A low-molecular-mass aspartic protease inhibitor from a novel *Penicillium* sp.: implications in combating fungal infections

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A low-molecular-mass aspartic protease inhibitor was isolated from a novel *Penicillium* sp. The inhibitor was purified to homogeneity, as shown by reversed-phase HPLC and SDS-PAGE. The $M_r$ of the inhibitor was 1585 and the amino acid composition showed the presence of D, D, D, E, A, K, L, Y, H, I and W residues. The steady-state kinetic interactions of *Aspergillus saitoi* aspartic protease with the inhibitor revealed the reversible, competitive, time-dependent tight-binding nature of the inhibitor, with IC$_{50}$ and $K_i$ values of 1.8 and 0.85 μM, respectively. Fluorescence spectroscopy and circular dichroism analysis showed that inactivation of the enzyme was due to binding of the inhibitor to the active site. The inhibitor was found to inhibit mycelial growth and spore germination of *Aspergillus fumigatus* and *Aspergillus niger* in vitro with MIC values of 1.65 and 0.30 μg ml$^{-1}$, respectively. This study will potentially open the way towards the development of a tight-binding peptidic inhibitor against fungal aspartic proteases to combat human fungal infections.

**INTRODUCTION**

Several proteases are essential for propagation of disease, and hence inhibition of different proteases is emerging as a promising approach in medical applications for the treatment of cancer, obesity, hepatitis and herpes, cardiovascular, inflammatory and neurodegenerative diseases, as well as various infectious and parasitic diseases (Rao et al., 1998). Aspartic proteases are a relatively small group of proteolytic enzymes. Over the last decade, they have received tremendous research interest as potential targets for pharmaceutical intervention, as many have been shown to play significant roles in physiological and pathological processes (Dash et al., 2003). Study of the kinetic properties of this class of enzymes has been motivated by their pharmaceutical and commercial importance, and has evoked considerable interest regarding the role of their inhibitors.

Invasive aspergillosis is a major threat to the long-term survival of immunocompromised patients (Denning & Stevens, 1990). Risk factors for invasive aspergillosis include prolonged and severe neutropenia, haematopoietic stem cell and solid organ transplantation, advanced AIDS, and chronic granulomatous disease (Segal & Walsh, 2006).

Aspergillus fumigatus is the most important airborne fungal pathogen. Although important advances in antifungal therapy have been achieved in the past decade, current treatment modalities remain limited in their therapeutic benefit (Walsh et al., 2007). Extracellular proteinases that can hydrolyse the structural components of the lung are thought to be possible virulence factors (Rhodes et al., 1988; Kothary et al., 1984). A combination of various proteolytic enzymes may contribute to the ability of *A. fumigatus* to degrade host tissue for nutritional acquisition and invasion (Dagenais & Keller, 2009). Aspartic proteases from *Aspergillus* sp. have been reported to be possible virulence factors in invasive aspergillosis (Lee & Kolattukudy, 1995; Hogan et al., 1996), although more recent studies have challenged the importance of secreted aspartic proteases in infection and reported that they are not important as virulence factors (Sharon et al., 2009; Hartmann et al., 2011). *A. fumigatus* aspartic protease is necessary for fungal growth in protein medium (Sriranganadane et al., 2011). Combinations of proteolytic enzymes that include aspartic proteases contribute to the ability of *A. fumigatus* to degrade host tissue for nutritional acquisition and invasion (Dagenais & Keller, 2009). Hence, the inhibition of aspartic protease might provide new insight into the prevention of invasive aspergillosis.

In the present work, fungal aspartic protease (PepA) was used as a prototypical aspartic protease, playing an important role as a model enzyme for the development of inhibitors for other aspartic proteases of therapeutic significance. There are few reports on the isolation of biological aspartic protease...
inhibitors and their mechanisms of inhibition. The present study describes for the first time the isolation and characterization of a low-molecular-mass peptidic PepA inhibitor from a novel Penicillium sp. Evaluation of kinetic parameters confirms that Penicillium aspartic protease inhibitor is a tight-binding, reversible and competitive inhibitor of fungal aspartic protease. Fluorescence microscopy and circular dichroism (CD) analyses show that binding of the inhibitor induces localized conformational changes in PepA. The new inhibitor is a potential lead compound for the development of molecules to combat human fungal infections.

METHODS

Materials. Acetonitrile was purchased from E-Merck, and ultra membranes UM 10, UM 3 and UM 0.5 were from Millipore. Aspartic protease from Aspergillus saitoi, trifluoroacetate (TFA) and haemoglobin were from Sigma-Aldrich. The Biogel P 2 column was from Bio-Rad Laboratories. All other chemicals used were of analytical grade.

