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Novel Bifunctional Inhibitor of Xylanase and Aspartic Protease: Implications for Inhibition of Fungal Growth

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A novel bifunctional inhibitor (ATBI) from an extremophilic *Bacillus* sp. exhibiting an activity against phytopathogenic fungi, including *Alternaria, Aspergillus, Curvularia, Colletotricum, Fusarium*, and *Phomopsis* species, and the saprophytic fungus *Trichoderma* sp. has been investigated. The 50% inhibitory concentrations of ATBI ranged from 0.30 to 5.9 μg/ml, whereas the MIC varied from 0.60 to 3.5 μg/ml for the fungal growth inhibition. The negative charge and the absence of periodic secondary structure in ATBI suggested an alternative mechanism for fungal growth inhibition. Rescue of fungal growth inhibition by the hydrolytic products of xylanase and aspartic protease indicated the involvement of these enzymes in cellular growth. The chemical modification of Asp or Glu or Lys residues of ATBI by 2,4,6-trinitrobenzenesulfonic acid and Woodward's reagent K, respectively, abolished its antifungal activity. In addition, ATBI also inhibited xylanase and aspartic protease competitively, with K_i values 1.75 and 3.25 μM, respectively. Our discovery led us to envisage a paradigm shift in the concept of fungal growth inhibition for the role of antixylanolytic activity. Here we report for the first time a novel class of antifungal peptide, exhibiting bifunctional inhibitory activity.

The primary current means for the identification of new antifungal agents are represented by screening of the vast biodiversity prevalent in natural resources such as soil samples, marine waters, insects, and tropical plants (6, 8). The need for safe and effective antifungal agents has triggered considerable interest in the isolation of new compounds from biological resources. The rapid emergence of fungal pathogens resistant to currently available antibiotics has further compounded the dearth of novel antifungal agents. The past decade has witnessed a dramatic growth in knowledge of natural peptides from plants, animals, and microorganisms. These peptides play an important role in the protection of plants from invasive infection and could prove to be useful tools for the genetic engineering of fungal resistance in transgenic plants (40).

Antifungal peptides are classified into two classes based on their mode of action (17). The first group acts by lysis, which occurs via several mechanisms (34). Lytic peptides may be amphipathic, having two faces, with one being positively charged and the other being neutral and hydrophobic. The second class of peptide interferes with the cell wall synthesis or the biosynthesis of essential components (14). The biological activities of a large number of peptide toxins have been rationalized in terms of the peptides' having the ability to adopt amphiphilic α-helical structures (15, 16, 21). Peptides are expected to have value as alternative agents in the fight against new resistant microbial strains as they have modes of action different from those of classical antibiotics. The characterization of such new antifungal and antimicrobial peptides and the design of analogues with improved activities have allowed better understanding of the structure-activity relationship of these peptides (33). The continuing development in the understanding of the mechanism of fungal resistance enables inhibition targets and pathways to be explored.

In the present paper, we have evaluated the antifungal potential of a novel peptidic inhibitor, ATBI, against phytopathogenic fungi in vitro. The kinetic studies have revealed the bifunctional characteristics of ATBI, as it was found to inhibit xylanase and aspartic protease. Chemical modification of the carboxylic and amine groups of ATBI resulted in the loss of inhibitory activity against xylanase and aspartic protease and also in the loss of antifungal property, indicating the correlation of these enzymatic activities to fungal growth. The unique sequence and potentially different secondary structure of ATBI probably suggest a specific mode of action distinctly different from that seen in the traditional peptide toxins, and thus, ATBI probably represents a new class. Here we report an antifungal peptide and its inhibitory activity against xylanase and aspartic protease and the correlation of these enzymatic activities to fungal growth inhibition.

MATERIALS AND METHODS

Purification of ATBI. The extremophilic *Bacillus* sp. was grown in a liquid medium containing soy meal (2%) and other nutrients at 50°C for 48 h as described (12). Briefly, about 1,000 ml of the extracellular culture filtrate was treated with 65 g of activated charcoal and the supernatant was subjected to membrane filtration using Amicon UM10 (M_r cutoff, 10,000) and UM2 (M_r cutoff, 2,000), membranes. The resulting clear filtrate was concentrated by lyophilization and loaded onto a prepacked Ultropac Lichrosorb RP-18 (LKB) column. The fractions detected at 210 nm were eluted on a linear gradient of 0 to 50% acetonitrile and water containing 0.01% trifluoroacetate. The fractions showing inhibitory activity were pooled and found to be homogenous by reverse-phase high-performance liquid chromatography.

Antifungal activity assay. The fungal strains *Alternaria solani* (NCIM 887), *Aspergillus flavus* (NCIM 535, 538, and 542), *Aspergillus niger* (NCIM 773), *Aspergillus oryzae* (NCIM 637, 643, 649, and 1032), *Claviceps purpurea* (NCIM 1046), *Colletotrichum* sp., *Curvularia fallax* (NCIM 714), *Curvularia lunata* (NCIM 716), *Curvularia cymbopogonis* (NCIM 695), *Fusarium oxysporum* (NCIM 1008, 1043, and 1072), *Fusarium moniliforme* (NCIM 1099, and 1100), *Helminthosporium* sp. (NCIM 1079), *Phomopsis* sp., *Penicilium fellulatum* (NCIM 1227), *Penicillium roqueforti* (NCIM 712), and *Trichoderma reesei* (NCIM 992, 1051, 1052, and 1186) were from our in-house culture collection unit, the Na-

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tional Collection of Industrial Micro-Organisms, Pune, India. Antifungal activity was assayed essentially by (i) hyphal extension inhibition assay, (ii) spore suspension assay, and (iii) microspectrometric assay. The hyphal extension assay was carried out as described (31), with some modifications. Freshly grown fungal mycelium was spot inoculated at the center of a petri plate containing potato dextrose (PD) agar medium and incubated at 28°C for 24 to 48 h. Sterile filter paper disks (5-mm diameter) impregnated with different concentrations of ATBI were placed in front of the growing fungal mycelium. The plates were further incubated at 28°C, and the crescent zones of retarded mycelial growth were observed. The antifungal activity was determined by the spore suspension assay as described (26). All manipulations were carried out under sterile conditions. Fungal spores were harvested from the freshly grown fungal culture and suspended in sterile water. The concentration of the spore suspension was adjusted to 1.0×10^5 to 2.5×10^5 spores/ml, depending on the fungus to be tested. To 1 ml of the freshly prepared spore suspension, 1 ml of half-strength PD agar was added and was immediately overlaid on petri dishes containing PD agar. To allow for spore germination and initial vegetative growth, plates were incubated at 28°C for 24 to 48 h. At this time, sterile filter disks were laid on the agar surface, and different concentrations of ATBI were applied to the disks. 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 (7). 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 (10^5 spores/ml) in half-strength PD 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 (9).

The purified ATBI was treated at 90°C for 5 min at pH 6.0, and the antifungal activity was determined by spore suspension assay. Similarly, the pH stability of ATBI was determined in the range from pH 2 to 10 at 40°C, and its effect on the antifungal activity was checked as described before.

MIC. The MICs for the fungal strains were determined by a broth dilution method (2). Serial dilutions of ATBI were made in half-strength PD 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 ATBI at which growth was inhibited.

