A low molecular weight aspartic protease inhibitor from a novel *Penicillium* sp: Implications in combating fungal infections

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Abstract

The article reports for the first time the isolation and characterization of a low molecular weight aspartic protease inhibitor from a novel Penicillium sp. The inhibitor was purified to homogeneity as shown by rp-HPLC and SDS-PAGE. The Mr of the inhibitor was 1585 and the amino acid composition showed the presence of D, D, D, E, A, K, L, Y, H, I, W residues. The steady-state kinetic interactions of Aspergillus saitoi aspartic protease with the inhibitor exhibited reversible, competitive, time-dependent tight binding nature of the inhibitor with $IC_{50}$ and $K_i$ values of 1.8 µM and 0.85 µM respectively. The fluorescence and CD analysis deciphered the inactivation of the enzyme due to binding of the inhibitor to the active site. The inhibitor was found to inhibit mycelial growth and spore germination of A. fumigatus and A. niger in vitro with MIC values of 1.65 and 0.30 µg/ml respectively. The finding of the inhibitor will potentially open the way towards the development of tight binding peptidic inhibitor against fungal aspartic protease to combat human fungal infections.

Keywords: Penicillium sp VM24, aspartic protease inhibitor, Biochemical characterization, Fungal aspartic protease, Competitive-tight binding inhibition, Fungal growth inhibition
Introduction

Several proteases are essential for propagation of diseases, and hence inhibition of different proteases is emerging as a promising approach in medicinal application for cancer, obesity, hepatitis, herpes, cardiovascular, inflammatory, neurodegenerative diseases, and various infectious and parasitic diseases (Rao et al., 1998). Aspartic proteases are relatively a 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). The study of the kinetic properties of this class of enzymes have been motivated due to their pharmaceutical and commercial importance and have evoked considerable interest for investigating the role of their inhibitors.

Invasive aspergillosis is a major threat to the long-term survival of immunocompromised patients (Dennings & Stevens, 1990). Risk factors for invasive aspergillosis include prolonged and severe neutropenia, hematopoietic 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 which can hydrolyze 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 were reported to be possible
virulence factor in invasive aspergillosis (Lee & Kolattukudy, 1995; Hogan et al., 1996), however recently several 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 shown to be necessary for fungal growth in protein medium (Sriranganadane et al., 2011). Combinations of proteolytic enzymes including 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 insights for 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 protease of therapeutic significance. There are only few reports on the isolation of biologic aspartic protease inhibitors and their mechanisms of inhibition. The current investigation envisage for the first time the isolation and characterization of a low molecular weight peptidic PepA inhibitor from a novel *Penicillium* sp. The evaluation of kinetic parameters affirms that *Penicillium* aspartic protease inhibitor is a tight-binding, reversible and competitive inhibitor of fungal aspartic protease. The fluorescence and circular dichroism studies reports that the binding of the inhibitor induces localized conformational changes in PepA. The new inhibitor will be a potential lead compound for the development of molecules to combat human fungal infections.
Materials and methods

Materials

Acetonitrile was purchased from E-Merck, Germany. Ultra membranes UM 10 and UM 3, UM 0.5 was from Millipore. Aspartic protease from *Aspergillus saitoi*, Trifluoroacetate, Hemoglobin were from Sigma-Aldrich, USA. Biogel P 2 was from Bio-Rad laboratories, CA. All other chemicals used were of analytical grade.