Isolation and identification of the isolate. The organism was isolated in the laboratory from a soil sample collected from Pashan, Pune district, India. Soil was suspended in sterile saline and serial dilutions of the soil sample were plated on potato dextrose agar (PDA) and incubated at 28 °C for 5–7 days. Fungal colonies obtained were purified by single colony plating and screened for aspartic protease inhibitor by growing them for 120 h in liquid broth (PDA) and incubated at 28 °C for 5–7 days. Fungal colonies obtained were purified by single colony plating and screened for aspartic protease inhibitor by growing them for 120 h in liquid broth containing soy meal (SBM) (2%, w/v) as an inducer and other nutrients [such as glucose (1%, w/v), beef extract (0.75%, w/v), peptone (0.75%, w/v), sodium chloride (0.3%, w/v), magnesium sulfate (0.1%, w/v) and dipotassium hydrogen phosphate (0.1%, w/v), pH 5.0]. Samples were withdrawn every 24 h and assayed for anti-PepA activity. The genomic DNA of the fungal culture producing the inhibitor was isolated according to the method of Makimura et al. (1994). The organism was identified based on the internal transcribed spacer (ITS) region of the 185 rRNA gene. The BLAST program was used for sequence searches of the National Centre for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLAST with default settings. The sequences were downloaded from the NCBI database and aligned with the CLUSTAL W program (http://www.genome.jp/tools/clustalw/). Sequences were trimmed using DAME software. A phylogenetic tree was generated via the neighbour-joining method (Saitou & Nei, 1987) using MEGA software (Kumar et al., 2001).

Production and purification of the inhibitor. Penicillium sp. VM24 was cultured in 250 ml liquid medium (as above) in 500 ml Erlenmeyer flasks for inhibitor production at 28 °C for 96 h. The extracellular culture filtrate obtained after centrifugation was treated with activated charcoal (6.5%, w/v) and centrifuged. The inhibitor was purified from the supernatant by ultrafiltration (UM 10, UM 3, UM 0.5), using a Biogel P 2 column and reversed-phase HPLC (Fluka RP-C8), pre-equilibrated with 10% (v/v) acetonitrile (CH3CN) and 0.1% (v/v) TFA. The fractions were eluted on a linear gradient of 0–90% (v/v) acetonitrile with H2O containing 0.05% (v/v) TFA at a flow rate of 0.5 ml min−1 and monitored at a wavelength of 210 nm. The eluted sample was lyophilized and dissolved in deionized water to check for inhibitor activity. The active fractions were rechromatographed via reversed-phase HPLC under similar experimental conditions.

Molecular mass and amino acid determination. The molecular mass of the inhibitor was determined by MS (MALDI-TOF) and tricine-SDS-PAGE. Analysis of amino acids was performed by hydrolysing 100 pM of inhibitor with 6 M HCl at 110 °C for 24 h in vacuum-sealed tubes. The hydrolysed amino acids were derivatized with AccQ Fluor Reagent (6-amino quinolyl-N-hydroxysuccinimide carbamate) and run on a prepacked reversed-phase HPLC column (3.9×150 mm; AccQ.Tag). The amino acids were eluted with an acetonitrile gradient (5–95%, v/v) and monitored with a fluorescence detector. To calculate the molar proportion of constituents, the peak areas of individual amino acids were compared with standards analysed under identical conditions. Total cysteine and tryptophan were estimated with intact peptide according to the methods of Cavallini et al. (1966) and Spande & Witkop (1967), respectively. The solutions for tricine-SDS-PAGE were prepared according to Schagger (2006). Protein concentration was determined according to the Bradford method, using BSA as standard (Bradford, 1976).

Fungal aspartic protease (PepA) inhibition assay. Proteolytic activity of PepA from A. saitoi was measured by assaysing enzyme activity using haemoglobin, as described by Dash et al. (2001a). Enzyme (1.5 μM) and inhibitor were incubated in glycine-HCl buffer (0.05 M, pH 3) for 10 min. The reaction was started by the addition of 1 ml haemoglobin (5 mg ml−1) and was incubated at 37 °C for 30 min. The reaction was quenched by the addition of 2 ml 10% (w/v) TCA acidified with 2.25% (v/v) HCl followed by centrifugation (10 000 g, 5 min) and filtration. The absorbance of the TCA-soluble products in the filtrate was read at 280 nm. One unit of PepA was defined as the amount of enzyme that produced an increase in absorbance of 0.001 at 280 nm min−1 under the conditions of the assay. One protease inhibitor unit was defined as the amount of inhibitor that inhibited one unit of fungal aspartic protease activity (Dash et al., 2001a).