Structural studies and homology search. Circular dichroism (CD) spectra of ATBI (25 μg/ml) were recorded on a J-715 spectropolarimeter (Jasco) using a quartz cell with a path length of 1 mm. Measurements were made over the range of 250 to 190 nm. All CD spectra were recorded at room temperature and obtained with a 1-nm bandwidth, a scan speed of 50 nm/min, and a time constant of 5 s. The spectra obtained were the averages of six scans to improve the signal-to-noise ratio. A baseline was recorded and subtracted after each spectrum. The data were expressed in terms of ellipticity as measured in millidegrees. A sequence homology search was undertaken after retrieving the sequences of all the antifungal peptides from the databases and aligning them manually.

Production of xylanase and acid protease from the fungal strains. Freshly grown *T. reesei* and *A. oryzae* were inoculated into a synthetic liquid medium having the following composition: KH_2PO_4 (2 g/liter), $(NH_4)_2SO_4$ (7 g/liter), urea (1.5 g/liter), $MgSO_4 \cdot 7H_2O$ (0.3 g/liter), $CaCl_2 \cdot 2H_2O$ (0.3 g/liter), $FeSO_4 \cdot 7H_2O$ (5 mg/liter), $MnSO_4 \cdot H_2O$ (1.56 mg/liter), $ZnSO_4 \cdot 7H_2O$ (1.4 mg/liter), $CoCl_2$ (1 mg/liter), and Tween 80 (1 g/liter); the medium also contained oat spelt xylan (10 g/liter) or soy meal (20 g/liter) for the production of xylanase or aspartic protease, respectively. Cells were incubated at 28°C for 72 h. The fungal cells were separated by filtration and centrifugation, and the extracellular culture filtrate was tested for the presence of xylanase and aspartic protease. The inhibition of the xylanase and acid protease in the culture filtrate was detected by plate assay. The above-mentioned synthetic medium was also used in agar plates for the fungal growth inhibition assay of *T. reesei* and *A. oryzae*, in the presence of xylan or casein at various concentrations of ATBI.

Xylanase inhibition assay. Xylanolytic activity of the purified xylanase from the extremophilic *Bacillus* sp. was determined at pH 6.0 and 50°C by measuring the amount of reducing sugar liberated following the hydrolysis of oat spelt xylan (1%), in a reaction volume of 1 ml. The reducing sugar released was determined by the dinitrosalicylic acid method as described (28), using D-xylose as the standard. One unit of xylanase activity is defined as the amount of enzyme which produced 1 μmol of xylose equivalent per min from xylan under the assay conditions. The xylanase inhibition assay was carried out as described above in the presence of increasing concentrations of the inhibitor. Inhibition kinetics was analyzed by Lineweaver-Burk reciprocal plot.

Protease inhibition assay. Proteolytic activity of the purified aspartic protease from *Aspergillus saitoi* (F-Prot) was measured by assaying residual enzyme activity after incubating the enzyme and the inhibitor. F-Prot activity was measured in the absence or presence of inhibitor. F-Prot (50 μl; 100 μg/ml) in glycine-HCl buffer (0.05 M; pH 3.0) was incubated with the inhibitor (25 μg/ml) for 5 min. The reaction was started by the addition of 1 ml of hemoglobin (5 mg/ml) and was allowed to proceed for 30 min at 37°C. The enzymatic activity was quenched by the addition of 2 ml of perchloric acid (PCA) (1.7 M) followed by 30 min of incubation at 28°C. The precipitate formed was removed by centrifugation and filtration. The optical absorbance of the PCA-soluble products in the filtrate was read at 280 nm. One unit of protease activity is defined by an increase of 0.001 at Δ280 nm per min at pH 3.0 and 37°C, measured as PCA-soluble products, with hemoglobin as the substrate. The inhibition kinetics was analyzed by Lineweaver-Burk's double reciprocal plot.

Chemical modification of ATBI with TNBS and *N*-ethyl-5-phenylisoxazolium-3′-sulfonic acid. ATBI (25 μg) and 0.25 ml of 4% sodium bicarbonate were incubated with various concentrations of 2,4,6-trinitrobenzenesulfonic acid (TNBS), a lysine group modifier (29), at 37°C in a reaction mixture of 0.5 ml in darkness. Aliquots were withdrawn at suitable intervals, and the reaction was terminated by adjusting the pH to 4.6. A control without the modifier was routinely included, and the residual activity at any given time was calculated relative to the control. The extent of inactivation of F-Prot and xylanase was determined with the modified inhibitor as described before.

N-Ethyl-5-phenylisoxazolium-3′-sulfonic acid (Woodward's reagent K [WRK]) has been known to react with the carboxyl group of aspartate and glutamate residues (3, 37). To modify the carboxylic groups, ATBI (25 μg) was incubated in the absence or presence of different concentrations of WRK at 28°C for 1 h. Aliquots were removed at different time intervals, and the reaction was quenched by the addition of sodium acetate buffer, pH 5.0, to a final concentration of 100 mM. Excess reagent was then removed by gel filtration on a Bio-Gel P2 column equilibrated with sodium phosphate buffer (0.05 M; pH 6.0). The fractions containing the modified ATBI were concentrated by lyophilization and the residual activity of the inhibitor was determined by assaying for the antixylanolytic and antiproteolytic activity.

The TNBS- and WRK-modified ATBI was used in the experiments to determine the antifungal potency against *T. reesei* and *A. oryzae*. Control experiments with the chemical modifiers were performed to observe the impact of these compounds on the fungal growth inhibition.

Rescue of fungal growth inhibition by the enzymatic reaction products. (i) Hydrolysis of xylan. The hydrolysis of xylan (1%) was carried out in a 5-ml reaction mixture containing 500 U of xylanase in potassium phosphate buffer (0.05 M; pH 6.0) at 50°C for 1 h. The hydrolysis was monitored by estimating the reducing sugar formed by the dinitrosalicylic acid method, using xylose as the standard. The hydrolyzed products were separated from the reaction mixture by passing through an Amicon UM10 membrane.

(ii) Hydrolysis of casein. Casein (1%) was subjected to enzymatic hydrolysis by F-Prot (100 μg/ml) in a reaction volume of 5 ml containing glycine-HCl buffer (0.05 M; pH 3.0) at 37°C for 1 h. The hydrolyzed products were separated by membrane filtration and centrifugation followed by monitoring at 280 nm.

The hydrolyzed products of xylan and casein were concentrated by lyophilization and used for the rescue of the cellular growth inhibition by spore suspension assay on the synthetic agar medium containing xylan or casein. ATBI was applied to the paper disks, and growth inhibition was observed after 12 h. The hydrolyzed products of xylan and casein were added to the plates of *T. reesei* and *A. oryzae* exhibiting inhibited mycelial growth, the plates were further incubated at 28°C overnight, and the vegetative growth of the fungi was monitored.