Isolation and identification of isolate

The organism was isolated in the laboratory from soil sample collected from Pashan, Pune district, India. Soil was suspended in sterile saline and serial dilution of the soil sample was plated on Potato dextrose agar (PDA) plates and kept for incubation at 28°C for 5 to 7 days. Fungal colonies obtained were purified by single colony plating technique and were screened for aspartic protease inhibitor by growing the fungal cultures in a 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)] at pH 5.0 for 120 h. Samples were withdrawn after every 24 h and were assayed for anti-PepA activity. The genomic DNA of the fungal culture producing the inhibitor was isolated by the method of Makimura et al (1994). The organism was identified based on the ITS region of the 18S rDNA. The blast searches of the sequences were performed at the National Centre for Biotechnology Information (NCBI) Gen-Bank data library (http://www.ncbi.nlm.nih.gov/BLAST/) using BLASTN with default settings. The sequences were downloaded from NCBI database and aligned using the CLUSTALW program (http://align.genome.jp/sit-bin/clustalw). Sequences
were trimmed using DAMBE software. Phylogenetic tree was generated by the neighbor-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 mentioned above) in 500 ml Erlenmeyer flask 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 was centrifuged. The inhibitor was purified from the supernatant by ultrafiltration (UM 10, UM 3, UM 0.5), Biogel P 2 and rp-HPLC (fluka RP-C 8), pre-equilibrated with 10 %(v/v) acetonitrile (CH₃CN) and 0.1 %(v/v) trifluoroacetate (TFA). The fractions were eluted on a linear gradient of 0 to 90 %(v/v) acetonitrile with H₂O containing 0.05 %(v/v) TFA at a flow rate of 0.5 ml/min and monitored at a wavelength of 210 nm. The eluted sample was lyophilized and dissolved in deionized water to check the inhibitor activity. The active fractions were rechromatographed on rp-HPLC under similar experimental conditions.

**Molecular mass and amino acid determination**

The molecular mass of the inhibitor was determined by the mass spectrometry (MALDI-TOF) and Tricine-SDS-PAGE. The amino acid analysis was done by hydrolyzing 100 pM of inhibitor with 6 N HCl at 110°C for 24 h in vacuum sealed tubes. The hydrolyzed amino acids were derivatized with AccQ Fluor Reagent (6-amino quinolyl-N-hydroxysuccinimide carbamate) and run on a prepacked rp-HPLC 3.9×150mm column AccQ.Tag. The amino acids were eluted with acetonitrile gradient [5 to 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 under identical conditions. Total cysteine and tryptophan were estimated with intact peptide according to the method 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 Bradford method, using bovine serum albumin as standards (Bradford, 1976).

**Fungal aspartic protease (PepA) inhibition assay**

Proteolytic activity of PepA from *Aspergillus saitoi* was measured by assaying the enzyme activity using hemoglobin as described by Dash et al (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 of hemoglobin (5 mg/mL) and incubated at 37°C for 30 min. The reaction was quenched by the addition of 2 mL of 10% (w/v) trichloroacetic acid (TCA) acidified with 2.25% (v/v) HCl followed by centrifugation (10000 g, 5 min) and filtration. The optical 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 per minute 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 of inhibitor towards trypsin, chymotrypsin, papain and subtilisin**

The inhibitory activities of the inhibitor against other class of proteases were also determined by assaying the enzyme activity using the specific chromogenic substrates at specific pH and temperature. Synthetic substrates Bz-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 the temperature stability experiments, the inhibitor (10 μM) was incubated at temperatures 25 to 50°C for 6 h and estimating its inhibitory activity against PepA at different intervals of time. The pH stability of the inhibitor was determined by pre-incubating the inhibitor (10 μM) in a range of pH values in appropriate buffers for 1 h and estimating the anti-PepA activity.