Assay for inhibitory activity towards trypsin, chymotrypsin, papain and subtilisin. Inhibitory activities of the inhibitor against other classes of proteases were also determined by assaysing enzyme activity using specific chromogenic substrates at various pHs and temperatures. The synthetic substrates Br−L-Arg-pNA, HCl for trypsin and papain, Bz-Tyr-pNA for chymotrypsin and Z-Ala-Ala-Leu-pNA for subtilisin were used (Arnon, 1970; Grant et al., 1970; Walsh, 1970).

Temperature and pH stability. For temperature stability experiments, the inhibitor (10 μM) was incubated at 25–50 °C for 6 h and its inhibitory activity was estimated against PepA at different intervals of time. The pH stability of the inhibitor was determined by pre-incubating the inhibitor (10 μM) at a range of pH in appropriate buffers for 1 h and estimating the anti-PepA activity.

Initial kinetic analysis for determination of Km and Ki. For analysis of initial kinetics, the kinetic parameters for substrate hydrolysis were determined by measuring the initial rate of enzyme activity. The IC50 value was determined by non-linear regression of the percentage inhibition data by using a four-parameter logistic equation (see equation 1), where p is percentage inhibition and is the relative decrease in enzyme activity due to the inhibitor concentration, p0/Km (Kulkarni & Rao, 2007). Regression analysis was performed using the software program Microcal Origin 8E.

\[
p = P_{\text{min}} + \left( P_{\text{max}} - P_{\text{min}} \right) / \left( 1 + \left( I / I_{\text{IC50}} \right)^n \right)
\]

The inhibition constant K_i was determined according to Dixon (1953) and also using a Lineweaver–Burk double reciprocal plot. For the latter, PepA (20 μM) was incubated with inhibitor at concentrations of 0, 2, 4 and 6 μM and assayed at increasing concentrations of haemoglobin (75–540 μM) at 37 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. In
Dixon’s method, the hydrolytic activity of PepA (20 μM) was measured in the presence of 250 and 450 μM haemoglobin at concentrations of inhibitor ranging from 1 to 10 μM at 37 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and $K_i$ was determined by fitting the data using Microcal Origin 8E.

Fig. 1. (a) Purified inhibitor was analysed for determination of $M_r$ by MALDI-TOF (1585). (Inset) Reversed-phase HPLC of purified inhibitor. Shown is the elution profile of the peak associated with anti-PepA activity. The peak detected showed a retention time of 6.27 min. (b) Tricine-SDS-PAGE of the inhibitor loaded on a 16 % tryptophan, 6 % cysteine Tris-Tricine gel. Lane 1, standard ultralow-molecular-mass markers; lane 2, HPLC-purified inhibitor. (c) Amino acid analysis of the inhibitor. Analysis was done by hydrolysing the inhibitor with 6 M HCl, and the hydrolysed amino acids were derivatized with AccQ Fluor Reagent and run on a prepacked reversed-phase HPLC column (AccQ.Tag). The amino acids were eluted with an acetonitrile gradient and monitored with a Waters fluorescence detector. The asterisk indicates tryptophan as determined by $N$-bromosuccinimide.
Initial apparent inhibition constants. Inhibition studies were performed by adding 100 µl of the enzyme (0.05 µM) to 300 µl of 250 µM haemoglobin solution in standard buffer containing varying concentrations of the inhibitor (1–10 µM) at 37 °C for 30 min. The product was estimated as described above. Relative enzyme activity \( R \) was calculated from the ratio of product amounts obtained in the presence and absence of inhibitors as \( R = 1 - [P^t]/[P^o] \). Relative inhibition was fitted by a non-linear least-squares regression to equation 2, where \( [I]_p \) is the total concentration of inhibitor and \( K_{app} \) (the fitting parameter) is the apparent inhibition constant:

\[
R = [I]_p/[K_{app} + [I]_p] \quad (2)
\]

Inhibitor progress curve analysis. The reaction time course in the presence of inhibitor was fitted to equation 3, which is a modification of the standard kinetic model:

\[
p = p_0 + V_a + (V_0 - V_a)[1 - \exp(-K_{app}t)]/K_{app} \quad (3)
\]