RESULTS

Purification, biochemical characterization, and antifungal activity of ATBI. The extracellular culture filtrate of the extremophilic *Bacillus* sp. was evaluated for its potency as a fungal growth inhibitor. To assess the antifungal activity of the compound we have purified the molecule (ATBI) from the culture filtrate. During the purification process, the antifungal property of ATBI was found to be copurified with its aspartic protease-inhibitory activity. The antifungal activity of the purified ATBI against 16 fungal strains was assessed in a variety of standard biological assays (Table 1). ATBI showed strong inhibitory activity against *A. flavus, A. oryzae, A. solani, F.*

TABLE 1. IC_{50}s and MICs of ATBI against the tested fungal strains

Fungus	IC_{50} (μg/ml) of ATBI	MIC (μg/ml) of ATBI
A. flavus	1.25	0.82
A. oryzae	2.12	1.12
A. solani	1.00	0.60
F. oxysporum	3.50	2.30
F. moniliforme	2.52	1.84
T. reesei	0.52	0.30
Phomopsis sp.	ND[a]	3.80
C. lunata	ND	3.92
C. cymbopogonis	ND	4.21
Colletotrichum sp.	ND	3.95
Helminthosporium sp.	ND	4.15
C. fallax	ND	4.03
A. niger	ND	3.85
P. roqueforti	ND	5.68
P. fellulatum	ND	5.90
C. purpurea	ND	ND

[a] ND, not determined.

oxysporum, F. moniliforme, and *T. reesei* and moderate activity against *A. niger, C. cymbopogonis, Phomopsis* sp., *C. fallax, C. lunata, P. roqueforti, P. fellulatum, Helminthosporium* sp., and *Colletotrichum* sp.

The antifungal activity of ATBI was indicated by the zone of inhibition that developed around the paper disks against the vegetative growth after the spore germination (Fig. 1a). Fungal growth inhibition was also monitored in microscopic assay, wherein the spores of different fungal strains were cultured in the presence of varied concentrations of the inhibitor. The morphological differences observed in the mycelial growth after 24 h at 28°C are shown in Fig. 1b. In the presence of the inhibitor, the germination of *T. reesei* spores was delayed, whereas in *F. oxysporum, F. moniliforme, A. solani*, and *A. oryzae*, the rate of growth of the mycelia was lower. As seen from the micrograph, lysis was not observed in mycelia in the presence of ATBI.

After 24 h, the concentration of ATBI required for 50% inhibition (IC_{50}) of fungal growth varied from 0.52 μg/ml for *T. reesei* to 3.5 μg/ml for *F. moniliforme*, whereas the MIC ranged from 0.30 μg/ml for *T. reesei* to 5.90 μg/ml for *P. fellulatum*. The saprophytic fungus *T. reesei* was found to be the most sensitive to ATBI, whereas *C. purpurea* was the least sensitive strain. Figure 2 describes the time-dependent dose-response curves of *T. reesei, F. oxysporum, F. moniliforme, A. solani, A. oryzae*, and *A. flavus*. As revealed from the figure the extent of growth inhibition tended to decrease with the increase in the incubation time. For example, in the case of *A. oryzae* the IC_{50} of ATBI (after 24 h) was increased from 2.125 to 2.25 and 2.375 μg/ml after 48 and 72 h, respectively. The time-dependent decrease in potency of ATBI was less pronounced in *T. reesei* and *A. solani* than it was in *A. oryzae, A. flavus, F. oxysporum*, and *F. moniliforme*.

The stability of the inhibitor towards fungal growth inhibition and aspartic protease-inhibitory activity was checked with respect to temperature and pH. The antifungal and aspartic protease-inhibitory activities of ATBI were resistant to heat treatment up to 90°C for 10 min and were stable over a pH range of 2 to 10.

Primary and secondary structure analysis of ATBI. The amino acid sequence of ATBI was determined to be Ala-Gly-Lys-Lys-Asp-Asp-Asp-Asp-Pro-Pro-Glu (13). Searches of the protein databases have failed to identify any antifungal proteins with significant homology to ATBI. The primary structure also revealed an unusually high content of aspartic acid (four residues per molecule). The net charge per molecule calculated from the amino acid composition is negative, indicating that ATBI is an anionic peptide. The secondary structure of ATBI as revealed from the CD spectrum exhibited a negative band at approximately 203 nm, which is a characteristic feature of random coil conformation (Fig. 3). The secondary structure content calculated from the data obtained from the CD spectrum by the algorithm of the K2d program (1, 27), showed no periodic structure in the peptidic inhibitor. Further, constructing the peptide by the Brookhaven protein-building method, using SYBYL software, also predicted a random coil structure of ATBI.

Role of xylanase and aspartic protease in fungal growth inhibition. To understand the mechanism of the fungal growth inhibition by ATBI, we have investigated the role of two essential hydrolytic enzymes, xylanase and aspartic protease, which are crucial for the growth of phytopathogenic fungal strains and, thus, in their biosynthetic pathway. The productions of xylanase and aspartic protease are well documented in *A. oryzae* (11, 41) and in *T. reesei* (5, 18). The growth of *T. reesei* and *A. oryzae* on the synthetic agar medium containing xylan or casein was inhibited by ATBI (Fig. 4a). In the presence of xylan the fungal cultures produced a considerable amount of xylanase, whereas the production of aspartic protease was negligible. Similarly, the selective production of aspartic protease was observed in the culture broth when soy meal was used. To investigate the effect of ATBI on xylanase and aspartic protease activities, the culture filtrate was added in the central well of the agar plate containing xylan or casein. ATBI was added in the peripheral wells, and the plates were incubated at 37°C. The xylanolytic or proteolytic activities were detected by the clearance zone observed around the central well and their inhibition was prominently seen as the crescent zone in front of the well containing ATBI (Fig. 4b). The retardation of the mycelial growth and the inhibition of xylanase and aspartic protease activities by ATBI suggested the correlation between the inhibitions of these enzymatic activities and the fungal growth inhibition.

To further investigate the role of xylanase and aspartic protease in fungal growth inhibition we have enriched the ATBI-treated fungal strains with the enzymatic products of xylan and casein. On the synthetic medium containing xylan or casein the spore suspensions of *T. reesei* and *A. oryzae* were inoculated and allowed to grow. ATBI was added to the disks, and zones of inhibition were observed after 12 h. To the ATBI-treated disks hydrolyzed xylan or casein was added, and the fungi were further allowed to grow. As a control sterile distilled water was added to an ATBI-treated disk. As observed from Fig. 5, growth inhibition was rescued by the addition of hydrolyzed xylan or casein, whereas the control did not show revival of the growth. The rescue of the fungal growth inhibition by the enzymatic reaction products suggested the role of xylanase and aspartic protease in cellular growth inhibition.