**Initial kinetic analysis for determination of $K_m$ and $K_i$**

For initial kinetic analysis, the kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The $IC_{50}$ value was determined by nonlinear regression of percent inhibition data by using the four parameter logistic Eq. (1) where $p$ is percent inhibition and is the relative decrease in enzymatic activity due to the inhibitor concentration $[I]_0$ (Kulkarni & Rao, 2007). The regression analysis was performed by the software Origin 8E.

\[
p = p_{\text{min}} + (p_{\text{max}} - p_{\text{min}})/1 + ([I]_0/IC_{50})^n \tag{Eq. 1}
\]

The inhibition constant $K_i$ was determined by Dixon method (Dixon, 1953) and also by the Lineweaver–Burk double reciprocal plot. For the Lineweaver-Burk analysis PepA (20 μM) was incubated with inhibitor concentrations of 0, 2, 4 and 6 μM and assayed at increasing concentration of hemoglobin (75 to 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, hydrolytic activity of PepA (20 μM) was measured in the presence of 250 and 450 μM of
hemoglobin 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 the $K_i$ was determined by fitting the data using Microcal Origin 8E.

**Initial apparent inhibition constants**

Inhibition studies were performed by adding 100 μl of the enzyme (0.05 μM) to 300 μl of 250 μM hemoglobin solution in standard buffer containing varying concentrations of the inhibitor (1 to 10 μM) at 37°C for 30 min. The product was estimated as mentioned above. Relative enzymatic activity $R$ was computed from the ratio of product amounts obtained in the presence and absence of inhibitors as $R=1−[P] / [P]_0$. The relative inhibition was fit by non-linear least squares regression to Eq. (2) where $[I]_0$ is the total concentration of inhibitor and $K_{app}$ the fitting parameter is the apparent inhibition constant.

$$R = [I]_0/(K_{app}+[I]_0) \tag{Eq. 2}$$

**Inhibitor progress curve analysis**

The reaction time course in the presence of inhibitor was fitted to the equation given below, which is a modification of the standard kinetic model.

$$p = p_0+V_{st}+(V_0-V_s)[1−exp(−k_{app}t)]/k_{app} \tag{Eq. 3}$$

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

\[
v = v_0([I]_0 - [I]_0 - K_{\text{app}} + ([E]_0 - [I]_0 - K_{\text{app}})^2 + 4[E]_0 K_{\text{app}})^{-1/2}
\]

(Eq. 3)

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

\[
IC_{50} = \frac{E_t}{2} + K_i^*
\]

(Eq. 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. The slit widths on both the excitation and emission were set at 5 nm, and the spectra were obtained at 1 nm/min. For inhibitor binding studies, PepA (20 µM) was dissolved in 0.05 N 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 smoothened. Corrections for the inner filter effect were performed as described by Eq. (6),

\[ F_c = F \frac{\text{antilog} \left[ \frac{A_{ex} + A_{em}}{2} \right]}{A_{ex} + A_{em}} \]  

(Eq. 6)

where \( F_c \) and \( F \) stand for 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 in 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. Spectra were averages of six scans with the base line 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 absence/presence of inhibitor or pepstatin (5 µM each). Secondary structure content of the PepA, the PepA-inhibitor complex was calculated using the algorithm of the K2d program (Andrade et al., 1993; Merelo et al., 1994).