Instrumental offset \( p_0 \) was treated as an adjustable parameter to account for the possibility of systematic errors in measuring the degree of product conversion. Each individual progress curve was fitted separately. The local fitting parameters were initial velocity \( V_0 \), steady-state velocity \( V_a \), steady-state velocity at time \( t \), and apparent first-order rate constant \( K_{app} \) and instrumental offset \( p_0 \). Equation 4 was used as the theoretical model and is applicable to a pure tight-binding inhibitor:

\[
v = V_0 - [I]_o - K_{app} + ([E]_o - [I]_o - K_{app})^2 + 4([E]_o - K_{app})^{-1/2} \quad (4)
\]

To fit data to this equation, the modified Marquardt–Levenberg least-square fitting equation was used. The rate constants were obtained by regression analysis of these data using the software Origin 8E. Another approach used to calculate the apparent \( K_i^* \) value for the inhibitor using equation 5:

\[
I_{C0} = E_t/(2 + K_i^*) \quad (5)
\]

\( E_t \) is the total enzyme concentration and \( K_i^* \) is the apparent enzyme–inhibitor dissociation constant. For competitive inhibition, the true \( K_i \) was obtained by dividing \( K_i^* \) by \((1 + S/K_m)\) (Dixon, 1953).

Fluorescence analysis of PepA–inhibitor interactions. Fluorescence measurements were performed on a Cary Varian eclipse fluorescence spectrophotometer connected to a Cary Varian temperature controller. Protein fluorescence was excited at 295 nm, and the emission was recorded from 300 to 400 nm at 25 °C. Slit widths on both excitation and emission were set at 5 nm, and the spectra were obtained at 1 nm min\(^{-1}\). For inhibitor binding studies, PepA (20 µM) was dissolved in 0.05 M HCl. Titration of the enzyme with inhibitor was performed by the addition of different concentrations of the inhibitor (0, 2.5, 5 and 10 µM) to a fixed concentration of enzyme solution. For each inhibitor concentration on the titration curve, a new enzyme solution was used. All the data on the titration curve were corrected for dilutions, and the graphs were smoothed. Corrections for the inner filter effect were performed as described by equation 6:

\[
F_t = F \left( \frac{A_{ex} + A_{em}}{2} \right) \quad (6)
\]

\( F_t \) and \( F \) are the corrected and measured fluorescence intensities, respectively, and \( A_{ex} \) and \( A_{em} \) are the absorbances of the solution at the excitation and emission wavelengths, respectively (Lakowicz, 1983). Background buffer spectra were subtracted to remove the contribution from Raman scattering.

Secondary structural analysis of PepA–inhibitor complexes. CD spectra were recorded on a Jasco-J715 spectropolarimeter at ambient temperature using a cell of 1 mm path length. Replicate scans were obtained at 0.1 nm resolution, 0.1 nm bandwidth and a scan speed of 50 nm min\(^{-1}\). Spectra were the averages of six scans with the baseline subtracted spanning from 260 to 200 nm in 0.1 nm increments. The CD spectrum of PepA (25 µM) was recorded in 0.05 M glycine-HCl buffer (pH 3) in the absence/presence of inhibitor or pepstatin (5 µM each). Secondary structure content of the PepA and the PepA–inhibitor complex was calculated using the algorithm of the K2d program (Andrade et al., 1993; Merelo et al., 1994).

Antifungal activity assay. A. fumigatus NCIM 902 and Aspergillus niger were obtained from our in-house culture collection unit, the National Collection of Industrial Micro-organisms, Pune, India. Antifungal activity was assayed essentially via (i) spore suspension and (ii) microspectrometric assays. Fungal spores were harvested from the freshly grown fungal culture and suspended in sterile water. The concentration of the spore suspension was adjusted to 1.0 × 10^8. To 1 ml of the freshly prepared spore suspension, 1 ml half-strength modified Mueller–Hinton (MH) agar (per litre: 2 g beef extract, 17.5 g acid hydrolysate of casein, 1.5 g starch and 25 g agar; Mueller & Hinton, 1941) (pH 5.5 ± 0.1) was added and was immediately overlaid on Petri dishes containing MH agar. To allow for spore germination and initial vegetative growth, plates were incubated at 28 °C for 24–48 h. At this time, wells were bored on the agar surface, and different concentrations of the inhibitor were applied. The plates were incubated at 28 °C and photographed after 24–72 h. All test solutions were filtered through a 0.22 µm pore-size membrane prior to application. A microspectrometric antifungal assay was performed for quantitative demonstration of antifungal activity (Broekaert et al., 1990). Briefly, routine tests were performed with 20 µl of filter-sterilized (0.22 µm pore-size) test solution and 80 µl fungal suspension (10^6 spores ml\(^{-1}\)) in half-strength MH broth. The control microculture contained 20 µl sterile distilled water and 80 µl fungal