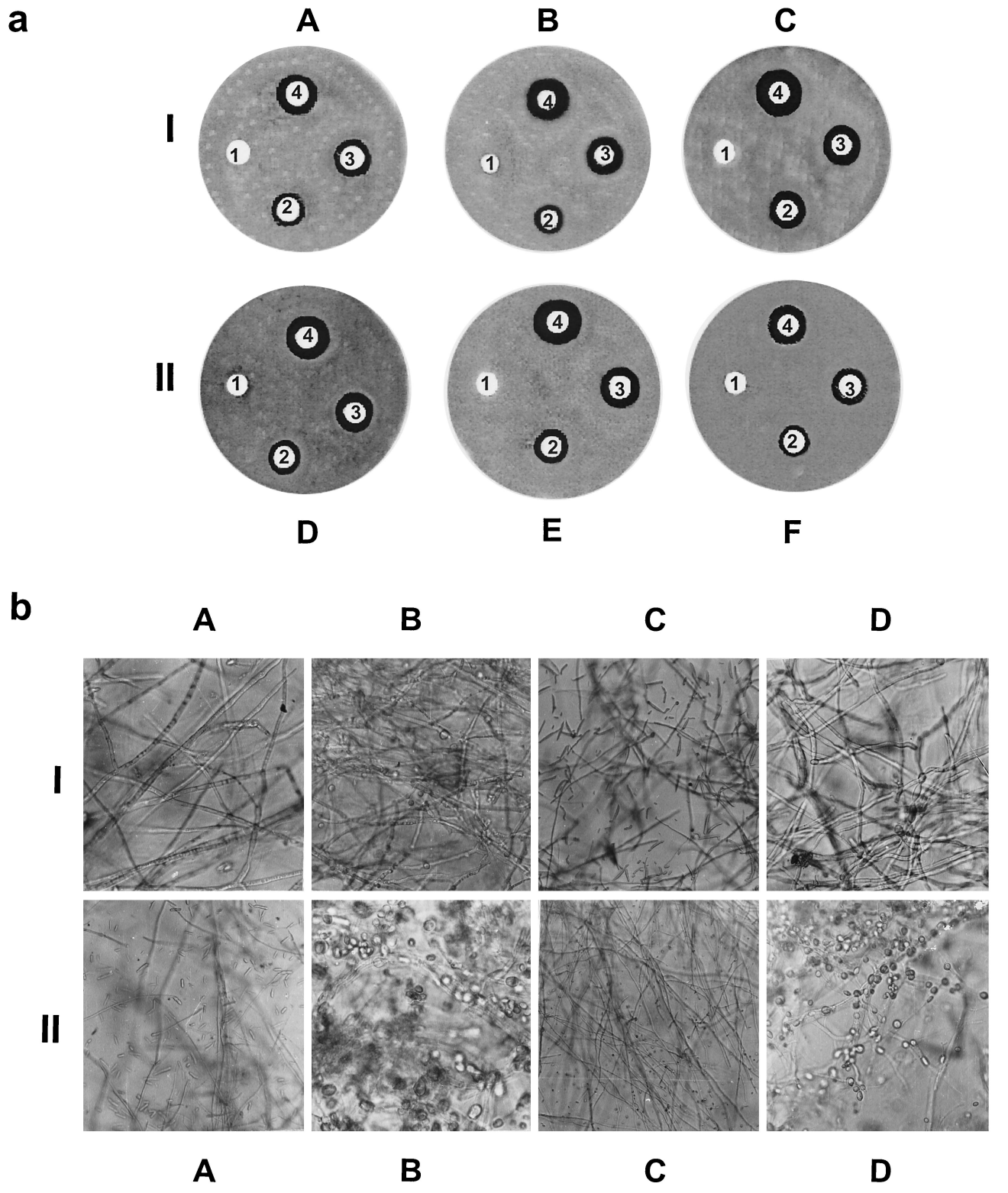

FIG. 1. Inhibition of fungal growth by purified ATBI. (a) Fungal spores were allowed to germinate on PD agar and grow for 24 h before the test solution was added. Subsequently, filter disks were placed on the agar, 40-μl aliquots of test solutions were added to the disks, and the fungi were allowed to grow for 12 h. The test solution (40 μl) contained 0 μg (1), 1 μg (2), 2 μg (3), and 3 μg (4) of ATBI. Fungal strains tested were *T. reesei* (A), *F. oxysporum* (B), *A. solani* (C), *A. flavus* (D), *A. oryzae* (E), and *F. moniliforme* (F). (b) Morphological changes induced in the mycelia of the fungal strains in the presence of ATBI. Fungal spores were germinated in half-strength PD broth in the absence (panels in row I) or presence (panels in row II) of ATBI, and growth was observed after 24 h. The fungal strains tested were *F. moniliforme* (A), *T. reesei* (B), *F. oxysporum* (C), and *A. oryzae* (D).

