for certain plant inhibitors (Wilson, 1986), is the regulation of proteolysis during specific stages of cellular development. The inhibitor described in the present paper may be involved in regulation of the 36000- M_r proteinase activity in the segmentation apparatus of endospore-spherule cells of *C. immitis*.

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