Table 1. Purification of the inhibitor

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units mg(^{-1}))</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>650</td>
<td>4083</td>
<td>607.61</td>
<td>6.72</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Activated charcoal</td>
<td>600</td>
<td>3498</td>
<td>280</td>
<td>12.49</td>
<td>1.86</td>
<td>85.67</td>
</tr>
<tr>
<td>Ultrafiltration 10 kDa cut-off</td>
<td>575</td>
<td>2942</td>
<td>86.25</td>
<td>34.11</td>
<td>5.08</td>
<td>72.05</td>
</tr>
<tr>
<td>Ultrafiltration 3 kDa cut-off</td>
<td>550</td>
<td>2648</td>
<td>48.03</td>
<td>55.13</td>
<td>8.12</td>
<td>64.85</td>
</tr>
<tr>
<td>Ultrafiltration 0.5 kDa cut-off</td>
<td>525</td>
<td>2458</td>
<td>35.62</td>
<td>69.03</td>
<td>10.28</td>
<td>60.19</td>
</tr>
<tr>
<td>Biogel P-2 column</td>
<td>12</td>
<td>1821</td>
<td>10.38</td>
<td>176.75</td>
<td>26.32</td>
<td>44.59</td>
</tr>
<tr>
<td>Reversed-phase HPLC</td>
<td>3.5</td>
<td>735</td>
<td>2.14</td>
<td>350</td>
<td>52.12</td>
<td>18.14</td>
</tr>
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</table>
spore suspension. Unless stated otherwise, incubation conditions for the experiments were 28 °C for 48 h. Antifungal activity is expressed in terms of percentage inhibition as defined by Cammue et al. (1992). The MICs for A. fumigatus and A. niger were determined by a broth dilution method (Amsterdam, 1991). Serial dilutions of the inhibitor were made in half-strength MH broth in microtitre plates. Each well was inoculated with 10 μl of the test organism at 10^5 spores ml^{-1}. The MIC was determined after overnight incubation of the plates and was taken as the lowest concentration of the inhibitor at which growth was inhibited.

**RESULTS**

Isolation and identification of the isolate

Sixty-eight fungal cultures were isolated on PDA plates from soil samples. Only one fungal isolate was found to produce an extracellular aspartic protease inhibitor. The isolated organism was identified on the basis of morphological and 18S rRNA gene sequence similarity. The ITS region of the 18S rRNA gene of Penicillium sp. VM24 was sequenced and comprised 433 bp, and BLAST search analysis showed 94 % homology to various Penicillium species. The neighbour-joining phylogenetic tree based on ITS sequences also confirmed that the isolate belongs to the genus Penicillium (Fig. S1 available with the online version of this paper). Based on BLAST search analysis, the isolate showed only 94 % similarity to the type strain of Penicillium pinophilum and of other Penicillium sp., and hence the new isolate may represent a novel species of the genus Penicillium. The isolate grew at pH 4–6 with an optimum at pH 5 and at an optimum temperature of 28 °C.

Purification and biochemical characterization of the inhibitor

Penicillium sp. VM24 produced the inhibitor in medium containing peptone beef extract with SBM as inducer at 28 °C and pH 5. The extracellular culture filtrate was subjected to activated charcoal treatment, ultrafiltration and gel filtration to remove high-molecular-mass impurities and salts. The concentrated inhibitor sample was further purified to homogeneity by reversed-phase HPLC, and the anti-PepA activity was associated with a single peak (Fig. 1a, inset). The purified inhibitor showed specific activity of 350 U mg^{-1} and a 52-fold increase in purification with a yield of 18 % (Table 1). The inhibitor was stable over a broad range of pH (2–6) and temperature (25–40 °C). The inhibitor was found to be specific for fungal aspartic protease (PepA) and exhibited no inhibitory activity against pepsin and other classes of proteases such as trypsin, chymotrypsin, papain and subtilisin (data not shown). The M_r of the inhibitor as determined by MALDI-TOF MS was 1585 (Fig. 1a). The inhibitor also showed a single homogeneous band with an M_r of 1580 on tricine-SDS-PAGE (Fig. 1b). The amino acid composition of the inhibitor revealed the presence of D, D, D, E, A, K, L, Y, H, I and W residues (Fig. 1c).