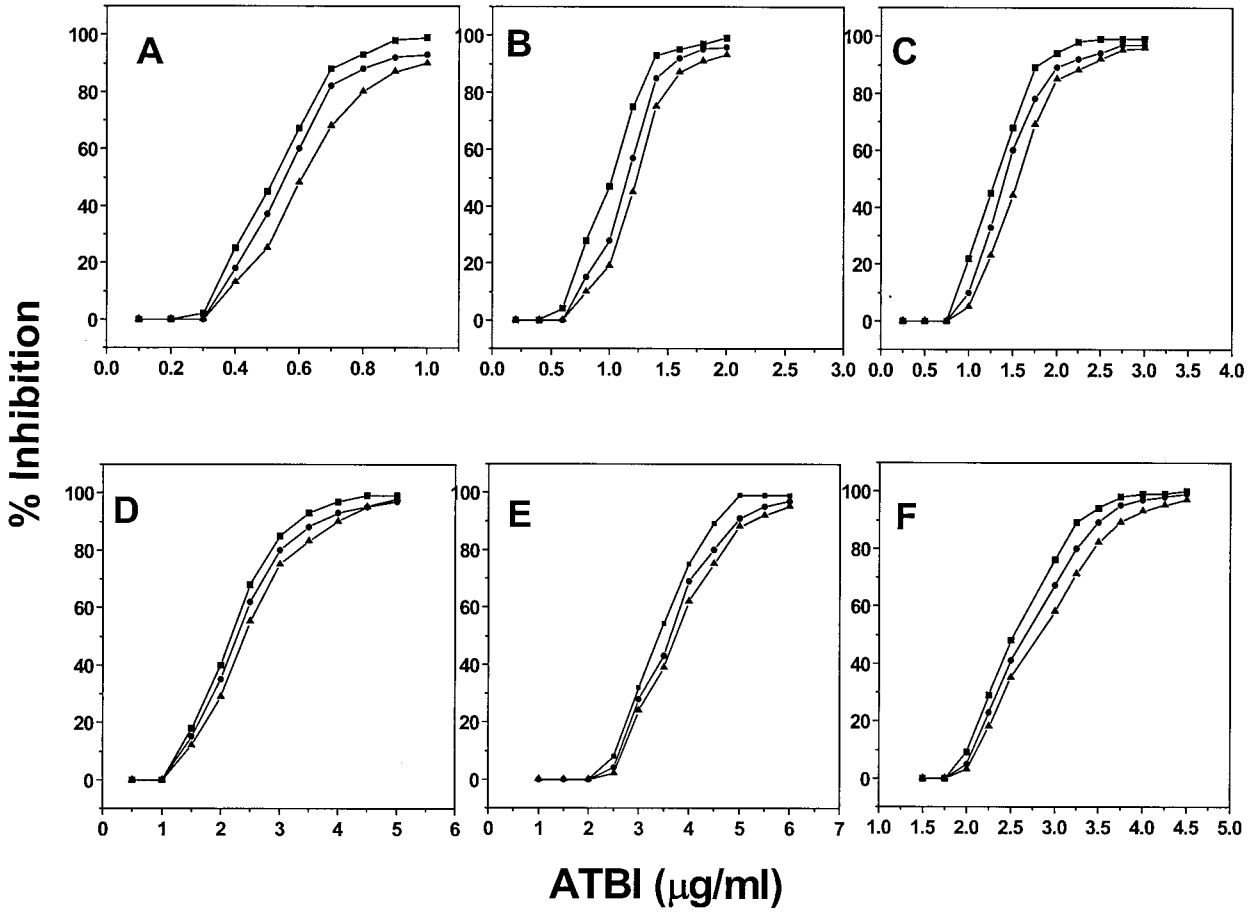

FIG. 2. Time-dependent dose-response growth inhibition curves. Growth inhibition of the fungal strains *T. reesei* (A), *A. solani* (B), *A. flavus* (C), *A. oryzae* (D), *F. oxysporum* (E), and *F. moniliforme* (F) at different concentrations of ATBI was recorded after 24 h (■), 48 h (●), and 72 h (▲). To 80 μl of spore suspension (10^5 spores/ml), 20 μl of test solution containing various concentrations of ATBI was added in a microculture plate. The control micro-culture plate contained 20 μl of distilled water and 80 μl of spore suspension. Antifungal activity of ATBI was estimated in terms of percent inhibition, and the IC_{50}s were calculated from the curves.

Chemical modification of ATBI and assessment of its antifungal activity. The role of functional groups for the inhibitory activity of ATBI was elucidated by employing chemical modifiers with specific reactivities. The amino acid sequence of ATBI revealed that Lys, Asp, and Glu are the amino acids containing ionizable side chains. The involvement of these groups in the mechanistic pathway was investigated using WRK, a carboxyl group modifier, and TNBS, an amine group modifier of lysine. Semilogarithmic plots of residual inhibitory activity against xylanase and aspartic protease as a function of time were linear (Fig. 5a), signifying that the inactivation process obeys pseudo-first-order kinetics. Loss of inhibitory activity was dependent on time and concentration of the reagent. The modification of the carboxyl groups of ATBI was monitored by the differential absorption at 210 and 340 nm. Analysis of the order of reaction for xylanase and F-Prot by the method described (24) yielded slopes of 1.67 and 1.64, respectively (insets of Fig. 5a), suggesting the involvement of two carboxyl groups of ATBI in the enzyme inactivation. The participation of the amine group of the lysine residues of ATBI was elucidated by use of the lysine modifier TNBS. TNBS caused time- and concentration-dependent loss of the inhibitory activity of ATBI. A reaction order of 0.75 and 0.79 for xylanase and F-Prot, respectively, with respect to the modifier was determined from the slope of the double-logarithmic plots (insets of Fig. 5b), indicating the involvement of a single amine group of ATBI in the enzyme inactivation.

In order to elucidate the mechanism of action of ATBI in fungal growth inhibition, the carboxyl- and amino-modified ATBI was tested for its potency towards the growth inhibition of *T. reesei* and *A. oryzae* on selective growth media. Figure 6, indicates that the TNBS- or WRK-modified ATBI did not inhibit the fungal growth, indicating the involvement of the amine and carboxylic groups of the Lys and Asp or Glu residues, respectively. Control experiments were carried out with the chemical modifiers WRK and TNBS and the synthetic agar medium. Interestingly, WRK inhibited the growth of *T. reesei* and *A. oryzae* (data not shown), as carboxyl groups are known to be present in the active site of xylanase and aspartic protease. TNBS failed to inhibit the fungal growth (data not shown), which is may be due to the absence of Lys residue in the active site of these enzymes.

Inhibition kinetics of xylanase and asparatic protease by ATBI. To elucidate the mechanism of inhibition of xylanase

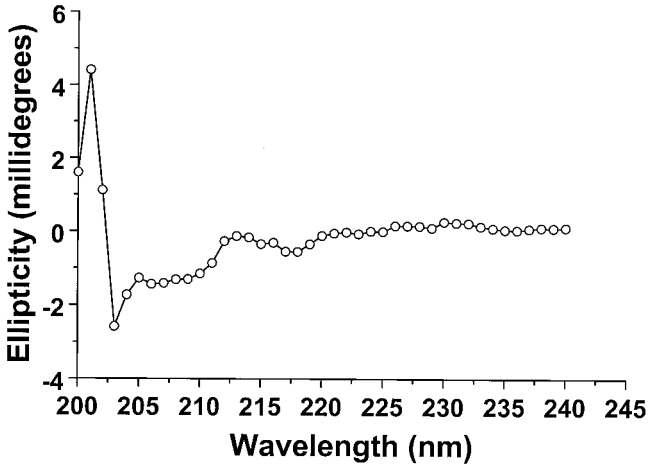

FIG. 3. Far-UV CD spectrum of ATBI. ATBI (25 μg/ml) was dissolved in KCl-HCl buffer (0.05 M; pH 3.0), and the CD spectrum was recorded from 280 to 200 nm at 25°C. The spectrum shown is the average of six scans with the baseline subtracted. The data are expressed in terms of ellipticity as measured in millidegrees.

and aspartic protease by ATBI, we have investigated the kinetics of inactivation. For the inhibition studies, we have used the purified xylanase from the extremophilic *Bacillus* sp. and F-Prot from *A. saitoi* as model systems. The enzyme activities were monitored in the presence of various concentrations of inhibitor and substrate, as a function of time. The double reciprocal plots of reaction velocity versus substrate concentration obtained for the enzymes demonstrated steady-state kinetic behavior and a competitive mode of inhibition (Fig. 7), suggesting the binding of ATBI to the active site of both the enzymes. The inhibition constants (K_is) determined for xylanase and F-Prot were 1.75 and 3.25 μM, respectively, revealing that the binding affinity of ATBI to the active site of xylanase was higher than to that of F-Prot.

DISCUSSION

The protease inhibitors play an important role in the protection of plant tissue from pest and pathogen attack by virtue of antinutritional interactions. Reports on cysteine and serine protease inhibitors, chitinases, glucanases, ribosome-inactivating proteins, and permatins as antifungal agents are well documented (7, 32). The present study is the first report of a bifunctional inhibitor, ATBI, which inactivates xylanase and aspartic protease as well as exhibiting antifungal activity. It is noteworthy that many of the fungal strains inhibited by the inhibitor are plant pathogens of significant importance to agriculture. ATBI was found to be active against a relatively broad spectrum of filamentous fungi, and its IC_{50}s indicated an exceptionally high potency. Significantly, in the cases of *T. reesei, A. solani*, and *A. flavus* the IC_{50}s were in the nanomolar