**Antifungal activity assay**

The fungal strain *Aspergillus fumigatus* (NCIM 902) and *Aspergillus niger* were from our in-house culture collection unit, the National collection of industrial microorganisms, Pune, India. Antifungal activity was assayed essentially by (i) spore suspension assay, and (ii) microspectrometric assay. 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 x 10^6. To 1 ml of the freshly prepared spore suspension, 1 ml of half-strength modified Muller-Hinton (MH) agar (beef extract 2 g/L; acid hydrolysate of casein 17.5 g/L; starch 1.5 g/L; agar 25 g/L) (Muller & Hinton, 1941) with 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 to 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 to 72 h. All test solutions were filtered through a 0.22 μm pore size membrane prior to the application. A microspectrometric antifungal assay was performed for the quantitative demonstration of antifungal activity as described (Broekaert et al., 1990). Briefly, routine tests were performed with 20 μl of (filter [0.22 μm pore size]-sterilized) test solution and 80 μl of fungal spore suspension (10^6 spores/ml) in half-strength MH broth. Control microculture contained 20 μl of sterile distilled water and 80 μl of the fungal spore suspension. Unless otherwise stated, the incubation conditions for the experiments were 28°C for 48 h. Antifungal activity is expressed in terms of percent inhibition as defined elsewhere (Cammue et al., 1992). The MIC 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 microtiter plates. Each well was inoculated with 10 μl of the test organism at 10^5 spores/ml. 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 isolate
68 fungal cultures were isolated on PDA plates from soil sample. Only one fungal isolate was found to produce extracellular aspartic protease inhibitor. The isolated organism was identified on the basis of morphological and 18S rDNA sequence homology. The ITS region of 18S rDNA of *Penicillium* sp VM24 was sequenced to 433 base pairs and BLAST search analysis showed 94% homology to various *Penicillium* species. The sequence was deposited in NCBI Gene Bank database with the accession number JN673378. The phylogenetic neighbor joining tree based on ITS sequence also confirmed that the isolate belongs to the genus *Penicillium* (Fig. S1). Based on the BLAST search analysis the isolate showed only 94% similarity with *Penicillium pinophilum* and other *Penicillium* sp, hence the new isolate may be a new species of *Penicillium*. The isolate grows over a pH range of 4 to 6 with an optimum at 5 and at an optimum temperature of 28°C.

**Purification and biochemical characterization of inhibitor**

*Penicillium* sp VM24 produced the inhibitor in a media 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 weight impurities and salts. The concentrated inhibitor sample was further purified to homogeneity by rp-HPLC and the anti-PepA activity was associated with a single peak (Fig. 1a inset). The purified inhibitor showed a specific activity of 350 U/mg and a 52 fold increase in purification with a yield of 18% (Table 1). The inhibitor was stable in a broad range of pH (2 to 6) and temperatures (25 to 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 like trypsin, chymotrypsin, papain.
and subtilisin (data not shown). The Mr of inhibitor as determined by mass spectrometry (MALDI-TOF) was 1585 (Fig. 1a). Further the inhibitor revealed a single homogenous band with a Mr 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, W residues (Fig. 1c).

The molecular mass of the inhibitor was determined to be 1585 Da. The peptide or isomers will have a molecular mass of 1404 Da. The difference in the molecular mass may be due to the presence of multiple groups such as acetyl, carboxyl, methyl, nitro attached to the side chains of the amino acids. Pepstatin, a biologic aspartic protease inhibitor from *Streptomyces* sp is known to contain a statin group (Umezawa, 1976). Several other synthetic peptide inhibitors are found to have similar groups increasing their potency (Nguyen, 2008). However structural studies of the inhibitor will provide more insights pertaining to the side groups and the differences in the molecular weight.

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

The kinetic analysis indicated an $IC_{50}$ value of 1.82 μM (Fig. 2a). The inhibition of PepA followed a hyperbolic pattern with increasing concentrations of the inhibitor. Initial kinetic assessments by the Lineweaver–Burk analysis revealed that the aspartic protease was competitively inhibited by the inhibitor (Fig. 2b). The $K_m$ value of the enzyme was 180 μM. The secondary plot of the slope versus inhibitor concentration gives the $K_i$ value of 2.24 μM (Fig. 2b inset) while with the Dixon plot a $K_i$ value of 1.2 μM was obtained.

**Progress curve analysis**

The reaction progress was analyzed by two different methods, one based on the rapid equilibrium assumption 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. S2a as the data did not fit into the other model. The progress curves obtained at 2, 4, 6, 8 and 10 μM inhibitor were fitted individually to the equation mentioned below. The best fit values of the individual parameters obtained are listed in Table S1. The results obtained corroborate with the one-step inhibition mechanism wherein the enzyme inhibitor complex is rapidly formed. 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 $V_0$ obtained is constant and the apparent rate constant $K_{app}$ increases linearly with the inhibitor concentration. The rate constants were obtained by fitting the data to Eqs. (7)–(9).