The molecular mass of the inhibitor was determined to be 1585 Da. The peptide or isomers would have a molecular mass of 1404 Da. The difference in the molecular mass may be due to the presence of multiple groups (e.g. acetyl, carboxyl, methyl, nitro) attached to the side chains of the amino acids. Pepstatin, a biological aspartic protease inhibitor from Streptomyces sp., is known to contain a statin group (Umezawa, 1976). Several other synthetic peptide inhibitors are known to have similar groups, increasing their potency (Nguyen et al., 2008). However,
Table 2. Kinetic rate constants for the inhibitor obtained by various methods

<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>Kinetic rate constant</th>
<th>Value obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor curve analysis</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.82 μM</td>
</tr>
<tr>
<td>Lineweaver–Burk plot</td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;m&lt;/i&gt;</td>
<td>180 μM</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>2.54 μM</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;m&lt;/i&gt;</td>
<td>0.61 μM min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dixon plot</td>
<td>&lt;i&gt;k&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>1.2 μM</td>
</tr>
<tr>
<td>Substrate kinetics</td>
<td>&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;m&lt;/i&gt;</td>
<td>168 μM</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>0.59 μM min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inhibitor progress curve</td>
<td>&lt;i&gt;k&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;*</td>
<td>0.71 μM</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;m&lt;/i&gt;</td>
<td>0.65 μM min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;k&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>6.25 × 10&lt;sup&gt;-3&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;k&lt;/i&gt;&lt;sub&gt;d&lt;/sub&gt;</td>
<td>11.84 × 10&lt;sup&gt;-3&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dissociation constant analysis</td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;*</td>
<td>1.48 μM</td>
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<tr>
<td>Cha analysis†</td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>1.25 μM</td>
</tr>
<tr>
<td></td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;d&lt;/sub&gt;</td>
<td>1.05 μM</td>
</tr>
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</table>

†Cha et al. (1975).

structural studies of the inhibitor will provide further insight into the side groups and differences in molecular mass.

**Kinetic analysis of the inhibition of PepA by the inhibitor**

Kinetic analysis indicated an IC<sub>50</sub> value of 1.82 μM (Fig. 2a). Inhibition of PepA followed a hyperbolic pattern with increasing concentrations of the inhibitor. Initial kinetic assessments via Lineweaver–Burk analysis revealed that the aspartic protease was competitively inhibited by the inhibitor (Fig. 2b). The <i>K</i><sub>m</sub> value of the enzyme was 180 μM. The secondary plot of the slope versus inhibitor concentration gave a <i>K</i><sub>i</sub> value of 2.24 μM (Fig. 2b, inset), while with the Dixon plot a <i>K</i><sub>i</sub> value of 1.2 μM was obtained.

**Progress curve analysis**

Progress of the reaction was analysed by two different methods, one based on the assumption of rapid equilibrium and the other on the assumption of slow equilibrium. The results of the analysis based on the assumption of rapid equilibrium are shown in Fig. S2(a), as the data did not fit the other model. The progress curves obtained at 2, 4, 6, 8 and 10 μM inhibitor were fitted individually to equations 7–9. The best-fit values of the individual parameters obtained are given in Table S1. The results corroborate the one-step inhibition mechanism, wherein the enzyme–inhibitor complex is formed rapidly. The onset of inhibition is rapid and the binding indicates a tight binding mechanism. The rate constants are in agreement with the model scheme outlined in Fig. S3 and rule out the possibility of a slow onset of inhibition. The initial velocity <i>V</i><sub>0</sub> obtained is constant and the apparent rate constant <i>K</i><sub>app</sub> increases linearly with inhibitor concentration. The rate constants were obtained by fitting the data to equations 7–9:

\[ V_0 = V_m[S]/(S_0 + K_m) \]  
\[ V_i = V_m[S]/(S_0 + K_m(1 + [I]/K_i)) \]  
\[ K_{app} = K_D + K_A[I]/(1 + [S]/K_m) \]

The parameters listed in Table 2 favour the one-step mechanism, because the initial velocity does not decrease with the concentration of the inhibitor, as predicted by equation 7. Also, the increase of the apparent rate constant with [I] is linear. The non-linear least-squares fit of <i>V</i><sub>0</sub>, <i>V</i><sub>i</sub> and <i>K</i><sub>app</sub> to equations 7–9 is shown in Fig. S2(b, c). The parameters obtained with the mechanism as outlined by Cha et al. (1975) corroborate well with the above-mentioned mechanism, yielding a <i>K</i><sub>i</sub>* of 1.075 μM and a <i>K</i><sub>i</sub> of 1.1245 μM.