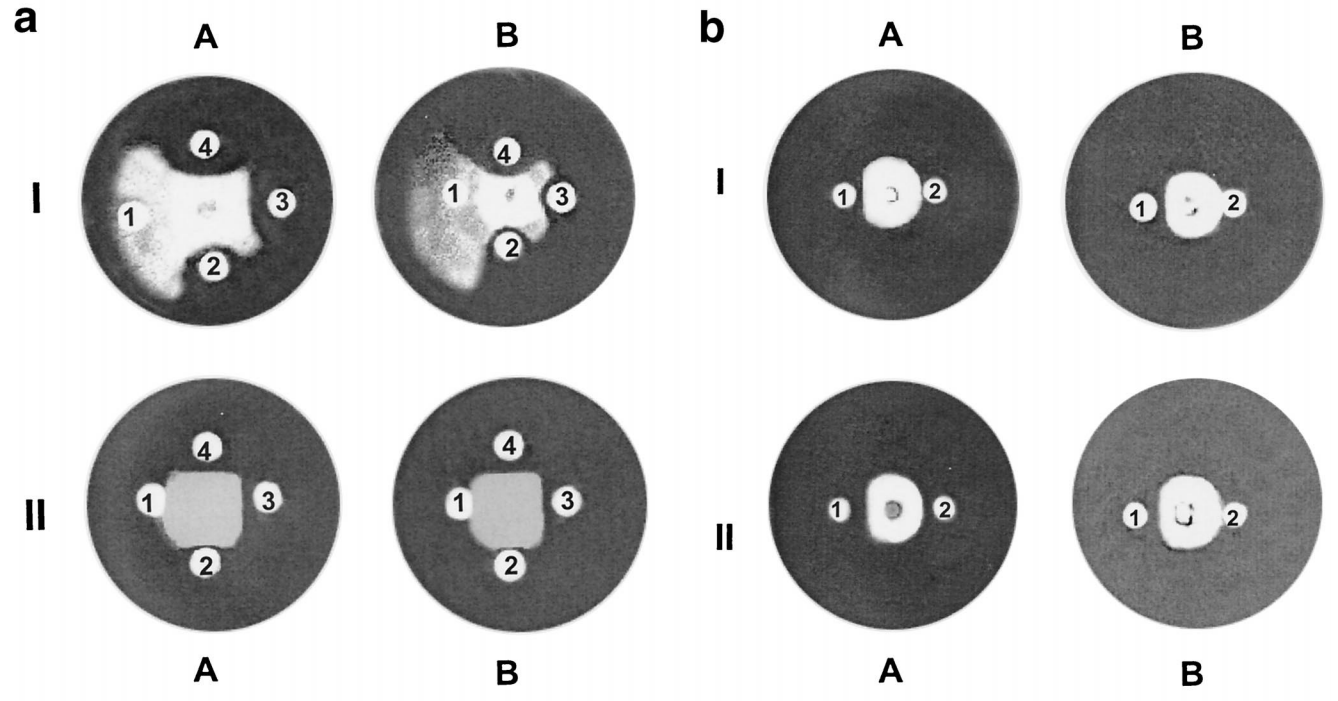

FIG. 4. Plate assay for growth inhibition and enzymatic activities. (a) Growth inhibition of *T. reesei* (panels in row I) and *A. oryzae* (panels in row II) on selective growth medium in the presence of ATBI. The fungal strains were spot inoculated on the synthetic agar media containing 0.5% xylan (A) or soy meal (B) as the carbon source. The paper disks at the periphery of the advancing mycelia contained 0 μg (1), 1 μg (2), 2 μg (3), and 3 μg (4) of ATBI. After the inhibitor treatment, the plates were incubated for 12 h. (b) Antixylanolytic and antiproteolytic activities of ATBI. *T. reesei*, (panels in row I) and *A. oryzae* (panels in row II) were grown in the synthetic medium containing 0.5% xylan or casein. The culture filtrates were added in the central well of the agar plate containing 0.5% xylan (A) or casein (B). The peripheral wells indicate the presence (1) or absence (2) of ATBI. The plate containing casein was preequilibrated with glycine-HCl buffer (0.05 M; pH 3.0) before addition of the culture filtrate. The plates were incubated at 37°C for 1 h.