\[
V_0 = V_m[S]_0/([S]_0+Km) \\ (Eq. 7)
\]

\[
V_S = V_m[S]_0/([S]_0+Km(1+[I]_0/K_i)) \\ (Eq. 8)
\]

\[
K_{app} = k_D+k_A[I]_0/(1+[S]_0/K_M) \\ (Eq. 9)
\]

The parameters listed in Table 2 favor the one-step mechanism, because the initial velocity does not decrease with the concentration of the inhibitor, as predicted by Eq. (7). Also, the increase of the apparent rate constant with $[I]_0$ is linear. The nonlinear least squares fit of $V_0$, $V_S$, and $K_{app}$ to Eqs. (7) to (9) are shown in Fig. S2b and S2c. The parameters obtained with the mechanism as explained by Cha et al (1975) corroborate well with the above mentioned mechanism yielding a $K_i^*$ of 1.075 μM and a $K_i$ of 1.1245 μM.

**Fluoremetric analysis of PepA-inhibitor interactions**
The localized changes induced in PepA due to interactions with the inhibitor were investigated by fluorescence spectroscopic analysis. 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 Trp residues. The fluorescence emission spectra of aspartic protease exhibited an emission maxima (λ<sub>max</sub>) at 340 nm as a result of the radiative decay of the π-π* transition from the Trp residues, confirming the hydrophilic nature of the Trp environment (Fig. 3a). The titration of the native enzyme with increasing concentrations of inhibitor resulted in a concentration dependent quenching of the tryptophanyl fluorescence. However, the λ<sub>max</sub> of the fluorescence profile indicated no blue or red shift negating any gross conformational changes in the three-dimensional structure of the enzyme due to inhibitor binding.

**Circular dichroism analysis of PepA-inhibitor complexes**

To elucidate the effects of inhibitor on the secondary structure of PepA, the CD spectra of enzyme-inhibitor complex was analyzed. The contents of α-helix, β-sheet, and β-turn of PepA were 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 of the native enzyme to 225 nm (Fig. 3b). This shift reveals a subtle change in the secondary structure of the enzyme upon inhibitor binding. Further, to throw light upon the mechanism of inactivation of PepA by the newly isolated inhibitor, we have analyzed the interactions of a representative competitive inhibitor, pepstatin (Richards et al., 1989) on the secondary structure of PepA. The 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 and were distinctly different from that of the unliganded
enzyme. The comparative analysis of the CD spectra of PepA upon binding of the inhibitor or the known active site-based inhibitor, pepstatin was found to be similar, suggesting the 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* were 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 of 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 microscopic assay, wherein the spores were cultured in the presence of varied concentrations of the inhibitor. The morphological differences observed in the mycelial growth after 48 h are shown in Fig. 4b. In the presence of the inhibitor, the 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 (*IC50*) of fungal growth was 0.58 μg/ml for *A. niger* and 2.34 μg/ml for *A. fumigatus*, whereas the MICs were 0.30 and 1.65 μg/ml respectively. The time dependent dose-response curves revealed that the extent of growth inhibition tends to decrease with the increase in the incubation time. The time dependent decrease in the
potency of the inhibitor was less pronounced in *A. niger* than it was in *A. fumigatus* (Fig. 5).

**Discussion**

The data reported 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. It was observed that the 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 under the “tight-binding inhibitor” group (William and 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 since 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 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 signifies that inhibitor binds rapidly to the enzyme but the rate of dissociation is slow. This indicates a fast inactivation of the enzyme in the presence of the inhibitor. The tight binding inhibitors combine at the active site and rapidly cause the enzyme to loose activity.