**Fluorometric analysis of PepA–inhibitor interactions**

The localized changes induced in PepA due to interactions with the inhibitor were investigated by fluorescence spectroscopy. The conformational changes induced in PepA upon binding of inhibitor were monitored by exploiting the intrinsic fluorescence by excitation of the π–π* transition in the tryptophan residues. The fluorescence emission spectra of aspartic protease exhibited an emission maximum (λ<sub>max</sub>) at 340 nm as a result of the radiative decay of the π–π* transition from the tryptophan residues, confirming the hydrophilic nature of the tryptophan environment (Fig. 3a). Titration of the native enzyme with increasing concentrations of inhibitor resulted in a concentration-dependent quenching of the tryptophanyl...
fluorescence. However, the $\lambda_{max}$ of the fluorescence profile indicated no blue or red shift, suggesting that there were no gross conformational changes in the 3D structure of the enzyme due to inhibitor binding.

**CD analysis of PepA–inhibitor complexes**

To elucidate the effects of the inhibitor on the secondary structure of PepA, the CD spectra of the enzyme–inhibitor complex were analysed. The $\alpha$-helix, $\beta$-sheet and $\beta$-turn content of PepA was 2.5, 59 and 18%, respectively. The CD spectrum of the PepA–inhibitor complex showed a pronounced shift in the negative band at 220 nm for the native enzyme to 225 nm (Fig. 3b). This shift reveals a subtle change in the secondary structure of the enzyme upon inhibitor binding. Furthermore, to elucidate the mechanism of inactivation of PepA by the newly isolated inhibitor, we analysed the interactions of a representative competitive inhibitor, pepstatin (Richards et al., 1989), on the secondary structure of PepA. Binding of pepstatin to PepA exhibited a similar pattern of negative ellipticity in the far-UV region, indicating that the newly isolated inhibitor causes similar structural changes making the bound enzyme distinctly different from the unliganded enzyme. Comparative analysis of the CD spectra of PepA upon binding of the inhibitor or the known active site-based inhibitor pepstatin showed that they were similar, suggesting binding of the newly isolated inhibitor to the active site of PepA.

**Antifungal activity of the inhibitor**

The inhibitory activity of the purified inhibitor against mycelial growth and spore germination of *A. fumigatus* and *A. niger* was assessed in various standard biological assays. *A. fumigatus* and *A. niger* were grown in MH medium (pH 5.5) at 28 °C and produced 80 IU extracellular PepA (data not shown). The antifungal activity of the inhibitor was indicated by the zone of inhibition that developed around the wells against the vegetative growth after spore germination (Fig. 4a). Fungal growth inhibition was monitored in a microscopic assay, wherein the spores were cultured in the presence of varied concentrations of the inhibitor. The morphological differences observed in mycelial growth after 48 h are shown in Fig. 4(b). In the presence of the inhibitor, germination of *A. niger* spores was delayed, whereas in *A. fumigatus*, the rate of mycelial growth was reduced. As observed from the micrographs, lysis was not observed in mycelia in the presence of inhibitor. After 24 h, the concentration of inhibitor required for 50% inhibition (IC$_{50}$) of fungal growth was 0.58 µg ml$^{-1}$ for *A. niger* and 2.34 µg ml$^{-1}$ for *A. fumigatus*, whereas the MICs were 0.30 and 1.65 µg ml$^{-1}$, respectively. The time-dependent dose–response curves revealed that the extent of growth inhibition tended to decrease with the increase in incubation time. The time-dependent decrease in the potency of the inhibitor was less pronounced in *A. niger* than in *A. fumigatus* (Fig. 5).

**DISCUSSION**

The data reported here showed that the newly isolated inhibitor from *Penicillium* sp. VM24 is a tight-binding inhibitor of PepA. *Penicillium* sp. grows well when it produces the inhibitor. Inhibitor production was growth-associated, and the highest inhibitor yield was obtained with maximum cell growth. During initial kinetic analysis the inhibitor showed competitive inhibition against the enzyme in vitro. The 1 : 1 molar ratio of the interaction of the inhibitor with the target enzymes classified it in the...
‘tight-binding inhibitor’ group (Williams & Morrison, 1979). As a rule, when comparing $K_i$ values for tight-binding inhibitors, it is essential to examine the mathematical methods used for their estimation, as different methods even when applied to the same data can yield $K_i$ estimates differing by several orders of magnitude (Szedlacsek et al., 1991; Reich, 1992). The Lineweaver–Burk reciprocal plot shows that the inhibitor is a competitive inhibitor of the fungal aspartic protease. In the region of $K_i$, the $k_A$ value is high, while the $k_D$ value is low. This indicates that the inhibitor binds rapidly to the enzyme but that the rate of dissociation is slow. This suggests fast inactivation of the enzyme in the presence of the inhibitor. Tight-binding inhibitors combine at the active site and rapidly cause the enzyme to lose activity.