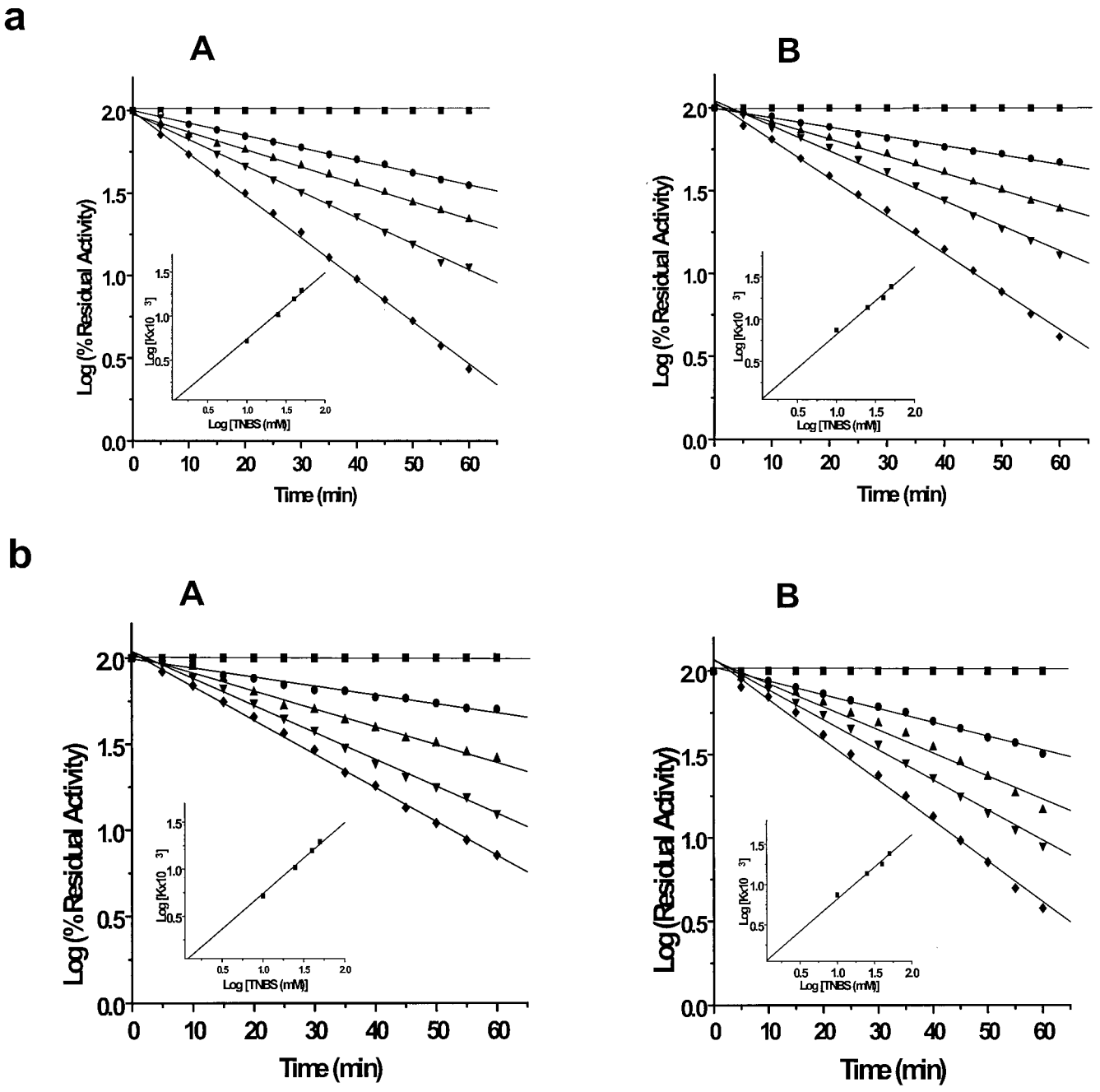

FIG. 5. Differential labeling of ionizable groups of ATBI with WRK and TNBS. (a) Inactivation of ATBI by WRK. ATBI (5 μg) was treated with 0 mM (■), 5 mM (●), 10 mM (▲), 20 mM (▼), and 30 mM (◆) WRK at 28°C for 1 h. Aliquots of the reaction mixture were removed at the times indicated, and the reaction was quenched with the addition of sodium acetate buffer to a final concentration of 100 mM. The residual WRK was removed by gel filtration on a Bio-Gel P2 column. Two hundred fifty microliters of the terminated WRK-modified ATBI was loaded on the Bio-Gel P2 column (preequilibrated with sodium phosphate buffer [0.05 M; pH 6.0]). The fractions were eluted at a flow rate of 12 ml/h. The active fractions were detected by the differential absorption at 210 and 340 nm and concentrated. (b) Effect of TNBS on the inactivation of ATBI. ATBI (5 μg) was incubated without (■) or with 10 mM (●), 25 mM (▲), 40 mM (▼), and 50 mM (◆) TNBS at 37°C in darkness for 1 h. Aliquots at specified time intervals were removed, and the reaction was stopped by adjusting the pH to 4.6. The inhibitory activity of the WRK- or TNBS-modified ATBI was determined by assaying against xylanase (A) and F-Prot (B), and the residual inhibitory activity at the given time was calculated relative to the control. The lines represent the best fit for the data obtained as the natural logarithm of percent residual inhibitory activity versus time. The insets are the double-logarithmic plots for the pseudo-first-order rate constants versus the concentration of the modifiers.

range. Our results documented that the specific activity of ATBI 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. ATBI at high concentrations was found to inhibit spore germination, and ATBI at lower concentrations delayed growth of the hyphae, which subsequently exhibited abnormal morphology.

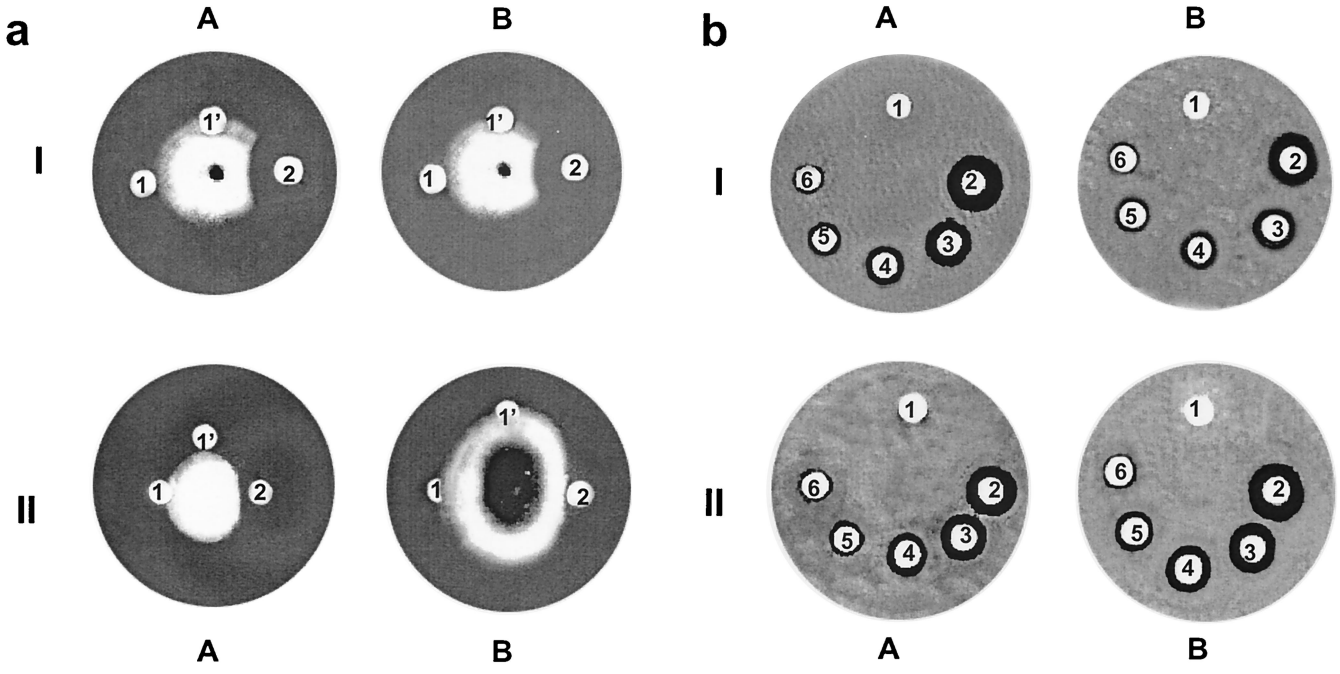

FIG. 6. Effect of chemically modified ATBI and enzymatic hydrolyzed products of xylan and casein on growth inhibition of *T. reesei* and *A. oryzae*. (a) Fungal growth inhibition in the presence of TNBS- or WRK-modified ATBI. *T. reesei* (panels in row I) and *A. oryzae* (panels in row II) were grown on synthetic agar media containing 0.5% xylan (A) or casein (B). The test solution containing TNBS-modified (1), WRK-modified (1′), or unmodified (2) ATBI was applied onto the paper disks at the periphery of the advancing mycelia, and fungal growth was observed after 12 to 24 h. (b) Rescue of fungal growth inhibition by the enzymatic reaction products. Fungal spore (10^5 spores/ml) of *T. reesei* (panels in row I) and *A. oryzae* (panels in row II) were allowed to germinate on the synthetic agar media containing 0.5% xylan (A) or casein (B). The filter disk on the agar contained 0 μg (1) or 3 μg (2 to 6) of ATBI. After 24 h the hydrolyzed products of xylan (A) or casein (B) at a concentration of 0, 0.5, 1, and 2 μg/ml were added to filter disks 3, 4, 5, and 6, respectively, and growth was observed after 12 to 24 h.

Antifungal peptides usually target the cytoplasmic membrane by using the self-promoting pathway due to their α-helical and β-sheet structure (35). To unravel the possible role of helicity of ATBI in the mechanism of fungal growth inhibition, we have investigated its secondary structure. Our results from the CD spectrum and from the structure prediction by SYBYL software revealed the absence of any periodic structure in ATBI. Furthermore, the structural features of the inhibitor could not be correlated with the biological activity against fungal growth, since lysis was not observed in the morphological changes in the hyphal growth. The primary structure of the anti-fungal peptide is completely different from those of any antifungal proteins so far isolated as indicated by the sequence homology studies. The structural novelty of ATBI probably depends on its high aspartic acid content and its unique sequence. The exceptional primary and secondary structure of ATBI suggested a different mechanism for fungal growth inhibition than the existing traditional antifungal compounds.