The tight-binding inhibitors induce conformational changes that cause the enzyme to clamp down in 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 the reversible enzyme-inhibitor complex. The
fluorescence quenching of the PepA by inhibitor revealed that the binding of inhibitor reduces the quantum yield of the Trp emission. The kinetically observable formation of enzyme-inhibitor complex does not involve a major alteration in the three-dimensional structure of the enzymes as reflected in the absence of any shift in the tryptophanyl fluorescence. Any changes in the environment of individual tryptophan residues may result in an alternation of fluorescence characteristics such as emission wavelength, quantum yield, and susceptibility to quenching (Pawagi and Deber 1990). Fluorescence quenching can also result from the energy transfer to an acceptor molecule having an overlapping absorption spectrum (Cheung 1991). As the inhibitor has no absorption in the region of 300-450 nm (data not shown), we ruled out the quenching of fluorescence due to the energy transfer between the inhibitor and the tryptophan residues. The effect of inhibitor concentration on the fluorescence quenching of the enzymes was also consistent with a 1:1 molar ratio. These findings indicated that the polarity of the Trp environment was negligibly altered after the binding of the inhibitor, suggesting minimal conformational changes in the tertiary structure of the fungal aspartic protease. Our interpretation for the changes observed in the secondary structure of the fungal aspartic protease due to the binding of classical inhibitor, pepstatin to the active site can be correlated to the similar pattern of changes observed due to the binding of the newly isolated peptidic inhibitor. Thus, we have concluded from the fluorescence and CD studies that the peptidic inhibitor 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 interventions
Analysis of proteolytic enzymes of pathogenic microorganisms might lead to design of inhibitors for controlling these pathogens (Santos 2011). To colonize in host, fungal microorganisms have evolved strategies to invade tissues, to optimize growth, and to propagate. To gain entrance, fungi generally secrete a cocktail of hydrolytic enzymes including protease, elastinolases and collagenases (Monod et al., 2002; Dagenais & Keller, 2009). However, there is a lacuna of literature on the inhibitors of aspartic protease exhibiting antifungal activity; such literature could provide further insight into the understanding of host-pathogen interactions. In the present study the newly isolated inhibitor was analyzed for its efficacy as an antifungal agent against *Aspergillus* sp. The inhibitor was found to be active against *A. fumigatus* and *A. niger* and its *IC*<sub>50</sub>s indicated an exceptionally high potency. Our results documented that the specific activity of inhibitor was decreased when the incubation time for the fungal growth was increased. A possible explanation for this phenomenon is that the germlings at the early stages of growth were more affected than the mycelium development at later stages. Inhibitor at high concentrations was found to inhibit spore germination, and at lower concentrations delayed growth of the hyphae, which subsequently exhibited abnormal morphology. The inhibition of fungal growth by the inhibitor is correlated to the anti-proteolytic activity of the inhibitor, leading to depletion of nutrients required for growth and propagation. As protease inhibitors play an important role in the protection of host from pathogen attack by virtue of antinutritional interactions (Dash et al., 2001b). Our result implicates the therapeutic application of the inhibitor in opportunistic fungal infections to circumvent host invasion.

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References


Dash, C., Phadtare, S., Desphande, V.V. & Rao, M. (2001a). Structural and mechanismics insights into the inhibition of aspartic proteases by a slow-tight binding inhibitor from an extremophilic *Bacillius* sp.: Correlation of the kinetic parameters with the inhibitor induced conformational changes. *Biochemistry* 40, 11525-11532.


Table 1 Purification of 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)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<tbody>
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<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>
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<td>12.49</td>
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<td>575</td>
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<td>Biogel P-2 column</td>
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<td>rp-HPLC</td>
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<td>735</td>
<td>2.14</td>
<td>350</td>
<td>52.12</td>
<td>18.14</td>
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**Table 2** Kinetic rate constants by various methods