Tight-binding inhibitors induce conformational changes that cause the enzyme to clamp down on the inhibitor, resulting in the formation of a stable enzyme–inhibitor complex. On the basis of our fluorescence studies, we propose that the rapid fluorescence loss was due to the formation of a reversible enzyme–inhibitor complex. Fluorescence quenching of PepA by the inhibitor revealed that binding of the inhibitor reduces the quantum yield of tryptophan emission. The kinetically observable formation of an enzyme–inhibitor complex does not involve a major alteration in the 3D structure of the enzymes, as reflected in the absence of any shift in tryptophanyl fluorescence. Any changes in the environment of individual tryptophan residues may result in an alteration of fluorescence characteristics such as emission wavelength, quantum yield and susceptibility to quenching (Pawagi & Deber, 1990). Fluorescence quenching can also result from energy transfer to an acceptor molecule with an overlapping absorption spectrum (Cheung, 1991). As the inhibitor shows no absorption in the region 300–450 nm (data not shown), we ruled out fluorescence quenching due to energy transfer between the inhibitor and the tryptophan residues. The effect of inhibitor concentration on fluorescence quenching of the enzymes was also consistent with a 1 : 1 molar ratio. These findings indicated that the polarity of the tryptophan environment was negligibly altered after binding of the inhibitor, suggesting minimal conformational changes in the tertiary structure of the fungal aspartic protease. Our interpretation of the changes observed in the secondary structure of the fungal aspartic protease due to binding of the classical inhibitor pepstatin to the active site correlates with the similar pattern of changes observed due to the binding of the newly isolated peptidic inhibitor. Our fluorescence spectroscopy and CD analyses suggest that the peptidic inhibitor

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Fig. 4. (a) Fungal spores were allowed to germinate on MH agar and grown for 24 h before the test solution was added. Subsequently, wells were bored on the agar, 30 µl aliquots of test solutions were added to the wells, and the fungi were allowed to grow for 12 h. The test solution (30 µl) contained 0 (1), 1 (2), 2 (3) and 3 µg (4) of the inhibitor. Fungal strains tested were A. fumigatus (I) and A. niger (II). (b) Morphological changes induced in mycelia of the fungal strains in the presence of inhibitor. Fungal spores were germinated in half-strength MH broth in the absence (panels in row I) or presence (panels in row II) of the inhibitor, and growth was observed after 24 h. The fungal strains tested were A. fumigatus (A) and A. niger (B).
binds to the active site of the fungal aspartic protease and causes inactivation.

Extracellular proteases of eukaryotic microbial pathogens have received tremendous research interest as potential drug targets for pharmaceutical intervention (Dash et al., 2003; Dos Santos, 2010). Analysis of proteolytic enzymes of pathogenic micro-organisms might lead to the design of inhibitors to control these pathogens (Dos Santos, 2011). To colonize a host, fungal micro-organisms have evolved strategies to invade tissues, optimize growth and propagate. To gain entrance, fungi generally secrete a cocktail of hydrolytic enzymes, including proteases, elastinolases and collagenases (Monod et al., 2002; Dagenais & Keller, 2009). However, there is a lacuna in the literature on inhibitors of aspartic proteases that exhibit antifungal activity; such literature could provide further insight into our understanding of host–pathogen interactions. Here the newly isolated inhibitor was analysed for its efficacy as an antifungal agent against Aspergillus spp. The inhibitor was found to be active against A. fumigatus and A. niger, and its IC_{50} values indicated exceptionally high potency. Our results documented that the specific activity of the inhibitor was decreased when the incubation time for fungal growth was increased. A possible explanation for this phenomenon is that the germlings at the early stages of growth were more affected than mycelium development at later stages. At high concentrations the inhibitor was found to inhibit spore germination, and at lower concentrations delayed growth of the hyphae, which subsequently exhibited abnormal morphology. Inhibition of fungal growth by the inhibitor is correlated with its anti-proteolytic activity, leading to depletion of nutrients required for growth and propagation. As protease inhibitors play an important role in protection of the host from pathogen attack by virtue of anti-nutritional interactions (Dash et al., 2001b), our results suggest therapeutic application of the inhibitor in opportunistic fungal infections to circumvent host invasion.

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REFERENCES


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