To colonize plants, fungal microorganisms have evolved strategies to invade plant tissues, to optimize growth in the plant, and to propagate. To gain entrance, fungi generally secrete a cocktail of hydrolytic enzymes including cutinases, cellulases, pectinases, xylanases, and proteases (19, 22). The ability of some proteinaceous plant inhibitors to modulate the activity of hydrolytic enzymes from plant pathogens has led to the theory or understanding that they play a role in plant defense as well as in the control of intrinsic enzyme activity. The data documented so far were not pertinent to the role of aspartic protease inhibitors in fungal growth inhibition. The roles of inhibitors of chitin synthase (10, 20), glucan synthase (4, 36), and proteases (25) as antifungal agents have been well established. However, there is a lacuna of literature on the inhibitors of xylanase, cellulase, and aspartic protease exhibiting antifungal activity; such literature could provide further insight into the understanding of host-pathogen interactions. In order to examine the contribution of the inhibition of xylanase and aspartic protease to the observed antifungal activity we have investigated the inhibition of these two enzymes by ATBI in vitro. The analysis of inhibition kinetics data revealed the binding of ATBI to the active site of xylanase and aspartic protease and a competitive mode of inhibition for both the enzymes. Further, to delineate the role of xylanase and aspartic protease in fungal growth inhibition, we have grown *T. reesei*, a saprophyte, and *A. oryzae*, a phytopathogen, as model systems on a synthetic medium containing xylan or casein as the sole carbon source. The retardation of mycelial growth by ATBI in the presence of xylan or casein implied the role of these enzyme activities in fungal growth inhibition. The kinetic constant K_i revealed that ATBI binds more effectively to the active site of the xylanase than to that of the aspartic protease, indicating the major contribution of antixylanolytic activity in fungal growth inhibition. This concurs with our thinking that, when a pathogen invades a host, on contact with the hemicellulosic or cellulosic surface the secretion of xylanolytic and/or cellulolytic enzymes would be essential for its survival. Hence, we visualize the functional role of a xylanase inhibitor in re-

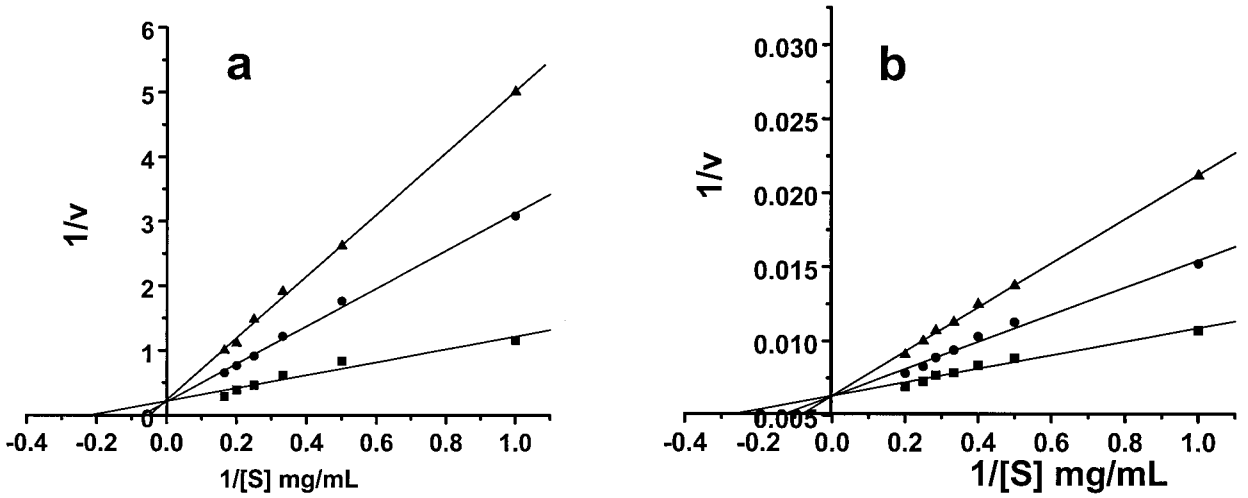

FIG. 7. Lineweaver-Burk reciprocal plots for inhibition of xylanase and F-Prot by ATBI. (a) Purified xylanase (25 μg/ml) in 50 mM potassium phosphate buffer (0.05 M; pH 6.0) was incubated without (■) or with the inhibitor at 10 μg/ml (●) or 20 μg/ml (▲) and assayed with increasing concentrations of xylan. (b) F-Prot (100 μg/ml) in glycine-HCl buffer (0.05 M; pH 3.0) was assayed in the absence (■) or presence of ABTI at 10 μg/ml (●) or 20 μg/ml (▲) with increasing concentrations of hemoglobin. The reciprocals of the rate of the substrate hydrolysis by xylanase and aspartic protease (1/v) for each inhibitor concentration were plotted against the reciprocals of the substrate concentration (1/[S]). The straight lines indicated the best fit for the data obtained. K_i was calculated from the formula for the competitive type of inhibition.

stricting the invasion of pathogens. There have been reports of bifunctional inhibitors of α-amylase and trypsin (38, 39). However, a bifunctional inhibitor of xylanase and aspartic protease, enzymes which are active under distinctly different physiological conditions, has not been reported so far. To our knowledge, ATBI represents the first report of a bifunctional aspartic protease inhibitor showing a broad spectrum of antifungal activity.

To determine the residues involved in the antixylanolytic or -proteolytic activity, we have modified the ionizable groups of Lys and Asp or Glu of ATBI. Modification of the amine group of Lys or the carboxyl group of Asp or Glu residues of ATBI by specific modifiers, TNBS or WRK, resulted in the loss of its inhibitory activity. The kinetic analysis indicated the participation of one amine and two carboxyl groups of ATBI in the inhibition of xylanase and F-Prot. It is well established that the catalytic site of xylanase and aspartic protease consists of two carboxyl groups and an essential lytic water molecule (23, 30). Although both the enzymes are active under entirely different physiological conditions, the structural and kinetic studies have revealed a similar mechanism in which the enzymatic reaction follows general acid-base catalysis with the direct participation of the lytic water molecule. Further, to decipher the role of ionizable groups of ATBI in fungal growth inhibition the Lys-, Asp- or Glu-modified ATBI was tested for its antifungal property on selective conditions. The modified ATBI failed to inhibit the growth of *T. reesei* and *A. oryzae* in the presence of xylan or casein, indicating the involvement of carboxyl and amine groups in fungal growth inhibition. This can be explained by the fact that abolishing the inhibitory property of ATBI resulted in the recovery of the xylanase and aspartic protease activities in the fungal strains. These results were further corroborated by the rescue of the growth inhibition by the addition of enzymatic products. Enrichment by the hydrolyzed products of xylan and casein to the ATBI-treated *T. reesei* and *A. oryzae* had substantially enhanced the growth. The rescue of fungal growth on the selective media containing xylan and casein has prompted us to propose the essential role of xylanase and aspartic protease in the cellular growth of fungal strains.

The bifunctional inhibitor ATBI was stable over a wide range of pH and temperature. Therefore, the direct application of ATBI as a biocontrol agent for the protection of plants against phytopathogenic fungi by encapsulation for surface application or by spray would be very useful. For more effective results, the seeds could be treated with the formulated preparation of ATBI; thus, they could be protected from fungal pathogenesis during germination. Moreover, being of microbial origin and an extracellular product, ATBI offers an attractive and economical process for commercial production. There have been reports that the inhibition of fungal growth was due to the inhibition of a single hydrolytic enzyme. However, as a bifunctional inhibitor, ATBI may act in concert to circumvent host invasion and make it difficult for the pathogens to acquire resistance.

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