<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>Kinetic rate constant</th>
<th>Value obtained</th>
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<tbody>
<tr>
<td>Inhibitor curve analysis</td>
<td>IC$_{50}$</td>
<td>1.82 µM</td>
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<tr>
<td>Lineweaver-Burk</td>
<td>$K_m$</td>
<td>180 µM</td>
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<td></td>
<td>$K_i$</td>
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<td></td>
<td>$V_m$</td>
<td>0.61 µM per min</td>
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<td>Dixon plot</td>
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<td>Substrate kinetics</td>
<td>$K_m$</td>
<td>168 µM</td>
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<tr>
<td></td>
<td>$V_m$</td>
<td>0.59 µM per min</td>
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<tr>
<td>Inhibitor progress curve</td>
<td>$K_i$</td>
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<td>$K_i^*$</td>
<td>0.71 µM</td>
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<tr>
<td></td>
<td>$V_m$</td>
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<td></td>
<td>$k_A$</td>
<td>$6.25 \times 10^{-3}$ per second</td>
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<td></td>
<td>$k_D$</td>
<td>$11.84 \times 10^{-3}$ per second</td>
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<td>Dissociation constant analysis</td>
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<td>Cha analysis</td>
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<td>$K_i^*$</td>
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Figure legends

Fig. 1 (a) The purified inhibitor was analyzed for the determination of Mr by MALDI TOF (1585). (inset) – rp-HPLC 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 16% T, 6% C trsi-tricine gel. Lane 1 show the ultra-low standard molecular weight markers and Lane 2 show the HPLC purified inhibitor. (c) Amino acid analysis of the inhibitor. The amino acid analysis was done by hydrolyzing the inhibitor with 6N HCl and the hydrolyzed amino acids were derivatized with AccQ Fluor Reagent and run on a prepacked rp-HPLC column AccQ.Tag. The amino acids were eluted with acetonitrile gradient and monitored with a Waters fluorescence detector. a Tryptophan determined by NBS.

Fig. 2 (a) The pepA activity (20 µM) was determined in the presence of increasing concentrations of inhibitor. The sigmoidal curve indicates the best fit for the percent inhibition data (average of triplicates) obtained, and the IC50 value was calculated based on the application of equation of Cha. (b) Estimation of kinetic constant by Lineweaver-Burk analysis. The enzyme activity was assayed at increasing concentrations of the substrate using various inhibitor concentration, 0 (■), 2 (●), 4 (▲), 6 (▼) µM. The reciprocal of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocal of the substrate concentration. The straight lines indicated the best fits for the data obtained by non-linear regression analysis and analyzed by Lineweaver-Burk’s reciprocal equation for competitive type of inhibition.

Fig. 3 (a) Protein fluorescence was excited at 295 nm, and emission was monitored from 300 to 400 nm at 25°C. Titration was performed by the addition of different
concentrations of the inhibitor to a fixed concentration of enzyme. (b) Far-UV circular dichroism spectra of the unliganded PepA and its complexes with the pepstatin and inhibitor are shown. PepA (25 µM) was dissolved in the buffer and the CD spectra were recorded in the absence (■) or in the presence of the inhibitor (●, 5 µM) or pepstatin (*, 5 µM) from 260 to 200 nm at 25°C. Each spectrum represents the average of six scans.

**Fig. 4 (a)** Fungal spores were allowed to germinate on Mueller-Hinton (MH) agar and grow 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 µg (1), 1 µg (2), 2 µg (3), and 3 µg (4) of the inhibitor. Fungal strains tested were *A. fumigatus* (I) and *A. niger* (II). (b) Morphological changes induced in the 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).

**Fig. 5** Time dependent dose-response growth inhibition curves. Growth inhibition of fungal strains (A) *A. fumigatus* and (B) *A. niger* at different concentrations of the inhibitor was recorded after 24 (■), 48 (●) and 72 h (▲). Antifungal activity of the inhibitor was estimated in terms of percent inhibition, and the *IC*₅₀ were calculated from the curves.
Fig 1

<table>
<thead>
<tr>
<th>Amino Acid</th>
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Mr = 1584 Da
Fig 2
Fig 3
Fig 4
Fig 5